IMMUNE RESPONSES OF THE MUCOSAL EPITHELIUM IN CHRONIC LUNG DISEASES

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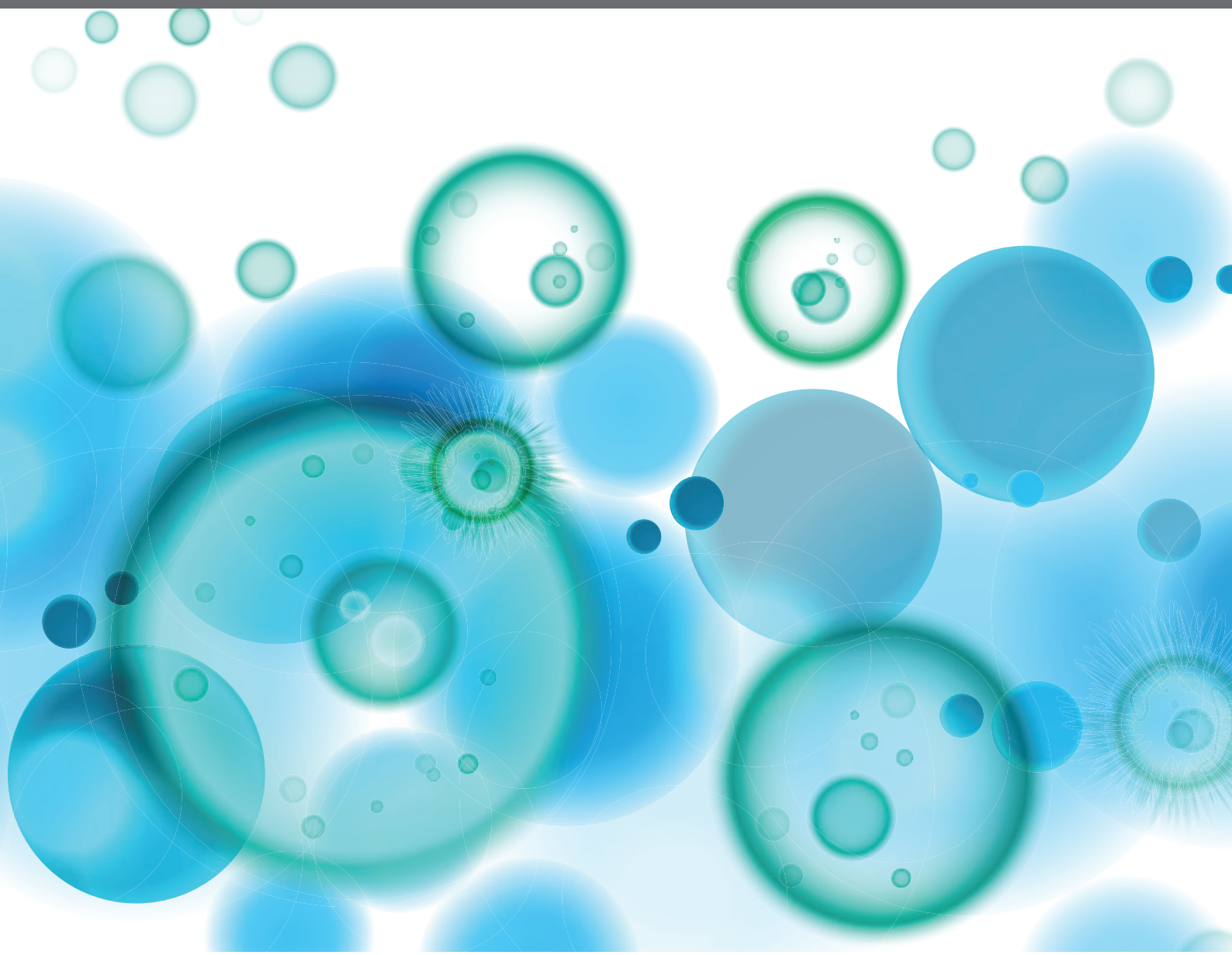

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IMMUNE RESPONSES OF THE MUCOSAL EPITHELIUM IN CHRONIC LUNG DISEASES

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EDITORIAL
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Editorial: Immune Responses of the Mucosal Epithelium in Chronic Lung Diseases

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Keywords: lung, epithelium, chronic lung disease, innate immunity, host defense

Editorial on the Research Topic

Immune Responses of the Mucosal Epithelium in Chronic Lung Diseases

The airway epithelium plays a prominent role in protecting us against detrimental agents since it is continually exposed to particles that are potentially harmful for the lungs including microorganisms, dust and air pollutants. However, *via* mucociliary clearance and coughing, these agents are usually eliminated and airway integrity is protected from these potentially damaging attacks. Until recently, the epithelium of the respiratory tract was only seen as a physical barrier, macrophages being considered as the main immune sensor of aggression. Although its structural integrity is essential, it is now well established that the airway epithelium plays a major role in triggering an innate immune response to protect the lung from infection and injury in various chronic respiratory diseases. A defect of these protections in the airways can induce some diseases as seen with the current health crisis caused by Severe Acute Respiratory Syndrome (SARS)-CoV2. Indeed, airway epithelium is playing a crucial role in the host defense against this virus (1). Defects in these mechanisms are also associated with chronic lung pathologies including cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthma. The 14 articles of this Research Topic highlight the latest advances regarding the role of the airway epithelium immune response in chronic respiratory disease.

In a mini-review of the literature, Guo-Parke et al. expose the cellular and molecular mechanism involved in COPD. COPD is a complex disease and it is estimated that around 50% of COPD acute exacerbations are related to respiratory viral infection. In this context, the authors especially address how respiratory viruses altered the immune response of the airway epithelium in the pathogenesis of exacerbations. They summarize the contribution of T cell exhaustion, NF-κB, TLR, EFGR, IFNs, and inflammasome.

Cystic fibrosis patients are often infected or colonized by the bacteria *P. aeruginosa*. It has been shown that *P. aeruginosa* acquire mutations during colonization, which promote their resistance, often associated with the acquisition of antibiotic resistance. Antimicrobial peptides (AMPs) are endogenous antibiotically active factors, produced by many different types of cells. Their antimicrobial activity mostly depends on their interaction with bacteria or host cell membranes but is also influenced by pH and ionic strength. Geitani et al. review the latest findings on the potential of antimicrobial peptides as therapeutics in CF.

Although bacterial pathogens play a prominent role in many pulmonary diseases, virus sometimes outperform their pathogenicity or predispose the host to a more severe course of the disease. This is the

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case for CF patients, who are often infected with viruses including *Influenza A* virus. In an original study Villeret et al. address what is the result of a concomitant infection with IAV and *P. aeruginosa*. They demonstrate with epithelial cells *in vitro* and *in vivo* that IAV promotes the deleterious effects of a secondary infection with *P. aeruginosa*. This damaging response is characterized by an increase of MMP9 activity and its inhibition supports lung resilience with no effect on the bacterial clearance. Also, the authors showed that IAV subverts the host response by inhibiting the antimicrobial/antiprotease molecule elafin/Trapin 2 transcript, known to possess beneficial anti-inflammatory properties. Altogether, the results of this study suggest that restoring tissue resilience could be a successful strategy in a coinfection situation. Montgomery et al. investigate rhinovirus (RV) induced airway epithelial cell necrosis in young children with CF. RV is indeed the commonest respiratory virus detected in the CF airways. These authors provide data that RV infection in airway epithelial cells from children with CF leads to more necrotic cell death and a higher IL-1R signaling. IL-1R in turn is a known driver for airway neutrophilia and mucin production. Thus, using IL1-Ra could mitigate the severity of the disease. Infection usually results in a complex signature of differentially expressed genes, not only involving IL-1 related pathways. With the advent of multiplex analysis tools, the complex nature of bacterial and viral infections has become even more evident. Applying a transcriptomic approach on primary tracheal CF human airway epithelial cell cultures infected with RV, Ling et al. provide detailed analysis of the biological pathways that are differently induced in comparison to non-CF cells. Their work confirmed the findings from Montgomery et al. showing that in response to RV infection, the genes for IL-1 signaling and mucin glycosylation were mostly dysregulated in fully differentiated CF airway epithelial cells.

Another hallmark of CF is the exaggerated neutrophil-dominated innate immune response. Indeed, Cystic fibrosis transmembrane receptor (CFTR) is broadly expressed on epithelial cells and cells of myeloid origin. Neutrophils contribute to the deleterious evolution of the CF lung disease. Cabrini et al. extensively review the hypotheses that there is an abnormal airway epithelial cell response in CF. This is due to the receptor-activated intracellular signaling pathways and the influence of epigenetic regulation of key chemokines. Laucirica et al. contributed a comprehensive overview on the latest progress of model systems to investigate mucosal inflammation in CF using cell and animal models. The focus of their review is on the function of neutrophils and their aberrant activation in the course of CF. Molloy et al. extends the topic to the secretome of *Stenotrophomonas maltophilia*, a Gram-negative opportunistic pathogen that can chronically colonize the lungs of people with CF and is associated with lethal pulmonary hemorrhage in immunocompromised patients. They show how bacterial proteases impair the airway epithelium integrity by disrupting the tight junctional complex in CFBE41o- bronchial epithelial cells, particularly ZO-1 and occludin.

In addition to viruses and bacteria, the airway epithelium is exposed to fungi including *Aspergillus fumigatus*. This filamentous fungus is found in the environment and can be pathogenic in immunocompromised patients or patients with altered mucociliary clearance such as CF patients. Bigot et al. reviewed the contribution of airway epithelial cells in the host immune response against *A. fumigatus*. The models to study *A. fumigatus*-airway epithelium are depicted as well as the different step of the host immune response: recognition, internalization, and host response of the bronchial epithelium.

Asthma is a complex respiratory disease with variable and complex symptoms. Frey et al. made a very comprehensive review describing the detrimental role of the airway epithelium in the formation, progression and acute exacerbation of asthma. They especially describe how the barriers (mucus and periciliary layers) and immune function (i.e. sIgA) of the airway epithelium are altered is asthma pathogenesis. In asthma, epithelial mesenchymal transition (EMT), which is necessary during lung development, is detrimental during asthma pathogenesis. Sun et al. provide data, that the exposure to house dust mite leads to an increased expression of IL-33 and CD146, which in turn induce EMT. The regulation of gene expression comprises multiple layers. Transcription factors, epigenetic modifications and small RNA-species potentially influence each other and in consequence the expression of their target genes. Epigenetically regulated gene expression in the context of asthma (or any other pathologic condition) is very interesting, since it is potentially hereditary and may impact the health of future generations. Besides epigenetic modifications, small RNA-species modify gene transcription. Alhamwe et al. review the latest findings that proof the influence of epigenetic modifications on the pathogenesis of asthma associated pulmonary inflammation.

Particles and compounds derived from cigarette smoke (CS), traffic, industry or open hearth play an important role in the development of many so-called "disease of civilization" like asthma, allergy, and COPD. Singh et al. contribute a very interesting study, highlighting physiological mechanisms by which maternal exposure to CS downregulate H_2S synthesizing enzymes in preclinical models and human placentas. H_2S synthesizing enzymes play a role in EMT during lung development and asthma pathogenesis. Interestingly, the CS-induced inhibition of H_2S synthesizing enzymes was transmitted to the F2 progeny and may increase risk for the development of asthma or bronchopulmonary dysplasia.

Interplay between metabolism and immunity/inflammation is an emerging domain of research. In this context, several associations between vitamin D deficiency and respiratory diseases and infections have been described. Schrumpf et al. reviewed the current knowledge about mucosal vitamin D metabolism and its signaling in chronic lung disease. Particularly, its metabolism in health and chronic inflammatory diseases is described, followed by the description of its protective effects on mucosal homeostasis. Finally, the last strategies of treatment with vitamin D are described.

AUTHOR CONTRIBUTIONS

CH, MC, and LG wrote the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Influenza A Virus Pre-Infection Exacerbates *Pseudomonas aeruginosa*-Mediated Lung Damage Through Increased MMP-9 Expression, Decreased Elafin Production and Tissue Resilience

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Individuals with impaired immune responses, such as ventilated and cystic fibrosis patients are often infected with *Pseudomonas aeruginosa* (*P.a*) bacteria, and a co-infection with the *Influenza* virus (IAV) is often present. It has been known for many years that infection with IAV predisposes the host to secondary bacterial infections (such as *Streptococcus pneumoniae* or *Staphylococcus aureus*), and there is an abundance of mechanistic studies, including those studying the role of desensitization of TLR signaling, type I IFN- mediated impairment of neutrophil chemokines and antimicrobial production, attenuation of IL1β production etc., showing this. However, little is known about the mechanistic events underlying the potential deleterious synergy between *Influenza* and *P.a* co-infections. We demonstrate here *in vitro* in epithelial cells and *in vivo* in three independent models (two involving mice given IAV +/– *P.a*, and one involving mice given IAV +/– IL-1β) that IAV promotes secondary *P.a*-mediated lung disease or augmented IL-1β-mediated inflammation. We show that IAV-*P.a*-mediated deleterious responses includes increased matrix metalloprotease (MMP) activity, and MMP-9 in particular, and that the use of the MMP inhibitor improves lung resilience. Furthermore, we show that IAV post-transcriptionally inhibits the antimicrobial/anti-protease molecule elafin/trappin-2, which we have shown previously to be anti-inflammatory and to protect the host against maladaptive neutrophilic inflammation in *P.a* infections. Our work highlights the capacity of IAV to promote further *P.a*-mediated lung damage, not necessarily through its interference with host resistance to the bacterium, but by down-regulating tissue resilience to lung inflammation instead. Our study therefore suggests that restoring tissue resilience in clinical settings where IAV/*P.a* co-exists could prove a fruitful strategy.

Keywords: *Pseudomonas aeruginosa*, *influenza* virus, elafin, metalloprotease, lung tissue resilience

INTRODUCTION

Individuals with impaired immune responses, such as cystic fibrosis (1–5) and ventilated patients (6, 7) demonstrate frequent respiratory viral infections with a variety of viruses, including the *Influenza A* virus (IAV). For example, clinical studies have shown correlations between viral infections with pulmonary exacerbations (1–3, 5), with the former often predisposing the host to secondary bacterial infections (8–11). Specifically, in a CF population study spanning over 6 years, which recruited in excess of 31,000 individuals and which reported an excess of 91,000 pulmonary exacerbations, the latter were associated with IAV activity (mostly of the H3N2 serotype) in both children and adults, with a *Pseudomonas aeruginosa* (*P.a)* prevalence of 48 and 82%, respectively. By contrast, an RSV association was only observed in adults (12). Surprisingly however, despite their simultaneous occurrence in such situations as acute nosocomial infections or chronic exacerbations in cystic fibrosis (see above) and COPD/emphysema, none of these studies have extensively tackled the interaction between *Influenza* and *Pseudomonas aeruginosa* (*P.a*). Indeed, mechanistic studies dealing with IAV and bacteria have mainly concerned *Staphylococcus aureus* and *Streptococcus pneumoniae*, for the latter, and a variety of mechanisms (often potentially contradictory) have been put forward to explain the deleterious consequences of such interactions.

These include, not exhaustively however, IAV-mediated desensitization of TLR signaling (13), type I interferon-mediated impairment of neutrophil chemokines or function (14–17), down-regulation of antimicrobial production (18–20), attenuation of IL1β production or IL1β-mediated alveolar macrophage activity (21, 22), exaggerated inflammatory responses (23, 24), or loss of lung repair potential (25, 26).

Mechanistically, we demonstrated here *in vitro* and *in vivo* in three independent models (two involving mice given IAV +/– *P.a*, and one involving mice given IAV +/– IL-1β) that IAV pre-treatment promoted secondary PAO1-mediated lung disease or augmented IL-1β-mediated inflammation, by enhancing deleterious inflammatory responses. These included increased matrix metalloprotease (MMP) activity, particularly MMP-9. Importantly, we showed that the MMP inhibitor batimastat improved lung resilience, and interestingly, this was not associated with an increase in bacterial clearance. Furthermore, we showed that IAV post-transcriptionally inhibited the antimicrobial/anti-protease molecule elafin/trappin-2, which we have shown previously to be anti-inflammatory in a variety of settings (27–29) and protects the host against maladaptive neutrophilic inflammation in *P.a* (27, 28, 30) infections, as well as against Plasmodium-mediated lung damage (29).

Altogether, out work highlights the capacity of IAV to promote further PAO1-mediated lung damage, not through its interference with host resistance to the bacterium (16, 19), but through down-regulating tissue resilience to lung inflammation instead. Our study therefore suggests that restoring tissue resilience in clinical settings where IAV/*P.a* co-exist could be a fruitful strategy.

MATERIALS AND METHODS

Materials

Phosphoramidon and batimastat were obtained from Sigma-Aldrich. Recombinant human and murine interleukin 1β were purchased from R&D Systems. Tace II metalloprotease substrate was obtained from Enzolife Science. Neutrophil elastase (NE) was obtained from Elastin products.

PAO1 and *Influenza A* Preparation

PAO1 WT (obtained from the ATCC; 15692), was grown overnight in Luria Broth (LB) medium (1% Bactotryptone, 0.5% Bacto Yeast Extract, 0.5% NaCl) under agitation. On the next day, an aliquot of PAO1 culture was grown over 3–4 h in an exponential phase and the OD was checked (600 nm). Bacteria were then centrifuged (4,000 rpm for 15 min) and pellets resuspended at the desired multiplicity of infection (moi) or colony forming units (cfu) in PBS.

The virus strain A/Scotland/20/74 (H3N2) was routinely amplified in MDCK cells (ATCC CCL-34), as described before Barbier et al. (31) and Villeret et al. (32). From the supernatants of infected cells, the *influenza* virus was purified by centrifugation in sucrose gradient, quantified by the virus plaque assay (32), aliquoted, and kept at −80°C until use.

When needed, purified samples were inactivated by heating at 95°C for 10 min in a block heater. Inability of heat-inactivated *influenza* virus (IAV*) to replicate was then confirmed by viral gene M2 q-PCR analysis (see below).

Adenovirus Constructs

The replication-deficient adenovirus (Ad) Ad-MCMV-elafin is described in Sallenave et al. (33).

Cells, Cell Cultures, and Protocols

NCI-H292 cells (ATCC reference number CRL-1848), a human pulmonary mucoepidermoid carcinoma cell line, BEAS-2B cells (ATCC CRL-9609), a SV-40 transformed bronchial epithelial cell line, and A549 (ATCC CCL-185) a cell line from a lung adenocarcinoma, were cultured in RPMI (NCI-H292) or F12/K Nutrient mixture (BEAS-2B and A549) medium supplemented with Glutamax, antibiotics, and 10% de-complemented fetal calf serum (all reagents from Gibco). Cells were incubated at 37°C in a water-jacketed CO_2 incubator. Cells were infected in serum-free medium with either IAV or PAO1. Alternatively, they were stimulated with either h-IL-1β, 5′ triphosphate double stranded RNA (5′ ppp dsRNA at 1.2 μg/ml) (Invivogen), complexed to lipofectamine 2000 (Invitrogen), with polyinosinic-polycytidylic acid (poly IC at 10 μg/ml) (Invivogen), or with combinations thereof.

Cell viability was assessed by measuring Lactate dehydrogenase (LDH) activity in cell lysates and supernatants, using the CytoTox 96 Nonradioactive Cytotoxicity assay (Promega).

Cells were washed twice with ice-cold PBS and lysed in TrisHCl 50 mM, NaCl 150 mM, NP40 1%, Glycerol 3%, EDTA 2 mM, and EGTA 2 mM buffer. After centrifugation (14,000 rpm, 15 min, 4°C) pellets were discarded. Cell supernatants and lysates were then recovered and stored at −80°C until further analysis.

In vivo Experiments

Procedures involving mice were approved by our Ethical Committee (Paris-Nord/No 121) and by the French ministry of Research (agreement numbers 4537.03 and 02012). Eight-week-old male C57Bl/6 mice and human elafin/trappin-2 transgenic mice (hereafter called eTg mice) were obtained from Janvier (Le Genest-Saint-Isle, France) and generated by our group (34), respectively. Mice were anesthetized using an intramuscular injection of ketamine 500 and xylazine 2% in 0.9% NaCl (20:10:70). Either the *Influenza A* virus (IAV), PAO1 bacteria, or m-IL-1ß recombinant protein were given intra-nasally (i.n) or through the oro-pharyngeal route, in a final volume of 40 µl instilled through a fine polypropylene tubing. Mice were then monitored for survival or were humanely killed (overdose of 100 µl intra-peritoneally-injected pentobarbital) for mechanistic studies. For the latter, bronchoalveolar lavages (BALs) fluid was obtained by cannulating the trachea and instilling 2 × 1 ml of PBS. Typically, a volume of 1.7 ml of BALF was retrieved and centrifuged at 2,000 rpm for 10 min. Supernatants were used for protein, cytokine/chemokine (ELISA), protease activity, and hemoglobin, as a surrogate for lung damage (absorbance reading at 405 nm) measurements. BAL cell pellets were used to perform cytospins for cell differential analysis (Diff-Quick, Dade Diagnostika GmbH, Unterschleissheim, Germany).

Lung tissues were used for RNA quantification, for assessment of bacterial count, after plating extracts on agarose plates, or for histological studies.

Cytokines/Chemokines/Antimicrobials Measurement

The concentration of mediators in cell cultures supernatants/lysates or murine BALs were quantified by sandwich ELISA kits following the manufacturer's indications (R&D Bio-Techne, Minneapolis, MN) or used in our in house ELISA (33).

BAL Protease Activities

BAL metalloprotease, trypsin-like, and neutrophil-elastase activities were measured using fluorogenic substrates, as

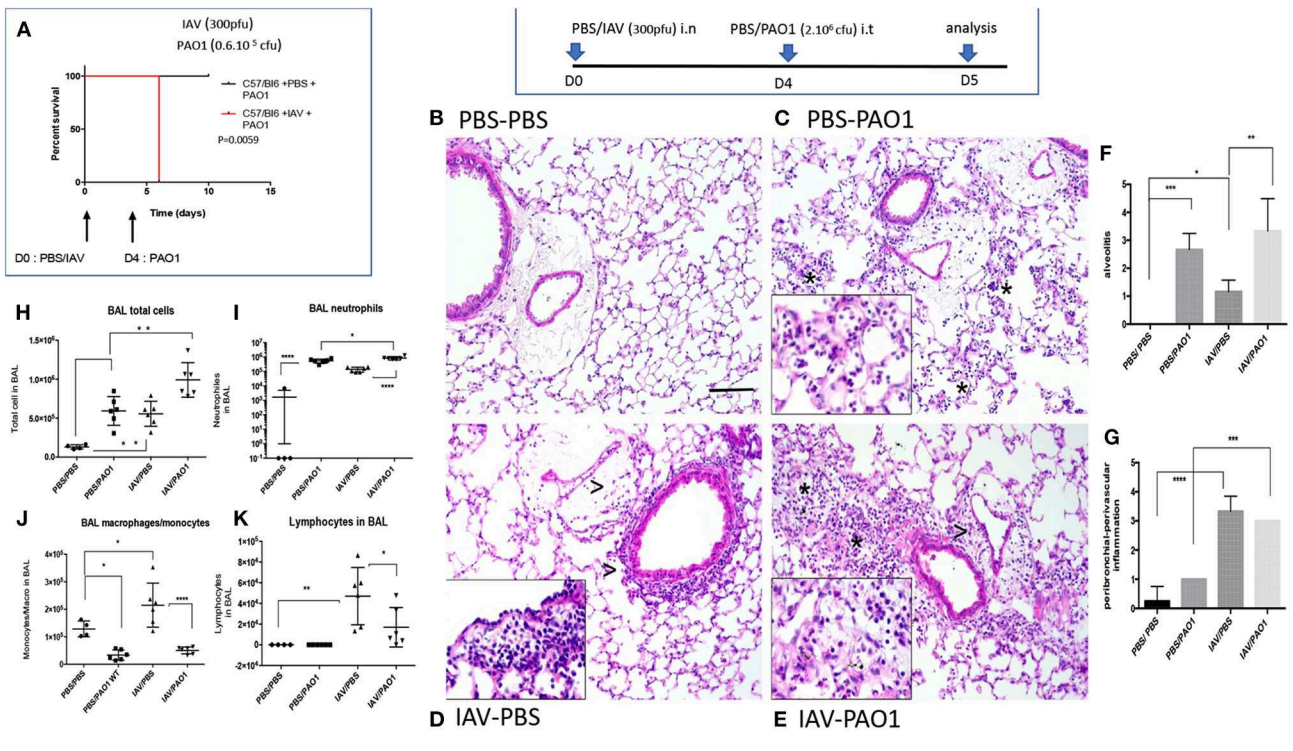

FIGURE 1 | IAV lung pre-infection sensitizes WT C57Bl/6 mice to further PAO1-mediated inflammation. **(A)** Survival experiment: C57Bl/6 WT mice were instilled i.n with either PBS or IAV (300 pfu) (two cohorts of six animals for each treatment). Four days later, mice were further instilled i.t with PAO1 ($0.6.10^6$ cfu), and mice survival was assessed, using Kaplan-Meier curves. Statistical tests were performed with the GraphPad Prism 6 package, using the Log-rank (Mantel-Cox) test, $p = 0.0059$. **(B–K)** Mechanistic experiment: at D0, C57Bl/6 WT mice were instilled i.n with either PBS or IAV (300 pfu). Four days later, mice were further instilled i.t with either PBS or PAO1 (2.10^6 cfu). At day 5, mice were sacrificed, and a bronchoalveolar lavage (BAL) was performed for cytospin cellular quantification **(H–K)**. Lungs were also obtained, inflated, cut and used for histological observation **(B–E)** and inflammation scoring **(F–G)**. NB: The three animals used for histology for the PBS/PAO1 and IAV/PAO1 groups all scored similarly, respectively 1 ($n = 3$) and 3 ($n = 3$). There are therefore no error bars. **(B–E)** (PBS/PBS; PBS/PAO1; IAV/PBS; IAV/PAO1, respectively): inflammatory lesions of treated lungs. Representative images of each condition on H&E slides at x20 original magnification. Scale bar: 200 µm. Stars indicate neutrophilic alveolar influx; arrow heads indicate perivascular and peribronchial mononuclear cell inflammation. Inserts are x40 magnification of cellular influx (mostly neutrophils in PBS-PAO1, mononuclear cells in IAV-PBS and a mix in IAV-PAO1). Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

described in Barbier et al. (31) Bastaert et al. (35), and Le Gars et al. (36), respectively. Alternatively, BAL MMP activity was measured by zymography (30).

RNA Preparation

Cell monolayers were directly lysed in RNA lysis buffer. RNA isolation was performed using the PureLink® RNA Mini Kit (12183018A, Ambion, Life technologies), following the manufacturer's instructions. For lung RNA assessment, frozen lungs were homogenized in RNA lysis buffer provided by the Pure Link RNA extraction kit (Life Technologies), using lysing matrix D tubes and the FastPrep-24 5G mixer (MP Biomedical) at 4°C (two cycles of 40s, level 5). Briefly, lysates were mixed with 70% ethanol and loaded onto a silica-membrane column. After different washings, total RNA was eluted in DNAse-RNAse-free water and stored at −80°C until use.

DNase treatment was performed prior to Reverse transcription polymerase chain reaction (RT-PCR) using RNAse-free DNAse I (Roche) at 37°C for 10 min. DNAse was then inactivated by increasing the temperature to 70°C for 10 min. Complementary DNA (cDNA) was synthesized from total RNA (500 ng) using M-MLV Reverse Transcriptase (Promega) as per the supplier's protocol (1 h at 37°C followed by 1 min at 70°C).

Real-time PCR was done in a 7500 Fast Real-Time PCR System (Applied Biosystems). Reactions were performed in a total volume of 15 µl using 2x Fast SYBR® Green Master Mix (Life Technologies), 2 µl of diluted cDNA, 2 µmol forward primer, and 2 µmol reverse primer in a 96-well plate. PCR was run with the standard program: 95°C 10 min, 40 times of cycling 95°C 15 s and 60°C 1 min in a 96-well plate. Triplicate Ct values were obtained, and results were expressed as dCT = CT gene of interest-CT HPRT/18S (with low and high values representing high and low levels of the gene of interest, respectively). Alternatively, the results were expressed using the comparative Ct (ΔΔCt) calculation and the following formula: Fold change $(RQ) = 2^{-(\Delta\Delta CT)}$, using "control cells" as calibrator (arbitrary unit =1).

The primers used were the following: M2 viral protein: (Fw: aagaccaatcctgtcacctct; Rw: caaagcgtctacgctgcagtc); CCL-5: (Fw: cagtcgtctttgtcacccgaa; Rw: tcccaagctaggacaagagca); IL-8 (Fw: agagacagcagagcacacaa; Rw: ttagcactccttggcaaaac); IL-6 (Fw: tcaatgaggagacttgcctg; Rw: tgtactcatctgcacagcctc); HPRT (Fw: ttgctttccttggtcaggca; Rw: atccaacacttcgtggggtc); 18s rRNA (Fw: cttagagggacaagtggcg; Rw: acgctgagccagtcagtgta).

Histology

Perfused and fixed lungs (4% PFA in PBS, overnight at 4°C) were embedded in paraffin and sectioned in slides (4 µm), stained

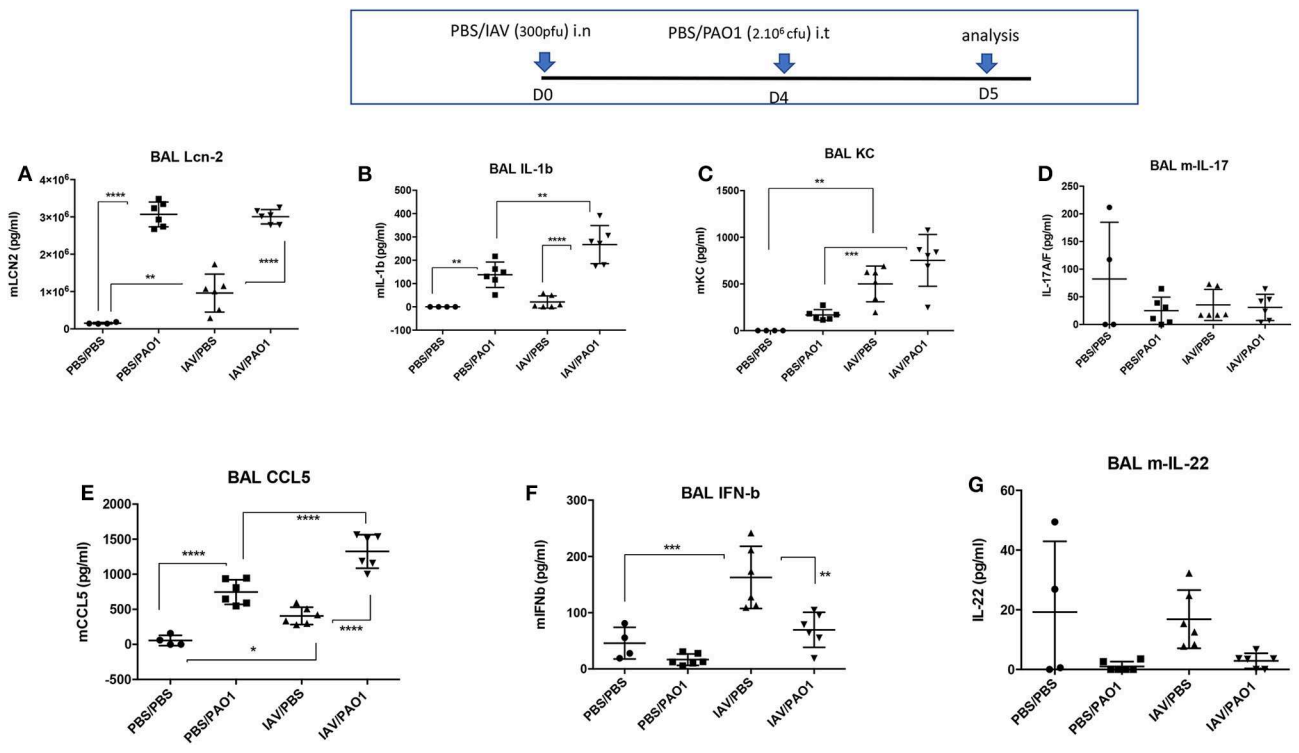

FIGURE 2 | IAV lung pre-infection sensitizes WT C57Bl/6 mice to further PAO1-mediated cytokine production. BAL supernatants (see **Figure 1** legend) were further used for assessment of cytokines and mediators. Each symbol represent an individual mouse. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. **(A)** Lcn-2 levels, **(B)** IL-1b levels, **(C)** KC levels, **(D)** IL-17 levels, **(E)** CCL5 levels, **(F)** IFN-b levels, **(G)** IL-22 levels.

with Hematoxylin-eosin. Inflammation was scored with a semi-quantitative scale (0: no inflammation to 4: severe inflammation with exudate) both in alveolar and in peribronchial/interstitial lung compartments.

Statistical Analysis

Data were expressed as means ± standard errors of the mean (SEM) unless otherwise stated. One-way ANOVA was used to determine statistically significant differences among groups followed by Tukey's multiple test for comparisons. Survival curves in murine model experiments were plotted using Kaplan-Meier curves and statistical tests were performed using the Log-rank (Mantel-Cox) test. All analyses were performed with Prism version 7, GraphPad.

RESULTS

IAV Pre-Infection Exacerbates *P. aeruginosa* Inflammation in C57Bl/6 Murine Lungs

In survival experiments, neither IAV (not shown) nor PAO1 alone (**Figure 1A**), induced any fatalities of C57Bl/6 mice, at the doses used. In contrast, IAV pre-treatment followed by PAO1 infection induced the death of all animals (**Figure 1A**).

In mechanistic experiments, C57Bl/6 mice infected with IAV (**Figures 1D,F,G**) or PAO1 (**Figures 1C,F,G**) exhibited increased lung tissue inflammation, compared to PBS mock-treated animals (**Figures 1B,F,G**). Neutrophils were the overwhelming cell type present after "PAO1 alone" infection, mainly in the alveoli (**Figures 1C,F**). By contrast, lymphocytes and monocytes (even though neutrophils were also present) were predominant in "IAV-alone"-infected animals and were mostly located in perivascular/peribronchial areas (**Figures 1D,G**).

Sequential IAV and PAO1 infections gave rise to increased neutrophilia, compared to "PAO1 alone" treatment (**Figures 1E–G**), especially in the peribronchial/perivascular areas, even though, notably, neither IL-17 nor IL-22 levels were increased over controls (**Figures 2D–G**).

This tissue inflammation was mirrored in BALs (**Figures 1H–K**), showing increased cytokine and inflammatory mediators in infected animals (IL-1β, KC, CCL-5, Lcn2, **Figures 2A–C,E,F**). IL-1β levels were only significantly increased following "PAO1-alone" and after "IAV+PAO1" infections (**Figure 2B**). With the notable exception of IFN-β, which was reduced, when compared to IAV alone (**Figure 2F**), and IL-17 and IL-22, which were not increased (**Figures 2D,G**), all inflammatory parameters

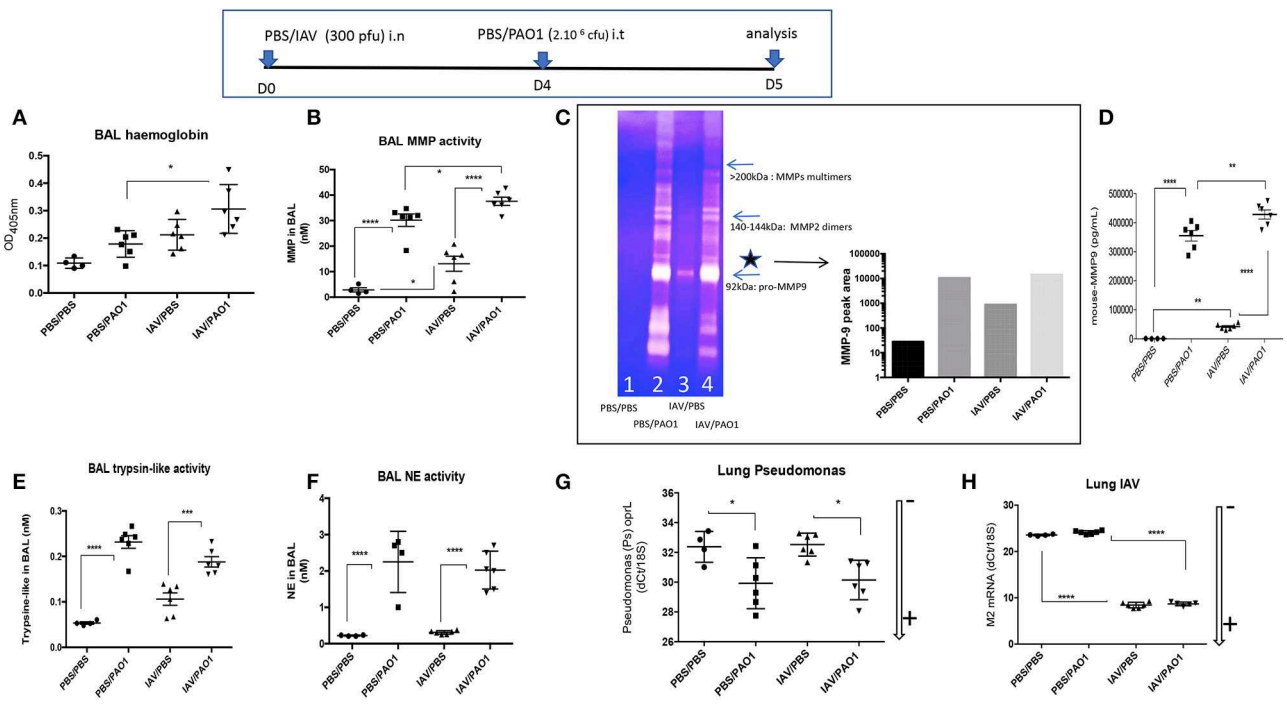

FIGURE 3 | IAV lung pre-infection sensitizes WT C57Bl/6 mice to further PAO1-mediated metalloprotease activity and lung injury. BAL supernatants (see **Figure 1** legend) was further used for hemoglobin content (absorbance at 405 nm, **A**) and protease activity **(B–H)**. **(B)** BAL metalloprotease bioactivity was assessed with the synthetic substrate 5-FAM-Ser-Pro-Leu-Ala-Gln-Ala-ValArg-Ser-Ser-Ser-Arg-Lys(5-TAMRA)-NH2 (35). **(C)** BAL metalloprotease bioactivity was also assessed by zymography. BALs from individual mice within the same experimental group (1: PBS/PBS; 2: PBS/PAO1; 3: IAV/PBS; 4: IAV/PAO1) were pooled and Pro-MMP-9 (*, left panel) intensity was assessed by densitometry (right panel). **(D)** BAL MMP-9 levels were measured by ELISA. **(E)** BAL trypsin-like activity was assessed with the synthetic substrate Boc-Phe-Ser-Arg-7-amido-4-méthylcoumarin (37). **(F)** BAL NE bioactivity was assessed with the synthetic substrate MeOSuc-Ala-Ala-Pro-Val-AMC (27). Lung extracts were also used for RNA preparation and RT-PCR analysis of M2 and oprL genes [as read-outs for IAV and PAO1 loads, respectively **(G–H)**]. Low and high RNA content is marked with an arrow indicating low (–) or high (+) level of expression. Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

were increased in IAV + PAO1-treated animals, compared to IAV alone. Compared to "PAO1" alone, IL-1β, KC and CCL-5 were significantly increased in the "IAV + PAO1" treatment.

Inflammation resulted in lung injury, demonstrated by increased BAL hemoglobin levels, and again IAV pre-treatment potentiated the latter (**Figure 3A**). Potentially explaining this, BALF metalloprotease activity (MMP), as measured with a synthetic substrate, was increased in IAV-alone- and PAO1-alone-treated mice (**Figure 3B**). This activity was again potentiated, when IAV preceded PAO1 treatment. Using zymography, we further showed that MMP-9 was present and was clearly the most abundant MMP in BAL of IAV-infected animals (*, 3C), which was confirmed by ELISA (3D). Importantly, and specifically, IAV/PAO1 induced more MMP-9 than PAO1 alone (3D).

Other protease activities were also present in BAL, albeit at much lower concentrations: trypsin-like activity was mostly increased in PAO1-alone and IAV+PAO1 arms of the experiment (**Figure 3E**), compared to MMP levels.

NE BALF activity was also increased in PAO1-alone and in IAV+PAO1-treated mice, but very poorly in "IAV-alone" mice (**Figure 3F**).

Notably, neither IAV nor PAO1 influenced each other's infection, using M2 and oprL genes as a read-out for IAV and PAO1 loads, respectively (**Figures 3G,H**), suggesting that dysregulated direct antimicrobial activity was not a major player here.

Epithelial Cell Modeling of IAV/PAO1/IL-1b Stimulations

Because epithelial cells are the main IAV targets, the interactions studied above *in vivo* were then modeled *in vitro* in these cells. After a comparative study in NCI-H292, BEAS-2B, and A549 lung cells, we found (not shown) that A549 cells were most responsive to IAV-, IAV PAMPs-, PAO1-, and IL-1ß-mediated infection/stimulation, and these cells were therefore further studied below:

(a) IAV differentially regulate inflammatory/anti-viral and antimicrobial mediators in A549 cells.

We either infected A549 cells with IAV (moi 1) or stimulated them with either poly IC, 5′ ppp ds RNA, synthetic ligands for TLR-3, RIG-I and MDA-5, respectively, or with IL-1ß, a sterile inflammatory stimulus. We then measured the RNA and protein levels of a variety of mediators (cytokines, antimicrobial/anti-inflammatory molecules), all relevant to the *in vivo* model described in **Figures 1–3**.

At the RNA level (**Figure 4**: NB: ΔdCT values are inversely proportional to RNA levels, and arrows indicate gene expression +/−), we showed that IL-8 was clearly induced by IAV and IL-1ß (ΔdCT of −4 and −7.5 respectively, **Figure 4B**), but modestly by

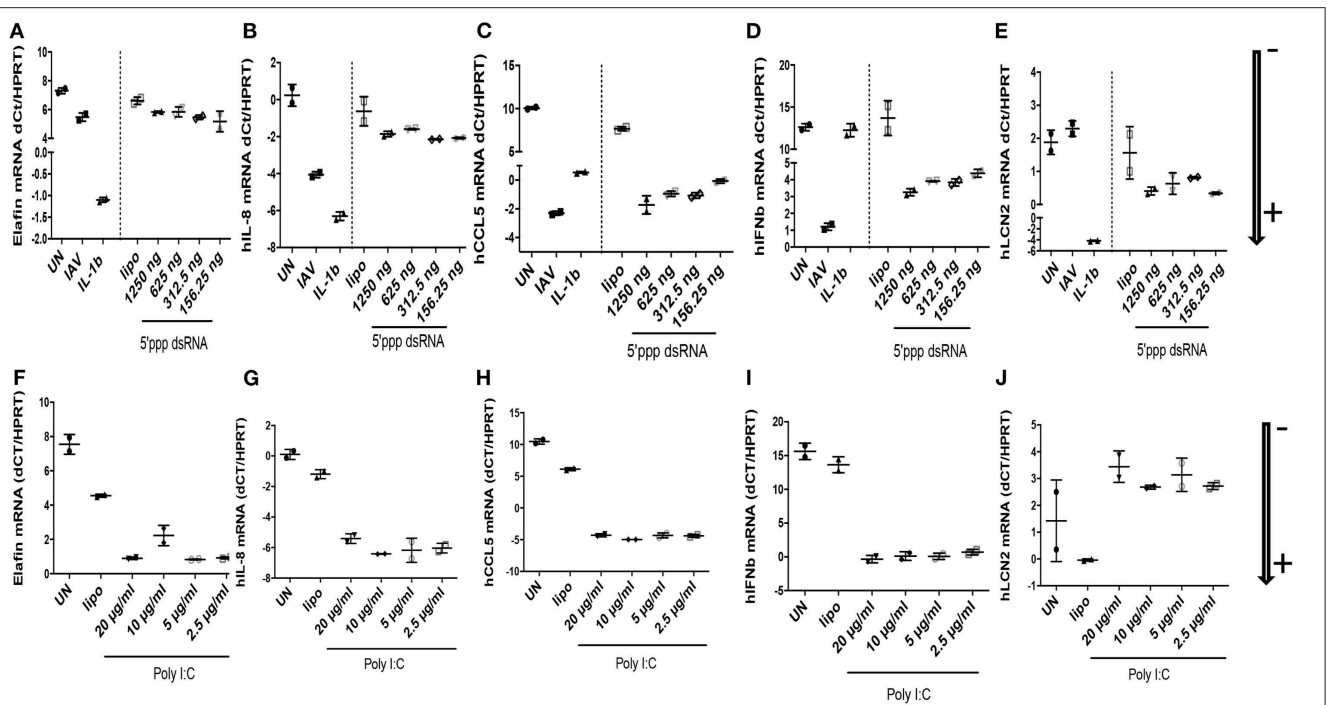

FIGURE 4 | IAV-, polyIC-, 5′ppp dsRNA-, and IL-1β- mediated induction of elafin, IL-8, CCL5, IFN- β, Lcn-2 RNA in A549 cells. At D0, A549 cells (24 wells plates, 70% confluence) were either left unstimulated (UN), infected with IAV (moi 1), or stimulated with either IL-1β (10 ng/ml), lipofectamine control (CLT lipo), 5′ ppp dsRNA (Invitrogen) or poly IC (Invitrogen), at various concentrations during 4 h at 37°C in OPTI-MEM medium. Cells were then washed, medium replenished, and 16 h later, cell lysates were recovered for further analysis of RNA **(A–J)**, respectively, for elafin, IL-8, CCL5, IFN- β, Lcn-2. q-PCR analysis was performed, and results expressed as dCT (CT gene of interest-CT HPRT), with low and high values representing high and low levels of the gene of interest, respectively (marked with an arrow indicating low (–) or high (+) level of expression). Two independent experiments (individual symbols) were performed. Results are shown as means ± SD.

5′ ppp dsRNA (ΔdCT of −1.0, **Figure 4B**) and at intermediate levels by poly-IC (ΔdCT of −4, **Figure 4G**). By contrast, CCL-5 RNA was robustly induced by all stimuli (ΔdCT of respectively −12, −10, −9.5, and −10, **Figures 4C,H**). Lcn-2 RNA was also strongly up-regulated by IL-1ß (ΔdCT of −7, **Figure 4E**), modestly by 5′ ppp dsRNA (**Figure 4E**) and even inhibited by poly IC (**Figure 4J**).

IFN-ß RNA stood alone since it was not induced by IL-1ß (**Figure 4D**), but was, as expected, strongly up-regulated by IAV, 5′ ppp dsRNA, and polyIC (ΔdCT of −11.5, −8, and −15, respectively, **Figures 4D,I**). When elafin, an antimicrobial molecule with anti-inflammatory/anti-NF-kb activity (27–30) was considered, IL-1ß was again clearly the greatest inducer of elafin mRNA (ΔdCT of −8, **Figure 4A**), with polyIC and IAV being strong and intermediate inducers (ΔdCT of −6.5 and −2, **Figures 4A,F**), respectively.

When protein levels were studied (**Figure 5**), we observed that IAV had important post-transcriptional regulatory activities: IAV clearly up-regulated IL-8, CCL-5, IFN-ß proteins (**Figures 5B–D**, respectively), in keeping with increased RNA levels, while having no effect on elafin and Lcn2 proteins (**Figures 5A,E**, respectively). Poly IC and 5′ ppp dsRNA effects were even more contrasted: poly IC slightly down-regulated the accumulation of IL-8 (**Figure 5G**) and drastically down-regulated that of elafin and Lcn-2 (**Figures 5F,J**) proteins, while very robustly inducing that of CCL-5 (**Figure 5H**) and IFN-ß (**Figure 5I**). Similarly, 5′ ppp dsRNA also sharply induced CCL-5 and IFN-ß protein accumulation (**Figures 5C,D**, respectively), in keeping with its effect on RNA, but had virtually no effect on elafin (**Figure 5A**), IL-8 (**Figure 5B**), or Lcn2 (**Figure 5E**) protein accumulation. These results demonstrated that IAV had specific down-regulatory post transcriptional activity on elafin and Lcn-2, and that its effect is mimicked by its nucleic acid analogs, either polyIC or 5′ppp dsRNA, suggesting an intra-cellular mode of action:

(b) IAV, but not PAO1, post-transcriptionally down-regulates elafin expression in A549 cells.

We then demonstrated in further *in vitro* independent experiments that the post-transcriptional regulation observed in **Figures 4**, **5**, in which IAV induced RNA, but not protein levels of elafin, was indeed "IAV-specific," since PAO1 up-regulated both RNA and protein levels of elafin and IL-8 instead (see **Figures S1G,H,J,K** and the **Supplementary Materials**).

(c) IAV exacerbates IL-1ß- and PAO1-mediated inflammatory responses and down-regulates elafin and Lcn-2 accumulation in A549 cells.

Having shown *in vivo* that IAV could exacerbate PAO1 responses (**Figures 1–3**) and that, studied individually, IAV alone (but not PAO1) could inhibit *in vitro* elafin and Lcn-2 protein production specifically (**Figure 5** and **Figure S1**), we set up a "multi-hit inflammatory model" in A549 cells, where combinations of "mixes" were studied together (**Figures 6**, **7**), similarly to the *in*

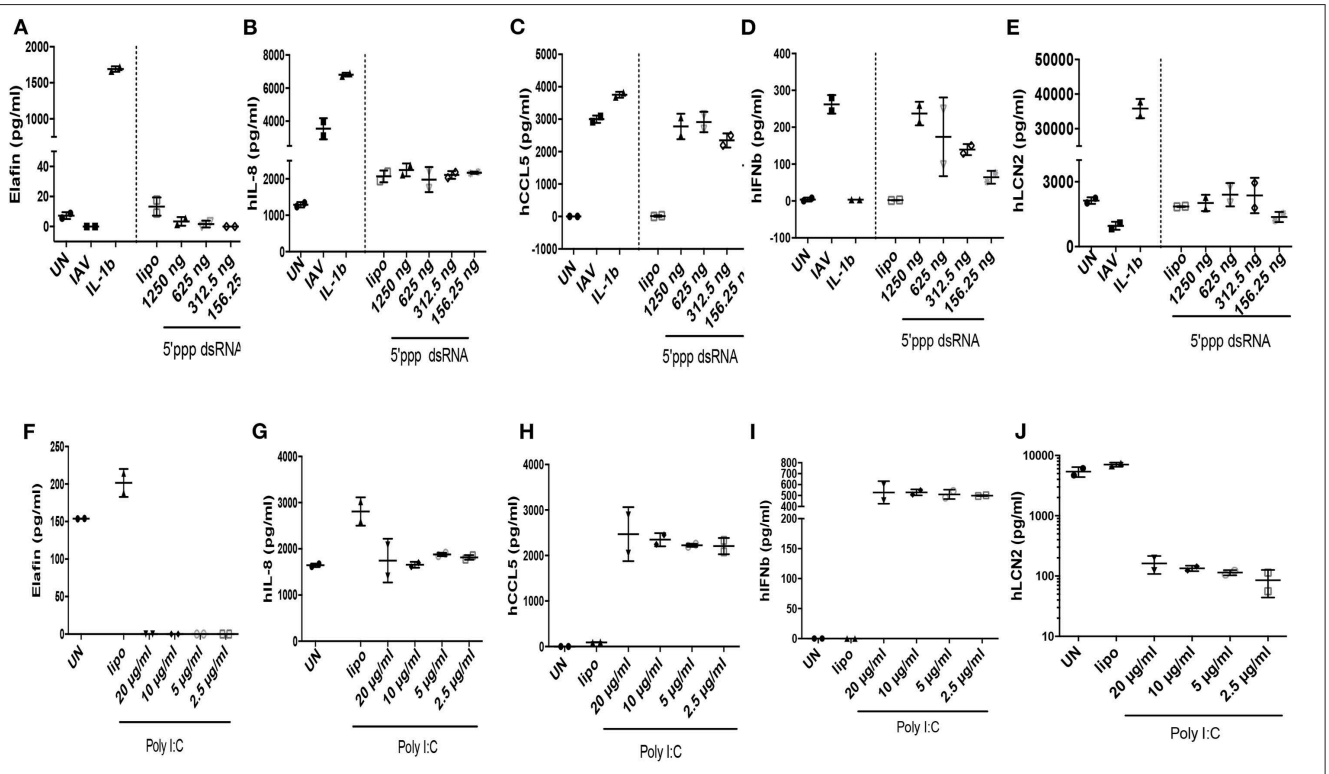

FIGURE 5 | IAV-, polyIC-, 5'ppp dsRNA- and IL-1β- mediated induction of elafin, IL-8, CCL5, IFN- β, Lcn-2 protein in A549 cells. The supernatants obtained from the experiments depicted in **Figure 4** were recovered and used for protein measurement (ELISA) of elafin, IL-8, CCL5, IFN- β, Lcn-2 **(A–J)**.

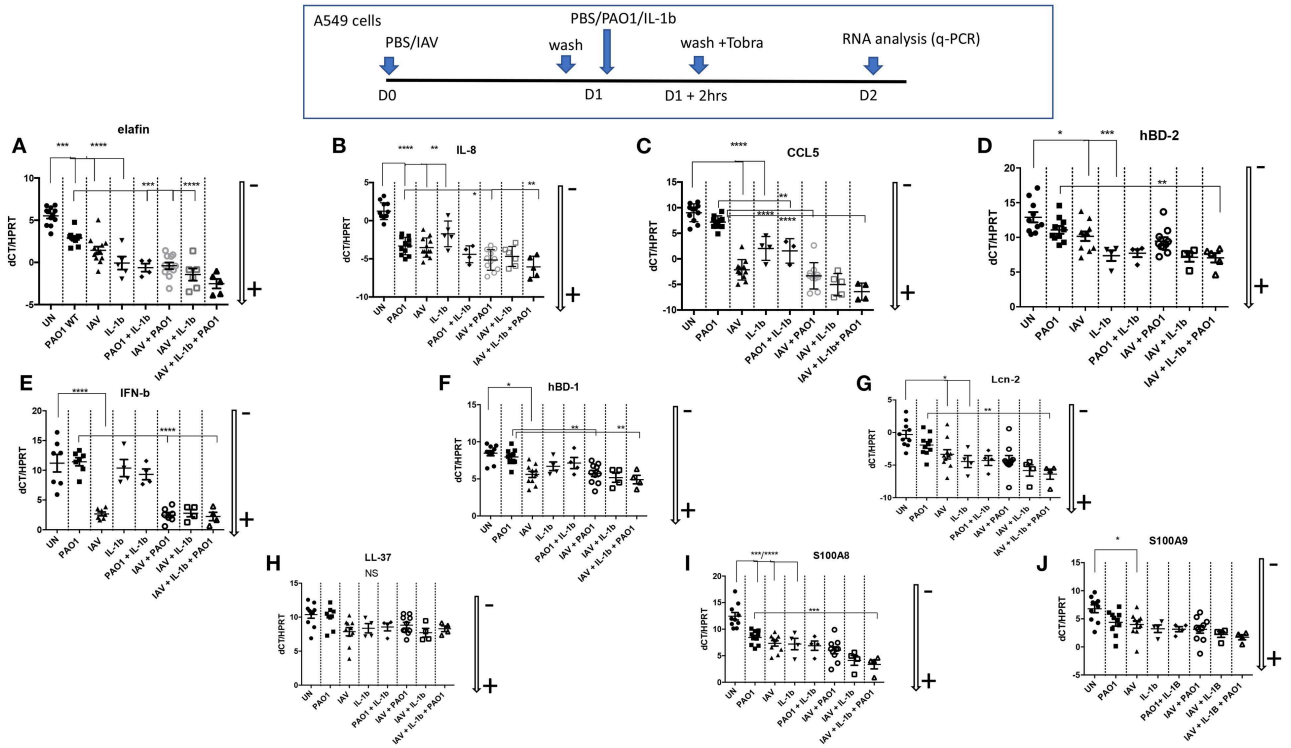

FIGURE 6 | Epithelial cell regulation of cytokines/chemokines and antimicrobials transcription in IAV/PAO1/IL-1β co-infection models. At D0, A549 cells (24 wells plates) were either uninfected or infected with IAV (moi 1) in serum-free medium for 1 h. After washing, and o/n incubation, cells were either replenished with fresh medium and left untreated (UN), or treated with either PAO1 alone (moi 1), IL-1β alone (10 ng/ml), or IL-1β + PAO1 (at the same doses). After a further 2 h, cells were washed with F12K medium containing tobramycin (40 μg/ml), to remove PAO1 load, and left for 24 h in medium. At the end of the incubation, at D2, supernatants and cell lysates were recovered, and cytokines/chemokines/antimicrobials were assessed for RNA (this figure) and protein levels (ELISAs, **Figure 7**). For RNA analysis, gene expression is marked with an arrow indicating low (–) or high (+) level of expression (see **Figure 4** for the full explanation). Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. **(A)** Elafin RNA, **(B)** IL-8 RNA, **(C)** CCL5 RNA, (D) hBD-2 RNA, **(E)** IFN-b RNA, **(F)** hBD-1 RNA, **(G)** Lcn-2 RNA, **(H)** LL-37 RNA, **(I)** S100 A8 RNA, **(J)** S100A9 RNA.

vivo protocol. In addition to IAV and PAO1, IL-1ß was added in this model, as an important "first wave" cytokine up-regulated *in vivo* (**Figure 2**).

Echoing these *in vivo* data, we showed that all these agents up-regulated the transcription of many inflammatory/antimicrobial molecules in A549 cells, as evidenced by a reduction in dCT levels (**Figure 6**). Notably, compared to "PAO1 alone," pre-infection with IAV followed by PAO1 infection further increased IL-8 (**Figure 6B**), CCL-5 (**Figure 6C**), hBD-1 (**Figure 6F**), and elafin (**Figure 6A**) mRNA levels.

IFN-ß inductions stood out as notable exceptions (**Figure 6E**), where only IAV-containing "mixes" were effective agonists, and LL-37 (**Figure 6H**), whose expression was relatively stable, in keeping with the described relative constitutiveness of this antimicrobial.

When protein levels were assessed in supernatants (**Figure 7**), IAV again demonstrated post-transcriptional regulation on elafin and Lcn-2; indeed, there was a trend for decreased elafin and Lcn-2 production in IAV + PAO1 treatments, compared to PAO1 alone, and IAV had a clear down-regulating effect on these mediators when "IAV+ IL-1β" and "IAV+ PAO1 + IL-1β" were compared to "IL-1β" and "IAV+ PAO1," respectively (**Figures 7A,B**). Again, this regulatory effect was specific since, as demonstrated above, IAV clearly up-regulated IL-8 and CCL-5 proteins, compared to untreated cells, and did either not change for IL-8 (**Figure 7C**) or even increased for CCL-5 (**Figure 7D**) protein production, when cells were infected with IAV and further treated with PAO1 and/or IL-1β.

To assess whether the IAV regulatory effect was acting intra- or extra-cellularly, the levels of the same mediators were measured in A549 lysates (instead of in supernatants). We found that, mirroring the effects observed in A549 supernatants, IAV again down-regulated the IL-1β-mediated intracellular accumulation of elafin and Lcn2 (**Figures 8A,B**), but not those of IL-8 and CCL-5 (**Figures 8C,D**).

In addition, we also studied the IAV regulation of an exogenously added Adenovirus-h-elafin construct in A549 cells (**Figures 8E–G**) and found that IAV also down-regulated elafin accumulation in both supernatants and lysates (**Figures 8E,F**).

Importantly, this IAV-mediated down-regulation was not due to an interference of IAV with Ad infection, since IAV

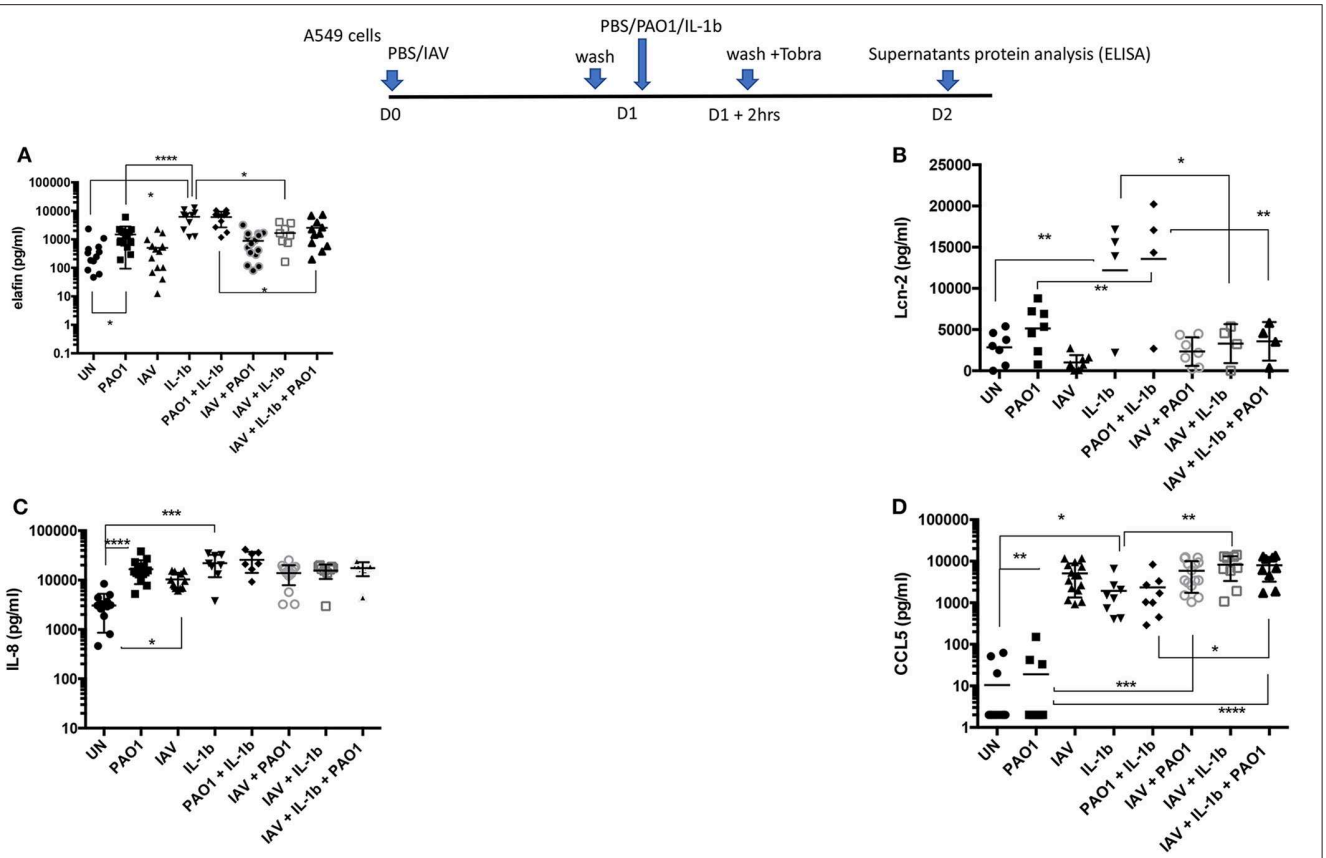

FIGURE 7 | Cytokines/chemokines and antimicrobials protein production in IAV/PAO1 and IAV/ IL-1β co-infection models. Elafin, IL-8, CCL5, and Lcn-2 protein content was assessed by ELISA in the supernatants from the experiment described in **Figure 6**. Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. **(A)** Elafin levels, **(B)** Lcn-2 levels, **(C)** IL-8 levels, **(D)** CCL5 levels.

+ Ad-h-elafin- RNA levels were even higher than in the Ad-elafin "alone" condition, as demonstrated with lower dCT levels; showing that the Ad vector efficiently delivered its genetic cargo (**Figure 8G**).

IAV Pre-Infection Down-Regulate Elafin Expression *in vivo* and Exacerbates *P. aeruginosa* Inflammation in Elafin-Over-Expressing Mice

Having demonstrated *in vitro* that IAV specifically inhibited elafin and Lcn-2 protein production in lung epithelial cells, two important antimicrobial/anti-inflammatory molecules (27–30, 38), we then tested whether this regulatory effect was also observed *in vivo*. Because C57Bl/6 WT mice are "natural KO" for elafin (34), this was tested in h-elafin over-expressers, using the Ad-h-elafin over-expressing system (33). Since elafin expression was only required in our protocol as a "read out target" for IAV, Ad-h-elafin was only given 16 h (at the same time as either PBS or PAO1) before animals were culled for analysis. Expectedly, given its anti-inflammatory nature as demonstrated previously (27–30), the PBS/Ad-elafin/PBS "Control" arm of the experiment did not induce any inflammation "per se," as assessed by a "typical" percentage of macrophages and neutrophils recovered in BALs, 99 and 1%, respectively, **Figures S2B–D**. Importantly, although IAV did not decrease either basal or PAO1-induced lung elafin RNA levels (**Figure 9A**), it drastically reduced elafin protein accumulation in BALs, akin to that observed *in vitro* (**Figure 9B**). In contrast, IAV up-regulated both Lcn-2 RNA and protein levels (**Figures 9C,D**) and did not significantly affect PAO1-mediated Lcn-2 protein up-regulation (**Figure 9D**), contrary to that observed *in vitro* in epithelial cells.

Irrespectively, infection of Ad-h-elafin-treated mice with either IAV or PAO1 exhibited increased inflammation, as assessed by BAL total inflammatory cells, neutrophilia (**Figures S2A–F**), increase in cytokine and antimicrobials levels (**Figures S2G–N**), protease activity (**Figures 9E–G,J**), and tissue injury (**Figure 9L**). This confirmed what was observed in WT C57Bl/6 mice (**Figures 1–3**), i.e., the exacerbated effect of IAV on PAO1 infection and the key involvement of metalloproteases, including MMP-9 (**Figures 9G,J**). Importantly again, as also demonstrated in WT C57Bl/6 mice (**Figure 3G**), neither IAV nor PAO1 influenced each other's infection (**Figures 9H,I**),

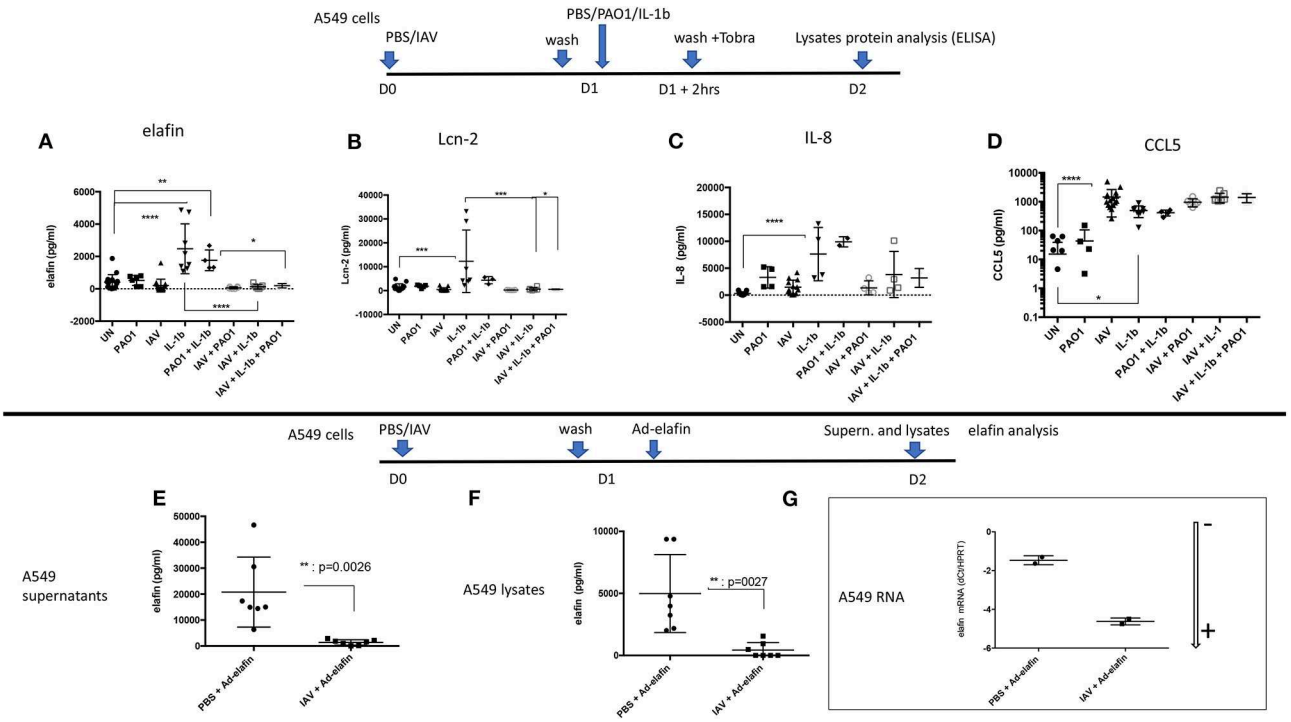

FIGURE 8 | IAV-mediated post-transcriptional regulation occurs intra-cellularly and in an Adenovirus-elafin reporter system in A549 cells. Top panel: A549 cells were treated as explained in the **Figure 6** legend. Cell lysates were recovered and protein levels for elafin, Lcn-2, IL-8, CCL-5 (**A–D**, respectively) were measured by ELISA. Bottom panel: A549 cells were mock-infected or infected with IAV (moi =1), as explained above. After 16 h, cells were further infected with replication-deficient Adenovirus-h-elafin (33). After a further 24 h, A549 supernatants and lysates were recovered and elafin protein levels measured by ELISA (**E,F** respectively). In a subset experiment, cell lysates were recovered for q-PCR assessment of elafin RNA content. The latter is marked with an arrow indicating low (–) or high (+) level of expression **(G)** (see **Figure 4** for full explanation). Results are shown as means ± SD. Statistical significance (except for **G**): ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).

reinforcing that dysregulated direct antimicrobial activity was not the major cause of IAV-induced inflammatory exacerbations. This was further strengthened by the use of the MMP inhibitor batimastat (see below, **Figure 10**). The latter was chosen because it does not inhibit LasB (39), quantitatively the most abundant PAO1 metalloprotease (30, 35), therefore allowing us to specifically address the effect of IAV on host (and not PAO1) metalloproteases. Importantly, because host proteases are known to be important for IAV replication (40, 41), and in order not to affect that cycle, batimastat was given "therapeutically" at D4 at the peak of IAV replication, at the same time as PAO1, and not "prophylactically" at the time of IAV infection (D0).

We showed that batimastat delayed the lethality of mice co-infected with IAV and PAO1 (**Figure 10A**), and down-regulated gelatinolytic activity, including MMP-9 expression, as shown by zymography (**Figure 10B**), and ELISA (**Figure 10C**). In addition, batimastat treatment down-regulated neutrophilic inflammation (**Figures 10D–G**) and tissue injury (**Figure 10L**), confirming that MMPs are indeed instrumental in the IAV-subversion of lung tissue resilience.

Interestingly, the beneficial effect of batimastat did not extend to rescuing elafin protein levels (**Figure 10I**), and even down-regulated Lcn-2 accumulation (**Figure 10J**), suggesting that proteolytic digestion of elafin by MMPs was not at play here, neither *in vivo* nor *in vitro* (not shown). Interestingly, batimastat was strikingly associated with a sharp increase in PAO1 load in lungs (**Figures 10M,O**), assessed by two independent methods, and in spleen (**Figure 10N**), demonstrating bacterial translocation into the periphery, and suggesting again a clear dissociation increase between tissue resilience and resistance to PAO1. In contrast, batimastat did not influence IAV load (**Figure 10P**). Some of the above results are described in more detail in the **Supplementary Material** (**Supplementary Material Results** and **Figure S2**).

DISCUSSION

Bacterial superinfections are an established risk of primary viral infections (e.g., rhinovirus, *Influenza* virus). Among the many potential mechanisms advocated, previous studies have suggested that IAV might promote further bacterial outgrowth by down-regulating neutrophilic function (14–17) or antimicrobial molecules (18–20). Notably, most

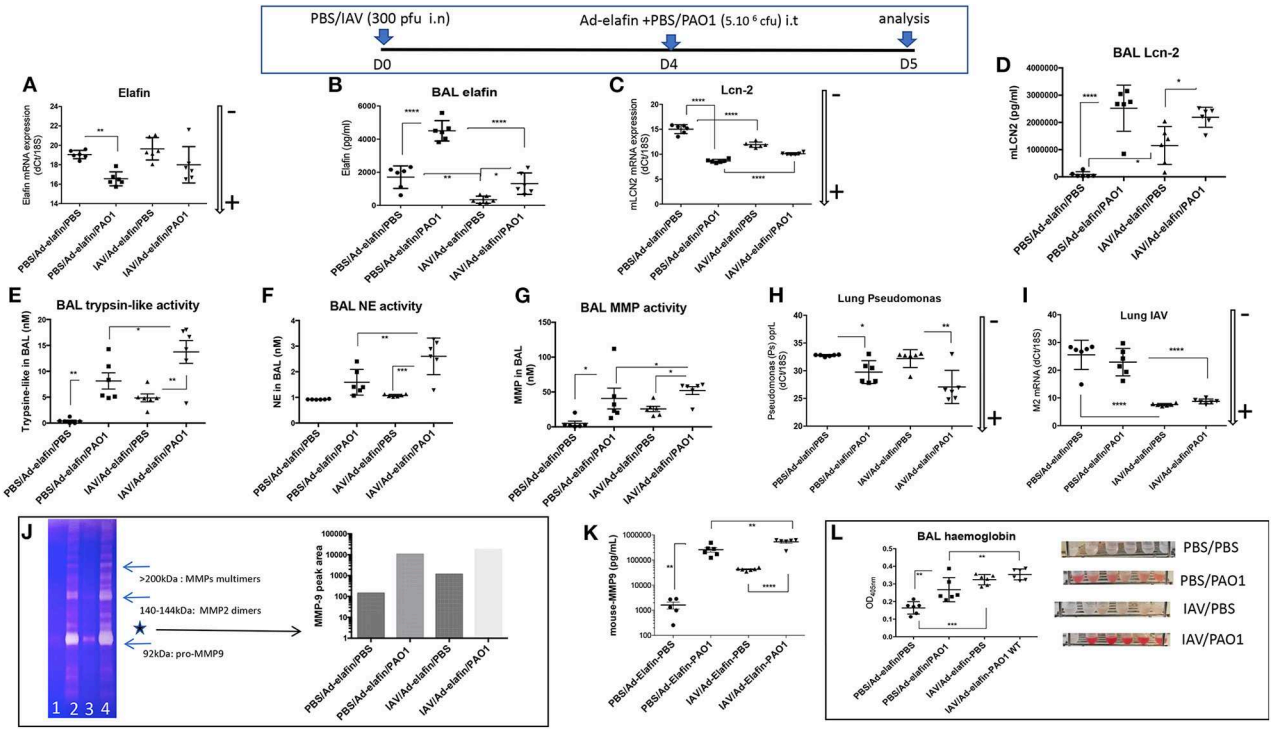

FIGURE 9 | IAV lung pre-infection sensitizes elafin over-expressing mice to further PAO1-mediated inflammation, metalloprotease activity, and lung injury. At D0, C57Bl/6 WT mice were instilled i.n with either PBS or IAV (300 pfu). Four days later, mice were further instilled i.t with Ad-elafin (3.10^6 pfu) plus either PBS or PAO1 (5.10^6 cfu). At day 5, mice were sacrificed, and a bronchoalveolar lavage (BAL) was performed. Lungs were also obtained, and RNA prepared for q-PCR assessment of elafin and Lcn-2 **(A,C)**. BAL was also used for elafin and Lcn-2 protein measurements **(B,D)**, and protease activity **(E–G)**, using synthetic substrates, or zymography **(J)**, as explained in the **Figure 3** legend. **(E)** BAL trypsin-like activity; **(F)** BAL NE bioactivity; **(G)** BAL MMP bioactivity; **(H)** Lung PAO1 load was assessed by q-PCR by measuring oprL expression; **(I)** Lung IAV load was similarly measured by q-PCR (M2 gene). As for other Figures, gene expression is marked with an arrow indicating low (–) or high (+) level of expression. **(J)** MMP bio activity was assessed by zymography: BALs from individual mice within the same experimental group (1: PBS/PBS; 2: PBS/PAO1; 3: IAV/PBS; 4: IAV/PAO1) were pooled and Pro-MMP-9 (*, left panel) intensity was assessed by densitometry (right panel). **(K)** BAL MMP-9 levels were measured by ELISA. **(L)** Hemoglobin levels were measured by absorbance at 413 nm. Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

authors have modeled their studies using *S. aureus* or *S. pneumoniae* as the secondary bacterial "hit," and despite its obvious clinical importance, no studies have, to our knowledge, comprehensively investigated mechanisms linking *Influenza* (IAV) and *P. aeruginosa* infections (16, 19). Using the latter combination, with the H3N2 and PAO1 strains, respectively, we show here in a variety of *in vitro* and *in vivo* models, that IAV dramatically down-regulates, at the post-transcriptional level, the antimicrobial/anti-inflammatory elafin/trappin-2 *in vitro* (**Figures 4–8** and **Figure S1**) and *in vivo* in PAO1- (**Figures 9**, **10** and **Figure S2**) and IL-1β-mediated models of inflammation (**Figures S3, S4** and **Supplementary Material Results**). Unfortunately, our attempts to determine whether other antimicrobial molecules might also be inhibited by IAV were thwarted by the previously reported unreliability of current available ELISA kits for antimicrobial molecules (not shown). An important exception was Lcn-2, which we also showed to be inhibited by IAV *in vitro* in epithelial cells (**Figures 4**, **5**, **7**), but not, unlike elafin, *in vivo* in mice lungs (**Figures 9**, **10** and **Figure S3**). Although the exact mechanism still remains obscure, IAV likely down-regulates, at least *in vitro*, an epithelial intra-cellular/cytosolic event, since elafin and Lcn2 intra-cellular protein levels were also drastically reduced (**Figure 8**).

Relatedly, in a previous study, Robinson et al. also showed that pre-infecting C57Bl/6 mice with PR8 H1N1 IAV down-regulated the antimicrobials Lcn2, RegIIIγ, and S100A8 mRNA levels upon further *S. aureus* infection, but the effect on protein levels was not reported, again likely because of the paucity of reliable ELISA kits (18). Relevantly, Mallia et al. showed that rhinovirus induced neutrophil elastase in COPD patients and suggests that the ensuing down-regulation of secretory leukocyte protease inhibitor and elafin is causative in triggering exacerbations in these patients (20). Relatedly, although no bacterial data were reported, in a transcriptomic study enrolling 1,610 individuals, 142 of which were followed for evaluation of acute viral respiratory illness, the elafin gene (PI3) was found to be the top downregulated gene in the acute phase of the *Influenza* infection, but not in the rhinovirus or other infection groups (42).

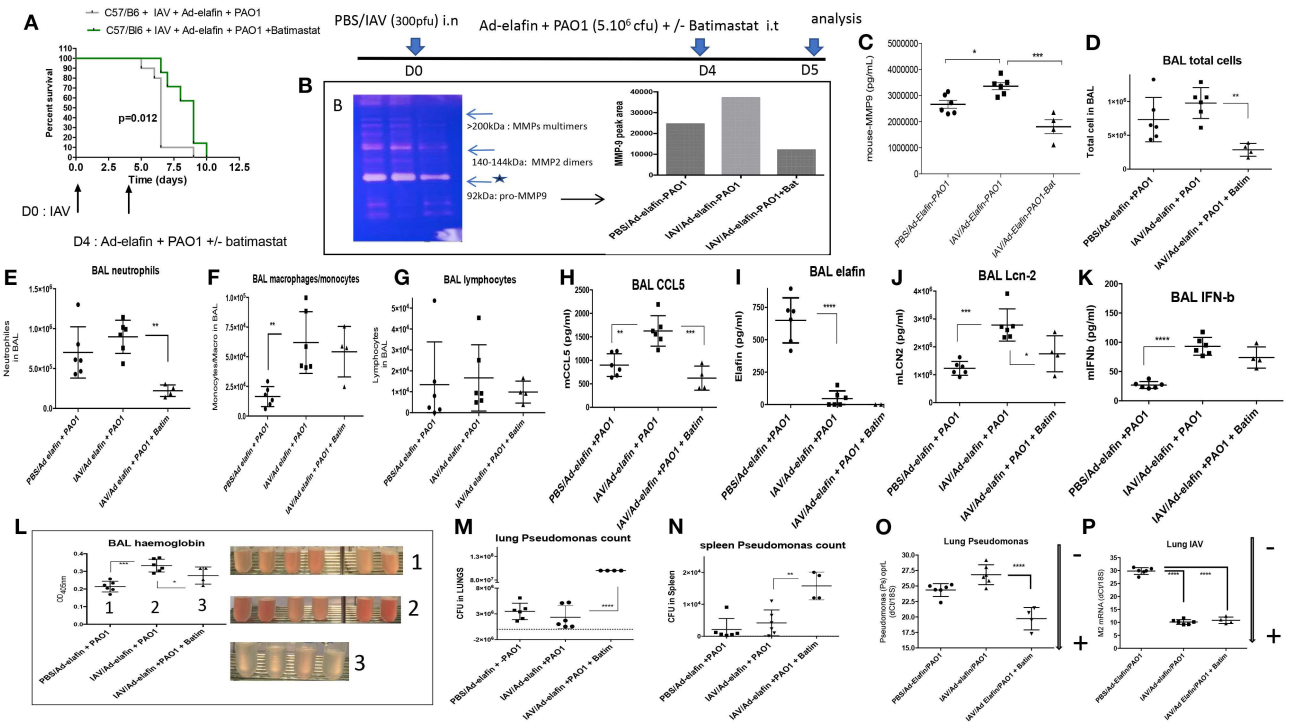

FIGURE 10 | The metalloprotease batimastat delays lethality in IAV + PAO1 infection and reduces inflammation and lung injury. **(A)** Survival experiment: C57/Bl6 mice were instilled i.n with IAV (300 pfu). Four days later, mice were further instilled i.t with Ad-elafin (3.10^6 pfu) and PAO1 (1.10^5 cfu) +/– the metalloprotease inhibitor batimastat (200 μgs). Survival was then followed and analyzed using Kaplan-Meier curves. Statistical tests were performed with the GraphPad Prism 6 package, using the Log-rank (Mantel-Cox) test. **(B–N)** Mechanistic experiments: C57Bl/6 WT mice were instilled i.n with either PBS or IAV (300 pfu). Four days later, mice were further instilled i.t with Ad-elafin (3.10^6 pfu) and PAO1 (5.10^6 cfu) +/– batimastat (200 μgs). At day 5, mice were sacrificed, and a bronchoalveolar lavage (BAL) was performed. BAL fluid supernatant was used for MMP measurements (zymography, **B**, and MMP-9 ELISA, **C**), cystospin cellular quantification **(D–G)**, ELISA assessment of a variety of mediators **(H–K)**, hemoglobin measurements **(L)**. Lungs and spleens were also harvested and used to measure PAO1 load (cfu counts on agarose plates, **M** and **N**, respectively). NB: in **(M)**, bacteria numbers were too high to be counted with the required precision. The value was therefore set at the maximum countable, considering the dilution used, i.e., 10^8 bacteria. PAO1 and IAV load were also independently assessed by measuring by q-PCR oprL and M2 gene expression, **O** and **P** respectively. Gene expression is marked, as above, with an arrow indicating low (–) or high (+) level of expression. Results are shown as means ± SD. Statistical significance: ANOVA, multiple comparison, Tukey's test, with each point representing an individual mouse, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Regardless of the mechanisms, a reduction in some antimicrobial molecule levels, like elafin in our study, is certainly a plausible mechanism to explain further sensitivity to bacterial infections. However, although it has long been assumed that these molecules only have a direct bacteriostatic/bactericidal activity on microbes, it is now apparent that they have more complex and pleiotropic activities (43). Specifically, we have previously demonstrated that a 5 day local over-expression of elafin protected mice lungs against maladaptive neutrophilic inflammation in *P.a* infections (27, 28, 30), and also against Plasmodium-induced inflammation, through the induction of anti-inflammatory pathways (29). Importantly, the focus of the present study was not to "re-demonstrate" the protective effect of elafin against *P.a* (see above, 27), since elafin expression was short-lived in our Ad-elafin *in vivo* protocol (16 h), but allowed us, as discussed above, to demonstrate for the first time that it is down-regulated by IAV.

Equally as important, and indeed the initial focus of our study, was the demonstration that IAV pre-infection exacerbated further PAO1-mediated inflammatory responses, regardless of elafin presence (Ad-elafin protocol, **Figure 9** and **Figure S2**), or of its absence (in C57Bl/6 WT mice, **Figures 1–3**). Indeed, in the context of IAV+ PAO1 infection, increased lung inflammatory cell influx, particularly neutrophils, which were activated, as evidenced by increased NE bioactivity, was associated with enhanced inflammatory markers, e.g., IL-1β, KC, and with an increase in protease (mainly MMP) activity, and with tissue injury.

Strengthening previously reported data that IAV can induce metalloproteases in the lung and other organs (44–48), we further demonstrated that induced MMP activity (including that of MMP-9) by IAV pre-infection was indeed likely an important factor in sensitization of mice to further PAO1-mediated lung damage, since the MMP inhibitor batimastat significantly delayed lethality (**Figure 10A**) and

diminished inflammatory responses (**Figures 10D–G**) and tissue damage (**Figure 10L**).

Although the cellular source of MMPs was not investigated here, neutrophils are known to secrete MMP-2 and 9 and they were likely a significant source (48). Interestingly, there was very little NE bio-activity in BALs from "IAV-alone"-infected animals, suggesting that either MMPs are more readily secreted than NE post-IAV, or that "IAV-alone" induced the secretion of elastase inhibitors capable of blocking NE activity.

Importantly, in contrast to previous IAV/bacteria associations studied in the past, e.g., IAV/*S.aureus*; IAV/*S. pneumoniae*, IAV pre-treatment did not condition the host to further PAO1 infection by restraining the IL-1β-IL-17 pathway (21, 22), and/or neutrophilic responses to bacteria (14–17). Although these differences may partly be explained by differences in the strains of *Influenza* used (H3N2 Scotland here), we believe that *P.a* may clearly respond differentially from other bacteria. This combination sets the scene for a furthered deleterious protease (MMP mainly) response and a down-regulation of elafin, a key anti-inflammatory molecule, resulting in increased tissue injury, where neutrophils probably play a major role (37, 49, 50).

In conclusion, as demonstrated by the fact that bacterial growth and dissemination does not equate with decreased survival (**Figure 10**), our data strengthen the concept (26, 51) that improvement of tissue resilience by inhibiting host proteases [(27–30), this study] and up-regulating antimicrobials/anti-inflammatory molecules inhibited by IAV, such as elafin (27–30) is not necessarily associated with bacterial clearance (specifically *P.a* in our study). Indeed, the MMP inhibitor batimastat even promoted bacterial dissemination, suggesting that MMPs might have anti-bacterial activities. This may have to be carefully considered in clinical situations where IAV/*P.a* co-infections are found (VAP, cystic fibrosis…).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Procedures involving mice were approved by our Ethical Committee (Paris-Nord/No 121) and by the French Ministry of Research (Agreement Nos. 4537.03 and 02012).

AUTHOR CONTRIBUTIONS

BV, BS, MS, and FL performed experiments. AC performed histological analysis. IG-V helped in the design of the experiments and critically appraised drafts of the document. J-MS designed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00117/full#supplementary-material

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Impaired Airway Epithelial Barrier Integrity in Response to *Stenotrophomonas maltophilia* Proteases, Novel Insights Using Cystic Fibrosis Bronchial Epithelial Cell Secretomics

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Stenotrophomonas maltophilia is a Gram-negative opportunistic pathogen that can chronically colonize the lungs of people with cystic fibrosis (CF) and is associated with lethal pulmonary hemorrhage in immunocompromised patients. Its secreted virulence factors include the extracellular serine proteases StmPR1, StmPR2, and StmPR3. To explore the impact of secreted virulence determinants on pulmonary mucosal defenses in CF, we examined the secretome of human CFBE41o- bronchial epithelial cells in response to treatment with *S. maltophilia* K279a cell culture supernatant (CS) using a liquid-chromatography-tandem mass spectrometry (LC-MS/MS) based label-free quantitative (LFQ) shotgun proteomics approach for global profiling of the cell secretome. Secretome analysis identified upregulated pathways mainly relating to biological adhesion and epithelial cell signaling in infection, whereas no specific pathways relating to the immune response were enriched. Further exploration of the potentially harmful effects of K279a CS on CF bronchial epithelial cells, demonstrated that K279a CS caused CFBE41o- cell condensation and detachment, reversible by the serine protease inhibitor PMSF. K279a CS also decreased trans-epithelial electrical resistance in CFBE41o- cell monolayers suggestive of disruption of tight junction complexes (TJC). This finding was corroborated by an observed increase in fluorescein isothiocyanate (FITC) dextran permeability and by demonstrating PMSF-sensitive degradation of the tight junction proteins ZO-1 and occludin, but not JAM-A or claudin-1. These observations demonstrating destruction of the CFBE41o- TJC provide a novel insight regarding the virulence of *S. maltophilia* and may explain the possible injurious effects of this bacterium on the CF bronchial epithelium and the pathogenic mechanism leading to lethal pulmonary hemorrhage.

Keywords: *Stenotrophomonas maltophilia*, cystic fibrosis, extracellular proteases, secretomics, tight junction, epithelial barrier dysfunction

INTRODUCTION

Culture supernatant (CS) from bacteria, most notably *Pseudomonas aeruginosa,* has been used extensively to study host-pathogen interactions in the cystic fibrosis (CF) lung. Studies have explored its effects on TLR-induced inflammation (1), pro-inflammatory cytokine production (2, 3), innate immunity proteins (4–6) and degradation of extracellular matrix components (5, 7, 8), amongst others. However, there is a paucity of information regarding the effects of the important emerging CF pathogen, *Stenotrophomonas maltophilia* on airway epithelial cells *in vitro*. Given that the pathogenesis of *S. maltophilia* is complex and multifactorial, high-throughput technologies such as proteomics can help decipher differences in protein expression in composite circumstances such as host-pathogen interactions. Secretome analysis is a promising area of research permitting novel insights into the pathogenesis of different infections. Proteins secreted by a pathogen are present at the interface between the pathogen and the host cells and can thus regulate or mediate the host responses and cause disease (9).

The cell secretome is a collection of proteins that have been shed and proteins secreted by cells into the extracellular space and are important for maintaining cell-cell communication and proliferation. Examples of secretory proteins include extracellular matrix proteins, digestive enzymes, cytokines, chemokines, and growth factors (10). Identification of proteins released by cells into culture supernatants *in vitro* may help to better understand pathological conditions and mechanisms *in vivo*. For example, using high-throughput subcellular proteomics Lietzen et al. showed a robust secretion of different danger-associated molecular patterns in human macrophages in response to influenza A, and that the P2X7 receptor and Src tyrosine kinase activity are essential for inflammasome activation (11). Secretome analysis of A549 cells infected with *Mycoplasma pneumoniae* revealed higher levels of IL-33 mimicking *in vivo* conditions whereby higher than normal IL-33 levels are evident in plasma and bronchoalveolar lavage fluid from patients with *M. pneumonia*-associated pneumonia (12). Analysis of the *in vitro* proteome response of a human bronchial epithelial cell line to *Aspergillus fumigatus* demonstrated previously unknown aspects of bronchial epithelial cell behavior in response to infection including both cellular defense mechanisms and immune reactions (13).

Airway epithelial cells provide the first line of defense following exposure to inhaled infectious agents. Virulence factors such as secreted proteases expressed by *S. maltophilia* are likely to be important mediators of the pathogenic interaction between *S. maltophilia* and these cells. Indeed *S. maltophilia* has been shown to preferentially adhere along intercellular junctions, raising the possibility that tight junction dysfunction may be an important pathogenic mechanism of this bacterium (14). *S. maltophilia* has also been shown to induce morphological changes in fibroblast monolayers resulting in the cell layer partially condensing, formation of cell-free areas, and detachment from the culture plate (15). StmPR1 is likely a causative factor leading to the clinical observation of lethal pulmonary hemorrhage in those who are immunocompromised (16). Moreover, purified StmPr1 induces cell rounding and detachment of A549 cells by targeting cell integrin-extracellular matrix connections (matrilysis) as well as adherence and tight junction proteins for degradation (17, 18).

In this study, using K279a as the reference clinical strain for *S. maltophilia* infection, we provide an insight into host-pathogen interaction using a liquid-chromatography-tandem mass spectrometry (LC-MS/MS) based label-free quantitative shotgun proteomics approach for global profiling of the K279a CS treated human CFBE41o- (cystic fibrosis airway epithelial cell line) cell secretome. Using data from this secretomic analysis we examine the effects of K279a CS on epithelial barrier integrity and degradation of components of CFBE41o- cell tight junctions.

MATERIALS AND METHODS

Reference Bacterial Strain

K279a was used as the reference clinical strain for this work (19). K279a was cultivated by scraping the surface of the frozen bacterial stock (−80°C) with a sterile 10 μL inoculating loop, placed in Luria-Bertani broth (LBB) and incubated overnight at 37°C on an orbital shaker at 200 rpm prior to use. Working stocks were maintained on agar plates at 4°C for up to 2 weeks. Cultures were regularly examined for purity using MALDI-TOF mass spectrometry (MS) analysis.

Preparation of K279a Culture Supernatant (CS)

We have previously shown that Dulbecco's modified essential medium (DMEM) low glucose (5.6 mM) medium (Invitrogen) is the optimal growth medium for inducing K279a protease activity (20). To prepare a stock solution of K279a CS, 10 μL of an overnight K279a culture was inoculated in 6 x 15 mls of DMEM low glucose (5.6 mM) medium and grown for 48 h at 37°C on an orbital shaker. K279a CS was passed sequentially through 0.45-μm and 0.2-μm filters millex filters (Millipore Corporation, Bedford, MA). Culture supernatant (90 mls) was then concentrated using 5-kDa nominal-weight limit (NMWL) cut-off Amicon® Ultra-15 filter devices (Millipore Corporation, Bedford, MA). All concentrates were centrifuged at 4,000 × g and subsequently diafiltered by centrifugation with sterile DPBS to remove any low molecular weight contaminants including glucose and amino acids present in DMEM. An equivalent volume of DMEM was used as a negative control and for correction during protein quantification using the BCA (bicinchoninic acid) assay.

Measurement of K279a CS Protease Activity

Protease activity was measured using the SensoLyte Red Protease Fluorometric Assay Kit (AnaSpec) as previously described (20). Prior to treatment of CFBE41o- airway epithelial cells, protease activity based on the known concentration of protein in K279a CS was adjusted based on a standard curve of K279a CS protease activity (measured in RFU/min).

CFBE41o- Cell Culture

CFBE41o–, an SV40-transformed human ΔF508 homozygote bronchial epithelial cell line was maintained in 75 cm^2 flasks at 37°C humidified CO_2 incubator in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen). The cell line was originally obtained as a gift from D. Gruenert (California Pacific Medical Center Research Institute, San Francisco). Prior to treatment, cells were washed twice with sterile DPBS to remove excess FCS and were placed in serum free medium for 6 h. Immediately prior to treatment with K279a CS, cells were placed in fresh serum-free media.

Romanowsky Stain of CFBE41o- Cells

To visualize morphological effects CFB41o- cells were untreated (control) or treated with K279a CS for 16 h at 37°C. Cells were stained using the Hema–Rapid staining set GURR® (VWR, UK), air dried and then fixed in methanol for 5 s following by staining with reagent one and reagent two for 3 and 6 s, respectively. Images were captured using an Olympus CKX41 and processed using CELL B by Soft Imaging System (Olympus, Tokyo, Japan).

LDH CFBE41o- Cell Viability Assay

LDH assays were performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) according to the manufacturer's instructions. Absorbance was measured 490 nm on a microplate reader (Victor™ X3 Multilabel Plate Reader, PerkinElmer, Massachusetts, USA).

CFBE41o- Cell Secretome In-Solution Digestion (ISD) and Mass Spectrometry

CFBE41o- cells were grown to 90% confluence, washed twice with warm DPBS and then either left untreated (control) or treated with K279a CS (protease activity: 500 RFU/min) for16 h. Supernatants were harvested, centrifuged at 4,500 × g for 10 min at 4°C and concentrated using Amicon-Ultra centrifugal filters (3-kDa NMWL) and protein concentration was determined by the BCA assay. Samples concentrations were adjusted to contain 20 μg of protein in 50 μL of 50 mM NH_4HCO_3 buffer for in solution digestion. Concentrated secretome samples were stored at −80° until processing for in solution digestion and mass spectrometry as described in the **Supplementary Methods**.

Bioinformatic Data Analysis

Methods by which proteomic analysis, cellular localization of identified proteins, gene ontology analysis, prediction of disease related proteins using candidate gene prioritization and CFBE41o- cell secretome and sub-network analysis are described in the **Supplementary Material**.

Tight Junction Studies

Monolayer Culture and TEER Measurement

CFBE41o- cells were seeded at 5 × 10^5 cells/cm^2 onto clear permeable filter inserts (Millipore Corporation, Bedford, MA, 6.5 mm diameter, 0.4 μm pore size), grown for 7 days prior to experiments, and medium was changed on alternate days. Prior to treatment, cells were washed with DPBS and the media replaced with serum free DMEM and allowed to equilibrate for 2 days prior to treatment. Tight junction integrity was assessed by measuring the TEER with an EVOM epithelial voltmeter in a Chopstick Electrode Set for EVOM (World Precision Instruments, Sarasota, FL, USA). The TEER of the background filter inserts was 20 Ω × cm^2.

Fluorescein Isothiocyanate (FITC) Dextran Permeability Assay

Inserts were gently washed twice with 200 μL of Hanks' Balanced Salt Solution (HBSS), pre-warmed to 37°C then transferred using sterile tweezers, to a fresh 24 well plate. FITC-labeled dextran (10 kDa) was added (200 μL) to the upper chamber at a concentration of 0.5 mg/ml and 1 ml of pre-warmed HBSS added to the lower chamber. Cells were incubated for 1 h at 37°C. FITC dextran permeability was then measured by transferring 100 uL from each of the basal chambers to a black 96-well-plate and read using a microplate reader (Victor™ X3 Multilabel Plate Reader, PerkinElmer, Massachusetts, USA) at excitation 485 nm and emission 535 nm.

Western Blotting

CFBE41o- cells were washed in ice cold DBPS and lysed for 15 min in 100 μL of RIPA buffer [50 mM Tris-Cl (pH 7.6), 1 mM EDTA (pH), 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 140 mM NaCl] in the presence of a protease inhibitor cocktail (Calbiochem, 539132), centrifuged at 14,000 × g for 5 min at 4°C and then heated to 95°C for 5 min prior to the addition of reducing sample buffer. Fifty microgram CFBE41o- lysate was separated using SDS-PAGE, transferred to PVDF using a semi-dry Novex XCell SureLock blotting system (ThermoFisher) and membranes were blocked (3% w/v Marvel skimmed milk, 1% bovine serum albumin in 0.1% PBS-Tween) then incubated with the respective primary antibodies for ZO-1 (1:500, rabbit polyclonal, ThermoFisher Scientific, #61-7300), Occludin (1:500, rabbit polyclonal, ThermoFisher Scientific, #71-1500), JAM-A (1:500, rabbit polyclonal, ThermoFisher Scientific, #36-1700), or Claudin-1 (1:500, rabbit polyclonal, ThermoFisher Scientific, #71-7800) overnight. β-actin was used a loading control (1:10,000 mouse monoclonal, Merck Millipore, #MAB1501). Visualization of immunoreactive protein bands was achieved using secondary antibodies to rabbit (Anti-rabbit IgG, HRP-linked antibody, 1:2,000, Cell Signaling, #7074S) or mouse (Anti-mouse IgG, HRP-linked antibody, 1:2,000, Cell Signaling, #7076S) and Immobilon Western chemiluminescent HRP substrate (Millipore) and the Syngene G:Box Chemi XL gel documentation system. Densitometry was performed using the GeneSnap Syngene program (Synoptics).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.0 software package (San Diego, CA). All experiments were performed in triplicate and results are expressed as the mean ± SEM and were compared by Student's *t*-test (two-tailed) or

analysis of variance where appropriate followed by Tukey *post-hoc* test for multiple comparisons where appropriate. Differences were considered significant at $p \leq 0.05$.

RESULTS

Morphological Effects of K279a CS on CFBE41o- Cells

There are many example reports using bacterial CS to study the behavior of virulence factors *in vitro* (3, 21–23). As a first step toward assessing the role, if any, of secreted proteases in the pathogenesis of *S. maltophilia* pulmonary infection, we examined the effect of differing concentrations of K279a CS (5 and 10% v/v) on CFBE41o- cell monolayers compared with untreated control cells (**Figure 1A**). Monolayers incubated with K279a CS displayed morphological changes including cell condensation, rounding and detachment after incubation for 16 h, the effects of which were most pronounced in those treated with 10% v/v K279a CS (**Figures 1B,C**). To determine whether extracellular serine protease(s) were responsible for these effects, K279a proteases were inhibited using 1 mM PMSF. The destructive effect of K279a CS was prevented by incubation with PMSF, which has been shown to be a potent inhibitor of K279a protease activity (**Figure 1D**).

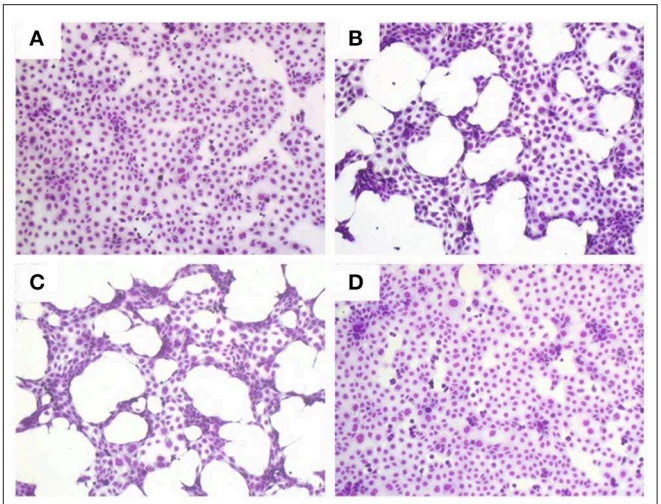


FIGURE 1 | Romanowsky stain (Hema-Gurr) of untreated and treated CFBE41o- cells with K279a CS in the absence and presence of PMSF. Wells were seeded with CFBE41o- cells (3×10^5 cells/ml) in MEM supplemented with 10% FCS and grown to 90% confluence in 24 well-tissue culture plates. The following day, medium was removed and washed twice with pre-warmed (37°C) DPBS to fully remove FCS. Cells were then placed in fresh serum free medium for 6 h prior to treatment. CFBE41o- cells were untreated (control, **A**) or incubated with 5% **(B)** or 10% v/v of K279a CS which was untreated **(C)** or treated with 1 mM PMSF **(D)** for 16 h. The following day the cell CS was removed and the remaining cells were stained using the Hema–Rapid staining set GURR® for hematology (VWR, UK). Cells were air dried and then fixed in methanol for 5 s following by staining with reagent one and reagent two for 3 and 6 s, respectively. Representative images were captured using an Olympus CKX41 and processed using CELL B by Soft Imaging System (Olympus, Tokyo, Japan).

Label-Free Quantitative (LFQ) Shotgun Proteomic Analysis of CFBE41o- Secretome Following Treatment With K279a CS

To further elucidate the potential pathological changes occurring in cystic fibrosis airway epithelial cells following *S. maltophilia* infection, we used label-free quantitative (LFQ) shotgun proteomic analysis of the CFBE41o- secretome following treatment with K279a CS. We compared the relative abundance of proteins between two experimental conditions, CFBE41o- cells left untreated (control) and those treated with K279a CS (with protease activity of 500 RFU/min). Cells were treated for 16 h in the presence or absence of K279a CS followed by collection of cell secreted proteins (24). A schematic representation of the experimental design is outlined in **Supplementary Figure 1**.

LDH cytotoxicity assay showed that treatment with K279a CS did not significantly affect CFBE41o- cell integrity within 16 h. Cells treated with K279a CS with the highest protease activity (5×10^3 RFU/min) released 40% greater LDH compared to control and the effect was prevented by PMSF. However, this was not statistically significant after correcting for multiple comparisons (**Supplementary Figure 2**).

Based on the LC-MS/MS data, 1290 proteins were identified, of which 972 ± 91 and 424.3 ± 9 were in the control group and the treatment group, respectively. A total of 376 proteins were included in the final analysis following filtration for proteins found in at least two out of three replicates in at least one group (**Figure 2A**). Among them, proteins were quantified on the basis of two or more peptides, with mean sequence coverage of 45.5 ± 17% (**Figure 2B**). Overall 271 statistically significant proteins were differentially expressed (Benjamini Hochberg false discovery rate <0.05) between treatment and control. Hierarchical clustering was also performed separately on the two groups (i.e., treated and untreated control), to determine proteins which were either up-regulated or down-regulated (**Figure 3**). Among those 271 proteins, 77 proteins were abundantly elevated in K279a CS treated cells, whereas 194 proteins were decreased (**Figure 2C**). A list of all identified and quantified proteins is presented in **Supplementary Table 1**.

Characterization of Classically Secreted Proteins

Proteins classically secreted via an ER/Golgi dependent pathway normally have an amino-terminal secretion signal peptide sequence (25). We screened for both non-classically secreted (ER/Golgi independent pathway) proteins using SecretomeP and classically secreted proteins utilizing SignalP software. Of the 271 proteins, 79 were categorized as non-classical whereas 101 were designated as classically secreted. The shared agreement among the algorithms was good, with 52 entries fulfilling the set criteria for secretion through the classical pathway (SignalP prediction). Only 3 proteins were predicted to have a transmembrane (TM) domain while 98 proteins were not using SignalP-TM and SignalP-noTM to predict those proteins that might include TM regions. In contrast, TMHMM (a transmembrane helix prediction tool) predicted that 42 proteins had a TM

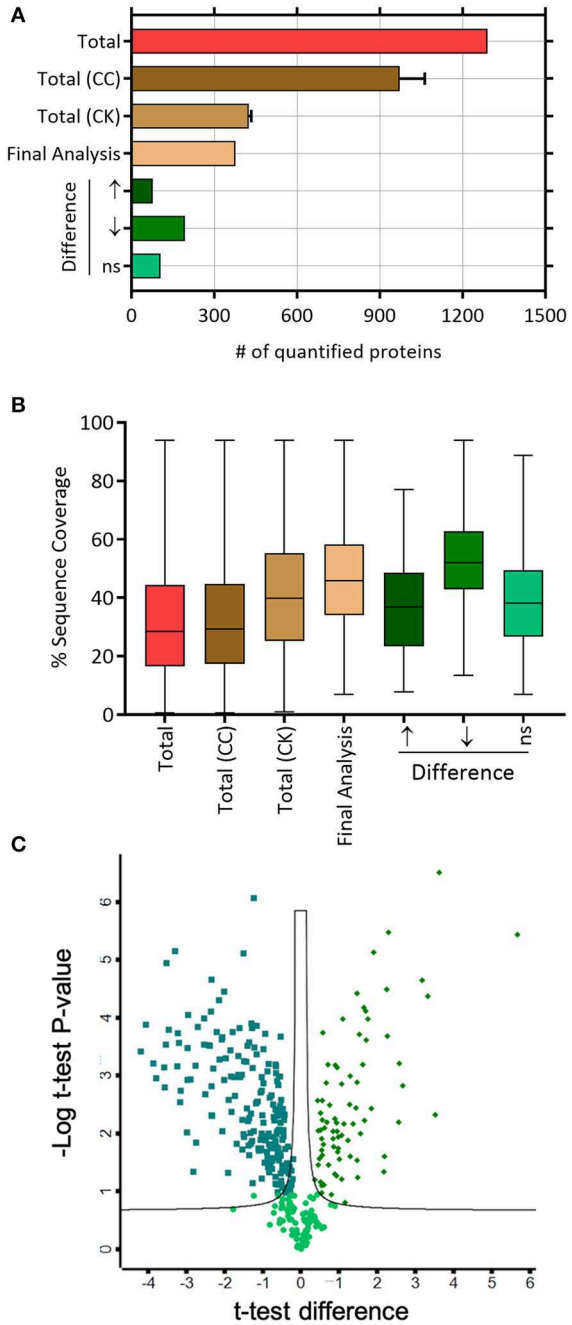


FIGURE 2 | Label-free mass spectrometry secretome analyses of CFBE41o- cells treated with K279a CS. **(A)** Number of quantified proteins in the indicated protein fractions and experimental conditions. The mean and standard deviation are shown where necessary. **(B)** Box and whisker plots depicting the distribution of protein sequence coverage (coverage of tryptic peptides per protein in %). **(C)** Of the 376 proteins identified, statistically significant differentially expressed proteins (Benjamini Hochberg false discovery ratio 0.05) are shown in the volcano plot. Volcano plot analysis reveals that 271 proteins are significantly differentially expressed. Among the 271 proteins, 77 proteins were significantly elevated in CFBE41o- cells treated with K279a CS (green diamonds), whereas 194 proteins were significantly decreased (green squares). Abbreviations: CC, CFBE41o- cell control; CK, CFBE41o- cells treated with K279a CS.

domain. Phobius, a combined transmembrane topology and signal peptide predictor, predicted that 32 proteins contained both a signal peptide and a TM domain. This correlated well with combined SignalP and TMHMM analysis with 34 overlapping proteins predicted to have both a signal peptide and a TM. WoLF Psort was used to determine the subcellular location of the identified proteins, 77 proteins were determined as extracellular and represented 28% of the total number of quantified proteins. The remaining proteins were located in the cytoplasm (32.84%), nucleus (11.44%), mitochondrion (9.59%), plasma membrane (5.54%), and endoplasmic reticulum (ER, 4.43%) (**Figure 4**).

Biological Significance of Identified Proteins

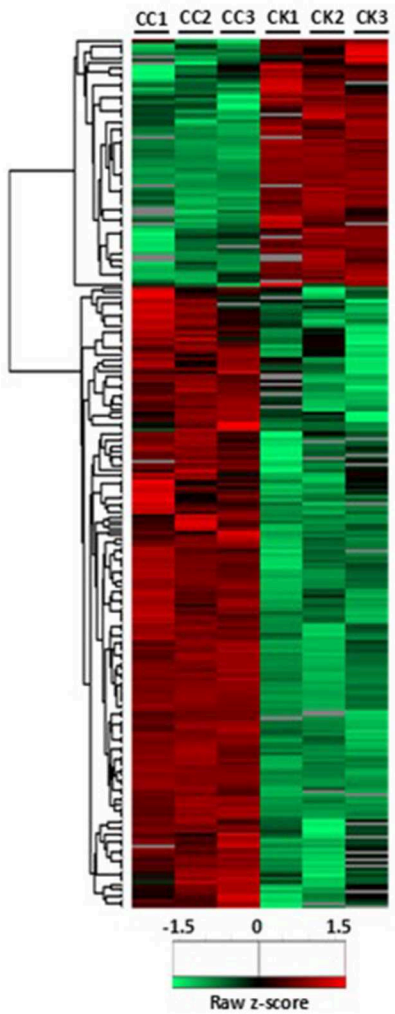
To understand the functional significance of the identified proteins, analyses of gene ontology for cellular component and biological function were classified using DAVID 6.8 (26). The analysis revealed major differences in the proteins that were differentially expressed in CFBE41o- cells treated with K279a CS compared to control. To enhance the power of disease-associated pathway detection, up-regulated and down-regulated proteins were analyzed separately [(27); **Supplementary Figure 3**]. Proteins residing in the extracellular region and extracellular matrix (ECM) were the most significantly altered in both groups but especially in the up-regulated proteins. Enrichment for cell junction was similar for both groups.

The terms biological adhesion, growth, and localization were enriched in the up-regulated proteins whereas cell killing, locomotion, and multi-organism process were enriched in down-regulated proteins. Notably, no significant differences in immune system processes were identified, indicating that the pathological response of CF bronchial epithelial cells in response to secreted *S. maltophilia* virulence factors may be structural rather than primarily immunostimulatory.

Identification of Candidate Disease Proteins From PPI Networks Based on Identified Differentially Regulated Proteins

Protein-protein interaction (PPI) networks are critical to comprehensively understand cellular mechanisms and function. They have emerged as an important resource for understanding data from proteomics experiments in order to identify proteins which could play important pathogenic roles in lung disease (28). The importance of PPIs in disease pathogenesis was recently demonstrated in CF. A detailed analysis of the CFTR interactome identified key novel interactors whose loss promoted enhanced CFTR channel function indicating that global remodeling of ΔF508 CFTR interactions is crucial for CFTR rescue (29).

Network analysis may reflect the biological processes more objectively than analyzing individual proteins. The first step is to identify proteins of interest and these inputs (or "seed proteins") are used to search for interactions from a curated PPI database. Constructing a PPI network (PPIN) using only the seed proteins may miss potentially important disease associated proteins and so completing the network with first order interactors by utilizing probabilistic PPINs improves the detection of



Increased

CD109	CD109	CTGF	FLNA	HNRNPC	LGALS3BP	PLXNB2	TFPI2
CD40	CD40	CYR61	FLNC	HNRNPH3	LTBP2	PPP1CA	TGFBI
CD44	CD44	DCBLD2	FN1	IGF2R	LYPD3	PRSS8	THBS1
CD59	CD59	EEF1G	FST	IGFL1	MAPRE1	PXDN	TMEM132A
CLDN6	CLDN6	EFNB2	GPR126	ILF2	NPM1	SEPT2	TNC
COCH	COCH	EGFR	H2AFY	LAMA3	NT5E	SFRP1	UGGT1
COPB2	COPB2	EPHA2	HIST1H2AJ	LAMB1	PAM	SOD1	XRCC6
CRIM1	CRIM1	FAM3C	HIST1H4A	LAMC2	PARP1	SSRP1	
CSNK2A1	CSNK2A1	FBLN2	HIST2H2BE	LCN2	PLAU	SUPT16H	
CSPG4	CSPG4	FKBP4	HIST2H3PS2	LGALS3	PLAUR	TAGLN2	

Decreased

ACTG1	CANT1	DDAH1	EZR	GLO1	HNRNPA2B1	ISYNA1	MYH9
AHCY	CFB	DDAH2	FBLN1	GLOD4	HNRNPK	KLK6	MYL6
AKR1B1	CFL1	DDT	FDPS	GNB2L1	HSP90AA1	L1CAM	NAP1L1
ALDOA	CLIC1	DKK3	FH	GOT1	HSP90B1	LAP3	NME1-NME2
ALDOC	CLIC4	DPP3	FKBP1A	GOT2	HSPA1B	LDHA	NPEPPS
APEX1	CLSTN1	DSG2	FLNB	GPC1	HSPA4	LDHB	NSFL1C
APOA1BP	CNBP	DSTN	FSCN1	GPI	HSPA5	LDLR	P4HB
ARHGDIA	COL4A2	DUT	FSTL3	GSN	HSPA8	LMNA	PAFAH1B1
ATRN	COL6A1	ECM1	FUCA2	GSS	HSPA9	LOXL2	PARK7
AXL	CS	EEF1A1P5	GANAB	GSTO1	HSPH1	LTA4H	PDIA3
B2M	CSRP1	EEF2	GAPDH	GSTP1	ICAM1	MAT2A	PDIA4
BCAM	CST3	EFEMP1	GDI1	HEXA	IGFBP7	MAT2B	PDIA6
BCAT2	CST6	EIF6	GDI2	HLA-A	ILF3	MDH1	PEBP1
CALM1	CTSD	ENO1	GGCT	HLA-B	INHBA	MDH2	PFN1
CALR	CTSZ	ERAP1	GGH	HNRNPA1	ISG15	MFI2	PGAM1

FIGURE 3 | Heat map of CFBE41o- cell secretome in response to K279a CS. Heatmap and dendrograms were produced by unsupervised hierarchical clustering (Euclidean distance with complete linkage) of the indicated 271 significantly differentially expressed proteins (two sample *t*-test <0.05). The row Z-score or scaled expression value of each feature is plotted in red–green color scale. The red color of the tile indicates high abundance and green indicates low abundance. Gray color in the heatmap indicates "not detected." The adjacent tables show the gene names of the proteins associated with the indicated clusters. CC, CFBE41o- cell control; CK, CFBE41o- cells treated with K279a CS.

candidate disease related proteins and disease pathways. This principle suggests that clues to the function of a protein can be obtained by seeing whether it interacts with another protein of known function (30). However, expanding a PPIN from a given set of "seed proteins" often leads to a complex PPIN lacking spatiotemporal consideration. To avoid this so-called "hairball" effect and to increase the robustness of our analysis we searched for shared candidate disease proteins among the top 100 interactors as ranked by three prioritization tools: NetworkAnalyst, GeneMANIA, and ToppGene.

In NetworkAnalyst, 161 up- and 348 down-regulated nodes were generated and the top 90 and 100 candidate proteins, respectively, were ranked. The interaction data from 100 related proteins generated using label propagation in GeneMANIA are summarized in **Supplementary Table 2**. Using ToppGene, 4,189 up- and 5,631 down-regulated first-order interactors of seed proteins were identified and ranked according to the network-based prioritization method (k-Step Markov, step size = 6) with a neighborhood distance of 1. The ranked list of the top 100 candidate proteins from the three prioritization tools are summarized in **Supplementary Tables 3–5**.

Overall comparison between the three networks identified 27 candidate proteins which were identified in two or more prioritization tools in both up-regulated and down-regulated networks. A list of these proteins and their basic information is described in **Supplementary Table 6**.

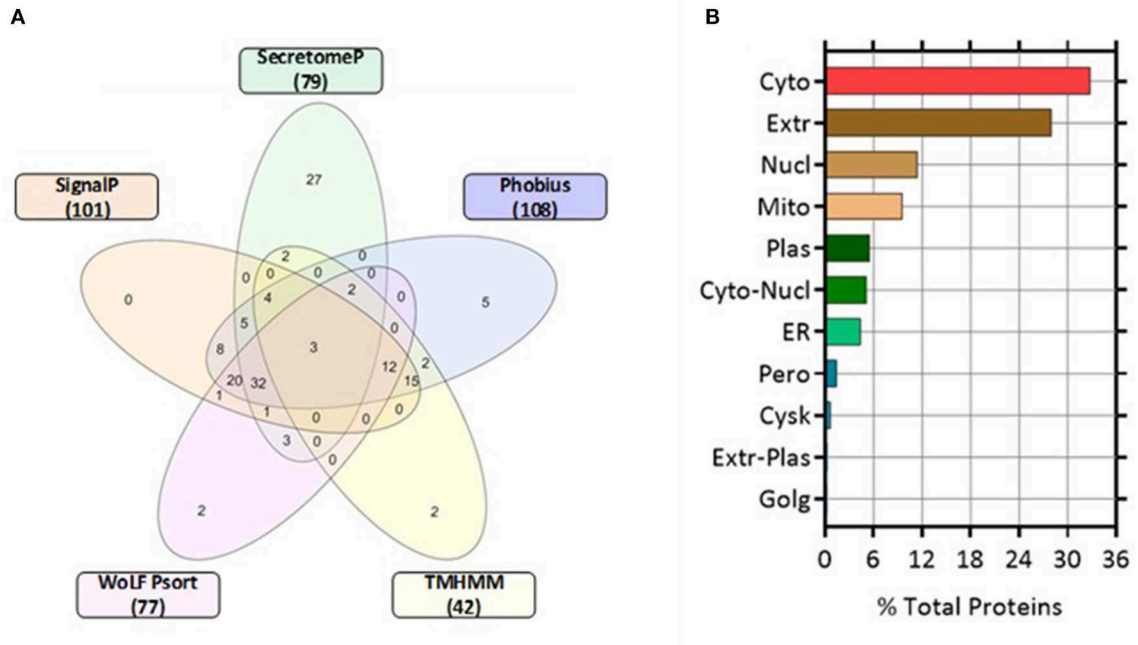


FIGURE 4 | Five-Venn diagram and cellular locations of the identified proteins as predicted by bioinformatic analyses. The significant differentially expressed proteins ($n = 271$) were submitted to SignalP, Secretome P, Phobius, TMHMM, and WoLF Psort servers as outlined in the Materials and Methods section. Results from each program were compiled and the numbers of proteins predicted by each program individually were tabulated (**Supplementary Table 1**). **(A)** The numbers of proteins predicted by each program and all possible combinations are indicated in the Venn diagram **(B)** Location analysis of the differentially secreted proteins was based on WoLF Psort. Results are represented as percentage of the total number of analyzed proteins. Cyto, cytoplasm; Extr, extracellular; Mito, mitochondrion; Plas, plasma membrane; ER, endoplasmic reticulum; Cysk, cytoskeleton; Golg, Golgi apparatus; Nucl, nucleus; Pero, peroxisome.

Functional Classification of Identified Proteins

To better understand the nature of the identified proteins, KEGG pathway annotations were obtained. Eighteen pathways were significantly over-represented in the KEGG database ($P < 0.01$), 12 in the up-regulated and 6 in the down-regulated network. In the up-regulated proteins focal adhesion, various cancers, ECM-receptor interaction, EGFR/GRB2/KRAS (annotated here as dorso-ventral axis formation), and Gap and adherens junctions featured, as did pathways related to bacterial and parasitic infection (**Supplementary Figure 4**). In contrast, in the down-regulated protein network, pathways related to metabolism, protein processing in the ER and Parkinson's disease featured (**Supplementary Figure 5**).

PPIN Module-Based Analysis Following Treatment of CFBE41o- Cells With K279a CS

Seed proteins and candidate proteins identified in both the up-regulated and down-regulated datasets were used for the construction of the final PPI network using STRING v10.0. Cluster analysis using the Reactome Functional Interaction (FI) app (Reactome FIViz) in Cytoscape v3.4.0 divided the up-regulated and down-regulated PPINs into five and seven modules, respectively (module 1 was excluded from further analysis secondary to an FDR > 0.01) (**Supplementary Figures 6**, 7). The most interesting terms are highlighted in bold; these relate to biological adhesion and bacterial infection. A summary of the over-represented KEGG pathways related to bacterial infection and their associated proteins can be found in **Table 1** and a summary of the over-represented KEGG pathways related to biological adhesion and their associated proteins can be found in **Table 2**.

Epithelial Barrier Integrity in CFBE41o- Cells Is Disrupted by Secreted K279a Serine Proteases

Using secretomics, we found that pathways related to biological adhesion and extracellular matrix components were significantly enriched following treatment with K279a CS. To extrapolate this further we sought to determine if serine proteases secreted by K279a had an effect on attachment of the CFBE41o- epithelial monolayer. Airway epithelial barrier function was determined by measuring TEER. In initial experiments, various concentrations of K279a CS with differing protease activity were added to the apical surface of CFBE41o- monolayers seeded in transwell permeable supports. Using repeated measures of analysis to evaluate the effect of K279a CS on TEER, apical treatment of CFBE41o- cell monolayers with K279a CS (protease activity of 5×10^3 RFU/min) significantly decreased monolayer resistance by 24 h ($p < 0.002$). The observed drop in TEER was protease

TABLE 1 | Proteins identified from KEGG pathways related to bacterial infection.

KEGG pathway	Network	Module	Proteins[a]	
			Seed	Candidate
Ep. cell signaling in *H. pylori* infection	↑	2	EGFR	SRC, TJP1
Legionellosis	↓	4	HSPA8, VCP	
Pathogenic *E. coli* infection	↓	5	ACTG1, EZR, TUBA1B	ACTB
		7	YWHAZ	YWHAQ
Shigellosis	↓	5	ACTG1, VCL, PFN1	ACTB
Salmonella infection	↓	5	ACTG1, MYH9, PFN1	ACTB
Bacterial invasion of epithelial cells	↓	5	ACTG1, VCL	ACTB

[a]*Proteins divided by (1) Seed proteins: identified from LFQ (label-free quantification) shotgun proteomics analysis and (2) Candidate proteins: Candidate disease proteins identified using prioritization tools.*
Ep, Epithelial; ↑, up-regulated network; ↓, down-regulated network.

TABLE 2 | Proteins identified from KEGG pathways related to biological adhesion.

KEGG Pathway	Network	Module(s)	Proteins[a]	
			Seed	Candidate
Focal adhesion	↑	2, 3, 5	COL4A2, EGFR, FLNA, FN1, LAMA3, LAMB1, LAMC2, PPP1CA, TNC, THBS1	COL1A1, CAV1, GRB2, LAMA5, SRC
Gap junction	↑	2	EGFR	GRB2, KRAS, SRC, TJP1
Tight junction	↑	2	CLDN6	KRAS, SRC, TJP1
	↓	5	ACTG1, MYH9, SPTAN1	ACTB
Adherens junction	↑	2	EGFR	SRC, TJP1
	↓	5	ACTG1, VCL	ACTB
ECM-receptor interaction	↑	5	AGRN, LAMA3, LAMB1, LAMC2	LAMA5
	↓	6	COL4A2, COL6A1, FN1	
ECM organization	↓	6	COL4A2, COL6A1, CTSD, EFEMP1, FBLN1, FN1, SERPINE1, SPARC, TIMP1	

[a]*Proteins divided by (1) Seed proteins: identified from LFQ (label-free quantification) shotgun proteomics analysis and (2) Candidate proteins: Candidate disease proteins identified using prioritization tools.*
↑, up-regulated network; ↓, down-regulated network.

mediated given the abrogation of the observed effects by PMSF ($p = 0.007$) (data not shown).

Further analysis of TEER kinetic curves demonstrated that addition of K279a CS to the apical surface of TEER monolayers resulted in a time-dependent decrease in monolayer resistance. TEER was significantly lower at 10 h in cells apically treated with K279a CS compared with controls cells ($p < 0.0001$) which indicated disruption of epithelial barrier integrity, the effect of which was prevented by PMSF ($p = 0.0004$). At 12 h, TEER was 60.1 ± 11.75% relative to the control ($p < 0.0001$; **Figure 5A**). In comparison, addition of K279a CS to the basolateral surface of CFBE41o- cell monolayers resulted in a significantly more rapid time-dependent decrease in monolayer resistance. After 4 h, cells treated with K279a CS had a lower TEER compared with controls ($p < 0.0001$). At 12 h, TEER was only 3.62 ± 0.09% relative to the control ($p < 0.0001$; **Figure 5B**). As observed in apically treated cells, this effect was abrogated in the presence of PMSF, which indicated that secreted serine protease(s) were responsible for this effect.

K279a CS Increase Paracellular Permeability to Macromolecular FITC-Dextran

We next assessed the permeability of K279a CS (protease activity = 5×10^3 RFU/min) treated CFBE41o- monolayers with the macromolecular tracer, FITC-dextran (10 kDa), which can only transverse the monolayer via the paracellular route. At 12 h, when the development of TEER was significantly reduced in both apically and basolaterally treated cells, the permeability of FITC-dextran was measured. Apically treated monolayers displayed a 2.393 ± 0.2108-fold increase in permeability to FITC-dextran compared with control ($p = 0.009$) whereas a 15.54 ± 2.882-fold increase was observed in basolaterally treated monolayers ($p = 0.002$). In both instances, the permeability of FITC-dextran was prevented by PMSF, linking K279a protease activity to increased paracellular macromolecular transport (**Figure 6**).

Secreted K279a CS Degrade the Tight Junction Proteins ZO-1 and Occludin

Given that we observed a decline in TEER and increase in paracellular macromolecular permeability in CFBE41o- cell monolayers following treatment with K279a CS, we further investigated the integrity of tight junction proteins. Secretome analysis of CFBE41o- cells treated with K279a CS showed that the tight junction protein claudin-6 was up-regulated and that TJP1 or ZO-1 were identified as candidate disease associated proteins by PPIN analysis. We chose to examine the expression of ZO-1, occludin, JAM-A and claudin-1. Claudins 1, 3, 4, 5, 7, 8, and 18 are expressed in human bronchi and bronchioles whereas claudin-6 expression may be an effect of immortalization of the CFBE41o- epithelial cell line as it has been reported in non-small cell lung cancer (31) and in developing lung tissue (32). Therefore, we focussed on claudin-1 rather than claudin-6 as the expression of former is more constitutive in the adult lung epithelium.

CFBE41o- cells were either untreated (negative control) or treated with K279a CS (protease activity 5×10^3 RFU/min) in the presence or absence of PMSF (1 mM) for 4 h. Following normalization to negative controls and correction to β-actin (**Figure 7A**), ZO-1 ($p < 0.0001$) and occludin ($p = 0.003$) were

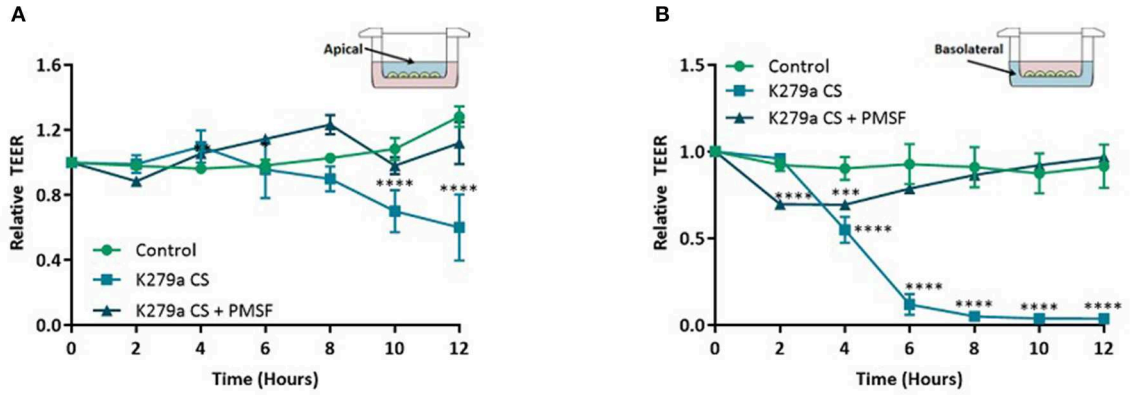


FIGURE 5 | TEER in CFBE41o- monolayers following treatment with K279a CS. CFBE41o- cells were seeded at a density of 5×10^5 cells/cm^2 onto clear permeable filter inserts (6.5 mm diameter, 0.4 μm pore size). Cells were grown for 7 days in MEM supplemented with 10% FCS and medium was changed on alternate days. Prior to treatment, cells were washed with DPBS to remove any residual FCS and the media replaced with serum free medium (SFM) and allowed to equilibrate for a further 48 h. The day before treatment cells were placed in fresh SFM overnight. CFBE41o- cell monolayers were left untreated (control) or treated with K279a CS (CS) (protease activity = 5×10^3 RFU/min) in the absence or presence of PMSF (1 mM). Transepithelial electrical resistance (TEER) was measured using an EVOM epithelial voltmeter in an Endohm-6 chamber. To construct TEER curves, measurements were taken every 2 h. **(A)** TEER curve of apically treated cells. **(B)** TEER curve of basolaterally treated cells. All results are representative of three independent experiments. Results are expressed as relative TEER to time zero (T0). Treatment vs. control: ****$p \leq 0.0001$, ***$p \leq 0.001$; Two-way-ANOVA followed by Tukey *post-hoc* test for multiple comparisons.

significantly degraded, effects that were prevented in the presence of PMSF (**Figures 7B,C**). No significant changes for JAM-A or claudin-1 were observed (**Figures 7D,E**). Therefore, we concluded that extracellular serine proteases from *S. maltophilia* degrade the tight junction proteins ZO-1 and occludin, but not JAM-A or claudin-1.

DISCUSSION

In this study, we analyzed the proteins secreted by CFBE41o- cells in response to treatment with *S. maltophilia* K279a CS. In total we identified 77 proteins which were significantly up-regulated and 194 proteins which were significantly down-regulated in response to treatment. Gene ontology and pathway analysis demonstrated that biological adhesion and assembly of extracellular matrix components were significantly enriched terms within the up-regulated proteins whereas cellular metabolism was over-represented in the down-regulated proteins. Using the seed proteins identified from this study we identified candidate disease proteins to determine the effect of *S. maltophilia* on CF bronchial epithelial cells using PPIN analysis from three prioritization tools: NetworkAnalyst, GeneMANIA, and ToppGene. Using modular analysis of the PPIN generated from the seed proteins and candidate disease proteins we identified six modules related to bacterial infection and five modules related to biological adhesion.

Stenotrophomonas maltophilia has been shown to preferentially adhere along intercellular junctions, raising the possibility that tight junction dysfunction may be an important pathogenic mechanism of this bacterium (14). In our analysis epithelial cell signaling related to *Helicobacter pylori* infection was significantly over-represented in the up-regulated PPIN. *H. pylori*, a spiral, gram negative rod plays an important

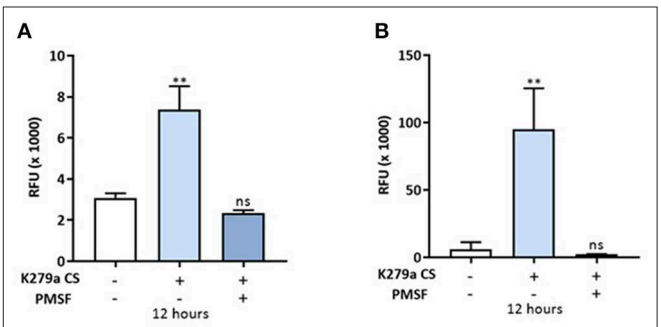


FIGURE 6 | *In vitro* permeability assay to FITC-dextran in CFBE41o- monolayers treated with K279a CS. FITC-dextran permeability (RFU × 1,000) in CFBE41o- cell monolayers was assessed in apically and basolaterally treated compartments after 12 h following treatment with K279a proteases (5×10^3 RFU/min) in the absence and presence of PMSF. Increased permeability to FITC dextran was observed in apically treated cells **(A)** but the effect was more pronounced when monolayers were treated basolaterally **(B)**. All measurements are means ± SEM from biological replicates. Treatment vs. control: **$p \leq 0.01$, one-way ANOVA followed by Tukey *post-hoc* test for multiple comparisons.

role in the pathogenesis of peptic ulcer disease and like *S. maltophilia*, it preferentially attaches to cell-cell interfaces (33). *H. pylori* can alter the function of the apical junctional complex resulting in changes of structure, function and morphology of gastric epithelial cells. Translocation of the protein CagA into these cells induces recruitment of the tight junction proteins ZO-1 and JAM to the sites of bacterial attachment and may serve to target and retain *H. pylori* at epithelial cell-cell junctions (34). In addition to alterations in tight junction assembly, *H. pylori* can alter expression of tight junction proteins. ZO-1 expression is decreased whereas claudin-4 is increased in *H. pylori* infected

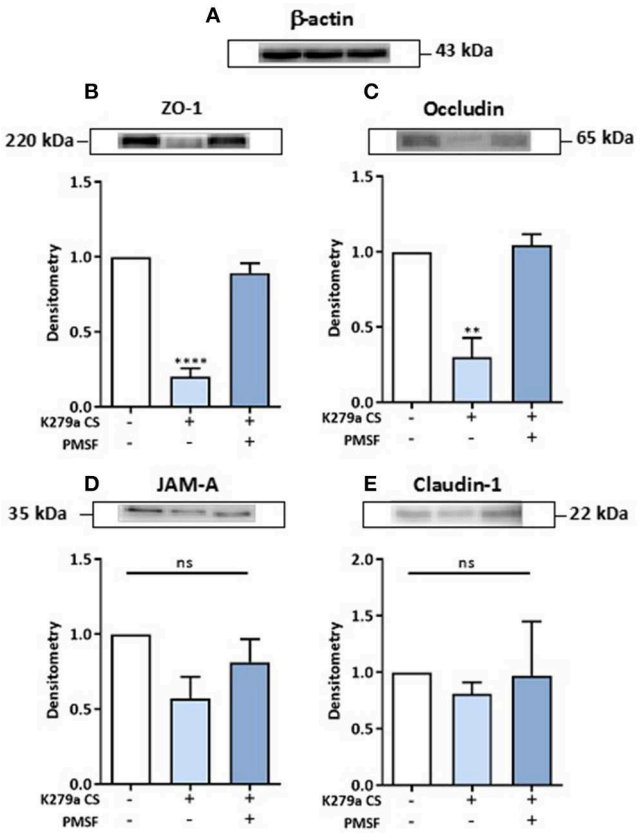


FIGURE 7 | Effect of K279a CS on the tight junction proteins: ZO-1, occludin, JAM-A and claudin-1. CFBE41o- cells were seeded at a density of 1×10^6 cells/ml onto 100 mm Triple Vented Tissue Culture Dishes. Cells were grown to 90% confluence in MEM supplemented with 10% FCS and medium was changed on alternate days. Prior to treatment, cells were washed with DPBS to remove any residual FCS and the media replaced with serum free medium (SFM) and allowed to equilibrate overnight. On the day of treatment, cells were placed in fresh SFM and treated for 4 h. Cells were left untreated (control) or treated with K279a proteases (5×10^3 RFU/min) in the absence or presence of PMSF (1 mM). Western blots were performed on whole-cell lysates (50 μg of protein) from CFBE41o- cells probed with antibodies to β-actin **(A)**, ZO-1 **(B)**, occludin **(C)**, JAM-A **(D),** and claudin-1 **(E)**. Histograms represent the densitometric fold change in relative protein expression (relative to untreated controls) in response to treatment following correction with β-actin. All results (expressed as relative densitometry units) are representative of three independent experiments. All measurements are means ± SEM from biological replicates. $^{****}P < 0.001$, $^{**}P \leq 0.01$; one-way-ANOVA with Tukey correction for multiple comparisons.

individuals indicating that damage to the gastric epithelial barrier function may be important in the pathogenesis of *H. pylori* peptic ulcer disease (35).

Stenotrophomonas maltophilia has been shown to induce morphological changes in fibroblast monolayers resulting in the cell layer partially condensing, formation of cell-free areas, and finally detachment from the culture plate (15). StmPR1 is a likely factor responsible for the clinical observation of lethal pulmonary hemorrhage in those who are immunocompromised (16). Although loss of structural components are important factors negatively affecting epithelial integrity, loss of function of key protease inhibitors and an increase in endogenous protease activity may also be relevant. TIMP1 and TIMP2, natural inhibitors of matrix metalloproteinases were down-regulated in response to K279a CS. We identified MMP2 (matrix metalloproteinase-2), a type IV collagenase as a candidate disease protein in the up-regulated protein network. *P. aeruginosa* has been shown to increase MMP-2 activity in CFBE41o- cells and a gain of functional MMP-2 and loss of function of TIMPs 1 and 2 are possible causes of epithelial damage in *S. maltophilia* lung disease (23). Other important anti-proteases which were down-regulated included alpha-1 antitrypsin and plasminogen activator inhibitor (PAI-1), an inhibitor of fibrinolysis, the absence of which predisposes the individual to a haemorrhagic diathesis. This is likely to have an important role in the pathogenesis of lung disease as *S. maltophilia* degrades the innate immune proteins: alpha-1 antitrypsin, secretory leukoprotease inhibitor and elafin (20).

In addition, the observed over enrichment of biological pathways involving biological adhesion indicated a possible causative role for secreted *S. maltophilia* proteases in disassembly of intercellular (tight, adherens, and gap) junctions. The cardinal work by Windhorst et al., examining the extracellular protease profile of *S. maltophilia* showed that the extracellular protease StmPR1 had significant pathological effects on fibroblasts and these effects were abrogated in the presence of the StmPR1 inhibitor chymostatin (15). Our observations using TEER measurements of CFBE41o- cell monolayers exposed to K279a CS are consistent with the suggested role of secreted extracellular proteases having a role in the pathogenesis of CF lung disease. Our conclusion is supported by several lines of evidence. Firstly, we demonstrated a significant disruption of the epithelial monolayer with morphological changes in cultured CFBE41o- cells which was abrogated in the presence of the protease inhibitor PMSF. Secondly, the TEER of CFBE41o- cells, which is higher than their non-CF counterparts, 16HBE14o- (36), was significantly reduced following treatment with K279a CS in comparison to the untreated control. Thirdly, the tight junction proteins ZO-1 and occludin but not JAM-A or claudin-1 were degraded in CFBE41o- cells following treatment with K279a CS, the effect of which was prevented by PMSF. The importance of this deleterious effect in the pathogenesis of CF has been demonstrated in other well-known CF pathogens, and disruption of epithelial barrier integrity may be one of the mechanisms inducing chronic inflammation in cystic fibrosis, similar to that observed in inflammatory bowel disease (37).

Like *S. maltophilia*, live *P. aeruginosa* is also capable of disrupting tight junctions in apically treated VA10 monolayers within 24 h of infection as measured by a gradual drop in TEER and a concomitant decrease in the expression of the tight junction protein, ZO-1 (22). *P. aeruginosa* have been shown to invade airway epithelial barriers by destroying tight junctions (38), while *Pseudomonas* elastase can disrupt the tight junction in human nasal epithelial cells by downregulating the transmembrane proteins claudin-1 and-4, occludin, and tricellulin (39). ER stress induced by *P. aeruginosa* has also been implicated as a cause of tight junction destruction in primary bronchial epithelial cells (40). More recently, a strong correlation between *in vitro* elastase

activity of clinical isolates of *P. aeruginosa* and mucosal barrier dysfunction has been demonstrated. These changes were seen in conjunction with degradation of ZO-1, occludin and β-actin and implicate *P. aeruginosa* exoproteins in the pathophysiology of *P. aeruginosa* associated chronic rhinosinusitis by severely compromising mucosal barrier structure and function (41).

Others have shown similar effects using xps mutants of K279a, a key regulatory gene of the type II secretory system from *S. maltophilia*. DuMont et al. demonstrated rounding, detachment, and death of A549 cells, an adenocarcinoma human alveolar epithelial cell line, mediated via degradation of ECM components such as type I collagen and fibronectin by the major and minor extracellular proteases StmPr1 and StmPr2 (17, 42). More recently, we and others have identified an intermediate protease, StmPR3, as an additional potential virulence factor of *S. maltophilia* (18, 20). Interestingly, StmPr3 showed xps-mediated rounding and detachment of A549 cells, as well as xps-mediated degradation of fibronectin, fibrinogen, and interleukin-8 (IL-8), similar to StmPR1 and StmPR2 (18). Additionally, purified StmPR1 has been shown to degrade the tight junction protein occludin and the basolaterally expressed adherens junction protein E-cadherin. The observed ability of StmPR1 to degrade E-cadherin within 1 h of co-incubation may in part explain the relative increased speeds of TEER reduction seen here following treatment of the basolateral compartment with K279a CS compared to the apical one (18). From a clinical perspective, continued exposure of the immunocompetent host epithelium to *S. maltophilia* proteases may induce microbleeds in the lung such as in the context of CF. However, in the immunocompromised host the inability of the host to control the virulence of the pathogen could permit access to the basolateral compartment of the pulmonary epithelium leading to pulmonary hemorrhage.

The ability of extracellular serine proteases from *S. maltophilia* to denude the airway epithelium likely confers a growth advantage for the bacterium. K279a and other clinical isolates of *S. maltophilia* have siderophore-like activity when grown at 37 °C in low-iron media and a mutation in one of the predicted biosynthesis genes (entC) impairs the production of the siderophore and reduces bacterial growth in low-iron conditions (43). The ability of *S. maltophilia* to cause microbleeds within the CF lung likely has beneficial effects for growth of the organism *in vivo* but harmful effects for the host. Degradation of host iron-containing proteins by neutrophil elastase in the CF lung is a source of iron (44) that can promote growth of *S. maltophilia* via FecA mediated transport of exogenous siderophore ferric citrate from the environment into the bacterial periplasm (45). Moreover, release of haem can be harmful to the CF patient as haem can stimulate IL-8 from CFBE41o- cells (46).

This study has a number of limitations. Firstly, K279a, the reference clinical strain of *S. maltophilia* used in this work was isolated from the blood of a cancer patient and its virulence determinants may differ from CF strains of the bacterium (47). However, there is considerable overlap in the major extracellular protease gene (StmPR1) between CF *S. maltophilia* isolates and K279a with 70% of CF-derived strains carrying the 1,621-bp allelic variant of StmPr1 present in the K279a reference genome (48). Secondly, we did not specifically examine the ability of endogenous antiproteases [e.g., alpha-1 antitrypsin (AAT)] to abrogate the effect of extracellular proteases in K279a CS on epithelial barrier integrity. Thirdly, by maintaining CFBE41o- cell monolayers in a submerged culture rather than at an air-liquid interface there may have been a dilutional effect which may have reduced the inhibitory ability of endogenous antiproteases on K279a CS. Alpha-1 antitrypsin is an endogenous inhibitor of neutrophil elastase with an extracellular pulmonary epithelial concentration ∼10% that of serum levels (49). While production of AAT and other antiproteases are normal in CF, the neutrophil elastase burden is so large that it overwhelms the normal anti–neutrophil elastase protection (50). We have recently shown that K279a CS is capable of degrading the endogenous proteases inhibitors AAT, SLPI, and elafin (20) and thus chronic colonization with *S. maltophilia* is an additional combatant to overwhelm the anti-protease armory within the CF lung. The use of aerosolised AAT is an attractive therapeutic option. Not only could it inhibit NE mediated IL-8, TNF-α, and LTB4 production (50), but it may also potentially inhibit extracellular bacterial proteases including those from *S. maltophilia*.

In conclusion, using secretomics we have provided a unique insight into the pathogenesis of *S. maltophilia* in CF lung disease. Using this data we have shown that one of the primary pathogenic mechanisms in *S. maltophilia* infection involves disruption of epithelial barrier integrity. We confirmed this by demonstrating a time dependent reduction in TEER and an increase in paracellular permeability, an effect mediated by degradation of the tight junction proteins ZO-1 and occludin. Future work to examine the relative contribution of purified StmPR1, StmPR2, and StmPR3 and mutant K279a strains lacking the aforementioned proteases will shed further light on the pathogenic potential of this emerging multi-drug resistant CF pathogen.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KM, CG, GC, ED, and NM contributed conception and design of the study. KM, GC, ED, and KW contributed to mass spectrometic analysis. KM performed the experiments, organized the data, performed the statistical analysis, and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00198/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Progress in Model Systems of Cystic Fibrosis Mucosal Inflammation to Understand Aberrant Neutrophil Activity

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In response to recurrent infection in cystic fibrosis (CF), powerful innate immune signals trigger polymorphonuclear neutrophil recruitment into the airway lumen. Exaggerated neutrophil proteolytic activity results in sustained inflammation and scarring of the airways. Consequently, neutrophils and their secretions are reliable clinical biomarkers of lung disease progression. As neutrophils are required to clear infection and yet a direct cause of airway damage, modulating adverse neutrophil activity while preserving their pathogen fighting function remains a key area of CF research. The factors that drive their pathological behavior are still under investigation, especially in early disease when aberrant neutrophil behavior first becomes evident. Here we examine the latest findings of neutrophils in pediatric CF lung disease and proposed mechanisms of their pathogenicity. Highlighted in this review are current and emerging experimental methods for assessing CF mucosal immunity and human neutrophil function in the laboratory.

Keywords: cystic fibrosis, neutrophil, inflammation, infection, model systems

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INTRODUCTION

Polymorphonuclear neutrophils are the most abundant immune cells in human blood and act as first responders to sites of infection. Their function is a key component of host defense against invading pathogens. In the autosomal recessive disorder cystic fibrosis (CF), persistent microbial colonization in the lungs induces abundant and continuous migration of neutrophils to the airways via powerful inflammatory signals of IL-6, IL-8, and leukotriene B4 (1). Recruited CF neutrophils secrete high levels of proteolytic compounds such as neutrophil elastase (NE), which damage airway tissue and highly correlate with disease severity (2, 3). Despite recognition of neutrophils in the progression of CF lung disease, mechanisms modulating their pathological role are not well-characterized. Past investigations have been hampered by a lack of widely available CF animal models and no suitable *in vitro* infection models that effectively incorporate multiple factors driving complex *in vivo* disease. New data from modern clinical studies are changing the view that neutrophils are a fixed population and are revealing a spectrum of functional phenotypes neutrophils employ to address the variety of pathogenic scenarios they encounter (4). Understanding neutrophil phenotypes and mechanisms in early CF disease, when the airway environment is less complex and more responsive to intervention, will require researchers to revisit or adapt many models of CF. With this review, we present new insights, challenges, and

considerations for researchers studying neutrophils in early CF lung disease.

PEDIATRIC CF LUNG DISEASE

The clinical picture of early life cystic fibrosis has changed significantly since CF was first identified in the mid twentieth century, when patients rarely survived to the age of 10 (5). Improvements in diagnostics, from sweat tests to genetic testing, and wider screening of newborns by immunoreactive trypsinogen, has permitted earlier detection of CF and management of the disease. Increased antibacterial therapy, mucolytic and osmotic agents, and the advent of CFTR modulators have further increased the lifespan of many CF patients to beyond 40 years of age (6). Despite these advances, signs of airway inflammation and lung damage are still evident in CF from an early age. In 2005, a pioneering study assessing bronchoalveolar lavage fluid (BALF) after CF newborn screening demonstrated that infection in the first year of life is linked to early airway inflammation (7). Subsequent surveillance studies have now established that for most children, inflammation, altered microbiome, active neutrophil proteolytic function, and lung damage all become evident within the first 2 years of life (8–11), before children are old enough to be treated with CFTR modulators (12, 13). Lung function declines can be evident in infants and continue into childhood (14–16). However, early airway disease can occur in the absence of overt respiratory symptoms (17) or infection (18, 19). Computed Tomography (CT) screening has revealed that CF associated structural changes diagnosed in early life persist into childhood and adolescence. Permanent bronchial wall thickening, or bronchiectasis, is detectable in about 8.5% of pediatric CF patients in the first year of life, and this increases to 36% by 4 years (8). In addition to cytokine release, neutrophil influx into early CF airways may be supported by chemotactic fragments from the extracellular matrix (20, 21). Overall, CF lung damage and declines in function are linked to neutrophil counts and levels of neutrophil proteases, that are often a response to early incidence of infection. Understanding the pathology of early lung disease as it appears today will be key to maximizing long-term benefits from subsequent CFTR modulator therapies.

NEUTROPHILS IN CF AIRWAYS

Neutrophil Elastase and Serine Proteases

Early in vertebrate immunity, neutrophils evolved a granule system to separately store enzymes and antimicrobial factors safely until fused with a phagosome (22). Neutrophil elastase (NE) and other serine proteases are a central component of the neutrophil antimicrobial arsenal, stored in the primary granules that are the last granule to mobilize and are highly resistant to fusion with the outer membrane (23). Yet uninhibited NE activity can be detected in over 30% of BALF samples from young children with CF (11, 24). Activity of NE is considered one of the most significant biomarkers in CF lung disease, as activity significantly correlates with lung damage and functional declines at all stages of life with CF (25–28). Unregulated activity is destructive to airway epithelial cells and the lamina propria, and can impede microbial clearance through destruction of host immune factors (29). *In vitro* and *in vivo* studies have demonstrated how elevated NE activity induces epithelial senescence in CF airway cells (30, 31), prevents epithelial repair mechanisms (24), and is a key driver of airway inflammation and mucus production (25–28). Neutrophil Elastase and other serine proteases digest a variety of host proteins, suggesting multiple mechanisms that implicate these compounds in CF airway pathology. Along with neutrophil serine proteases cathepsin G and proteinase 3, NE directly interacts with cytokines, including IL-8 and IL-1α, increasing their potency (32–34). Counter-intuitively, serine proteases also degrade antimicrobial peptides (AMPs), including lactoferrin, midkine, and surfactant protein-A (SP-A) (35–37). In particular, NE has been shown to degrade pattern recognition receptors including toll-like receptor 4 (TLR4), reducing bacterial lipopolysaccharide (LPS) sensitivity and increasing inflammation (38). In addition to modulating mucosal immunity, serine proteases may promote airway epithelial dysfunction in CF. For example, NE cleaves E-cadherin, an important component of adherens junctions, compromising epithelial integrity (39). It also induces CFTR protein degradation by calpain activity in both *in vitro* epithelial cells and *in vivo* mouse models, resulting in impaired channel function as well as increasing sodium transport into cells through proteolytic activation of sodium ion channels (ENaC) (40–42).

Cysteine Proteases, Matrix Metalloproteinases, and Reactive Oxygen Species

In addition to NE and other enzymes found in primary granules, additional neutrophil derived compounds may contribute to CF airway pathology. Crucial for intracellular degradation of pathogens, secreted cysteine proteases have similar deleterious effects as their serine counterparts. Cathepsins B and S positively correlate with clinical markers of inflammation in pediatric CF airways, including NE, IL-8, and TNFα (43, 44). They selectively maintain neutrophil influx through activation of chemokines containing glutamic acid-leucine-arginine (ELR) motifs and inactivation of lymphocyte attracting non-ELR chemokines (45). Similar to serine proteases, cathepsins can compromise immunity through degradation of AMPs such as lactoferrin, LL-37, SP-A, and β-defensins (46–49). Cathepsins B and S are also implicated in airway mucus dehydration through induction of ENaC activity (50, 51). Matrix metalloproteinases (MMPs) are additional proteases implicated in CF associated with airway remodeling following lung injury. They can originate from any tissue, but neutrophil derived MMP-9 is particularly linked to airway damage, inflammation, and lung function decline in early CF (52, 53). Furthermore, MMP-9 sustains airway neutrophilia through potentiation of IL-8 and generation of proline-glycine-proline (PGP) matrikine fragments from breakdown of collagen (21, 54). Upon phagocytosis of pathogens, neutrophils produce large amounts of superoxide radicals for microbial killing. Broadly termed reactive oxygen species (ROS),

neutrophils are among the most potent producers of these compounds (55). Oxidative stress as shown by elevated airway ROS is observed in chronic obstructive pulmonary disease (COPD) as well as CF (56–58). Increased ROS production results in destruction of antiproteases, which are crucial for protecting tissue from unregulated proteolysis (59, 60). In the context of CF, ROS may impede the function of antiproteases such as alpha-1-antitrypsin, an important NE inhibitor, prolonging airway neutrophil proteolytic activity (24, 61).

CFTR in Neutrophils

A central conundrum of CF is why proteolytic activity develops in such early, mild stages of CF lung inflammation. One obvious area of investigation has been whether neutrophil dysfunction in CF airways is exclusively influenced by factors in the lung environment or is also a consequence of inherent CFTR defects. Since the discovery of the CFTR gene, there have been studies suggesting CFTR protein is routinely expressed in cells of myeloid origin and has a role in microbial clearance within phagosomes (62–64). Hypochlorous acid (HOCl) is an important antimicrobial component of neutrophil phagosomes whose formation is proposed to be dependent on CFTR-mediated chloride ion transport (65). CFTR is reported to traffic to phagosomal membranes in peripheral blood neutrophils, with CFTR mutation resulting in defective phagosomal chlorination, affecting clearance of microbes such as *P. aeruginosa* (66–68). Contrasting findings have shown normal respiratory burst activity and production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase components in CF blood neutrophils and no detectable CFTR protein in these cells (69). Additional evidence for the role of CFTR in neutrophils comes from a small number of studies showing restoration of CF neutrophil functions including CFTR phagosomal trafficking (67), leukocyte activation (70), and intracellular ion regulation (71) in response to CFTR modulator treatment. Still, further research is needed to clarify the presence and function of CFTR in neutrophils, and how defects in the gene influence the pathological activity of CF airway neutrophils. One consistent observation is a CFTR mutation dependent effect on *in vivo* neutrophil lifespan, with CF neutrophils displaying delayed apoptosis compared to non-CF neutrophils, possibly preventing resolution of neutrophilic inflammation (72–74). The most recent of these studies demonstrated a link between delayed apoptosis by CF neutrophils and propensity to form neutrophil extracellular traps (74).

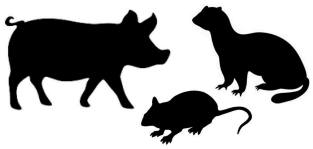
Neutrophil Extracellular Traps

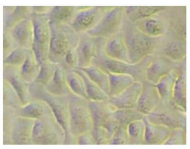
The identification of neutrophil extracellular traps (NETs), extracellular networks of DNA containing azurophilic granules, neutrophil elastase and other antimicrobial components, was a significant event in neutrophil biology (75). NET formation was initially viewed as a form of active cell death upon which nuclear and granular membranes were disintegrated, contents ejected and mixed in the cytoplasm, then released upon deterioration of the cell membrane (76). The process was later termed NETosis and proposed to be an alternative strategy used by neutrophils upon failing to clear infection via traditional phagocytosis. While NETs can trap and neutralize invading pathogens, the extent of their microbe killing abilities is debated (77, 78). A significant amount of research into NETosis has been undertaken, as recently reviewed by this journal (79). Multiple studies have described forms of NETosis that result in mitochondrial DNA release rather than nuclear DNA, or allow neutrophils to remain viable and motile after NET formation (80–83). The ability of NETs to harbor NE, the presence of NET derived DNA in CF sputum, and increased pathogen resistance in response to NETs, suggest NETosis is likely to play a role in CF lung disease (84, 85). Yet the question remains on how frequently NETosis occurs during early CF airway inflammation, prior to significant biofilm formation that reduces availability of bacteria to neutrophils.

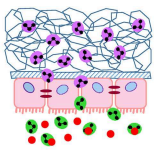
Neutrophil Exocytosis

Perhaps the most intriguing hypothesis explaining early airway neutrophil proteolytic activity is that upon recruitment to CF airways, neutrophils reprogram toward an aberrant granular-releasing, immunoregulatory, and metabolically distinct (GRIM) phenotype that includes exocytosis of primary granules—as evidenced by high CD63 expression (86–88). The GRIM phenotype is specific to recruited neutrophils as peripheral blood neutrophils from CF patients exhibit a normal phenotype (89). However, when naïve neutrophils from either CF or non-CF donors are stimulated in an *in vitro* transmigration model of neutrophil recruitment by adult CF BALF or sputum, cells from both groups of donors undergo GRIM reprogramming (89). While factors such as tumor necrosis factor-alpha (TNF-α) can prime exocytosis of neutrophil azurophilic granules (90), Forrest and colleagues observed GRIM reprogramming only upon stimulation with *ex vivo* CF samples but not with exogenously added chemokines, suggesting a yet unidentified factor in CF airways is responsible for changes in neutrophil activity (89).

Most significantly, GRIM neutrophils were also found to have reduced bacterial killing capacity, which aligns with the apparent disconnect between NE release and inability to resolve infection in CF airways (89). More recent studies have reported how Staphylococcal superantigen-like protein 13 (SSL13) from *Staphylococcus aureus*, a common early CF pathogen, can induce neutrophil exocytosis (91) and whose production is evident in the CF microbiome (92). In an age related cohort of non-CF children admitted for acute respiratory distress syndrome (ARDS), neutrophil exocytosis and reduced bacterial killing was observed in individuals co-infected with virus and bacteria but not viral infection alone, suggesting that neutrophil exocytosis may be linked to responses against polymicrobial infection (93). This relationship with infection is yet to be established in early CF disease, however neutrophil exocytosis markers correlate positively with disease severity more so than free NE activity (11). Therefore, changes in airway neutrophil functional markers may be more reliable indicators of disease progression in children with CF and should be a focus of early CF lung disease research.







	CF Animals	Primary Airway Cells	Transmigration Models
Current Applications	Observe gross CF disease phenotypes.	Assess CFTR function, effects of modulator therapy, and innate immunity of airway epithelium.	Study PMN recruitment and activity in the airways.
Advantages	Study *in vivo* disease across multiple organ systems. Depending on animal, models full to partial pathology of CF lung (infection, inflammation, mucus plugging, structural changes, etc).	Responses are measured in disease relevant, non-cancerous, human cells. Different ages, sex, and mutation types can be tested in culture systems representative of native airway tissue.	Allows close, controlled, testing and observation of human PMN in response to infectious and non-infectious stimuli.
Disadvantages	Disease phenotype and progression not wholly representative of CF in humans, limited mutation types for testing, care of animals is costly and laborious.	Results are variable depending on sampling and culture of cells among other factors. Limited by access to human donors.	Reliance on cancer cell lines to act as epithelial barrier during migration. Also limited by access to *ex vivo* BALF/sputum.
Potential Applications	Study *in vivo* PMN exocytosis and proteolytic activity? NETosis? CFTR function?	Screen early CF infection scenarios or insults that trigger PMN pathological activity?	Study coordinated CF epithelial/ PMN responses to infection?

FIGURE 1 | Comparison of model systems for studying CF lung disease.

MODELING INFECTION AND INFLAMMATION

Characterizing the early mechanisms that trigger phenotype shifts in airway neutrophils may be key for preventing progressive lung disease. Clinical surveillance gives valuable insights into disease phenotypes *in vivo*, however, basic science is crucial for understanding the biology of CF lung disease and the role of the airway epithelium. Over the years, researchers have developed a variety of approaches for this purpose (**Figure 1**). The following is a summary of some of the more important, biologically relevant models currently in use to study infection and inflammation in CF airways.

Animal Models

While CFTR mutant and knockout mice were developed shortly after discovery of the CFTR gene, their use as animal models for CF lung disease is controversial as they lack a robust CF lung phenotype of spontaneous infection and disease (94–97). Despite extensive similarity, mouse immune cells can behave differently to human counterparts in their response to pathogens, for example, murine neutrophils are not activated by SSL13 (91). Mice also express toll-like receptor 11 (TLR11), a TLR not expressed in humans, that detects profilin and bacterial flagellin (98). Still, mouse models of induced airway infection have provided insights into CF airway inflammation and disease. Studies of acute *Pseudomonas aeruginosa* infection have observed poor growth, increased mortality, and reduced bacterial clearance in CF vs. wild-type mice (99, 100). Additionally, CF mice have exaggerated levels of murine inflammatory cytokines and airway neutrophilia in response to infection, as well as prolonged inflammation compared to wild-type mice (101, 102). Most of these studies have inoculated animals through intratracheal delivery of agarose beads embedded with bacteria, an unrealistic representation of how CF patients normally acquire these organisms. Of interest has been the observation that environmental acquisition of *P. aeruginosa* can be modeled in mice through inoculated drinking water, with CF mice more susceptible to chronic colonization via this route (103). Chronic exposure of CF mice to *P. aeruginosa* LPS also results in increased airway inflammation, neutrophilia and airway remodeling (104, 105). A common theme emerging from these studies, is that neutrophils and their products play a central role in CF lung pathology. With the development of Cre recombinase mice targeting the neutrophil-specific locus Ly6G (106), future studies utilizing this model will continue to play a very useful role in elucidating CF airway neutrophil biology.

The more physiologically relevant animal model for studying CF lung disease include CFTR disrupted pigs and ferrets, as they recapitulate the CF phenotype across all organ systems implicated in human disease (107). Both models were developed just over a decade ago using adenoviral vectors, generating CFTR full or partial knockout animals in both species via exon 10 disruption, as well as a ΔF508 pig (108–110). Pigs are suitable human disease models due to their analogous physiology, and in the case of respiratory disease, similar bronchial structure and distribution of submucosal glands (111). CF pigs have CFTR protein similar to that of humans (112). Neonatal CF pigs have little airway inflammation and normal levels of IL-8 and neutrophil counts in BAL compared to non-CF pigs (109). Neonatal CF pigs also

have increased presence of microbes in the lungs as shown by culture from *ex vivo* tissue samples, and are less likely to have sterile BAL samples compared to non-CF pigs (113). In the months following birth, CF pigs develop signs of lung disease such as mucus accumulation, inflammation, infection, and airway remodeling (113). While lung disease progression in the CF pig model reflects progression in humans, there are obvious drawbacks of cost and time of pig husbandry and the need of adequate facilities and resources. Furthermore, virtually all CF pigs develop meconium ileus and require early surgical intervention; in contrast, the condition is present in only 20% of infants with CF (109, 113, 114).

Ferret CFTR protein length, amino acid sequence, and function is also similar to that of humans (115). Like CF humans, CF ferrets are prone to spontaneous airway infection; however, infection in these animals is far more severe, with CF ferrets requiring continuous antibiotic treatment immediately after birth to survive (116). Additionally, CF ferrets demonstrate abnormally high levels of lung inflammation from birth, and lung disease progresses rapidly upon cessation of prophylactic antibiotics (117, 118). As a result, CF ferrets may not be an ideal system to model the slow progressive lung disease observed in humans, as their disease phenotype develops too quickly. However, a recent study developed homozygous $CFTR^{G551D/G551D}$ ferrets to test effects of *in utero* treatment with VX-770 (ivacaftor) (119). Prenatal and early postnatal administration ameliorated CF multi-organ disease, posing new research questions around CFTR in early development, the possibility of prenatal modulator therapy, and disease attenuation in CF animals to further study effects of modulator treatment or model mild disease in humans. While neutrophil counts and elastase activity in CF animals trend similarly to human disease, neutrophil reprogramming has yet to be evaluated. Future studies must assess airway neutrophil exocytosis and lung disease severity in CF animals to determine if they are suitable models for characterizing this process in humans.

Primary Airway Epithelial Cells

While animal models allow observation of gross phenotype of disease, *in vitro* studies permit experimentation in a highly controlled environment and are important for understanding mechanisms of disease at the cellular level. The accepted gold standard for *in vitro* CF research are patient derived primary airway epithelial cells (pAEC). As a barrier that protects the lung from direct environmental exposure, the airway epithelium has long been recognized for its role in host defense and respiratory disease (120–125). Cells are typically isolated from epithelial brushings of the nose or lower airways, or less frequently from explanted lungs (126, 127). Yields from brushings are variable and *ex vivo* pAEC have limited proliferative capacity; they become senescent after only a few passages making them difficult to expand in culture (126, 127). The adaptation of conditionally reprogrammed airway epithelial cells (CRAEC) through co-culture with irradiated fibroblast feeder cells has significantly increased passage number capacity of pAEC, while maintaining lineage specific characteristics (128). Additionally, CRAEC can be seeded from co-culture into air-liquid interface culture (ALI) to form a differentiated pseudostratified epithelial layer (128, 129). This has enabled many CF research groups to look to CF primary airway epithelial cell models in order to understand the cellular drivers of progressive lung disease, and more recently to evaluate the efficacy of CFTR modulators in restoring CFTR function in target cells (130, 131). Nasal pAEC are increasingly being used in epithelial CFTR studies, since their growth, differentiation, CFTR activity, and response to modulators are similar to lower airway cells, and have the advantage of being more readily accessible (129). Nasal cells have also been adapted to three dimensional spheroid cultures that are representative of native epithelium and mature more quickly than traditional ALI cultures (132). These spheroids have then been used to quantify CFTR function via spheroid swelling in cultures from CF patients across different mutation classes, to assess individual responses to modulator treatment (133, 134). As such, they have potential as a preclinical screening tool to identify responses to modulator therapies in a personalized medicine approach.

Despite increased airway inflammation in CF patients, there is still debate as to whether the CF airway epithelium is inherently pro-inflammatory (135–137). Baseline expression of neutrophil chemoattractants including IL-8, IL-6, and IL-1β is reported in some studies to be similar in CF vs. non-CF pAEC (138–140), but others report increases in CF cells at baseline (141, 142). Increased airway inflammation could also be a result of dysfunctional CF epithelial innate immunity, a major topic in CF research, as the airway epithelium has an important role in responding to infection and neutrophil recruitment (143–147). Studies have shown IL-8 release and NF-κB activity are increased in CF vs. non-CF pAEC following *P. aeruginosa* infection (142, 148, 149). CF pAEC have also been shown to display differential gene expression at the transcriptional level compared to non-CF pAEC in response to *P. aeruginosa* infection, which may be further evidence of CF aberrant immune responses (150, 151). In response to infection with respiratory viruses, studies have also observed increased IL-8 production in CF vs. non-CF pAEC (152, 153), which is analogous to *in vivo* findings in pediatric CF patients with rhinovirus infection (154). However, other studies have reported no difference in inflammatory cytokine production as a result of *in vitro* viral infection (155, 156). The filamentous fungi *Aspergillus fumigatus* is emerging as an important early life CF pathogen increasingly detected in pediatric CF airways (157, 158), with *A. fumigatus* infection associated with increased air trapping among 5 year old CF patients (159). Two *in vitro* studies to date have used immortalized cell lines and reported altered cytokine production, though specific data were conflicting (160, 161). Assessing innate immune responses to fungal pathogens in CF will be key to determining treatment priority, but innate immune mechanisms have yet to be corroborated in CF pAEC.

Variability amongst findings from pAEC infection studies could be attributed to the originating cohorts, sampling differences, age and disease severity of subjects, culture methods, and use of differentiated vs. undifferentiated cultures. Additionally, the selection of pathogens can affect outcomes, as pAEC responses can be heterogeneous to individual strains or isolates of the same species (162). Primary cells will continue

to be important tools for addressing unanswered questions in CF lung disease, including how epithelial immunity is linked to neutrophil inflammation, how the epithelium responds to fungal and polymicrobial infection, how the airway microbiome affects epithelial homeostasis, and whether CFTR modulators have effects on airway innate immune signaling. Researchers must think carefully about the above factors and how they influence experimental outcomes in pAEC, especially if findings are translated to lung disease pathogenesis in CF patients.

Neutrophil Transmigration to the Airways

The epithelium is not only a barrier to external pathogens, but also presents an obstacle to responding neutrophils. *In vitro* replication of this mucosal physiology was established by early studies assessing neutrophil transmigration across the intestinal mucosa (163–166). These studies established polarized epithelial cultures onto inverted permeable inserts, which were turned over prior to migration for direct loading of naïve isolated neutrophils and thus model basolateral to apical neutrophil migration. It has since been adapted to characterize transmigration across lung epithelium (167), assess the role of neutrophils in β-catenin mediated airway epithelial repair (168, 169), as well as describe responses to infection with respiratory syncytial virus (RSV) and *P. aeruginosa* (170–172). The model previously mentioned in this review uses Alvetex™ 3D scaffolds rather than permeable membranes, which better replicate neutrophil swarming (89, 93), to study neutrophil responses to CF sputum. To understand factors driving early neutrophil fate including exocytosis, this same model could be applied with pediatric BALF. However, pediatric *ex vivo* samples are difficult to obtain, often of limited volume compared to samples from adults. One approach yet to be fully utilized is to apply material from infected CF pAEC as surrogates for human samples. This has multiple benefits. Robust models of pAEC infection responses are well-established and because pAEC can be bio-banked for downstream culture and infection, material can be generated as needed. This approach also facilitates a more focused assessment of factors influencing neutrophil functions, such as epithelial responses to specific infection scenarios.

One caveat of past transmigration studies is the dependence on lung cancer derived cells lines, such as A549, H292, H441, 16HBE, and Calu-3 cells, as a substitute for primary cell derived epithelium. Advantages include easy access to cell lines and robust growth in culture, but at the cost of interpreting epithelial responses during migration through cancer cells. This is critical, since neutrophils directly change airway epithelium via microRNA (173) or exosomes (174) and neoplastic cells may not reproduce CF pAEC responses. As limitations on pAEC culture expansion are overcome, transmigration studies are increasingly incorporating pAEC (175). A remaining challenge is that established methods for differentiating pAEC traditionally employ 0.4 μm pore size inserts, but a 3.0μm pore size or larger is required to permit neutrophil transmigration, which can result in significant loss of primary cells during seeding. One study has managed to address this issue by coating both faces of a transwell insert with extracellular matrix and providing seeded cells with laminins to improve attachment (175). Primary cells differentiated into pseudostratified epithelial layers on a 3.0 μm insert, similar to how they would on a conventional 0.4 μm transwell insert, and permitted neutrophil transmigration upon apical infection with *P. aeruginosa* (175). Future integration of CF pAEC in models of neutrophil transmigration will be required for studying coordinated immune responses of the CF airway

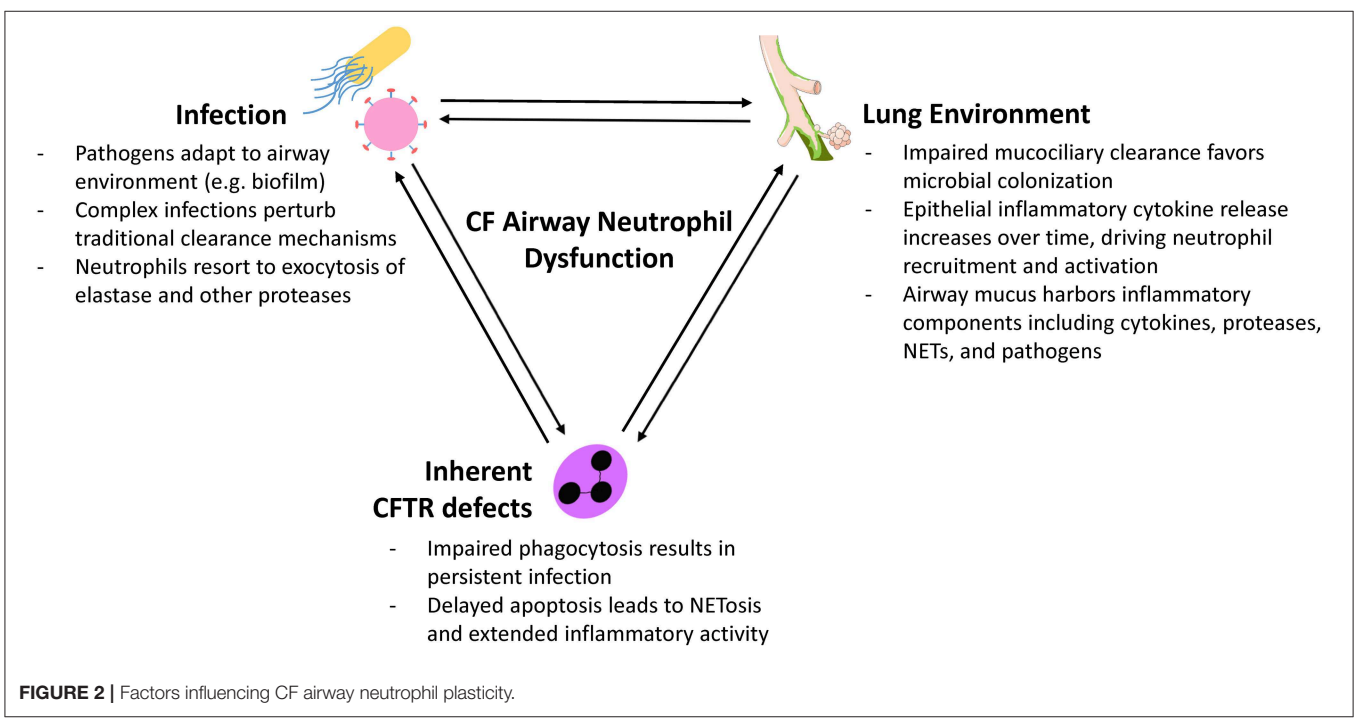


FIGURE 2 | Factors influencing CF airway neutrophil plasticity.

epithelium and recruited neutrophils in a single translational system. If designed with high throughput screening in mind, there is great potential to facilitate much needed pre-clinical testing of anti-inflammatory drugs in CF.

CFTR MODULATOR THERAPY AND AIRWAY INFLAMMATION

Depending on their mechanism of action, CFTR modulators are characterized as correctors that improve defective CFTR trafficking to the cell surface, or potentiators that enhance defective CFTR function. Studies of modulators have shown improvements in patients as measured by sweat chloride levels and FEV_1, however, efficacy against infection and airway inflammation is poorly investigated. In placebo controlled studies of lumacaftor-ivacaftor and tezacaftor-ivacaftor in CF patients $\geq$12 years of age, infective pulmonary exacerbations occurred at similar rates in both treatment and placebo groups (176–178). Phase 3 trials of lumacaftor-ivacaftor in CF patients aged 6–11 also found that incidence of infection associated pulmonary exacerbations was similar between patients receiving treatment (18%) and patients receiving a placebo control (19%) (179). The recently FDA approved elexacaftor-tezacaftor-ivacaftor triple therapy roughly halves the incidence of infective pulmonary excacerbations compared to a placebo (180), but the drug is not yet approved for patients under 12 years of age. Multiple studies have shown that administration of modulators reduces bacterial colonization within the first year of treatment and delays acquisition in uncolonized patients; however, bacterial isolates present in the airways prior to treatment persist and may eventually rebound over longer periods (12, 13, 181, 182). Whether CFTR modulators reduce levels of inflammatory cytokines is still not certain, as there is evidence of both reduction and no effect on clinically relevant biomarkers including NE (12, 13). Altogether, current findings suggest that modulator therapy alone may not be sufficient to manage infection and airway inflammation in this population, especially over the long term.

CONCLUSION

Neutrophils have a major role in CF lung disease but our ability to treat the underlying mechanisms is still limited. Modern approaches are revealing new perspectives on neutrophils as plastic, programmable drivers of airway disease who both respond to and actively shape the local airway environment (**Figure 2**). These novel neutrophil functions are occurring even in mild and largely asymptomatic pediatric CF lung disease and precede structural lung changes. Even with the advent of combination CFTR modulator therapy, which improves lung function but perhaps not infection and inflammation, continued investigation of initial neutrophil pathological activity is necessary to identify much-needed interventions that can address this problem. Researchers now have available a diverse number of tools for understanding the complex interplay between infection, the airway epithelium, and recruited neutrophils (**Figure 1**). Moving forward, basic studies will need to consider the advantages of various approaches, caveats, and carefully select appropriate models when exploring the beginnings of CF airway neutrophilic disease.

AUTHOR CONTRIBUTIONS

DL, LG, and AK conceived the review. DL and LG conducted literature review and wrote the manuscript. LG and AK provided critical review.

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Conflict of Interest: LG and AK are co-investigators with some of the authors cited within the review.

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Rhinovirus Infection Is Associated With Airway Epithelial Cell Necrosis and Inflammation via Interleukin-1 in Young Children With Cystic Fibrosis

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Introduction: The responses of cystic fibrosis (CF) airway epithelial cells (AEC) to rhinovirus (RV) infection are likely to contribute to early pathobiology of lung disease with increased neutrophilic inflammation and lower apoptosis reported. Necrosis of AEC resulting in airway inflammation driven by IL-1 signaling is a characteristic finding in CF detectable in airways of young children. Being the most common early-life infection, RV-induced epithelial necrosis may contribute to early neutrophilic inflammation in CF via IL-1 signaling. As little is known about IL-1 and biology of CF lung disease, this study assessed cellular and pro-inflammatory responses of CF and non-CF AEC following RV infection, with the hypothesis that RV infection drives epithelial necrosis and IL-1 driven inflammation.

Methods: Primary AEC obtained from children with ($n = 6$) and without CF ($n = 6$) were infected with RV (MOI 3) for 24 h and viable, necrotic and apoptotic events quantified via flow cytometry using a seven-step gating strategy (% total events). IL-1α, IL-1β, IL-1Ra, IL-8, CXCL10, CCL5, IFN-β, IL-28A, IL-28B, and IL-29 were also measured in cell culture supernatants (pg/mL).

Results: RV infection reduced viable events in non-CF AEC ($p < 0.05$), increased necrotic events in non-CF and CF AEC ($p < 0.05$) and increased apoptotic events in non-CF AEC ($p < 0.05$). Infection induced IL-1α and IL-1β production in both phenotypes ($p < 0.05$) but only correlated with necrosis (IL-1α: $r = 0.80$; IL-1β: $r = 0.77$; $p < 0.0001$) in CF AEC. RV infection also increased IL-1Ra in non-CF and CF AEC ($p < 0.05$), although significantly more in non-CF AEC ($p < 0.05$). Finally, infection stimulated IL-8 production in non-CF and CF AEC ($p < 0.05$) and correlated with IL-1α ($r = 0.63$ & $r = 0.74$ respectively; $p < 0.0001$).

Conclusions: This study found RV infection drives necrotic cell death in CF AEC. Furthermore, RV induced IL-1 strongly correlated with necrotic cell death in these cells. As IL-1R signaling drives airway neutrophilia and mucin production, these observations suggest RV infection early in life may exacerbate inflammation and mucin accumulation driving early CF lung disease. Since IL-1R can be targeted therapeutically with IL-1Ra, these data suggest a new anti-inflammatory therapeutic approach targeting downstream effects of IL-1R signaling to mitigate viral-induced, muco-inflammatory triggers of early lung disease.

Keywords: cystic fibrosis, airway epithelium, rhinovirus, interleukin-1, necrosis

INTRODUCTION

Cystic Fibrosis (CF) lung disease is progressive, evolves within the first months of life, and is characterized by mucus obstruction and inflammation observable on CT even in the absence of clinical symptoms and often in the absence of detectable respiratory infection (1, 2). Neutrophilic inflammation is a key risk factor for airway disease resulting in bronchiectasis and loss of lung function (3). However, the link between mucus obstruction and airway inflammation has not yet been clearly identified.

Recent evidence from the Australian Respiratory Early Surveillance Team for CF (AREST CF) implicates mucin accumulation as the initial trigger of neutrophilic inflammation in the CF airway (4), and suggests respiratory viral infection may trigger the muco-inflammatory phenotype observed in CF since the heterogeneity of early CF lung disease mirrors the heterogeneity of childhood viral infection (5, 6). Human rhinovirus (RV) appears to be able to manipulate host responses switching from apoptotic to necrotic cell death in airway epithelial cells (AEC) (7, 8). Studies investigating non-bacterial inflammation in the CF airway microenvironment have linked interleukin (IL)-1R signaling driven by IL-1α released from necrotic AEC to neutrophilic inflammation (9, 10). As RV is the most common early life viral infection observed in children with CF (11) and IL-1R signaling has already been detected in the airways of young children with mild disease (12), we hypothesize that resultant neutrophilic inflammation may be driven via this signaling pathway triggered by RV-induced AEC necrosis. However, this proposed mechanism has yet to be investigated.

Given our previous observations of defective responses to RV (8) and IL-1 driven inflammatory responses to necrosis in the pediatric CF airway (12), this study aimed to investigate the direct relationship between RV infection, the type of induced cell death, and IL-1R-driven inflammation *in vitro* using primary AEC from infants and young children with CF. We obtained primary AEC from young children with and without CF and assessed viable, necrotic and apoptotic events following RV infection utilizing flow cytometry. Using experimental supernatants; IL-1α, IL-1β, IL-1Ra, sIL-1R2, IL-8, CXCL10, CCL5, IFN-β, IL-28A, IL-28B, and IL-29 were measured and subsequently correlated to viable, necrotic and apoptotic responses.

MATERIALS AND METHODS

Please also refer to the **Supplementary Data** for full details.

Study Population and Establishment of Primary Cell Culture

This study was approved by the relevant institutional Human Ethics Committees with written consent obtained from parents or guardians. This study included samples from six clinically stable infants and children with CF (mean age 2.9 ± 1.8 years old; **Table 1**) participating in the AREST CF early surveillance program (2), and samples from six children without CF (mean age 3.8 ± 1.9 years old; **Table 1**) recruited upon admission to hospital for elective non-respiratory related surgery. Cystic fibrosis transmembrane conductance regulator (CFTR) genotype was determined as part of newborn screening (**Table 1**). Current bacterial infection in CF samples was determined as part of standard clinical practice using gold-standard microbiological screening, with previous infection the presence of a bacterial infection at any previous visit. Prior wheeze was determined by parent-reported wheeze in the three-months prior to recruitment. Children without CF had no respiratory symptoms observed at time of recruitment. Samples were attained by brushing of the tracheal mucosa of children with a single-sheathed nylon bronchial cytology brush as previously described (8, 13). After collection, primary AEC cultures were established as previously described (14).

TABLE 1 | Demographics of the study population.

	Non-CF	CF
Number of subjects	6	6
Age (mean ± standard deviation)	3.52 ± 1.6 years	3.16 ± 0.98 years
Sex (% Male)	66.6%	50%
Prior wheeze status (% Wheeze)	50%	0%
Genotype (% Phe.508del homozygous)	N/A	83.3%
Current bacterial infection	N/A	50%
Previous bacterial infection	N/A	33.3%
Neutrophil elastase presence	N/A	33.3%

Human RV Infection

Human rhinovirus 1b (RV1b) was propagated as previously described (15). To simulate an acute RV infection *in vitro*, primary AEC were infected with ∼2.95 × 10^5 $TCID_{50}$/mL. To ensure responses were due to actively replicating virus, controls were exposed to an UV-inactivated RV1b at the same $TCID_{50}$ as previously described (16). After 24- and 48-h cells were collected for analysis via flow cytometry and supernatant collected for cytokine measurement. As the peak concentration of RV viral load following infection is observed 24 h post-infection (17, 18), this timepoint was chosen for analysis. Viral load was assessed via qPCR as previously described (19). Infection with RV1b induced typical viral cytokine production from both non-CF and CF AEC (**Table S1**). Data from 48 h of RV1b infection is presented in the **Supplementary Data**.

Flow Cytometry

A flow cytometry methodology to measure cell death and disassembly was adapted for use with AEC (20). Briefly, primary cells were detached from culture surfaces via gentle trypsinization, combined with cells obtained from supernatant following centrifugation, and resuspended at a concentration of 10^6 cells/mL in annexin binding buffer (ThermoFisher Scientific, Scoresby, VIC, Australia). Tubes containing 100 μL of cell suspension were stained for 15 min with 100 μL of Annexin V/AlexaFluor488 (ThermoFisher Scientific, Scoresby, VIC, Australia) (1:40 v/v) and TO-PRO-3 (10 μM final concentration) (ThermoFisher Scientific, Scoresby, VIC, Australia) in annexin binding buffer and flow cytometry performed via a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of 20,000 events were recorded during acquisition for each sample. Analysis was performed using FlowJo software v10.4 (FlowJo LLC, Ashland, OR, USA) using a seven-step gating strategy to separate events into viable, necrotic, A5+ apoptotic, A5- apoptotic, apoptotic bodies and cellular debris as previously described (20) (**Figure S1**). Cutoffs used for positive forward scatter (FSC) and side scatter (SSC) were 50 k. Events were grouped into "viable", "necrotic", and "apoptotic" for further analysis. Data are presented as percentage of total events (% total).

Cytokine Measurement

Interleukin (IL)-1α, IL-1β, and interferon- beta (IFN-β) protein production was determined using commercially available AlphaLISA kits (Perkin Elmer, Waltham, MA, USA) in cell-free culture supernatant. Similarly, IL-8 (BD Biosciences, San Diego, CA, USA), IL-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor 2 (sIL-1R2), C-X-C motif chemokine 10 (CXCL10), Chemokine (C-C motif) ligand 5 (CCL5), IL-28A, IL-28B, and IL-29 protein production (R&D Systems, Minneapolis, MN, USA) were all determined using commercially available ELISA kits performed according to manufacturer's instructions. Samples below the detection range were arbitrarily reported as half the lower limit and included in the analysis with all other samples as previously described (21).

Statistical Analysis

Data were analyzed using GraphPad Prism v7.04 (GraphPad Software, La Jolla, CA, USA). Data were natural log transformed where appropriate. Comparisons between paired data were performed using Wilcoxon matched pairs signed rank test and Friedman's test with Dunn's multiple comparisons test presented as mean ± standard deviation. Comparisons between unpaired data were performed using Mann-Whitney tests presented as mean ± standard deviation. Associations between flow cytometry events and cytokines measured were assessed using Spearman's rank-order correlations. A two tailed P value < 0.05 was considered statistically significant.

RESULTS

Demographic data for the study populations are summarized in **Table 1**. Sex and age were similar between cohorts, with most children with CF homozygous for the p.Phe508del mutation. Infection with RV1b resulted in increased rhinovirus load measured via qPCR compared to UV-inactivated RV1b (31.3 ± 29.8 copy #/ng RNA vs. 2.37 × 10^7 ± 1.46 × 10^7 copy #/ng RNA; $p < 0.05$), increased typical pro-inflammatory viral cytokines CXCL10 and CCL5 (**Figure S2**), and type I and III interferon responses (**Figures S3, S4**).

Rhinovirus Infection Increases Necrosis but Not Apoptosis in CF AEC

To determine the cellular response to rhinovirus infection, we measured viable, necrotic, and apoptotic events in non-CF ($n = 6$) and CF ($n = 6$) AEC (**Figure 1**). Infection with RV1b resulted in reduced viable events in non-CF AEC (57.6 ± 9.8% vs. 35.4 ± 9.8%; $p < 0.05$) and CF AEC (65.1 ± 17.5% vs. 49.8 ± 19.4%; $p < 0.05$) (**Figure 1A**), and significantly elevated necrotic events in non-CF AEC (8.7 ± 2.3% vs. 12.6 ± 4.9%; $p < 0.05$) and CF AEC (8.5 ± 1.8% vs. 11.8 ± 3.5%; $p < 0.05$) (**Figure 1B**). RV1b infection significantly increased apoptotic events in non-CF AEC (28.8 ± 8.5% vs. 36.9 ± 6.1%; $p < 0.05$), however, this was not observed for CF AEC (23.5 ± 16.6% vs. 26.2 ± 11.9%) (**Figure 1C**). Similarly, infection with RV1b for 48 h decreased viable events, increased necrotic events, and increased apoptotic events in both non-CF and CF AEC (**Figure S5**).

IL-1α and IL-1β Are Increased in Supernatant and Correlate With Cell Death Following Rhinovirus Infection

We next investigated the role of IL-1 signaling in the inflammatory response following rhinovirus-induced cell death *in vitro* by measuring IL-1α and IL-1β protein following RV1b infection and correlated these with viable, necrotic, and apoptotic events in non-CF and CF AEC (**Figure 2**). Infection with RV1b increased IL-1α in non-CF (61.6 ± 31.7 pg/mL vs. 511 ± 252 pg/mL; $p < 0.05$) and CF AEC supernatant compared to controls (46.2 ± 32.7 pg/mL vs. 236 ± 93.1 pg/mL; $p < 0.05$) (**Figure 2A**). IL-1α was higher in supernatant from non-CF AEC when compared to CF AEC ($p < 0.05$). Similarly, IL-1β protein was significantly elevated post infection in both

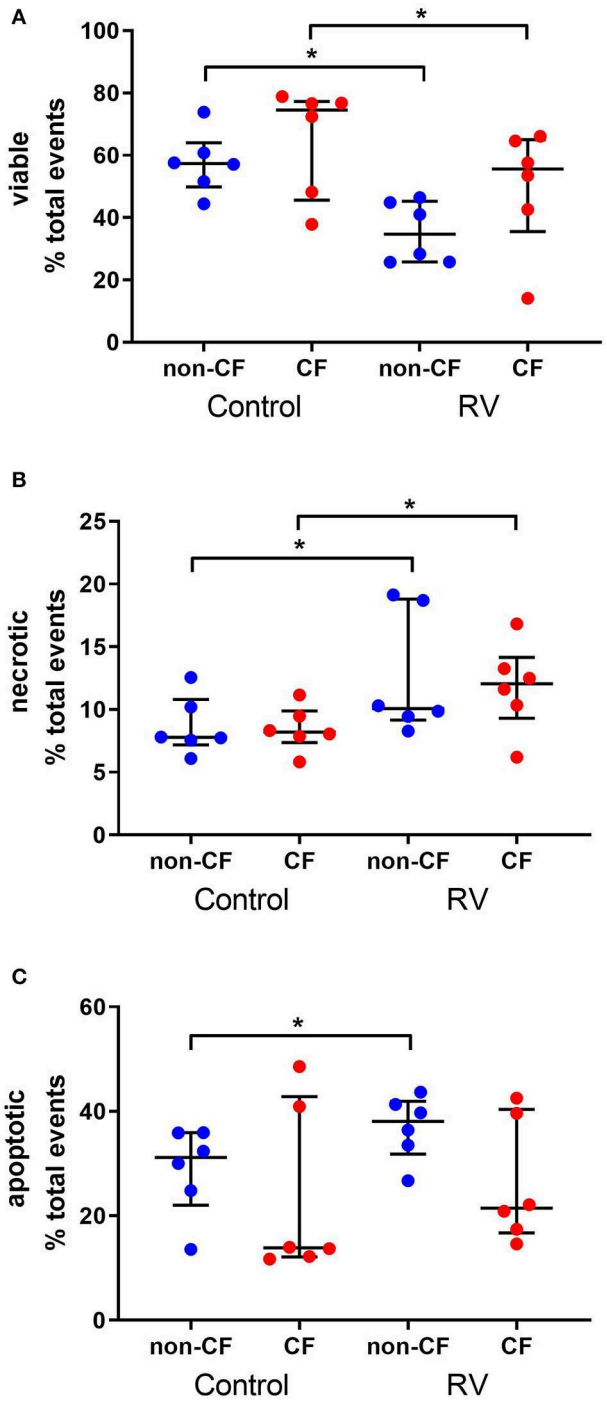

FIGURE 1 | Rhinovirus infection of non-CF and CF AEC decreases viable events, increases necrotic events, and increases apoptotic events in non-CF AEC only. Non-CF ($n = 6$) and CF ($n = 6$) AEC infected with RV1b for 24 h were assessed for changes in viable **(A)**, necrotic **(B)**, and apoptotic **(C)** events measured via flow cytometry. Infection with RV1b for 24 h resulted in **(A)** decreased viable events in non-CF and CF AEC compared to controls, **(B)** increased necrotic events in non-CF and CF AEC compared to controls, and **(C)** increased apoptotic events in non-CF AEC compared to controls. *$p <$ 0.05.

non-CF (4.4 ± 2.3 pg/mL vs. 20.9 ± 9.9 pg/mL; $p < 0.05$) and CF AEC (3.9 ± 3.6 pg/mL vs. 24.2 ± 18.7 pg/mL; $p <$ 0.05) (**Figure 2B**) supernatant when compared to controls. Upon analysis, IL-1α was found to be negatively correlated with viable events measured in non-CF AEC only ($r = -0.63$, $p < 0.0001$), positively correlated with necrotic events measured in CF AEC ($r = 0.80$, $p < 0.0001$), as well as apoptotic events measured in non-CF ($r = 0.47$, $p = 0.0011$) (**Figures 3A–C**). Similarly, IL-1β was negatively correlated with viable events measured in non-CF ($r = -0.47$, $p = 0.0029$), strongly positively correlated with necrotic events measured in CF AEC ($r = 0.77$, $p < 0.0001$). A weak correlation was also observed between IL-1β and apoptotic events measured in non-CF AEC only ($r = 0.37$, $p < 0.05$) (**Figures 3D–F**). Infection with RV1b for 48 h produced similar responses, with increased IL-1α and IL-1β following infection (**Figure S6**) significantly associated with necrotic events only in CF AEC, but with apoptotic events in non-CF and CF AEC (**Figure S7**).

IL-1Ra Is Increased in Supernatant Following Rhinovirus Infection

Since we observed differential responses in IL-1 signaling, we next assessed IL-1R regulatory protein expression, namely IL-1Ra and sIL-1R2, by non-CF and CF AEC following infection with RV1b (**Figure 4**). Rhinovirus infection resulted in increased IL-1Ra production from non-CF (1368.2 ± 205.6 pg/mL vs. 8149.0 ± 3013.1 pg/mL; $p < 0.05$) and CF AEC (1930.4 ± 870.4 pg/mL vs. 5334.1 ± 1425.4 pg/mL; $p < 0.05$) compared to control, with significantly higher IL-1Ra observed in non-CF AEC after infection compared to CF AEC ($p < 0.05$) (**Figure 4A**). There was no difference in sIL-1R2 protein production between non-CF or CF AEC, however, sIL-1R2 was significantly induced after infection in CF AEC when compared to non-CF AEC (16.5 ± 2.1 pg/mL vs. 49.8 ± 38.1 pg/mL; $p < 0.05$) (**Figure 4B**). Similarly, infection with RV1b for 48 h increased IL-1Ra but not sIL-1R2 production in both non-CF and CF AEC (**Figure S8**).

IL-8 Is Increased in Supernatant and Associated With IL-1α and Necrotic Events Following Rhinovirus Infection

We next measured inflammation downstream of IL-1R activation by measuring levels of the main neutrophil chemoattractant, IL-8, by non-CF and CF AEC following RV1b infection. Viral infection resulted in a significant increase in IL-8 protein by both non-CF (4890.5 ± 2426.7 pg/mL vs. 15656.4 ± 4102.1 pg/mL; $p < 0.05$) and CF AEC (3915.3 ± 1262.1 pg/mL vs. 8762.8 ± 3919.0 pg/mL; $p < 0.01$) compared to relevant controls (**Figure 4C**), with significantly higher IL-8 produced by non-CF AEC compared to CF AEC ($p < 0.05$). After infection for 48 h, IL-8 was significantly increased in non-CF and CF AEC (**Figure S9A**). When analyzed for associations with IL-1 signaling and cell death, IL-8 was positively correlated with necrotic events in non-CF and CF AEC ($r = 0.35$, $p < 0.05$ and $r = 0.60$, $p =$ 0.0001 respectively) (**Figure 4D**), and IL-1α in non-CF and CF AEC ($r = 0.63$ & $r = 0.74$ respectively; $p < 0.0001$) (**Figure 4E**).

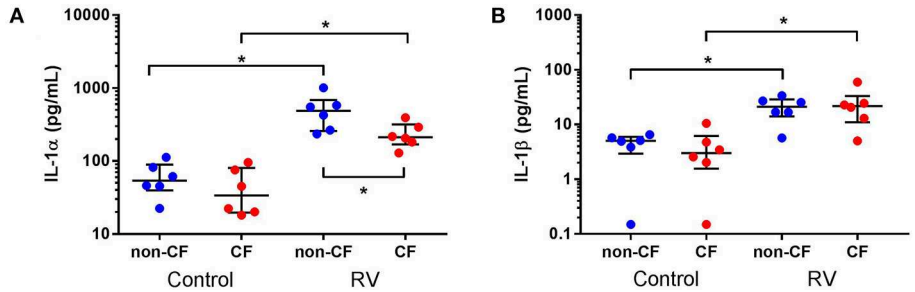

FIGURE 2 | IL-1α and IL-1β is increased in supernatant from non-CF and CF AEC following rhinovirus infection. Supernatant from non-CF ($n = 6$) and CF ($n = 6$) AEC infected with RV1b at for 24 h was assessed for levels of IL-1α and IL-1β protein. Infection with RV1b for 24 h resulted in **(A)** increased IL-1α from non-CF and CF AEC compared to control, with higher levels in non-CF supernatant compared to CF supernatant post-infection, and **(B)** increased IL-1β from non-CF and CF AEC compared to controls. *$p < 0.05$.

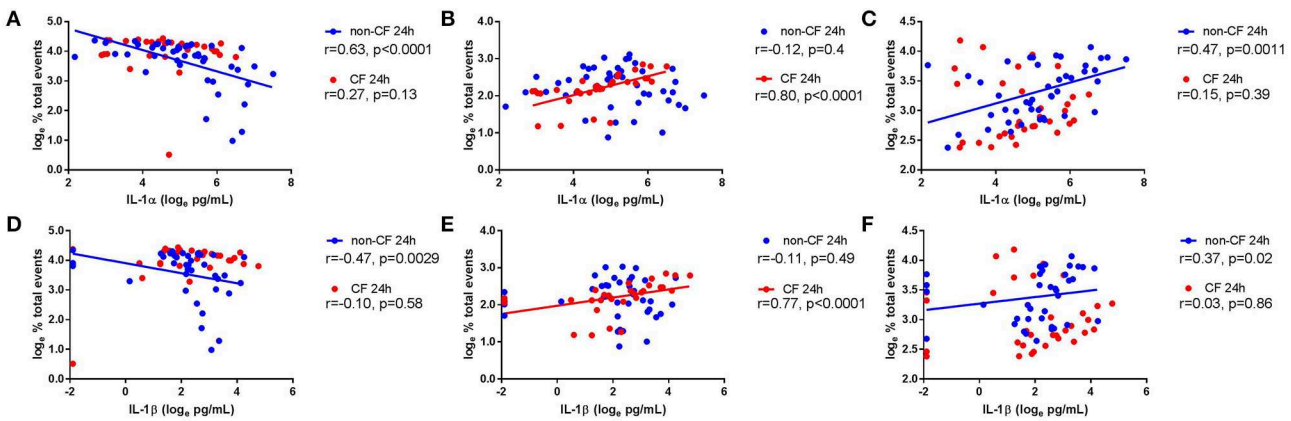

FIGURE 3 | IL-1α and IL-1β in supernatant are associated with necrotic events in CF AEC but not non-CF AEC following 24 h of rhinovirus infection. IL-1α and IL-1β protein in supernatant from non-CF ($n = 44$) and CF ($n = 32$) AEC following RV1b infection for 24 h were assessed for correlations with the corresponding changes in viable, necrotic and apoptotic events measured via flow cytometry. IL-1α protein in supernatant was **(A)** significantly correlated with decreased viable events in non-CF AEC but not CF AEC, **(B)** significantly correlated with increased necrotic events in CF AEC but not non-CF AEC, and **(C)** significantly correlated with increased apoptotic events in non-CF AEC but not CF AEC. Similarly, IL-1β protein in supernatant was **(D)** significantly correlated with decreased viable events in non-CF AEC but not CF AEC, **(E)** significantly correlated with increased necrotic events in CF AEC but not non-CF AEC, and **(F)** significantly correlated with increased apoptotic events in non-CF AEC but not CF AEC.

Similar responses were observed following 48 h of infection, with significant associations between IL-8 and IL-1α and necrotic events in non-CF and CF AEC (**Figures S9B,C**).

DISCUSSION

Our previous work demonstrated a defective response of CF AEC to RV infection (8), and an inflammatory response to epithelial necrosis in CF driven by IL-1R signaling (9) that is already detectable in the airways of infants and children with CF in the absence of bacterial infection (12). In the current study, we add to these earlier findings by conducting a series of *in vitro* experiments on AEC from children with and without CF focusing on the response of the epithelium to RV infection. Utilizing flow cytometry we observed increased necrosis in CF AEC associated with IL-1R signaling, but increased apoptosis in non-CF AEC associated with IL-1R signaling. When we assessed the IL-1 receptor antagonist IL-1Ra, we found that RV induced IL-Ra production in both phenotypes however this was significantly higher in non-CF AEC. This corresponded with increased IL-8 following RV infection that was significantly higher in non-CF AEC. Furthermore, production of IL-8 was associated with IL-1α and epithelial necrosis in non-CF and CF AEC.

This study provides several novel insights into the mechanisms surrounding pro-inflammatory responses and cell death following RV infection in the CF airway. Our data shows RV infection directly increases necrotic events in both non-CF and CF AEC supporting previous data where rhinovirus protease 3C increased necrosis in nasal AEC (7). The lack of apoptosis in CF AEC supports previous work in our laboratory where dampened apoptosis was observed following RV infection (8). This study supports data suggesting RV infection drives lytic cell death (7), potentially responsible for the increased viral load observed in CF (8, 22).

Delayed apoptosis was also observed in CF AEC following RV infection in this study. Defective apoptotic responses have

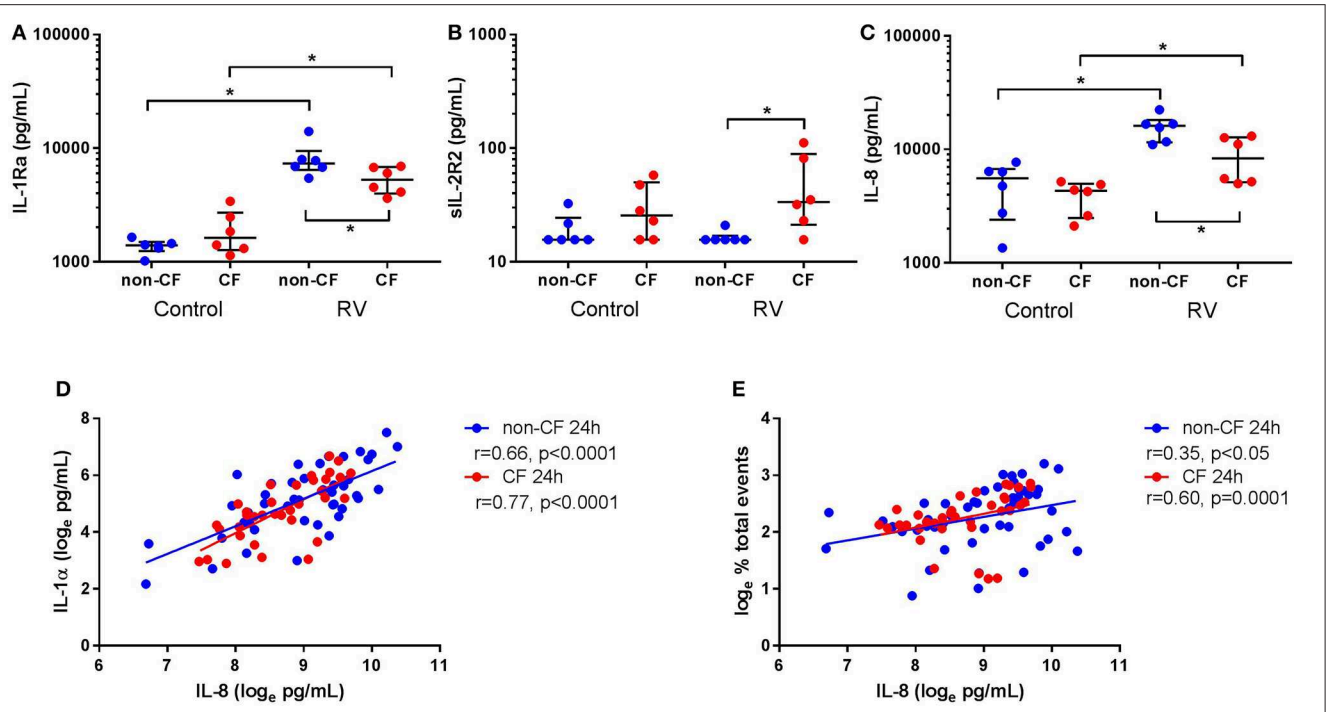

FIGURE 4 | Rhinovirus infection increases IL-1Ra and IL-8 signaling in supernatant from non-CF and CF AEC. Supernatant from non-CF ($n = 6$) and CF ($n = 6$) AEC infected with RV1b for 24 h was assessed for levels of IL-1Ra, sIL-1R2, IL-8 protein, and correlations between IL-8 and IL-1α protein or necrotic events measured via flow cytometry. Infection with RV1b for 24 h resulted in **(A)** increased IL-1Ra from non-CF and CF AEC compared to control, with significantly higher IL-1Ra in supernatant from non-CF AEC compared to CF AEC, **(B)** no change in sIL-1R2 from non-CF and CF AEC compared to control, but significantly higher sIL-1R2 after infection in supernatant from CF AEC compared to non-CF AEC, and **(C)** increased IL-8 in supernatant from non-CF and CF AEC compared to control, with significantly higher IL-8 in supernatant from non-CF AEC compared to CF AEC which **(D)** significantly correlated with IL-1α levels in supernatant from non-CF and CF AEC and **(E)** significantly correlated with increased necrotic events in both non-CF and CF AEC. *$p < 0.05$.

been observed in AEC and neutrophils in CF (8, 23, 24), we hypothesize reported accumulation of apoptotic cells in the CF airway may be suggestive evidence of defective efferocytosis (25, 26). Cleavage of the phosphatidylserine receptor by neutrophil elastase specifically disrupts phagocytosis of apoptotic cells (26, 27) and as free neutrophil elastase is increased in the CF airway (28, 29), it may explain the reduced apoptotic response and defective efferocytosis observed in the CF airway. Additionally, as suggested by the data in this study, a delayed apoptotic response following RV infection of AEC may also contribute to the defective apoptosis and increased viral load observed in CF (8, 22). The study by Vandivier et al. also found evidence of secondary necrosis following delayed apoptosis, potentially further exacerbating inflammation in the airway via release of DAMPs such as IL-1 signaling (26). As phagocytosis of apoptotic cells can induce anti-inflammatory cytokine production (30, 31), impaired clearance of apoptotic cells may have an additive effect on airway inflammation via reduced anti-inflammatory capacity.

Neutrophilic inflammation is a key risk factor for airway disease resulting in bronchiectasis and loss of lung function (3) which is observed in the absence of detectable bacterial infection (1, 2, 10). It is therefore important to elucidate triggers of early inflammation prior to bacterial colonization of the CF airway. As IL-1R signaling has been investigated as a key pathway driving neutrophilic and eosinophilic inflammation in the airway (9, 12, 32, 33), we next investigated IL-1α and IL-1β signaling following RV infection of AEC. As IL-1α is constitutively active, it can be released directly from necrotic cells in the airway epithelium (9) or actively secreted following activation of the NLRP3 inflammasome and caspase-1 (34, 35) which is required for IL-1β cleavage and release. Activation of the NLRP3 inflammasome has been reported following RV infection resulting from calcium flux resulting from RV ion channel protein 2B activity (36), potassium efflux from lytic cell death such as necrosis or pyroptosis (37), and dysregulated sodium transport due to ENaC upregulation (38). It has also been observed in other inflammatory respiratory diseases with RV associated exacerbations as a hallmark of disease like asthma or COPD, where viral-induced cell death likely contributes to morbidity (36, 39, 40). In this study, we found increased IL-1α and IL-1β alongside increased necrotic cell death suggesting NLRP3 activation could potentially exacerbate the inflammatory cascade following RV infection. This finding supports previously reported data that both IL-1α and IL-1β are released from AEC following RV infection and implicated active secretion via NLRP3 activation (41, 42). Additionally, IL-1α and IL-1β in supernatants of airway mucopurulent secretions have been shown to regulate both MUC5B and MUC5AC through IL-1R (43–45). Release of

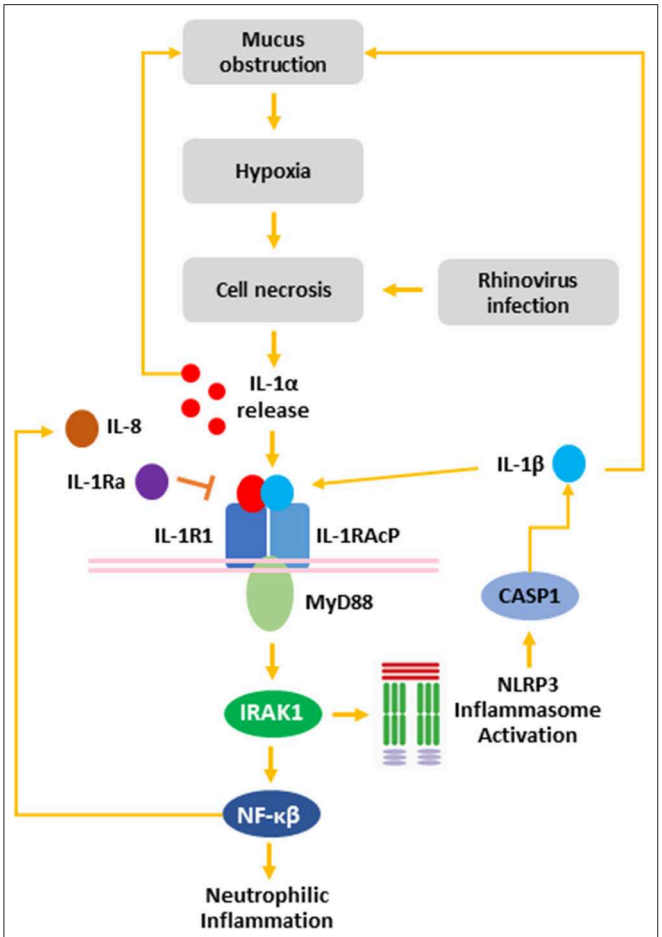

FIGURE 5 | The role of rhinovirus infection in the IL-1 inflammatory response in the CF airway. Mucus obstruction in the CF airway leads to hypoxia of the airway epithelium and epithelial necrosis. Additionally, rhinovirus infection induced necrosis of AEC resulting in release of IL-1α from necrotic cells. Binding of IL-1α to IL-1R recruits MyD88 to the IL-1R:IL-1RAcP complex. Activation of MyD88 leads to IRAK1 activation, which activates the NLRP3 inflammasome leading to CASP1 activation and IL-1β secretion. Additionally, IRAK1 activates NF-κβ, which induces IL-8 release leading to neutrophilic airway inflammation. Both IL-1α and IL-1β induce mucin secretion, which leads to further mucus obstruction creating a positive feedback loop capable of exacerbating CF airway disease. IL-1R activation can be blocked by IL-1Ra to inhibit signaling downstream of IL-1R. IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-1R1, interleukin-1 receptor 1; IL-1Ra, interleukin-1 receptor antagonist IL-1RAcP, interleukin-1 receptor accessory protein; IL-8, interleukin-8; IRAK, interleukin-1 receptor-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NFκB, nuclear factor kappa beta; NLRP3, nod-like receptor protein 3.

IL-1α is primarily through AEC while IL-1β in the CF lung is mainly released from macrophages and interstitial mononuclear cells (46, 47), potentially explaining the differences between IL-1α and IL-1β levels observed in this study when compared to levels reported in other studies in *ex-vivo* samples (43). This data suggests IL-1α and IL-1β observed following RV-A infection may exacerbate mucus hyperconcentration and obstruction evident in the CF airway (4, 43).

Furthermore, we found IL-1α and IL-1β significantly correlated with necrotic events in CF AEC only, while IL-1α and IL-1β correlated with apoptotic events in non-CF AEC only. Studies utilizing the β-ENaC murine model of CF-like lung disease have observed the presence of mucus obstruction and airway neutrophilia in germ-free conditions (48, 49), with "sterile" inflammation in the CF airway triggered by IL-1α released from necrotic AEC (9, 50). IL-1α is measurable in the airways of young children with CF with mild lung disease and associated with structural lung disease measured via CT in the absence of detectable bacterial infection, suggesting a role for IL-1α in the inflammatory cascade in the CF airway environment in the absence of detectable bacterial infection (12). The current study observed levels of IL-1α higher than measured in BALf in young children with CF, suggesting clinically relevant amounts of IL-1α are released from AEC following RV infection. There was higher IL-1α detected in non-CF AEC compared to CF AEC in response to RV infection suggesting IL-1α release from CF AEC occurs predominantly via necrotic cell death post-infection, and release from non-CF AEC via apoptotic cell death. Additionally, IL-1α is associated with viability of non-CF AEC, suggesting overall cell death had a greater effect on IL-1α release in non-CF AEC. Apoptotic cell death is considered immunologically silent due to efficient phagocytosis (51), however, in an *in vitro* monoculture there is a lack of clearance which results in secondary necrosis and cellular breakdown (52). While epithelial cells can self-phagocytize to reduce inflammatory consequences (53, 54), clearance of apoptotic cells relies on professional phagocytes like macrophages (55) and failure leads to release of immunostimulatory danger associated molecular patterns such as IL-1α (56). Secondary necrosis of AEC *in vitro* may potentially explain the differences in IL-1α detected between phenotypes, likely due to the observed and reported lack of apoptosis in CF AEC following RV infection (8). Defective apoptosis due to cleavage of apoptotic signaling receptors by neutrophil elastase and manipulation of phagocytic ability by *Pseudomonas aeruginosa* in monocytes has been reported in CF (26, 57). As IL-1α is increased in the CF airway during bacterial infection (12), we hypothesize defective apoptotic signaling and efferocytosis may play a role in IL-1R-activated neutrophilic inflammation in the CF airway before and after bacterial colonization of the CF airway.

Several recent studies have shown the potential for anti-inflammatory therapy by blocking of IL-1R via genetic deletion of the receptor or pharmacological inhibition via IL-1Ra to inhibit IL-8 expression and neutrophilic inflammation (9, 43). Deletion of IL-1R and IL-1Ra treatment in the βENaC-transgenic mouse significantly reduced IL-1β, neutrophils present in the airway and levels of keratinocyte chemoattractant—a murine IL-8 ortholog (9). This finding was also observed in primary AEC grown at air-liquid interface after stimulation with supernatants of airway mucopurulent secretions, with IL-1Ra treatment reducing IL-8 mRNA (43). The present study found increased IL-1Ra following RV infection in both non-CF and CF AEC, although IL-1Ra was higher in non-CF AEC when compared to CF AEC. This did not correspond with a reduction in IL-8 signaling likely as a result of the amount measured being dramatically lower than

the therapeutic concentrations used in other studies (9, 43). RV infection increased IL-8 in both non-CF and CF AEC, however it was significantly higher in non-CF AEC post-infection. This contrasts with previous data by Sutanto et al. which demonstrated significantly higher IL-8 from CF AEC post-RV infection (8). However, differences in the viral titer used for infection and shorter timepoint may have contributed toward the differences in the observed findings.

There are number of unique strengths to the current study. Firstly, primary AEC from pediatric patients were used for experiments in this study, as most immortalized cell lines that are commonly used in CF research are derived from adult donors and may not accurately recapitulate phenotypic differences observed following RV infection in primary AEC isolated from the pediatric airway (8). Secondly, primary cell cultures were passaged before use in this study to distance *in vitro* cultures from the inflammatory environment from which they were isolated to minimize any pro-inflammatory influences from the *in vivo* airway milieu (58). While using freshly isolated AEC for *in vitro* studies may more accurately recapitulate the environment in the CF airway, it could obfuscate mild and virus-specific inflammatory responses. Finally, the use of a more robust flow cytometry methodology that captures events related to apoptotic cell disassembly to analyze cell death allows us to have greater confidence in data generated (20), as conventional methodologies utilizing propidium iodide staining are suggested to have a false positive rate of up to 40% (59).

For this study, we used a submerged monolayer culture model that doesn't fully represent the physiological features of a differentiated respiratory epithelium (60). However, as the basal cells are epithelial progenitors, they are likely to represent intrinsic properties of the respiratory epithelium. Additionally, since viral replication and pro-inflammatory responses are elevated in air-liquid interface compared to monolayer culture (61) subtle phenotypic and mechanistic differences might be more easily identified in an air-liquid interface system. Rhinovirus species affect viral replication and inflammatory responses differently (17, 62), thus the implications of the findings from this study are limited to RV-A infections. However, RV-A has been reported as the most common strain present in adults with CF and associated with more severe clinical outcomes (63). We used a laboratory strain of RV-A (RV1b) that has been reported to induce cytotoxicity more readily than community-derived strains (8, 64, 65) and therefore future work will focus on corroborating the findings of this study using community RV strains of various serotype in order to determine if all RV induce inflammation via IL-1 signaling (66).

In summary, we have demonstrated that RV-A infection of non-CF and CF AEC drives necrotic cell death specifically associated with IL-1α and IL-1β in CF AEC. Viral infection also drove increased IL-8 release associated with necrotic cell death, implicating necrotic cell death following RV infection as a trigger of IL-1R-mediated neutrophilic inflammation in the CF airway. Collectively, these results suggest a role for RV infection as a trigger of IL-1R-driven neutrophilic inflammation in the early life CF airway (**Figure 5**). Mucin accumulation and hyperconcentration has been identified as the earliest trigger of cystic fibrosis lung disease (4), and linked to IL-1 signaling *in vitro* (43) creating a positive feedback cycle capable of inducing neutrophilic inflammation in the absence of bacterial infection. Previous studies have highlighted the potential translation of IL-1Ra as a novel anti-inflammatory therapy in CF (9, 12, 67, 68), with the aim to prevent further mucus obstruction and viral-induced, muco-inflammatory triggers of early lung disease in young CF children.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of Western Australia Human Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SM, MM, SS, and AK contributed conception and design of the study. SM and DF acquired the data and performed the statistical analysis. SM, DF, MM, SS, and AK contributed to data analysis and interpretation. SM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00596/full#supplementary-material

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REVIEW
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More Than Just a Barrier: The Immune Functions of the Airway Epithelium in Asthma Pathogenesis

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Allergic bronchial asthma is a chronic disease of the airways that is characterized by symptoms like respiratory distress, chest tightness, wheezing, productive cough, and acute episodes of broncho-obstruction. This symptom-complex arises on the basis of chronic allergic inflammation of the airway wall. Consequently, the airway epithelium is central to the pathogenesis of this disease, because its multiple abilities directly have an impact on the inflammatory response and thus the formation of the disease. In turn, its structure and functions are markedly impaired by the inflammation. Hence, the airway epithelium represents a sealed, self-cleaning barrier, that prohibits penetration of inhaled allergens, pathogens, and other noxious agents into the body. This barrier is covered with mucus that further contains antimicrobial peptides and antibodies that are either produced or specifically transported by the airway epithelium in order to trap these particles and to remove them from the body by a process called mucociliary clearance. Once this first line of defense of the lung is overcome, airway epithelial cells are the first cells to get in contact with pathogens, to be damaged or infected. Therefore, these cells release a plethora of chemokines and cytokines that not only induce an acute inflammatory reaction but also have an impact on the alignment of the following immune reaction. In case of asthma, all these functions are impaired by the already existing allergic immune response that *per se* weakens the barrier integrity and self-cleaning abilities of the airway epithelium making it more vulnerable to penetration of allergens as well as of infection by bacteria and viruses. Recent studies indicate that the history of allergy- and pathogen-derived insults can leave some kind of memory in these cells that can be described as imprinting or trained immunity. Thus, the airway epithelium is in the center of processes that lead to formation, progression and acute exacerbation of asthma.

Keywords: asthma, inflammation, barrier, mucus, polarization, imprinting, trained immunity

INTRODUCTION

With more than 300 million people affected bronchial asthma is one of the most common chronic inflammatory diseases worldwide (1). Actually, 1 out of 250 deaths is associated with asthma and it causes annual direct medical (drugs, care, hospitalization) and indirect economic (productivity loss, early retirement) costs of about €34 Billion for the EU (2) and over 80 Billion for the United States (3, 4), making it a major burden for public healthcare systems (5).

Asthma is characterized by acute broncho-obstruction, in combination with additional symptoms such as cough, chest tightness, shortness of breath, and wheezing, which vary in extent and over time. These symptoms arise on the basis of chronic airway inflammation in response to a trigger, most commonly inhaled allergen(s), that causes airway hyperresponsiveness (AHR), airway remodeling and mucus hypersecretion (6). Due to the complexity and variation of the symptoms along with its pathogenesis, asthma is nowadays described as a heterogeneous syndrome with distinct sub- or endotypes. However, the majority of asthma patients displays allergic inflammation of the airways, which can be classified by profiles of several characteristic mediators in "TH2 high" or "TH2 low" subtypes (7). Thus, in sensitized individuals T helper 2 (TH2) cells orchestrate allergic inflammation by releasing a typical array of cytokines including interleukins (IL)-4, -5, -9, and -13 and granulocyte-macrophage colony stimulating factor (GM-CSF). These mediators induce the production of allergen-specific immunoglobulin (Ig) E, TH2 cell development, goblet cell differentiation, submucosal gland activity, as well as recruitment, maturation, and activation of eosinophils and its precursors (8). Activation of mast cells and eosinophils via IgE-bound allergens results in their degranulation and, thus, in the release of a plethora of effector molecules and growth factors that on the one hand destroy airway tissue and on the other hand conduct its repair. Chronic activation of these processes ultimately lead to signs of airway remodeling such as increased smooth muscle mass, subepithelial fibrosis, and epithelial desquamation, which in turn give rise to the pathological changes and clinical symptoms characteristic for asthma (9).

Allergic sensitization against aeroallergens represents the strongest factor predisposing for the development of asthma, indicating a hyperreaction of the immune system to be the central event within the pathogenesis of this disease. Nevertheless, structural cells and particularly airway epithelial cells also appear to be of critical importance. This is not surprising since these cells represent the barrier that first encounters environmental stress factors like air pollutants, bacterial and viral pathogens, as well as allergens, and markedly contributes to their neutralization by a mechanism called mucociliary clearance (MCC). Besides these barrier and cleaning functions airway epithelial cells also exert a number of immunological tasks interweaving the role of the epithelium with that of the above-mentioned cells of the immune system. Here we aim to review these immune functions of the airway epithelium against the background of asthma pathogenesis.

THE BARRIER FUNCTION OF THE AIRWAY EPITHELIAL CELL LAYER ITSELF

The main purpose of mucosae is to separate the body from its environment and therefore they are essential for the maintenance of the inner homeostasis. Though this task is not commonly regarded as an "active" or "typical" immune function, it is absolutely central for the defense against allergens, pathogens and other harmful environmental factors. In order to fulfill this function the airway epithelium forms a continuous, self-cleaning barrier with a considerable resistance against biological, chemical or physical stressors (10). Together with the physical barriers of the MCC and glycocalyx, this is achieved by three types of intercellular epithelial junctions that form the structural adhesion forces of the airway mucosa by linking the intracellular structures of the cytoskeleton of one epithelial cell to that of its neighbors. These junctions involve adherens junctions (AJs), hemidesmosomes, and tight junctions (TJs).

AJs can appear as spots (adhesion plaques) or as bands encircling the cell (zonula adherens). In the junctional zone AJs interconnect the actin filaments of the adherent cells via homotypic transmembrane E-cadherin adhesions and anchor proteins like actinin, vinculin, and α-, β-, and p120 catenins, while adhesion plaques attach the cells to the extracellular matrix (11).

Similarly, hemidesmosomes are focal structures that form adhesive bonds between the cytoskeleton of epithelial cells and the lamina lucida, which is a part of the lamina propria. Hemidesmosomes utilize integrin α6β4, plectin 1a and the tetraspanin CD151 connecting laminin and fibronectin of the extracellular matrix to the intermediate filaments of the cytoskeleton (12).

In contrast, TJs form a multiprotein junctional complex called zonula occludens (ZO) that in turn appears as the main regulator of the paracellular permeability. These complexes are formed by several transmembrane and cytoplasmic proteins that are attached to actin filaments of the cytoskeleton. The main components of TJs are claudins and occludins, proteins with four transmembrane domains, as well as so-called junctional adhesion molecules (JAMs) belonging to the immunoglobulin superfamily with only one transmembrane domain. These proteins are connected to actin filaments by cingulin and ZO proteins 1, -2, and -3 (13).

In the airways of healthy individuals, the TJs of the zonula occludens and AJs of the zonula adherens constitute dense protein networks that interconnect the basolateral sides of epithelial cells in such a way that they prevent the paracellular passage of basically all molecules, including water, ions and proteins, as well as of pathogens or other inhaled particulate matter. Several findings strongly indicate that in asthma patients the barrier function is impaired by epithelial disruption. For example, endobronchial biopsies revealed a fragile or even

injured airway mucosa with partially or completely uncovered areas and detachment of columnar, ciliated cells (14). Epithelial desquamation is further indicated by the presence of epithelial cells in bronchoalveolar lavage (BAL) and of creola bodies (epithelial cell aggregates) in sputum of asthmatics (15). Furthermore, bronchial biopsies of asthmatic subjects displayed patchy disruption of TJs (16) and the expression of a number of proteins that are essential for the formation of TJs and AJs has been shown to be markedly reduced. Among these proteins are α-catenin (17), β-catenin (18), occluding (16), ZO-1 (16, 17), and E-cadherin (17, 19). The levels of the latter one in sputum also correlate with asthma severity (20). These data are further supported by *in vitro* studies where primary bronchial epithelial cells are kept in air liquid interface (ALI) culture, a method that allows the cells to differentiate and form a pseudo-stratified epithelial monolayer largely resembling the physiological structure of the airway mucosa. Once this structure has been established, *in vitro* barrier integrity can be assessed by measuring the transepithelial electrical resistance (TEER), a characteristic that is indicative of the tightness of a cell layer (21). Several studies showed that ALI cultured airway epithelia from asthma patients display a decreased TEER in comparison to epithelia derived from healthy controls (16, 22, 23).

IMPAIRMENT OF CELLULAR BARRIER FUNCTIONS IN ASTHMA PATHOGENESIS

To date, three different factors are discussed to have a harmful impact on the barrier integrity of the airway epithelium in asthma pathogenesis: allergens themselves, viral infection, and (allergic) inflammation. According to the "protease hypothesis" allergens with an inherent protease activity are capable of cleaving the protein components of the aforementioned intercellular epithelial junctions so that the barrier function is disrupted and allergens can penetrate the airway mucosa on the paracellular route, which eventually could result in sensitization against them. Accordingly, a considerable number of allergens has been tested *in vitro* for proteolytic potential and for an effect on epithelial barrier integrity. Several studies provided evidence for a direct cleavage of e.g., occludin and ZO-1 proteins by the major allergen from house dust mites (*Dermatophagoides*), Der p 1 (24, 25). House dust mite extracts as well as Der p 1 have been shown to increase the permeability and to decrease TEER of epithelial layers *in vitro* (23, 25, 26). Comparable effects have been shown for extracts of the allergenic fungus *Alternaria alternata* that reduced TEER of human bronchial epithelial cells *in vitro* (27) or the *Aspergillus fumigatus*-derived alkaline protease 1 (Alp-1) (28). Similarly, a variety of different pollen extracts has been investigated for their effect on the barrier integrity of epithelial cells *in vitro*. Diffusates of Italian cypress (*Cupressus sempervirens*), Orchard grass (*Dactylis glomerata*), Olive (*Olivia europaea*), and *Scots pine* (*Pinus sylvestris*) have been shown to affect claudin-1, E-cadherin, and occludin expression and thus to disrupt epithelial junctions in ALI cultures of Calu-3 cells, an effect which could be suppressed by protease inhibitors (29). Japanese hop (*Humulus japonicus*) extract also reduced expression of occludin in a comparable setting (30). Another study provided evidence for proteolytic activity of Giant ragweed (*Ambrosia trifida*), Kentucky bluegrass (*Poa pratensis*), and White birch (*Betula pendula*) as shown by reduced expression of claudin-1, occludin, and ZO-1 in Calu-3 as well as in MDCK cells (31).

However, an inherent protease activity appears not to be the only way, by which allergens can impair the barrier integrity of the airway epithelium. Cockroach, HDM, fungus, and mold extracts have also been shown to activate the protease-activated receptor (PAR-) 1 and/or 2, which in turn leads to degradation of AJ components (25, 32–34).

The effect of viral infections on airway barrier function is even more pronounced than that of allergens. Respiratory viruses cause junction dysfunction by different mechanisms: human rhinoviruses (HRV), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), influenza and parainfluenza viruses bind to their entry receptor, which are typically protein or sugar structures expressed on the cellular surface for other purposes, leading to endocytosis of the virus. Once the virus has been internalized, it uncoats and initiates the viral replication process, which has certain consequences for infected cells. On the one hand, the cell starts with the production of type I interferons (IFN) in order to slow down the internal virus replication and to activate the cellular immune response against the virus. In consequence, infected airway epithelial cells are killed by virus-specific, cytotoxic CD8 + T cells. On the other hand, the virus itself also kills epithelial cells, since it induces morphological alteration of the cells summarized as cytopathic effect (CPE). For HRV and influenza viruses, the CPE manifests in rounding and detachment of airway epithelial cells that are ultimately lysed by the virus in order to release freshly produced viruses. Paramyxoviruses such as RSV and HMPV induce cell fusion so that four or more cells form typical syncytia (35, 36). Additionally, at least HRV and RSV affect the barrier integrity of the airway epithelium by reducing the expression of epithelial junction proteins (37–40). It could be shown that HRV increases epithelial permeability by a reduction of occludin and ZO-1 expression (41, 42). RSV also disrupts junctional complex structures by fostering the activity of protein kinase D (PKD) (43).

The antiviral immune response also includes the release of cytokines that directly affect epithelial barrier function as well. This is especially true for IL-1β, IFN-γ and tumor necrosis factor (TNF). These cytokines have been shown to support epithelial permeability and to decrease expression of claudins, JAM, occludin, and ZO-1 in several *in vitro* studies (44–46). In case of asthma, these effects are even more pronounced because of the allergic inflammatory response that already exists before the viral infection of the airway epithelium. Hence, TH2 type cytokines like IL-4 and IL-13 also increase barrier permeability by inhibiting the surface expression of β-catenin, E-cadherin, occludin, and ZO-1 (45, 47). In addition to cytokines, mast cell derived mediators also appear to have an effect on the barrier function of

the airway mucosa. Histamine for example has been shown to contribute to transient disruption of apical junctional complex integrity and thus to increase epithelial permeability *in vitro* (48).

Allergens, viruses, and the inflammatory response to their exposure represent extrinsic factors that impair the barrier integrity of the airway epithelium. However, some studies suggest that epithelial cells of asthma patients inherently predispose for an increased permeability. As already mentioned above, airway epithelial cells that have been isolated from asthmatics and propagated *in vitro* to form an epithelial monolayer under ALI culture conditions, display a decreased TEER as compared to cells from healthy donors (23, 45). This observation indicates that the cellular properties leading to an increased barrier permeability are somehow imprinted within the cells. Whether this is a matter of genetic predisposition encoded in epithelial stem cells or whether epithelial cells from asthma patients "remember" previous insults by epigenetic modifications that predispose for asthma development in later life remains elusive.

PASSIVE LUMINAL BARRIER STRUCTURE ON THE AIRWAY EPITHELIUM

Besides the barrier function of the epithelium provided by the mere presence of the sealed cell layer itself, two additional barrier structures are "exported" onto the luminal surface by the airway epithelium, a static one dubbed glycocalyx or periciliary layer (PCL) and a mobile one termed mucus.

STRUCTURE AND FUNCTION OF THE PERICILIARY LAYER

The glycocalyx or PCL is a sponge/fleece-like, cell membrane-anchored layer of glycolipids and glycoproteins – mainly mucins (see below) – that vertically stick out of the apical epithelial cell membrane. Although mainly attributed to the gut epithelium where it can extend up to 1500 nm (49) and to the vascular endothelium (50, 51) a glycocalyx/PCL is also present throughout the airway epithelium even down to the alveoli (52), again with heights up to 1500 nm in certain areas (53) (**Figure 1**). This static glycoprotein and glycolipid coat stores water to control mucus hydration but also serves as a protective zone against the compression of the mucus lying above in order to allow persistent cilia beating for ongoing functionality of the mucociliary clearance (MCC; see below) (53, 54). Beyond that, the glycocalyx/PCL regulates receptor specificity by architectural means and prevents the progression of viruses through the occasionally patchy mucus layer. It has been shown that the height and density of the epithelial glycocalyx can determine whether a ligand-equipped nanoparticle may attach to its membrane receptor or not (49, 55). In line with this, the inefficiency of adenovirus-mediated gene transfer into the airway epithelium was found to be caused by the membrane-tethered glycocalyx/PCL proteins that put a halt to the advance of the viral vectors (56, 57). Consequently, the susceptibility of the airway epithelium toward infection is at least to some extent controlled by the glycocalyx/PCL. Very small viruses such as bocavirus (HBoV1) and HRV, which are 20–30 nm in size (58, 59) should readily advance through the PCL to the epithelial plasma membrane as has been observed with nanoparticles of the respective size (53). Consequently, those viruses should be able to luminally infect airway epithelial cells as long as their receptor is present on the apical side. Little is known about receptor distribution *in vivo* but at least on cultured airway epithelial cells apical receptor expression and/or infectivity has been demonstrated for both viruses (60–63). Larger particles of about 100 nm and above, on the other hand, are efficiently blocked by the PCL (53). Hence, viruses such as RSV, HMPV, influenza and parainfluenza viruses, adenovirus or coronavirus, which are in this size range (64–67), should be hindered efficiently by the PCL to infect the host. Yet, those viruses often are associated with respiratory infections and asthma exacerbations (68, 69). One possibility for them to infect the airway epithelium may be the presence of the viral receptor on structures that extend from the PCL such as the tips of the cilia. An example for this is chemokine receptor CX3CR1 via which RSV can infect its host. In differentiated human airway epithelial cells this molecule is highly abundant on cilia (70, 71). Another possibility is the preceding action of a door-opener such as HBoV1 which may pave the way for further viral infections. HBoV1 was shown to persist for several months in the human airway epithelium (72) and causes pyroptotic cell death, epithelial cell hypertrophy, loss of cilia and disruption of the tight junction barrier (60, 61). Such a predamaged epithelial barrier may then readily fall victim to an influenza, parainfluenza or HPMV infection. With up to 13% of asthma exacerbations in small children found to be associated with HBoV1 infection (73) it may be worthwhile to further investigate possible coinfections with HBoV1 in asthma exacerbation cases. In this context, it may also be of interest that the treatment of chronic inflammatory diseases of the airways such as asthma with corticosteroids (CS) seems to reduce the glycocalyx/PCL height on the alveolar epithelium (74) thereby rendering the lung more susceptible to e.g., *Pneumocystis carinii* infection. Consequently, alleviating chronic inflammation in asthma with CS may make the patient more susceptible to certain infections, which in turn may enhance inflammation again, clearly a two-edged outcome of CS therapy in asthma.

Although the glycocalyx appears to be static on the architectural level, it may not be invariant in terms of its molecular composition. It was shown that lipopolysaccharide exposure could lead to heparan sulfate shedding from the airway epithelium thereby causing increased lung permeability (52). Allergen exposure of experimental animals resulted in different glycosylation patterns of the glycocalyx (75), which may result in a deviant presentation of viral and bacterial receptors on the cell surface. In light of the above, the airway epithelial glycocalyx seems to play a so far underestimated but possibly important role in airway epithelial defense. Whether or not the molecular composition

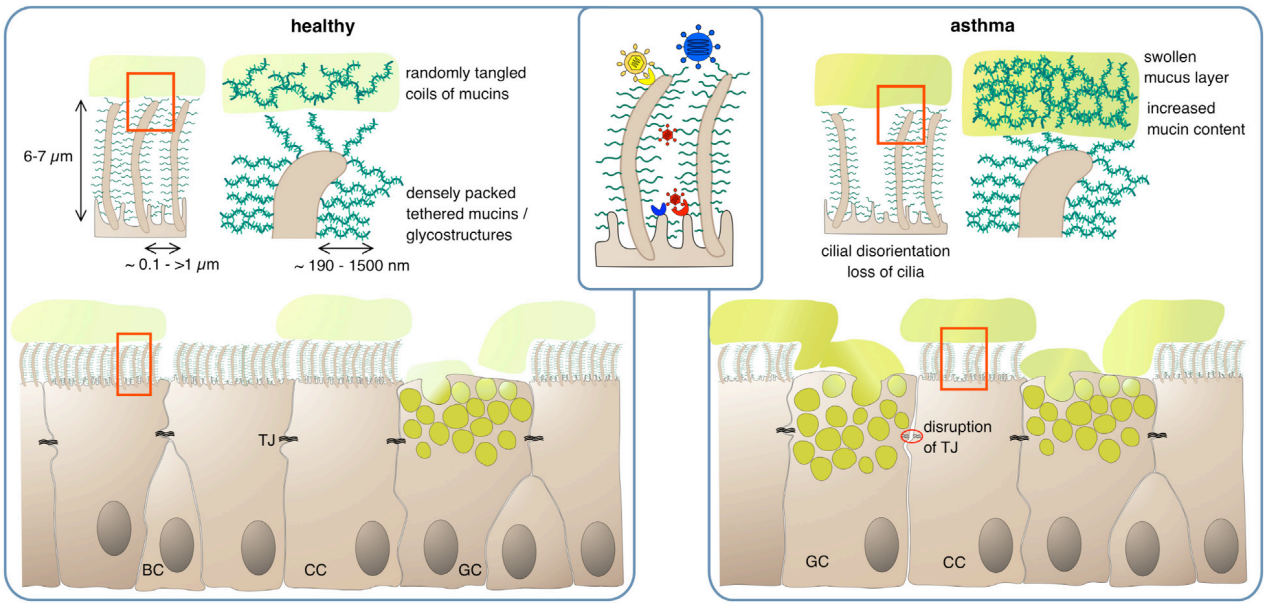

FIGURE 1 | Protection of epithelial surfaces by physical barriers. In the healthy state, ciliated cells (CC) form a tight epithelial layer where paracellular passage is prevented by sealing of lateral intercellular spaces with tight junctions (TJ). The apical epithelial cell surface, including the cilia, is covered by a layer of membrane-anchored glycoproteins and glycolipids, the glycocalyx. The dense meshwork of glycostructures restricts access of luminal matter to the apical cell surface; depending on their size, larger pathogens can be cut off from their receptor if it is not present on cilia (inset). Goblet cells (GC) secrete mucus, consisting of highly glycosylated mucins which absorb large quantities of water to form a viscous gel. The mucus – and any matter trapped within – is transported upward in the airway lumen by the coordinated beating of the cilia. BC, basal cells. In the asthmatic state, barrier functions can be compromised by partial disruption of tight junctions and gaps in the PCL/glycocalyx meshwork due to loss of cilia. Mucus clearance is impeded by increased mucus viscosity and swelling of the gel matrix, and by disturbance of ciliar beating due to disorganization and dykinesia of cilia.

of this static cell coat is different in asthma, remains to be investigated.

COMPOSITION AND FUNCTION OF MUCUS

While the role of the glycocalyx/PCL is still subject of debate, the importance of mucus in airway luminal defense is unchallenged. Mucus is an unstirred discontinuous sheet of secreted mucous hydrogel which floats on top of the epithelium and is transported toward the oral cavity like the cargo on a conveyor due to the constant and coordinated beating of underlying ciliated cells (76). The mucus carried upward by this mucociliary clearance (MCC) mechanism can be swallowed or expectorated. As the mucus layer separates the airway lumen from the epithelium only objects that diffuse faster "vertically" toward the epithelial surface than the mucus is transported "horizontally" toward the oral cavity can reach the epithelial cell membranes. Thus, only nanoscalar objects and smaller, like gases, water, salts and nutrients are able to reach the epithelial cells (77, 78). This way the mucus carpet provides a protective line of defense against pathogens, dust and other harmful objects that might be inhaled by an individual. In addition to that, the sticky texture of the mucus slows down airborne objects and further prevents their advance to the epithelial cell layer. In order to exert these functions properly, mucus requires a specific composition. Mucus consists mainly of water, further components are salts, lipids and proteins. Among those, antimicrobial proteins like lysozyme, immunoglobulins and antimicrobial peptides are major molecular scavengers distributed within the mucus layer (79–81). The characteristic viscous, elastic and sticky properties of the mucus are provided by a group of macromolecules named mucins (82, 83).

To date, 21 genes coding for mucins have been described (84). Their protein products are secreted either to form mucus or remain immobile on the apical membranes of the airway epithelial cells where they become part of the glycocalyx/PCL. MUC5AC and MUC5B are the major secreted mucins in the airways. In addition, MUC2 and MUC19 are also part of airway mucus, albeit to a considerably smaller proportion, and thus belong to the family of secreted mucins (83). In contrast, MUC1, MUC4 and MUC16 are tethered to the cells of the airway epithelium (**Figure 1**) thereby contributing to the static luminal epithelial barrier, which resides underneath the mobile mucus layer (85, 86).

The viscous and elastic properties of the mucous gel are primarily given by the secreted polymeric mucins MUC5AC and MUC5B (83). These mucins are highly O-glycosylated proteins enriched with amino acids like proline, serine or threonine (87). Although both have a similar structure, MUC5B and MUC5AC differ in charge due to differential glycosylations (88). Their production depends on cell type and site of production. In the upper airways, MUC5AC is produced by epithelial goblet cells while MUC5B is secreted from mucous cells in

submucosal glands from secretory cells in the tracheal and bronchial epithelium. In the distal airways, MUC5B is also produced by secretory cells of the epithelium and seems to be the major mucin of this airway region (83, 89, 90). Before secretion, polymeric proteins are stored in secretory granules in a compacted, dehydrated state. Upon release, they switch to a hydrated form, which is necessary for the mucous gel-like properties (91, 92). Whether the two different mucins have different functions restricted to their site of production or whether the two mucins mingle to create a novel type of barrier structure is not clear yet. At least some studies analyzing airways of piglets have shown that MUC5B strands are becoming coated with MUC5AC to some extent after release at the epithelial surface (93, 94). A possible role of MUC2 and MUC19 has not been identified yet.

Howsoever, under healthy conditions, the viscous mucus traps noxious substances, which are then removed from the airway via cilial beating by the MCC (95). In asthma, the MCC is impaired leading to mucus plug formation which in turn results in the characteristic obstruction observed in asthmatics. This is already a feature of mild stable asthma and the dysfunction worsens during aggravation of asthma and in asthma exacerbations (96–98). One reason is an increased mucin content of the mucus thereby disturbing its regular composition. Normally, the airways' mucus consists of ∼98% water and only ∼2% solid factors mainly mucins. In obstructive diseases, the amounts of mucins rise up to 8–15% (54, 86). Due to its hygroscopic nature, this leads to acquisition of water from the underlying PCL/glycocalyx and shrinking of this static layer. The now protruding cilia either project into the mucus or get bend (54). Both effects impede passing on of the mucus to the next cell. Loss and/or disorientation of cilia as it is typical for asthmatics will further disturb the "bucket chain"-like transport process (99). On the cargo side enhanced intermolecular crosslinking of mucus constituents by oxidative processes may further complicate forwarding. It will also increase mucus viscosity eventually leading to plug formation. Oxidative intramolecular crosslinking of biomolecules is predominantly caused by cysteines whose thiol side chains can form disulfide bridges. All mucins are rich in cysteines, especially in their less glycosylated carboxy- and amino terminal regions. In the "normal" mucous gel of healthy individuals these cysteines are believed to be only moderately crosslinked, forming a lightly entangled network. In asthma, however, the degree of crosslinking and the density of the mucin network increases considerably (100, 101) (**Figure 1**). Increased oxidative stress appears to play an important role in this respect with eosinophils being the main suspects for oxidant production. The abnormally high levels of eosinophil peroxidase detected in the sputum of asthma patients may form an oxidative milieu. This would also bring the widely observed correlation between airway eosinophilia and airway obstruction into a causative relationship (102). Lastly, the MUC5AC of asthmatics tends to tether to the epithelium, which also complicates mucus forwarding (103).

In asthmatics not only the amount but also the composition of the mucus changes, especially the ratio of MUC5AC to MUC5B as well as the posttranslational modification of MUC5B. Mucus from healthy individuals contains predominantly MUC5B, which is essential for the MCC and protection against pathogens (104–106). In asthma, the proportion of MUC5B relative to MUC5AC often decreased (104, 105) along with the expression of a low-charge form of MUC5B. Consequently, there was a changed ratio between the two differently glycosylated forms of MUC5B (104, 107). The importance of MUC5B is indicated by *Muc5b*-deficient mice, which showed an accumulation of undesired substances e.g., bacteria, resulting in severe inflammation and airway obstruction (106).

The ratio between MUC5B and MUC5AC changes dramatically in asthma because MUC5AC expression and protein production are substantially upregulated in asthmatic patients (104, 105, 107). Especially patients with an eosinophilic type 2 asthmatic phenotype showed a shifted ratio toward higher MUC5AC concentrations (105, 108). This is in line with the assumption that MUC5AC seems to be important for the defense against enteric nematodal and influenza infections (109, 110). The increased expression of MUC5AC seems to depend on substantially increased levels of IL-13. The IL-13 signaling pathway activates the signal transducer and activator of transcription 6 (STAT6), which induces the expression of MUC5AC via various regulators (111) and appears to be involved in AHR development (112, 113). Several studies using *in vitro* systems with human epithelial cells or murine models validated this mechanism (114–117). Furthermore, EGFR, which is also overexpressed in asthma, also induces the expression of MUC5AC (118–121). This excessive production of mucins in the asthmatic airway epithelium leads to an increased volume of intracellular stored mucins, a mucus metaplasia (122). Thus, a higher number of goblet cells compared to the healthy situation appears in case of asthma (122). It is not exactly understood, whether this switch from a muco-ciliary phenotype to a mucous metaplastic phenotype develops from goblet cell hyperplasia, metaplasia or both as reviewed before (123). In animal models, goblet cell metaplasia/hyperplasia arises from an increased expression of primarily IL-13, but also of IL-4 and IL-9 (124–127). These cytokines are highly upregulated in asthmatic individuals (128–131). One important factor for the development of the goblet cell metaplasia is Notch2 regulated by IL-13 (132). Studies analyzing the function of SAM-pointed domain containing ETS transcription factor (SPDEF) highlighted its essential role in the development of goblet cell differentiation, hyperplasia and mucous metaplasia (133–135). Therefore, SPDEF seems to inhibit the expression of Forkhead box protein A2 (FOXA2) which is an important negative regulator of genes associated with mucous metaplasia and goblet cell hyperplasia (111, 121, 133, 136, 137).

Thus, the physical barriers provided by the airway epithelial layer seem to be deeply disturbed in asthmatic individuals. Although mucin is one of the most important barrier molecules in the airways its unbalanced overproduction is clearly detrimental to the desired outcome. Mucus plugging impedes egress of the active luminal defense molecules necessary to eliminate invaders.

ACTIVE LUMINAL DEFENSE MECHANISMS ON THE AIRWAY EPITHELIUM

Although strong walls (tight junctionally sealed epithelial cell layer) surrounded by a glacis (pericilial layer/glycocalyx) and a moat filled with flowing liquid (MCC) are crucial to prevent invaders from entering a castle, active defenses are necessary to end the siege. This is of particular importance when the besieger can replicate and thus may increase continuously by number, as is the case when pathogenic bacteria colonize the luminal side of the airway epithelium.

DEFENSIVE MOLECULES PRODUCED BY THE EPITHELIUM

In order to get rid of a potential invader the airway epithelium possesses a battery of defense molecules, with which a potential microbial enemy can be attacked, destroyed or removed out of the airway lumen. Prominent innate molecular scavengers are lysozyme, transferrin and antimicrobial peptides. Lysozyme is produced in large amounts (20 mg/day) by serous cells of the upper human airway epithelium (138) and is able to destroy the polysaccharide capsules of many bacterial species. It has been shown that the production of lysozyme by serous cells residing in the serous glands of the upper airways is crucial for defending against bacterial airway invaders (139). Once the polysaccharide capsule is destroyed or damaged, so called defensins or antimicrobial peptides may exert the lethal hit to the invader. Defensins can form holes or pores into a bacterial cell membrane thereby killing a pathogen that aims to enter the body (79, 140, 141). In addition, lactoferrin is produced and secreted by serous cells (142), and transferrin is expressed by alveolar type I cells (143). These ferrins are iron-binding proteins, which deplete their environment from iron ions that are essential for the growth of a self-replicating organism (143, 144). Consequently, the pathogen is starved out.

THE ROLE OF SECRETORY IgA IN EPITHELIAL DEFENSE

Besides this innate "rapid response team," the polarized epithelium of the human airways is also able to transport and apically release immunoglobulins that carry a J-chain (joining chain) by using its poly Ig receptor (pIgR) (145–147) that is expressed by all non-stratified epithelial cells (**Figure 2**). Only IgM and multimeric IgA are equipped with J-chains (148, 149). These two immunoglobulin classes not only circulate in the bloodstream but are also produced directly underneath the airway epithelium by B cells, given those lymphocytes express the J-chain (150, 151). Functionally, IgM can substitute for multimeric IgA. For that reason IgA-deficient individuals do not show a strong phenotype concerning susceptibility to infection. Nevertheless, secreted IgA (sIgA) outperforms IgM in terms of mucosal protection as it usually displays a higher affinity toward its antigen and, more importantly, is able to crosslink with mucins upon target binding (152, 153). This way an incoming viral or bacterial pathogen becomes trapped in mucus and is removed from the airway surface via the MCC. The protective function of secreted IgA has been demonstrated with various model systems, both for the gastrointestinal mucosa as well as for the airways, using passively administered monoclonal IgA (154–157), injected hybridoma cells whose target specific, dimeric IgAs are then transported across the mucosae ("backpack tumor model") (158–160) and by neutralization of preexisting mucosal IgA immunity with mucosally administered anti-IgA immunoglobulins (80). Although adaptive multivalent target binding via its hypervariable regions is probably the main mode of protection in those models, sIgA is also able to bind in an innate manner to luminal pathogens via its carbohydrate components by presenting decoy structures that mimic target cell surface receptors (161). If both modes of repelling fail and a pathogen has nonetheless invaded an epithelial cell, dimeric IgA may still be able to protect the infected cell, this time from inside. This is possible whenever the respective pathogen does not directly infect the cytosol of its target cell or inject its nucleic acids directly into the cytosol but rather uses an initial endocytosis step for infection. Depending on the infected organelle, vesicles, which concurrently translocate IgA toward the apical site, may fuse with the infected organelle, bind to the invader and carry it away into the lumen. In addition to this removal activity, mucus crosslinking and the tricking of pathogens by offering decoy receptors, sIgA also scavenges IL-8 and thereby inhibits IL-8-driven neutrophil chemotaxis (162).

In addition to these molecular interactions with a pathogenic target, IgA also binds to numerous cell types that patrol at the airway epithelium. The most important cellular partner seems to be the eosinophil as this cell possesses a total of five different receptors for IgA: FcαRI (CD89), transferrin receptor (TfR) (CD71), pIgR, asialoglycoprotein receptor (ASGPR) and a receptor for secretory component (SCR) with the integrin Mac-1 (CD11b/CD18) serving as a coreceptor for FcαRI (163, 164). Depending on the receptor addressed and the form of IgA offered, i.e., soluble versus target-bound and cross-linked, eosinophils are either calmed down or activated (165–167). Yet, eosinophils are not only manipulated by IgA, they also influence IgA production themselves (168, 169). Thus, immunoglobulin A and eosinophils share a really intimate relationship.

Equipped with less receptors but still responsive to IgA are neutrophils, dendritic cells, macrophages, basophils and even epithelial lining cells that express the transferrin receptor such as alveolar-type 2 cells (170). An additional, so far unidentified receptor is present on M cells (microfold cells) (171, 172). M cells are a specialized epithelial lining cell type that is responsible for antigen sampling at mucosal surfaces and predominantly occurs in the epithelium above organized mucosa-associated lymphoid tissue (173, 174). This receptor senses the distance between two heavy chain domains in IgA. Thus, it is not able to bind IgA1, an IgA subclass present only in primates. IgA1 is different from IgA2 in that it contains a mucin-like, highly glycosylated

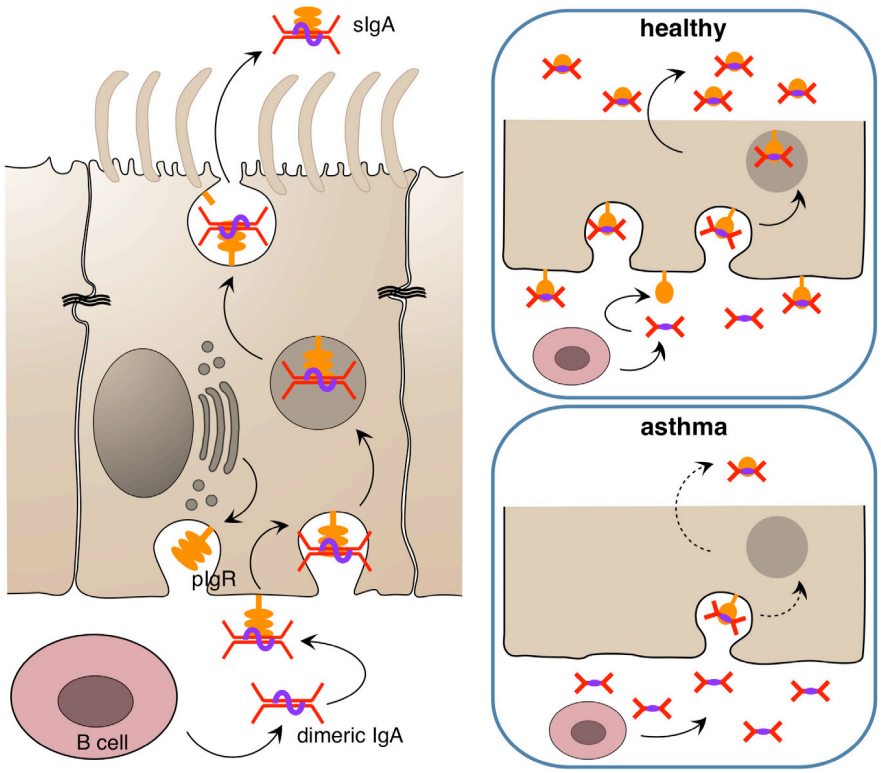

FIGURE 2 | Release of immunoglobulin A at epithelial surfaces. Dimeric IgA is released submucosally by B cells and binds to the poly Ig receptor (pIgR) which is found in high amounts in the basolateral membrane of the epithelium in the healthy state. The complex of pIgR and dimeric IgA is transported in secretory vesicles to the apical side, where it is released into the lumen as secreted IgA (sIgA) to bind target antigens/allergens/pathogens and entrap them in mucus. In the asthmatic state, the amount of pIgR in the epithelium appears to be reduced, resulting in diminished secretion of sIgA and futile accumulation of IgA in the subepithelial compartment.

extension of its hinge region. It is believed that this hinge region also serves as a ligand for yet other IgA receptors (163). If this holds true, subclass switching may be another adjusting wheel for IgA function. The class switch from IgA1 to IgA2 depends on the presence of the cytokines APRIL and BAFF which were shown to be produced by the epithelial layer itself, at least in case of the gut, upon bacterial stimulation (175). This way the microbiome as sparring partner of the epithelium comes into play as the true master of this adjusting wheel. In contrast to the gut where IgA2 prevails, IgA1 is the predominant IgA subclass in the airways (176). This, however, does not imply that IgA2 is of less importance for airway defense. IgA1 simply may be the first class formed after pathogen challenge. Those initial secretory IgA (sIgA) responses are believed to be not very mature. Upon pathogen challenge the human body apparently rapidly switches its current IgM repertoire to IgA even if most of such "first line of defense" IgA are of low affinity (177). This can be regarded as just a "better than nothing" attempt; yet it creates a window of opportunity for the body to develop more powerful sIgA via affinity maturation. Such optimized immunological scavengers are then able to block and eventually clear a microbial infection. Thus, the IgA system in which the transporting epithelium plays a key role is a complex defense machinery that combines innate with adaptive immune responses. It is therefore not surprising that the role of secretory IgA attracted attention in asthma research in recent years.

sIgA IN CHRONIC INFLAMMATION OF THE LUNG

The role of sIgA in chronic inflammatory lung diseases is still ambiguous. Some studies show that sIgA is necessary to maintain immune homeostasis, other reports claim that sIgA may play a detrimental role in asthma. A harmful effect of IgA in asthma may be explained by its ability to activate eosinophils and neutrophils because both cell types play a central role in the pathogenesis and persistence of asthma (178). When IgA is able to activate those cell types, this would readily lead to the hypothesis that in case of allergic asthma, allergen-specific IgA is responsible for this activation upon allergen exposure. This assumption is supported by the finding that increased levels of both, allergen-specific IgE and IgA were observed in the airway mucosa of patients with atopic asthma and/or rhinitis (179–183), and it was shown that allergen-specific IgA levels were positively correlated to eosinophil activation marker release after segmental lung challenge of asthmatic patients (166). Yet, coincidence and correlation do not necessarily imply a causative relationship.

In the abovementioned study, where a positive correlation of allergen-specific IgA and eosinophil activation was observed, the non-allergic control patients also displayed allergen-specific IgA in their airways; but in contrast, they did not have any allergen-specific IgE as was the case for asthmatics. Either so the allergen-specific IgE was responsible for eosinophil activation in asthmatics or the eosinophils of asthmatics underwent some kind of imprinting or immune training that rendered them more sensitive to allergen-specific IgA. With the expression of five different IgA receptors on the eosinophil described so far (163), locked-in differences in IgA receptor expression in eosinophils of asthmatics versus healthy individuals are not impossible. On the other hand, a coincidence of allergen-specific IgA and IgE does not necessarily imply a pathological role of IgA either. It may still be the case that IgA are beneficial to chronic airway inflammation, and the concomitant production of allergen-specific IgA can also be interpreted as a rescue attempt of the body to counteract the allergen-specific IgE.

In fact, more evidence points toward a beneficial role of IgA in asthma and other chronic airway inflammations. It was shown for instance that upon aging IgA knockout mice tend to develop chronic airway inflammation that resembles chronic obstructive pulmonary disease (COPD) in humans (184). A COPD-like phenotype also develops in pIgR knockout mice upon exogenous bacterial challenge (185), and it has been shown in the past that COPD patients have an impaired pIgR expression (186) and reduced sIgA levels on the airway epithelium (187). Recently a similar phenomenon was reported for asthma (188) and rhinosinusitis (189). In the study of Ladjemi et al., asthmatics show a reduced immunostaining of pIgR in airway epithelia, with IL-4 and IL-13 being the suppressors of pIgR formation in the airway epithelium. Notably, there were no significant differences in the pIgR gene expression rate among asthmatics and healthy individuals (188). Thus, a posttranslational event such as proteolytic degradation of pIgR in the epithelium may be responsible for the observed differences.

A beneficial effect of allergen-specific IgA in the airway lumen was highlighted by Schwarze et al. (190). They showed that local application of a human monoclonal IgA antibody directed against the ragweed allergen Amb a attenuated the proinflammatory response to allergen inhalation in mice sensitized to Amb a I, whereas a control IgA against ovalbumin did not. Notably, the instilled anti-ragweed IgA induced the formation of Amb a I-specific IgG2a in the animals upon allergen challenge which indicates a shift toward Th1. Thus, IgA residing in the airways may have an anti-allergic/anti-asthmatic immunomodulatory activity. This effect may be explained by the IgA feedback loop, via which a secretory IgA response is adjusted to current needs. In order to provide an optimal defense against luminal noxa luminal IgA are continuously sampled at the epithelium and transported to the basolateral side, where it is inspected by immune cells whether it is loaded with antigen or not. If this is the case, an immune response is mounted or boosted (191). Although this type of transcytotic event has been attributed primarily to M cells, the set-up of the ragweed-study rather precludes that route in this specific case in as much as a human IgA1 against Amb a I was used and this type of IgA does not bind to murine M cells (172). However, with a plethora of IgA receptors known, other epithelial cell types may have taken over the task. The IgA-binding transferrin receptor, for instance, is expressed by type II pneumocytes and was shown to transport transferrin conjugates to the basolateral site (192). In addition, similar to the gut, airway dendritic cells, which also carry IgA receptors, send protrusions to the epithelial layer via which luminal antigen can be sampled (193, 194). Yet, sampling antigen-loaded IgA from the airway lumen and driving the airway immune response toward Th1 requires the presence of IgA in the lumen, which is reduced by the Th2 micro-milieu in allergic asthma. This results in a vicious circle of a locked-in Th2 environment where a lack of IgA causes a further lack of IgA.

This is in line with clinical observations on asthmatic patients that suggests a critical role for IgA in asthma pathogenesis. Patients with selective IgA deficiency tend to bronchial hyperresponsiveness (195) and children that show a delay in maturation of IgA production display atopic manifestations more often (196). Moreover, immunotherapy against the respective aeroallergen result in higher specific mucosal IgA levels along with lower skin prick test sensitivity (197) or lower airway hyperreactivity (198). Nevertheless, most of the above suggests a prominent role of sIgA or, more precisely, the lack thereof in the pathogenesis and chronicity of atopic asthma. Whether a lack of sIgA also plays a prominent role in asthma exacerbations remains to be elucidated.

THE AIRWAY EPITHELIUM AS MEDIATOR OF AN ACUTE INFLAMMATORY REACTION

In addition to all the homeostatic defense functions like the maintenance of barrier integrity, transcytosis, and the mucosal clearance the airway epithelium also plays a major role against inhaled materials by producing several defense proteins such as mucins, defensins, antimicrobial peptides, cytokines, and chemokines (199). Thus, it contributes to local acute inflammatory reactions by regulating early inflammatory events via transcription and secretion of antimicrobial and pro-inflammatory proteins and by activating of mucin production (200, 201). Consequently, it is also a critical player during sensitization processes, asthma pathogenesis and acute exacerbations of the established disease (**Figure 3**).

DANGER AND PATHOGEN ASSOCIATED MOLECULAR PATTERN (DAMP AND PAMP) RECOGNITION BY PATTERN RECOGNITION RECEPTORS (PRR) OF THE AIRWAY EPITHELIUM

Inhaled pathogens that are not cleared by MCC are recognized by airway epithelial cells (202). Equipped with a large number of PRRs such as cytoplasmic NOD like receptors (NLR) and transmembrane toll like receptors (TLR) that can respond to

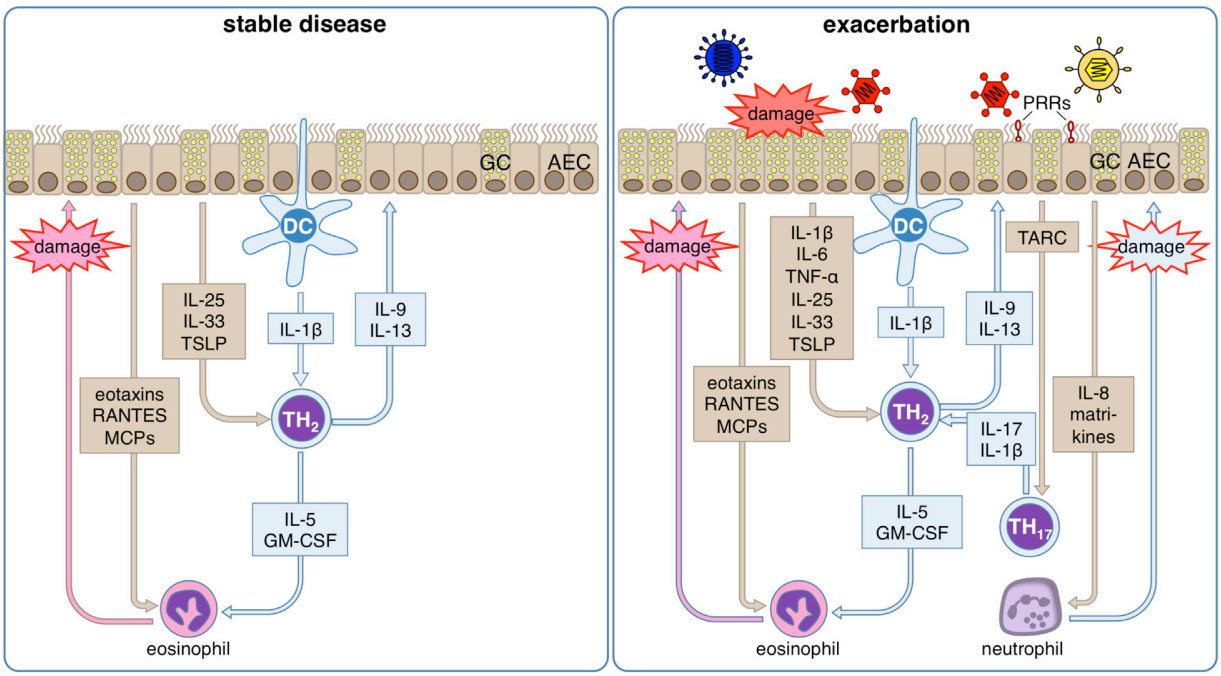

FIGURE 3 | Inflammatory response of the airway epithelium during stable allergic asthma and exacerbation. During stable allergic asthma airway epithelial cells (AECs) release IL-25, IL-33 and TSLP supporting differentiation of T helper (TH) 2 cells that are activated by dendritic cells (DCs). Th2 cells in turn secrete IL-5 and GM-CSF that together with AEC-derived eotaxins, RANTES and MCPs regulate the production, maturation, recruitment and activation of eosinophils. Local degranulation of eosinophils in the lung eventually leads to damage of the airway epithelium. In parallel, the TH2-type cytokines IL-9 and IL-13 induce goblet cell (GC) metaplasia in airway epithelium. During viral induced asthma exacerbations, several other additional factors lead to an aggravation of this inflammatory response. Viral infection can be detected by the airway epithelium via pattern recognition receptors (PRR). Subsequently, AECs secrete on the one hand TARC, the main chemokine for the recruitment of Th17 cells that amplifies the proinflammatory effects of Th2 cells via release of IL-17, and on the other hand IL-8, which leads to the recruitment of neutrophils. Local degranulation of neutrophils in the lung eventually leads to additional damage of the airway epithelium. Viral infection of AECs also directly leads to damage of the airway epithelium. In summary, these conditions result in a markedly increased damage of the airway epithelium compared to the stable disease, which further impairs barrier integrity and leads to release of matrikines further amplifying the ongoing inflammation.

DAMPs and PAMPs AECs represent the first line of cells, which can respond to pathogens and other danger signals like cell stress and cell death in the lung (203).

DAMPs are molecules that are release from injured cells. Their presence is a clear sign for the loss of homeostatic integrity of specific cell compartments or even whole cells. Hence, they originate from the cytoplasm (S100 proteins, heat shock proteins, defensins, galectins, uric acid), the nucleus (High-Mobility-Group-Protein (HMGB1)), the endoplasmic reticulum (calreticulin), mitochondria (ATP, mitochondrial DNA, N-formylated peptides) or from the extracellular matrix (fibronectin, hyaluronan, versican) (204). Airway epithelial cells are both, responder to and producer of DAMPs (205, 206). The DNA binding protein HMGB1 for example, set free during necrosis of one cell can be detected from nearby cells by binding to their receptor AGE (RAGE), which leads to activation of nuclear factor kappa b (NFκB) and thereby to the production of pro-inflammatory mediators and consequently the recruitment of immune cells.

In turn, PAMPs are preserved molecules and structures from pathogens and toxins. They can originate from such different sources as bacteria, mycobacteria, viruses, fungi and parasites (207).

PAMPs and DAMPs activate signaling pathways resulting in the transcription and production of cytokines and chemokines. In brief, signal transduction through MyD88 and MyD88-independent mechanisms leads to the activation of NFκB, mitogen-activated protein (MAP) kinases, and interferon regulatory factor (IRF) 3 (203). Based on the nature of the triggering PAMPs and DAMPs and a possible preexisting inflammatory environment in the lung its signals can result in protective effects or pathological effects for the host organism (208). Repeated cell stress and exposure to pathogens trigger chronic activation of PRR pathways in airway epithelial cells that are highly active and play an important role in chronic airways diseases (204).

PRR-TRIGGERED CYTOKINE AND CHEMOKINE REACTION OF THE AIRWAY EPITHELIUM

Activation of PRRs by DAMPs leads to a massive secretion of proinflammatory mediators like IL-6, CXCL8, TNF that consequently entail infiltration of activated immune cells. Some of these cells like dendritic cells (DC), lymphocytes and mast

cells are also involved in the pathogenesis of asthma (204, 209, 210). After contact for example with HDM extracts, representing a major source of asthma associated allergens, TLR4 dependent activation of NFκB and protease induced injuries in airway epithelial cells lead to secretion of chemokines and cytokines like thymic stromal lymphopoietin (TSLP), GM-CSF, IL-25, and IL-33 (211–215). This results in the activation and infiltration of DCs, innate lymphoid cells type 2 (ILC2) and Th2 cells (216–218).

During infection with bacterial pathogens airway epithelial cells can sense bacterial cell wall components via TLR2 (recognizing e.g., LTA), TLR4 (recognizing e.g., LPS), nucleotide-binding oligomerization domain-containing protein 1 (Nod1) and Nod2 (recognizing peptidoglycans) leading to activation of NFκB and subsequent immune responses and consequently to regulation of bacterial clearance (219, 220). Nucleic acid patterns arising during viral infection can be sensed via TLR3, TLR7/8, retinoic acid inducible gene I (RIG-1), and melanoma differentiation-associated protein 5 (MDA-5) (221–225). In response to TLR activation airway epithelial cells can also produce antimicrobial peptides such as human β-Defensin 2 (HBD-2) after TLR2 activation (201, 226).

The signals of different PRRs like TLRs, NLRs, and RAGE cooperate to regulate cellular immune responses to cell stress, infection and inflammation, which can amplify or dampen their effects (227).

Airway epithelial cells are very potent producers of cytokines and chemokines. The presence of aggressors like toxins and pathogens leads to production and fast and early secretion of IL-1β, IL-6, TNF, CXCL8, CCL11, and CCL20 (202, 228–230). Thereby, airway epithelial cells regulate and orchestrate local immunity by interacting with the recruitment of DCs, T-cells, and B-cells (CCL20), eosinophils (CCL11), and neutrophils (CXCL8). During viral infections they constitutively produce IFNβ to reduce viral replication and to support epithelial apoptosis (231). Thereby, airway epithelial cells represent the frontline of antiviral defense mechanisms. As mentioned earlier, increased concentrations of proinflammatory cytokines like IL-1β, IL-4, IL-13, and TNF can directly lead to damage of the barrier function of the airway epithelium (44–47, 232, 233).

In allergic asthma airway epithelial cells are one of the main producers of proinflammatory cytokines and chemokines like IL-13, IL-33, TSLP, CCL5 (Rantes), CCL7 (MCP-3), CCL17 (TARC), CCL22, and several eotaxins. All of these cytokines strongly direct or support the development of a Th2 polarized inflammation (234). The chemokines CCL17 and CCL22 play a prominent role in the recruitment of Th2 cells by binding to the CCR4 receptor, since activation of it is a key event for Th2 cell specific chemoattraction (235, 236). As a highly potent producer of TSLP the airway epithelium can create a local micro-milieu that supports and maintains a Th2 polarized inflammation (234, 237). In response to different epithelial injuries, airway epithelial cells secrete so-called alarmins like TSLP, IL-25, and IL-33 that direct T helper cell differentiation toward an Th2 phenotype (238). Additionally, secreted GM-CSF from airway epithelial cells leads to maturation and survival of eosinophils (239–241). Both effects are supporting allergic inflammation in asthma.

Taken together the airway epithelium plays a major role for the recognition of PAMPs and DAMPs in the lung. Binding of these molecules to their respective receptors enables the airway epithelium to regulate pathways important for barrier function, MCC and local immune responses. Functional disorders of the airway epithelium in the ability to answer the presence of PAMPs and DAMPs favor the development of chronic airways diseases. Viral infections and exposure to bacteria in early life modulate the acquisition of Th1 and Th2 immunity during further development and influence the responses to following exposures. These effects could play an even greater role in patients with asthma since they show disrupted MCC that could amplify the disease morbidity (242, 243). A cytokine induced Th2 polarization of the epithelium in combination with a barrier dysfunction induced by the same cytokines augments barrier impairment, further infiltration of proinflammatory cells, and enhanced penetration of inhaled allergens, which can be described as a self-reinforcing mechanism that predisposes for the development and perpetuation of allergic asthma (244–246).

Consequently, it has been suggested that an abnormal programming of the airway epithelium in general paired with an impaired capability to produce anti-inflammatory mediators such as IL-37 or α melanocyte stimulating hormone (α-MSH) may be the origin of chronic inflammatory airway diseases (247, 248).

THE AIRWAY EPITHELIUM IN VIRAL EXACERBATIONS OF ASTHMA

Viral infections of the airways is of critical importance for the pathogenesis of allergic bronchial asthma: On the one hand recurrent respiratory viral infections during early childhood represent one of the strongest factors increasing the risk for the development of asthma in later life (249–253). On the other hand such infections are by far the most common cause for acute exacerbation of already established asthma leading to acute aggravation of disease symptoms and necessitating increased medication, GP visits, and can lead to hospitalization and critical care measures under certain conditions (254). Indeed, the airway epithelium is in the center of action during such an exacerbation, since it is not only the barrier that first comes into contact with viral pathogens, but its cells are also the target for their infection and the site of their replication. Thus, viral infection of the airway epithelium does not only impair the barrier integrity as already mentioned before, but also triggers the release of chemokines, cytokines, alarmins, and matrikines of the epithelial layer that affect the pre-existing inflammatory response in the asthmatic airway, which largely contributes to the formation of an acute exacerbation.

The viruses that have been implicated in asthma pathogenesis and especially the formation of acute exacerbation are HRV, RSV, influenza and parainfluenza viruses, human metapneumovirus, corona and adenoviruses, however, HRV infections appear to be the most common cause (255, 256). HRV is a non-enveloped, icosahedral virus, which belongs to the family of picornaviridae (genus enterovirus) and is subdivided into three clades (A, B,

and C). The single-stranded positive RNA genome of HRV is constituted of ca. 7200 nucleotides (257, 258).

Clades A and B, which comprise the 100 most common serotypes, are further subdivided into a major and minor group. The major group utilizes the intracellular adhesion molecule-1 (ICAM-1) to bind to and transfect the host cell (259–261). The minor group HRV bind to the low density lipoprotein (LDL) receptor (262–265) and are considered to be more infectious. Clade C consists of 50 serotypes (266), which all bind to the cadherin-related family member 3 (CDHR3). All species of HRV have been shown to infect and replicate in airway epithelial cells (267).

Viral engagement with the specific receptor leads to transfection of the host cell (e.g., bronchial epithelial cells) and subsequently to a multitude of cellular responses. It is this cellular response that is believed to facilitate acute asthma exacerbation.

The cellular response to the virus is initiated by the detection of single-stranded RNA via TLRs -3, -7, and -8, MAD5, and RIG-I (221, 268). Activation of these receptors ultimately leads to the secretion of cytokines such as IL-1, IL-6, IL-8, IL-11, chemokines like CXCL10, CCL11 (eotaxin), CCL5 and anti-viral interferons of type I and III (268–274). The interferons have not only innate but also adaptive immune-system functionality to keep the viral infection locally at bay by mobilizing the adaptive immune response for effective viral clearance (275). Furthermore, proinflammatory cytokines like IL-1β and IL-6 are not specific for special types of immune response and thus, not only support the immune response against the invading virus but also promote the allergic immune response already established in the airways. Consequently, augmentation of the allergic immune response results in acute amplification of tissue damage, mucus production, and mediator release, and therefore in acute symptom aggravation.

Especially, the interferon response is thought to be an early post-infection event. In asthmatics epithelial interferon responses are believed to be hampered and as a consequence antiviral responses lack sufficient clearance (275). Not only interferons seem to be differently expressed in epithelial cells from asthmatics but also TSLP, which promotes Th2 responses (276). Another critical cytokine elevated in humans after HRV infections is IL-33, which also augments Th2 cell development (277).

AIRWAY REMODELING IS A FEATURE OF HUMAN RHINOVIRUS INFECTION

It is of note that viral infections not only initiate an immune response but also drive remodeling of the epithelial barrier and the subepithelial extracellular matrix (ECM). Hence, HRV16 induces perlecan, collagen V, tenascin c and matrix-associated (ma-) VEGF expression in an either TLR-3 or TLR-3/-7 associated manner *in vitro* (278, 279). Elevated expression of ma-VEGF and tenascin c was replicated in a mouse model of HRV infection, in which also Collagen I and fibronectin was found to be increased (278, 279). In addition, our group found human nasal epithelial cells infected with RV-16 *in vitro* to significantly downregulated genes associated with ECM receptor interaction and focal adhesion (280).

After infection and viral replication, the release of a vast array of mediators is among the earliest responses. In tissue culture a pneumocyte cell line expresses large amounts of IL-8 and CCL20 readily after 6 h post-infection (281). Also, the interferon response is thought to be an early post-infection event. In asthmatics epithelial interferon responses have been suggested to be hampered and as a consequence antiviral responses lack sufficient clearance (275). But not only interferons seem to be differently expressed in epithelial cells from asthmatics but also TSLP, which promotes Th2 responses (276). Another critical cytokine elevated in humans after HRV infections is IL-33. In line with that, Jackson et al. impressively showed, that the release of IL-33 by bronchial epithelial cells induces IL-4, IL-5, IL-13, and GATA3 expression in Th0 cells. An effect, which could be entirely blocked by an antibody against the IL-33 receptor (277).

Recently, active fragments from epithelial deposited ECM molecules have gained some recognition in asthma and asthma exacerbation. Matrikines are a class of molecules derived from ECM proteins (e.g., via proteolysis) with different properties from the parent molecule (282–284).

In 2010, Burgess et al. reported diminished levels of the collagen IV isoform alpha 3 (COL4A3) in airways from asthmatic subjects. The non-collagenous domain of COL4A3 is referred to as tumstatin and a biologically active matrikine. Treatment of mice with experimental allergic asthma with human recombinant tumstatin let to a significant reduction of hallmark disease features (e.g., airway hyperresponsiveness, ma-VEGF, eosinophil influx, IL13) (285). In another study, Van der Velden et al. identified an anti-angiogenic effect of tumstatin in a sheep model of asthma (286). Further investigations revealed a novel active region in Tumstatin (CP17), which significantly reduced neutrophil influx, mucus production in a mouse model of viral asthma exacerbation and reduced migrational speed and production of reactive oxygen species of neutrophils *in vitro* (287, 288). While the matrikine tumstatin conveys protection from experimental features of asthma and asthma exacerbation, a collagen I derived matrikine (PGP, Acetylated-(Ac) PGP) has been shown to be a more potent inducer of neutrophil chemotaxis then IL-8 and is found to be increased in severe asthmatics, a group of patients prone to develop exacerbations (283, 289).

Albeit ECM derived matrikines follow a different kinetical pathway (deposited first, released during inflammation) as compared to cytokines and chemokines (*de novo* production after viral infection), they can serve as protective or aggravating factors in asthma exacerbations.

In addition, the notion of epigenetic modification due to HRV infection in epithelial cells in asthma has gained attraction. McErlean et al. infected nasal epithelial cells from asthmatics and found evidence of reproducible changes to the methylome (290). We confirmed these results, which may unriddle how asthmatic airway epithelial cells may be able to respond differently to the same stimuli as compared to healthy epithelial cells (280). First studies to investigate this effect *in vivo* are underway (291, 292).

E2-POLARIZED AIRWAY EPITHELIUM

Infection associated stimuli appear not to be the only factors that imprint mucosal immune reactions of the airway epithelium. In addition, the interaction between epithelial cells and leukocytes can lead to sustained alteration of respiratory epithelial cell biology. Even though these cells are definitely not able to constitute an immunological memory, it becomes more and more obvious that especially epithelial cells somehow memorize their exposure to certain environmental factors and the following insults and thereby develop some kind of trained immunity. We are just at the beginning to understand these processes and questions of which parts of the epithelium are trained and of how long the training effects sustain in the mucosa remain to be answered.

Thus, respiratory epithelial cells are constantly exposed to many types of challenges, including pathogens, allergens and environmental pollutants. Consequently, they are able to respond quickly and effectively to cellular damage such as the local cytokine production, lateral transport by ion exchanges, wide arrays of mucus compositions, secretion of antimicrobial peptides, and epithelial shedding. To date, it appears possible that different inflammatory environments as originated by for example typical Th1- or Th2-directed immune responses have a different impact on the biology of the respiratory epithelium and lead to some kind of E1- or E2- polarization of the respective epithelial cells (293) (**Figure 4**).

There is *in vivo* evidence for the inhibitory role of IFN-γ on asthma pathogenesis at the epithelial level indicating that type-1 responses counteract allergy (294, 295). The direct implication of airway epithelial cells was demonstrated by selective transgenic expression of the IFN-γ receptor on the airway epithelium and showed that IFN-γ inhibits mucus secretion, release of chitinases and eosinophilia independent of the activation of Th2 cells (296). In turn, GATA-3 inhibition causes an increase of T-BET und IFN-γ expression levels, leading to a dampened allergic phenotype (297). In addition, an increase in DNA-methylation of IFN-γ was observed during allergic sensitization (298), while perinatal prevention of allergy mediated by *Acinetobacter* does not show the anticipated drop in H4 acetylation in the IFN-γ promoter (299). The immunological consequence of epithelial differentiation becomes increasingly interesting, as sensitization, but also recovery processes and airway remodeling could open new options for intervention and prevention of lung damage. Increasing evidence of mechanisms involving epithelial cytokine production such as CCL-26, and the epithelium-derived alarmins TSLP and IL-33 are substantiating the current focus on the cross talk between airway epithelium and immune cells in allergy research.

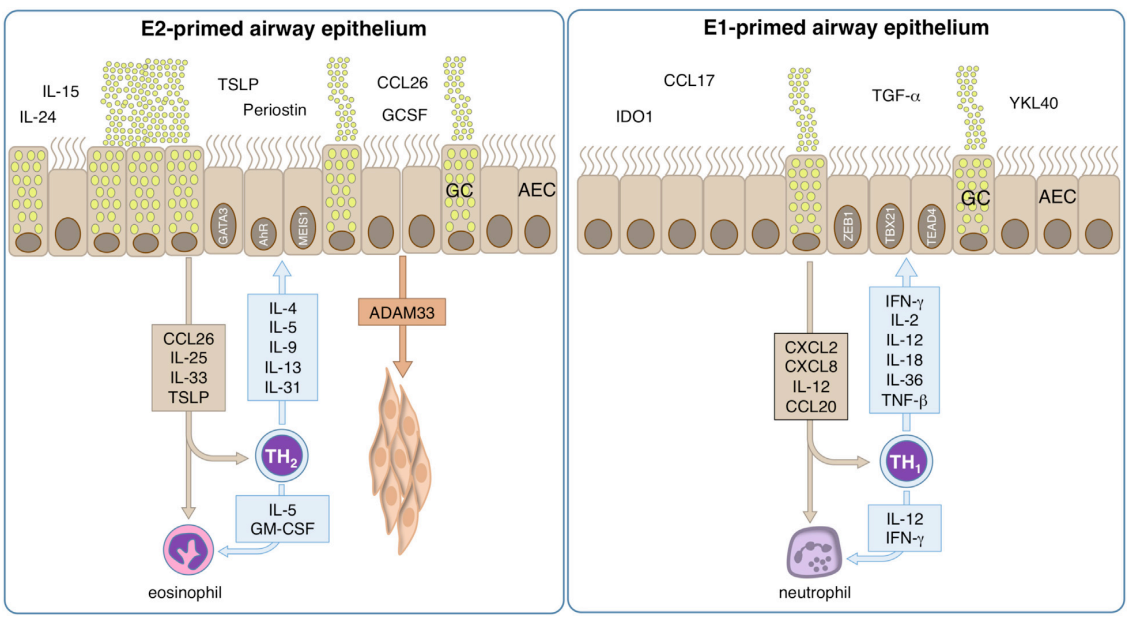

FIGURE 4 | Mechanisms of allergic inflammation in epithelial cells. The role of tissue cells in the early phase of disease is largely unknown, but could provide important information about the pathologic development and could help to identify the causal relationships. However, bronchial epithelial cells are pre-committed to a type-2 (E2) or type-1 (E1) like phenotype. E2 epithelial cell activation by allergens takes place and their pro-inflammatory cytokines and chemokines induce inflammation and contribute to an epithelial type-2 response, so called "E2 response" with epithelial alarmins TSLP, IL-31, CCL-26, IL-25, and IL-33. Local type-2 responses involve multiple cytokines such as IL-4, IL-5, IL-9, IL-13, IL-25, IL-33, and increase of eosinophils. A series of chemokines are produced and migration of inflammatory cells to the allergic tissues takes place. The activation of e.g., smooth muscle cells by ADAM33 lead to remodeling. Bronchial hyperreactivity takes place leading to an enhanced susceptibility to bronchoconstriction. E1 epithelial cells respond to an infection releasing CXCL2, CXCL8, IL12 and CCL20, thus stimulating the local synthesis of IFN-γ, IL-2, IL-12, IL-18, IL-36, and TNF-α that present a wide range of antiviral activities, inducing up-regulation of MHC-I molecules and antiviral resistance in uninfected cells. Neutrophils respond to the infection signals IL-12 and IFN-γ by releasing pro-inflammatory cytokines, leading to the containment of the infection, rise of body temperature and to the recruitment of further phagocytic cells.

However, it is unknown whether epithelial cells are influenced by IL-4 prior or with entry into terminal differentiation. This early influence could imprint the offspring cells that populate the epithelial surface and therefore have major consequences for the physiology of the airways. IL-4 was shown to have a major effect on the epithelium, as mice overexpressing this cytokine under the Club cell-secretory protein 10 (CC10) promoter show increased cellular infiltration, epithelial hypertrophy, mucus cell hyperplasia, secretion of gastric mucins and surfactant proteins (SP) A and B (300). While this model effectively demonstrated all hallmarks of experimental allergic asthma, it did not demonstrate whether IL-4 itself is inducing differentiation of basal cells or whether secondary effects trigger epithelial differentiation. However, the differentiation effects could also be observed in human primary epithelial cells of the nose, where IL-13 modulates the differentiation toward less ciliated and more secretory cells (114). To date, it is controversially discussed, whether IL-4 and IL-13 can also affect fully differentiated epithelial cells in air liquid-interphase cultures or whether this is only possible in immature submerged cultures (301, 302). However, during the epithelial differentiation process induced by air-liquid exposure, the addition of IL-4 enhances expression of certain antimicrobial peptides (303) and eicosanoids (304). Furthermore, it was demonstrated that IL-4 and IL-13, through inhibition of TLR3 expression and signaling (IRF3), impair immune responses to HRV infection (305). This is in line with the finding that chronic house dust mite exposure in the airways not only causes a strong Th2-directed inflammation but also diminishes anti-rhinovirus responses and local IFN expression, particularly of epithelial IFN-λ (306). In line with this, transgenic IL-4 expression in the lungs reduces cytotoxic T cell responses against Influenza viruses (307). On the level of secreted factors, it was shown that cytokines such as Wnt5a (Wingless-Type MMTV Integration Site Family, Member 5a) or IL-24 are expressed as response to IL-4 stimulation only, while proteins with known pathological roles such as the IL-4 induced protein CCL-26 or periostin were shown to be upregulated by IL-4 and down-regulated in IFN-γ environment. These results were consistent when comparing upper and lower airway secretions, thus confirming nasal lining fluids as a proxy for the lower respiratory tract, particularly for epithelial type-2 biomarker like CCL-26 and IL24 (308, 309). The E2-related transcription factor network contained the E2 hub-transcription factors *GATA3, NFE2, MEIS1, HEY2,* and *AHR*: *GATA3* is well known as the master transcription factor of type-2 response in immune cells, however it was also shown to be expressed in airway epithelial cells. *NFE2* was demonstrated to have a cytoprotective activity against epithelial cell injury by cigarette smoke, which could hint on a protective role in an IL-4 dominated micro-milieu (310). For *MEIS1,* it has been demonstrated that its inactivation produces an increase in airway smooth muscle mass and a corresponding decrease in cartilage and suggesting an important role in allergic airway diseases. A loss of Hox gene function, however, does not preclude airway repair, but regenerated epithelium displays goblet cell metaplasia and less SCGB1A1-positive cells, demonstrating the essential role of Hoxa5 for correct differentiation. This goblet cell metaplasia is further associated with increased Notch signaling activity. Consistent with these findings, expression levels of activated NOTCH1 and the effector gene HEY2 are in turn enhanced in patients with allergic disease (293, 311, 312). Taken together, E2-polarization has a marked impact on the barrier and especially immune functions of the airway epithelium and at least supports impairment of the MCC and antiviral responses, both factors that are critical in asthma pathogenesis.

CONCLUSION

In summary, the airway epithelium exerts a broad variety of immune functions that range from passive barrier over MCC, active production and transport of pathogen-neutralizing molecules to pathogen recognition and targeting as well as cytokine and chemokine release. The airway epithelium is usually the first tissue that is exposed to inhaled allergens, pathogens or pollutants. Since it is able to react on this contact by inducing local inflammatory reactions, it is clearly a central part of the local immune response and bridges innate and adaptive immune functions against all types of harmful intruders entering the respiratory system. Therefore, the airway epithelium is a key factor in asthma pathogenesis and plays a critical role in the development as well as in the progression and exacerbation of the disease: Hence, a disturbed cellular barrier enables allergens to enter the body and to induce a sensitization reaction, which is widely regarded as the starting point of an asthma career. Down the line, the protective mucus and PCL layers provided by the epithelium as physical barriers are compromised and the release of pathogen deterring molecules such as secretory Iimmunoglobulins becomes impaired. As a consequence, this frontline toward invading pathogens can be breached and airway epithelial cells are infected and even destroyed by respiratory pathogens. A vicious cycle is started where barrier disturbance and infection promote each other. The latter, in particular with certain viruses, is also one of the strongest factors predisposing toward asthma development in early childhood.

Once asthma has been established, the airway epithelium responds to further viral infection with the release of manifold factors promoting not only the antiviral response but also augmenting the already present allergic inflammation and thus promoting acute asthma exacerbations. Finally yet importantly, it is also impaired and polarized by products released from cells of the chronified allergic immune response in the airways, which leads to impairment of mucus production, MCC, and the anti-inflammatory IFN-response in E2-polarized airway epithelial cells. Recent studies indicate that, in addition to this E2-polarization, also the history of allergy- and pathogen-derived insults does not only have a transient effect on the airway epithelium, but leaves some kind of memory in these cells that can be described as "imprinting" or "trained immunity." Understanding the mechanisms underlying these processes would not only help to further understand the key role of airway epithelial cells in asthma pathogenesis but also could identify new targets for the treatment or even prevention of allergic asthma.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Bronchial Epithelial Cells on the Front Line to Fight Lung Infection-Causing *Aspergillus fumigatus*

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Aspergillus fumigatus is an environmental filamentous fungus that can be pathogenic for humans, wherein it is responsible for a large variety of clinical forms ranging from allergic diseases to life-threatening disseminated infections. The contamination occurs by inhalation of conidia present in the air, and the first encounter of this fungus in the human host is most likely with the bronchial epithelial cells. Although alveolar macrophages have been widely studied in the *Aspergillus*–lung interaction, increasing evidence suggests that bronchial epithelium plays a key role in responding to the fungus. This review focuses on the innate immune response of the bronchial epithelial cells against *A. fumigatus*, the predominant pathogenic species. We have also detailed the molecular interactants and the effects of the different modes of interaction between these cells and the fungus.

Keywords: bronchial epithelial cells, *Aspergillus fumigatus*, innate immunity, lung infection, mucociliary machinery

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INTRODUCTION

Aspergillus spp. are saprophytic filamentous fungi capable of colonizing different ecological habitats. They are usually isolated from soils, decaying organic matters, and plants but are also present in the air and indoor environments (1). *Aspergillus* spores, or so-called conidia, represent the disseminating form of the fungus that spreads through the air. These conidia are produced through asexual reproduction by differentiated fungal cells called phialides, which are themselves carried on a conidiophore. Conidia remain "dormant" or metabolically inactive until they encounter favorable environmental conditions. In this case, the conidia swell, germinate to produce hyphae that grow into a mycelium that harbor conidiophores, and then form conidia (2).

Aspergillus genus encompasses several hundred of species (3). *Aspergillus fumigatus* is by far the most frequent pathogenic species, responsible for about 90% of the cases of *Aspergillus* diseases, followed by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans* (3, 4). Indeed, *A. fumigatus* is the predominant fungal species isolated from the ambient air able to grow at 37°C, the human body temperature.

Humans inhale around a few hundred conidia daily (5). Due to their small size (2- to 3-μm diameter) they can reach the lower respiratory tract (4) but, in most of cases, this phenomenon does not lead to any symptoms thanks to their evacuation by the mucociliary machinery of the tracheobronchial epithelium. However, depending on the immune status of the host, this contamination can be followed by a wide spectrum of manifestations (1). Concisely,

immunocompromised patients are at risk for invasive infection, so-called invasive pulmonary aspergillosis (IPA) and patients with pre-formed lung cavity (typically following previous pulmonary tuberculosis) are prone to chronic pulmonary aspergillosis, of which aspergilloma is one of the main presentations. Finally, patients with altered mucociliary clearance, such as cystic fibrosis (CF) patients, may be colonized which can turn, in patients with exacerbated immune response, into allergic bronchopulmonary aspergillosis (ABPA).

The essential role of neutrophils and monocytes in anti-*Aspergillus* immunity has been emphasized by the high rate of incidence of *Aspergillus* invasive infection in patients with quantitative (neutropenia) or qualitative (corticosteroid therapy, chronic granulomatous disease) deficiency of these cells (6–8). However, the role of the bronchial epithelium should not be underestimated as it represents the first physical and biological barrier preventing fungal implantation.

While studies looking at the interactions between *Aspergillus* and leukocytes (alveolar macrophages and recruited neutrophils) are numerous (6, 9–11), data on the role of bronchial epithelial cells (BECs) in anti-*Aspergillus* defense are still limited. Yet, BECs seem to play a crucial role in the innate immune response against *Aspergillus* particularly in preventing the bronchial colonization. The high prevalence of *Aspergillus* bronchial colonization in patients suffering from CF (12, 13), a disease characterized by the thickening of the bronchial mucus, highlights this phenomenon. Bronchial *Aspergillus* colonization, whose role in the subsequent development of IPA is still debated, may have deleterious consequences as it is the starting point of *Aspergillus* bronchitis and immuno-allergic forms (14, 15). In CF patients, while remaining superficial, bronchial colonization is associated with the occurrence of bronchial exacerbations, a decline in lung function, and ABPA with a prevalence ranging between 1 and 15% (16). Fungal sensitization to *Aspergillus* antigens may also occur in allergic patients (17) but the role of the bronchial epithelium in these diseases won't be analyzed in this review.

Thanks to experimental studies, there is increasing knowledge on the interactions between the different morphotypes of *A. fumigatus* and BECs. This review aims to decipher these interactions at the molecular level and their effect on anti-*Aspergillus* immunity.

STUDY MODELS OF THE INTERACTION BETWEEN *ASPERGILLUS FUMIGATUS* AND BRONCHIAL EPITHELIAL CELLS

The respiratory tract is lined by epithelial cells whose types vary according to the anatomic structure of the airways. Trachea, bronchi, and bronchioles are lined by the pseudostratified epithelium, while type I and II pneumocytes constitute the alveolar epithelium. At the bronchial level, the pseudostratified epithelium is mostly composed of ciliated, secretory, and basal cells from which the first two derive.

To understand the interactions between *Aspergillus* and BECs, different cell lines (immortalized or tumor) have been commonly used. Among the most popular bronchial cell lines used and commercially available, we can cite BEAS-2B and 16HBE, both isolated from normal human bronchial epithelium and secondarily immortalized through transfection of a replication-defective SV40 plasmid (18, 19). NCI-H292 cells derive from a lymph node metastasis sample of a pulmonary mucoepidermoid carcinoma. But other respiratory cell lines are occasionally used in some studies. All these cell lines have major advantages such as easy to maintain (cultured in simple and inexpensive culture media), capable of growing at high densities, and exhibiting an extended life span (20). However, these cells represent only one donor, and many cellular processes are deregulated due to immortalization. Refinement of the model consists of the use of commercially available primary bronchial cells that are free from any genetic modification and whose physiological functions are intact. However, before they are used, those cells must undergo antibiotic, antifungal, and growth factor treatment. Usually, these cells have a limited life span with limited proliferation capacity and are more difficult to culture than cell lines requiring more complex, specialized, and expensive cell culture media. Irrespective of the cell type, cells are usually cultured under submerged conditions, i.e., in flat-bottom plastic wells filled with culture medium, that hamper cells to differentiate. To better mimic physiological conditions, air-liquid interface cell culture (ALI) systems have been developed (21). In this case, primary BECs and also some cell lines such as 16HBE, differentiate until they develop the mucociliary phenotype characteristic of a pseudostratified epithelium and express mucins (20). Basal surface is therefore in contact with the liquid medium and the apical part of the cellular layer is exposed to the air. Obtaining this type of differentiated epithelium is time-consuming and requires specific technical skills but such cell culture systems mimic the required *in vivo* conditions in the best way.

Different approaches can be used to mimic an *Aspergillus* infection. *Aspergillus*, mostly in the form of dormant conidia can be inoculated in cell culture supernatants and then recovered after defined incubation intervals to measure the parameters of interest (such as cytokine level, cytotoxicity, etc.). In these conditions, *A. fumigatus* hyphae are usually obtained after 15 h of incubation. Killed (UV or irradiated for example) resting, swollen conidia and hyphae have also been used as inoculum. Differences between experimental protocols, especially the use of different multiplicity of infection, likely far from reality, could explain some discrepancies in the results obtained in different studies.

In addition to *in vitro* models, *in vivo* models of *Aspergillus* infection have already been used. Mice, rats or rabbits are the animals the most commonly used. They are immunosuppressed or not, and infected with *A. fumigatus* through inhalation of conidia administered either intranasal or *via* intratracheal route. In addition to the measurements of mortality rate and/or fungal

Abbreviations: BECs, Bronchial epithelial cells; IPA, Invasive pulmonary aspergillosis; CF, Cystic fibrosis; PCD, Primary ciliary dyskinesia; ABPA, Allergic bronchopulmonary aspergillosis; ALI, Air-liquid interface; DHN, Dihydroxynaphthalene; HBE cells, papilloma virus–immortalized bronchial epithelial cell line; PAMPs, Pathogen-associated molecular patterns; PRRs, Pathogen recognition receptors; IL-1Ra, Interleukin-1 receptor antagonist; NLRP3, NOD-, LRR-, and pyrin domain-containing 3; ROS, Reactive oxygen species; IL, Interleukin; AMPs, Antimicrobial peptides; hBD, Human β-defensins; NAGase, N-Acetyl-β-d-glucosaminidase.

load in the lungs, more precise descriptions of the immune response have also been reported, looking at the immune cells recruitment or inflammatory response (22). *In vivo* models have the considerable advantage of most closely imitate lung infection and immunity as a whole, however, there is a paucity of *in vivo* models (conditional and inducible transgenic mice targeting bronchial/airway epithelial cells) allowing the study of BECs against *Aspergillus* challenge specifically.

ANTI-*ASPERGILLUS* PHYSICOCHEMICAL ACTIVITY EXHIBITED BY THE BRONCHIAL EPITHELIUM

Inhaled conidia first face the physical barriers of the upper airways that include the mouth, nose, larynx, and pharynx. Mucociliary clearance from the nasal walls and mechanical defenses such as coughing and sneezing help eliminate most of the inhaled particles. If the conidia pass these first barriers, they then arrive in the lower airways consisting of the trachea that divided into two-stem bronchial tubes, which in turn are subdivided into several smaller bronchial tubes, followed by bronchioles that end with the alveoli. The bronchial epithelium participates in the clearance of inhaled conidia to prevent their germination and growth locally. Secretory cells, including serous and goblet cells, together with submucosal glands, participate in the formation of mucus, which protects the epithelium from the inhaled particles. Basically, the mucus traps the inhaled particles, which are then actively transported by the beating of the cilia to the oropharynx where they are swallowed or expectorated. Under healthy conditions, mucus is composed of 97% water and 3% of mucins, non-mucin proteins, salts, lipids, and cellular debris (23). Mucins, namely MUC5AC and MUC5B, are the major macromolecular constituents of the mucus. They are large glycoproteins with serine-/threonine-rich domains linked by their hydroxyl side groups to sugar chains forming a polymeric gel that ensure the properties of the mucus (24). Ciliated cells also play a fundamental role in the elimination of particles engulfed in the mucus because they mechanize the movement of the mucus blanket (25). The role of these physicochemical barriers associated with the bronchial epithelium can be better understood in patients suffering from CF or primary ciliary dyskinesia (PCD) in whom mucus properties and/or ciliary beating are impaired. Hence, CF patients are frequently colonized by *A. fumigatus* (14), and a similar trend has been noticed in patients with PCD (26). This machinery can also be altered by pathogens. Indeed, mycotoxins secreted by *A. fumigatus*, damage epithelial cells and inhibit ciliary beating (27). Among those toxins, gliotoxin has been extensively studied because it is the most abundant one produced by *A. fumigatus* and it exhibits immunosuppressive properties that have been described extensively in a previously reported review (28).

RECOGNITION OF *ASPERGILLUS FUMIGATUS* BY BRONCHIAL EPITHELIAL CELLS

Schematically, the interaction between *Aspergillus fumigatus* and the BECs leads to three main types of cellular response: internalization, synthesis of cytokines/chemokines and release of bioactive molecules potentially active against *Aspergillus*. Specific interactions between *A. fumigatus* and BECs require close contact between the fungus and the cell-surface ligands. Fungal cell-wall polysaccharides and, to a lesser extent, some proteins or the genetic material (ADN or ARN) act as pathogen-associated molecular patterns (PAMPs). They are sensed through pathogen recognition receptors (PRRs), several of which but not all, have been identified in BECs (5) (**Table 1**).

TABLE 1 | Pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) involved in the recognition of *Aspergillus fumigatus* by bronchial epithelial cells and the consequences of their activation.

PRRs	*A. fumigatus* PAMPs	Cellular study model	Role in Anti-*Aspergillus* immunity on BECs	References
Dectin-1	β-D-Glucan	HBE cells	Internalization Pro-inflammatory cytokines and chemokines release Inflammasome activation ROS generation	(29)
TLR2	β-D-Glucan	HBE cells	IL-6 and IL-8 release Increase in Dectin-1 expression	(29)
MR	Mannose-rich polysaccharides		Not elicited	
Unknown	FleA (conidia)	BEAS-2B cells	IL-8 synthesis Inhibition of *Aspergillus fumigatus* germination of extracellular conidia Binding of conidia to mucins and to macrophages	(30–32)
TLR3	dsRNA (resting conidia)	Primary human BECs	Release of inflammatory mediators, interferon (IFN)-β and IFN-γ-inducible protein (IP)-10	(33)
TLR4	Unknown		Not elicited	(34, 35)
TLR9	Hypomethylated DNA		Not elicited	(34, 35)
Pentraxin 3	Galactomannan		Not elicited	(36)

BECs, Bronchial epithelial cells; IL, Interleukin; dsRNA, double stranded RNA; ROS, Reactive oxygen species; HBE cells, Papilloma virus–immortalized bronchial epithelial cell line.

A. fumigatus cell wall encompasses an inner and outer layer whose composition varies along with the fungus' life cycle (**Figure 1**). The outer part of the conidial surface is composed of hydrophobic RodA proteins that conceal an underlying fungal pigment, dihydroxynaphthalene (DHN) melanin (37). This outer layer plays a key role in conidial dispersion, their protection against external stress factors such as desiccation, physical damage, drugs, and UV radiation. They also mask the epitopes present in the underlying layer by inhibiting their recognition by the host's innate immune system (38–40). Whereas, dormant conidia are described as immunologically inert, the FleA lectin, a fucose-binding lectin expressed on their surface (30, 41), has been shown to mediate their binding both to the airway mucins produced by the epithelial cells and also to macrophages (31). Moreover, stimulation of BEAS-2B cells by FleA has been reported to lead to an increase in interleukin (IL)-8 synthesis and contributed to the inflammatory response (30). Thus, FleA acts as a PAMP-like molecule whose cellular ligands remain to be identified.

Under favorable conditions, the dormant conidia become metabolically active and ensue an increased intracellular osmotic pressure followed by water uptake and isodiametric growth (42). The resulting swollen conidia lose their rodlet layer by proteolytic degradation, and consequently, their hydrophobicity. This phenomenon is associated with a rupture of the melanin layer by a still-unknown mechanism (43). Then, the swollen conidia grow in a polarized way that leads to the formation of a germ tube. At this stage, the melanin layer is disrupted but the inner layer of the cell wall remains unchanged and participates in hyphal formation. The hyphae are mainly composed of galactosaminogalactan (GAG) that allows adhesion of the filaments to various biotic or abiotic surfaces (44, 45). Recent studies have also shown that GAG, expressed during conidial germination, exhibits a possible anti-inflammatory effect. Indeed, GAG induces the release of the IL-1 receptor antagonist (IL-1Ra), a potent anti-inflammatory cytokine that blocks IL-1 signaling (46), by macrophages and neutrophils. Furthermore, in a mouse model of aspergillosis, treatment by GAG before and during intranasally induced *A. fumigatus* infection inhibits neutrophil infiltration in the lung at the site of infection (6, 44, 46).

Regardless of the *A. fumigatus* morphotype, the inner layer, the so-called fibrillary core, is continuously composed of branched β-(1, 3)-glucan/β-(1, 4)-glucan, chitin, galactomannan, and α-(1, 3)-glucan (43). A few studies have shown that, as already demonstrated for macrophages and digestive epithelial cells (47), Dectin-1 is a major but not the unique receptor for β-glucan on a papilloma virus-immortalized BEC cell line (HBE cells) (29). Dectin-1 is a transmembrane receptor and member of the C-type lectin receptor family (11). The role of Dectin-1 in the immune response against *A. fumigatus* has been highlighted in different *in vivo* studies. For example, immunocompetent mice lacking Dectin-1 are more sensitive to intratracheal challenge with *A. fumigatus* than control mice (48). In humans, mutations in Dectin-1 are associated with increased susceptibility to IPA (49). It has also been shown that immunocompromised mice, transfected to upregulate Dectin-1 expression in airway epithelial cells, have a lower fungal burden, an increase in the recruitment of neutrophils into the lungs and a greater survival rate in response to intratracheal injection of *A. fumigatus* conidia compared to the controls (50). After ligation of β-glucan to Dectin-1, two distinct signaling pathways are activated through the spleen tyrosine kinase (SYK)-caspase recruitment domain-containing protein 9 (CARD9) or through RAF-1 (51). These pathways act synergistically to induce nuclear factor-κB

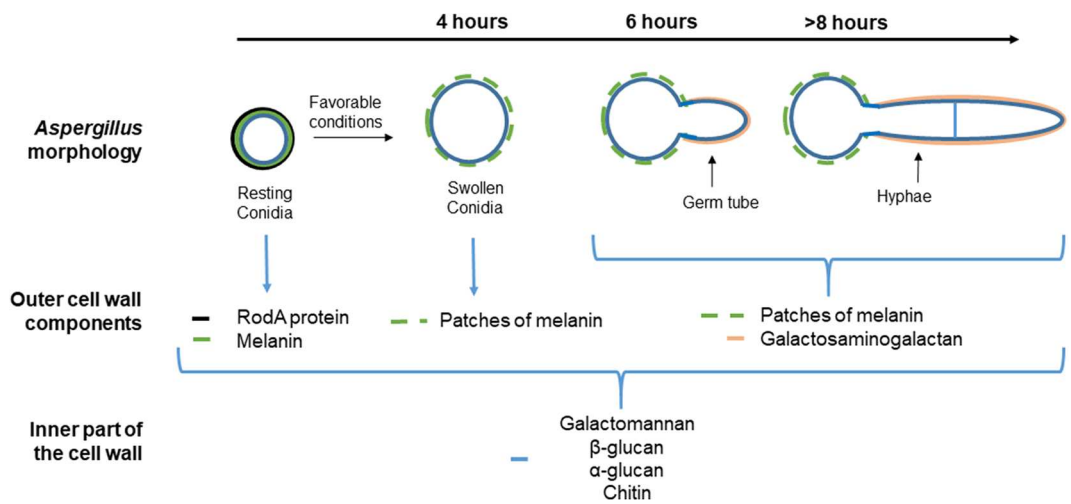

FIGURE 1 | Different morphological stages and compositions of the cell wall during the vegetative cycle of *Aspergillus fumigatus*. The cell wall of the resting conidia consists of (the most external part to the inner) (i) the rodlet layer (black), composed of the hydrophobin RodA, (ii) the melanin layer below (green), and (iii) polysaccharides (blue). Under favorable conditions, the resting conidia begin to swell after 4 h. The rodlet layer is lost by proteolytic degradation and conidium swelling is due to an increase in the internal osmotic pressure. The melanin layer is then disorganized and the inner layer of the swollen conidium forms the mycelium cell wall. After 8 h, hyphae are apparent. Then, an extracellular matrix mainly composed of galactosaminogalactan (GAG) (pink) covers up the hyphae. Specific and universal components of each morphotype are shown below each image.

activation and pro-inflammatory gene expression (52). The SYK–CARD9 pathway also activates the NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome, which results in the proteolytic activation of the pro-inflammatory cytokines IL-1β and IL-18 by caspase 1. The role of *A. fumigatus* antigens in this activation cascade has been demonstrated by Jeong et al. They showed that sensitized mice intratracheally challenged with *A. fumigatus* crude antigens displayed an increased in immunofluorescence intensities of NLRP3 and caspase-1 in lung tissue, particularly in epithelial cell layers, leading to an increase in IL1-β concentration in the lung tissue (53). Similar results were obtained by using an *in vitro* model utilizing primary human BECs stimulated with the same *A. fumigatus* antigens (53). The mannose receptor (MR) is another C-type lectin receptor involved in fungal, and notably *Aspergillus* conidia, recognition and is expressed by 16HBE cells and primary BECs (54). The MR recognizes carbohydrates rich in mannose typically produced by many microorganisms including fungi (55) but its immune-specific role against *A. fumigatus* in association with BECs has not been investigated.

Toll-like receptors (TLRs) are another family of conserved PRRs (56). So far 10 different TLRs have been identified and described. TLR2, TLR4, and TLR9 are the main molecules involved in sensing fungal components (11, 57, 58). These receptors possess extracellular leucine-rich repeat ligand-binding domains and a conserved intracellular toll/IL-1R (TIR) signaling domain that induces specific signaling cascades through intracellular TIR containing adaptors such as MyD88. Interestingly, the expression of the 10 TLRs has been detected by reverse transcription–quantitative real-time polymerase chain reaction in two independent studies using human primary BECs (34, 35). Using a silencing method, it has been demonstrated that TLR2, in a heterodimer form with TLR1 or TLR6, is required for the expression of Dectin-1 by HBE cells in response to *A. fumigatus* infection (29). Moreover, blocking TLR2 with antibodies results in the inhibition of the release of IL-6 and IL-8 by BEAS-2B cells infected with *A. fumigatus* hyphal fragments (59). Certain polymorphisms in TLR4, receptor for the bacterial lipopolysaccharide, are clearly associated with increased susceptibility to invasive aspergillosis (60). TLR4 expressed by mouse macrophages, recognizes *A. fumigatus* conidia and hyphae and induces the release of pro-inflammatory molecules. However, after blocking TLR4 with a specific monoclonal antibody, Øya et al. failed to detect any significant change in the levels of IL-6 and IL-8 released by BEAS-2B cells infected with X-ray-treated hyphal fragments of *A. fumigatus* (59). Hypomethylated DNA, the natural ligand of TLR9, has been extracted in *A. fumigatus* hyphae (61). In murine model of invasive aspergillosis, Leiva-Juarez et al. revealed that therapeutic stimulation of lung epithelial defenses by inhalation of a synergistic combination of TLR 2/6 and TLR9 agonists robustly protects against the development of *A. fumigatus* lung infection despite the profound immune dysfunction (62). TLR9 is expressed by BECs (16HBE cells) (63) but its role in response to *Aspergillus* challenge remains to be investigated in the context of these cells. TLR3 is localized onto endosomal membranes and recognizes double-stranded (ds)RNA. It is primarily involved in the recognition of viruses but has also been implicated in the recognition of *A. fumigatus* dsRNA of resting conidia (33). In this study, the infection by *Aspergillus* of human primary BECs cultivated either in submerged or in ALI culture system, induced the release of inflammatory mediators, notably interferon-β and interferon-γ-inducible protein-10, through TLR3 signaling. Interestingly, this induction is internalization dependent, as demonstrated by the use of an actin-polymerization inhibitor and observed only with killed resting conidia (heat- or UV light-inactivated).

Finally, pentraxin 3, a soluble PRR, plays a key role in the recognition, uptake, and killing of *Aspergillus* conidia by macrophages and dendritic cells through binding to galactomannan (64). This molecule can act as an opsonizing factor for activating the complement system and subsequent phagocytosis by macrophages (65). Pentraxin 3 is secreted by both human primary bronchial and BEAS-2B cells but, again, its precise role in these cell types has yet to be determined (36).

INTERNALIZATION OF *ASPERGILLUS FUMIGATUS* CONIDIA BY BRONCHIAL EPITHELIAL CELLS

The alveolar epithelial cell line A549 has been extensively used to study conidia internalization by non-professional phagocytic cells (66–68). Wasylnka and Moore described that internalized conidia fused with lysosomes and colocalized with lysosomal protein (Lysosomal-associated membrane protein 1 and CD63). Nonetheless, a significant percentage of internalized conidia persist and germinate in A549 epithelial cells. Little is known about the internalization by BECs and conflicting results have been published (**Figure 2A**). First, Paris et al., showed by microscopic observations that rabbit tracheal epithelial cells were able to internalize *A. fumigatus* conidia after 6 h of incubation and that conidia were enclosed in membrane-bound vacuoles (69). Then, other studies reported that the conidia of *A. fumigatus* were taken up *in vitro* by a human bronchial epithelial cell line cultured in monolayers (70, 71). Indeed, Clark et al. observed the internalization of 10 to 20% of the conidia in contact with BEAS-2B cells at 6 and 9 h after challenge. In comparison, 70% of conidia in contact with macrophages were internalized after 1 h incubation (71). A 41% internalization rate has also been reported with 16HBE cells after 6 h incubation (70). Overall, these results contrast with those from other studies that have considered the internalization by BECs a very minor phenomenon. By using a model of primary BECs grown in ALI culture system, Toor et al. showed that only 1% of the bound conidia were internalized 6 h after exposure (72). Similarly, Fernandes et al. were unable to demonstrate any case of internalization using primary BECs cultured in ALI (73). However, these authors described the formation, within the cell, of an actin tunnel not altering the viability of the penetrated cells (**Figure 2A**). The authors suggested that this event could explain the penetration of the hyphae into the underlying

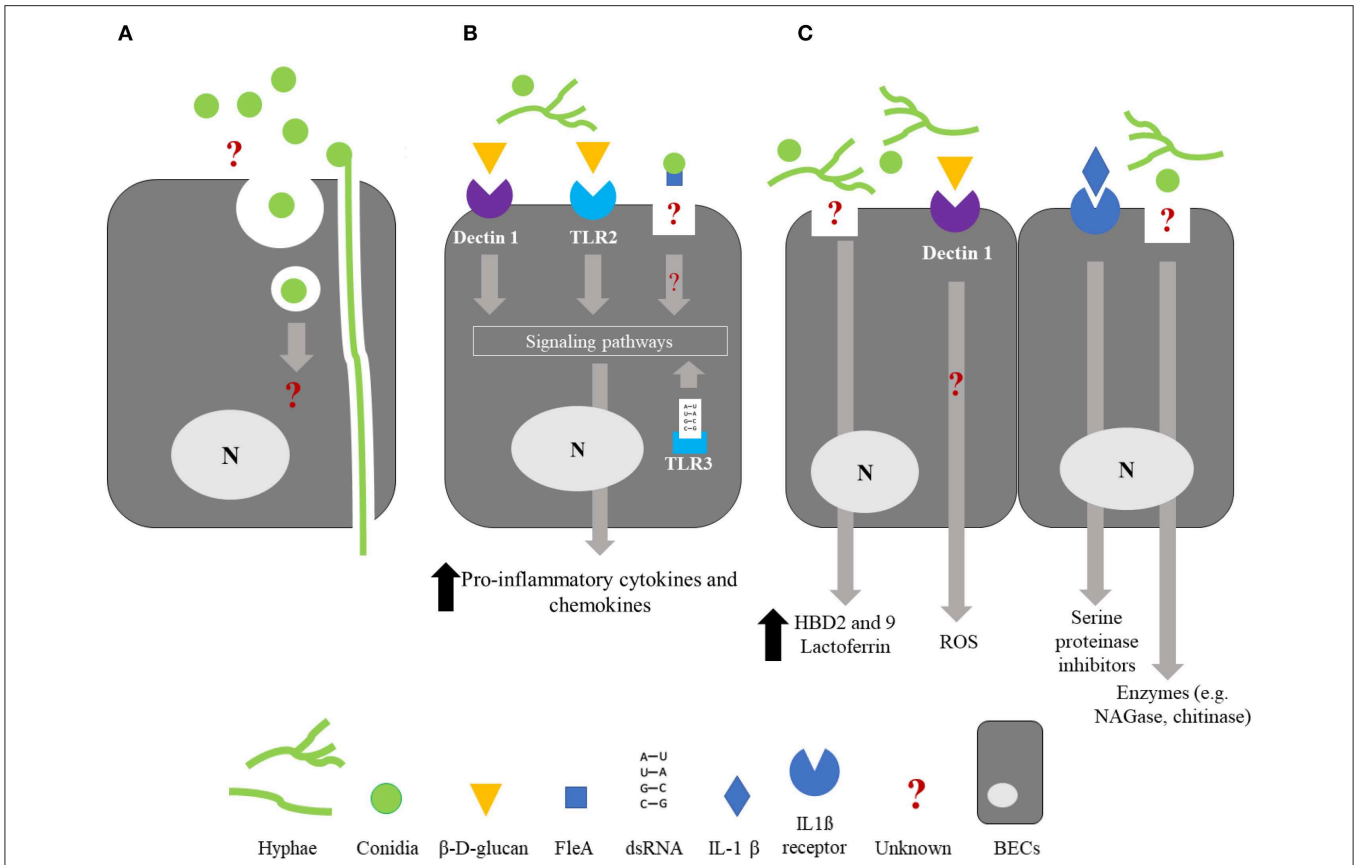

FIGURE 2 | Summary of the interactions between bronchial epithelial cells and *Aspergillus fumigatus*. For clarity, it should be noted that the schematized epithelial cells are not polarized. **(A)** Internalization; BECs are able to internalized *A. fumigatus* conidia but the molecular mechanisms involved in this phenomenon and the fate of the internalized conidia are still unknown. An alternative way of penetration of the hyphae through an actin channel within the cell has been proposed. **(B)** Cytokine/chemokine release; Recognition of β-D-glucan by BECs through the membrane receptors Dectin-1 and TLR2, and recognition of *A. fumigatus* dsRNA by the endosomal receptor TLR3 result in the activation of intracellular signaling pathways and a significant increase in the release of proinflammatory cytokines and chemokines, leading to phagocyte recruitment. FleA lectin, expressed on the surface of *A. fumigatus* conidia, induces IL-8 synthesis by BECs and binding of the conidia to airway mucins. Recognition of FleA by BEC induces the inhibition of conidium germination. **(C)** Bioactive molecules potentially active against *A. fumigatus*; BECs synthetize antimicrobials molecules such as defensins HBD2 and HBD9, lactoferrin, reactive oxygen species (ROS), SLPI, ESI, and chitinase in response to *A. fumigatus* infection or inflammatory challenge. Until now, an antifungal activity has only been described for lactoferrin, ROS, SLPI, ESI, and chitinase. BECs, Bronchial epithelial cells; SLPI, Secretory leukocyte proteinase inhibitor; ESI, Elastase-specific inhibitor; NAGase, N-Acetyl-β-d-glucosaminidase; ROS, Reactive oxygen species; N, nucleus.

parenchyma leading to the development of invasive infection in immunocompromised patients (73). It is noteworthy that the internalization of conidia by BECs was not seen 18 h after an intratracheal challenge in an immunosuppressed mouse model (74). The molecular mechanism(s) involved in the internalization of *Aspergillus* conidia by BECs remains incompletely understood. Adhesion of conidia onto BECs induces actin polymerization (75), a phenomenon dependent on the activity of the human actin reorganization complex 2 and 3, regulated by Wiskott-Aldrich syndrome protein-interacting proteins (76). Using the BEAS-2B cell line, Clark et al. identified 7 host markers—caveolin, flotillin-2, RAB5C, RAB8B, RAB7A, 2xFYVE, and FAPP1—that consistently localized around the internalized conidia (71).

Even if the internalization of *Aspergillus fumigatus* conidia by BECs is a rare event, however, it could represent a starting point of invasive forms in immunosuppressed patients. The fate of the internalized conidia is still unknown and one can postulate that some of them remain quiescent within the cells until they reactivate, thanks to iatrogenic immunosuppression (77). This could explain the breakthrough of some invasive aspergillosis cases occurring in immunocompromised patients otherwise protected from contamination from the ambient air using a high-efficiency particulate air filter chamber (78).

SYNTHESIS AND RELEASE OF BIOACTIVE MOLECULES BY BRONCHIAL EPITHELIAL CELLS INFECTED WITH *ASPERGILLUS FUMIGATUS*

Cytokine/Chemokine Synthesis

One of the most studied consequences of PAMP/PRR interaction is the induction of cytokine/chemokine synthesis. Several studies

have shown that infection of BECs with *Aspergillus fumigatus* leads to the release of pro-inflammatory cytokines, mostly IL-6, IL-8, or tumor necrosis factor-α provided that the infection time is more than 6 h and allows conidia germination (79–81) (**Figure 2B**). Hence, after 6 h of incubation, expression (mRNA) of not only tumor necrosis factor-α and IL-8 but also granulocyte macrophage-colony stimulating factor (GM-CSF) in HBE cells exposed to *A. fumigatus* conidia, is significantly increased by 8 to 14 times in regards to non-infected cells (29). Similarly, BEAS-2B cells release an increased amount of IL-8, 8 h after having been infected with *A. fumigatus* conidia, at a time corresponding to the hyphal formation (79) (**Figure 1**). In contrast, IL-8 synthesis was not triggered 15 h post-infection in BEAS-2B cells infected with a mutant strain of *A. fumigatus* unable to germinate (79). Considering the differences in the cell-wall content between the conidial and hyphal stages, these observations strongly suggest a role for some parietal molecules in masking resting conidia, avoiding their recognition by the BECs. RodA hydrophobin, whose masking role toward immune cells has been mentioned above, could be involved but, to the best of our knowledge, this hiding role toward BECs has never been checked.

Inflammatory mediators act synergistically to establish an organized and regulated host response against *Aspergillus fumigatus*. For example, IL-8, also known as CXCL-8, is a chemokine exhibiting a pleiotropic effect on neutrophils: strong chemotactic influence, degranulation of lysosomes with release of enzymes within the phagosome, production of reactive oxygen species (ROS), and increased expression of adhesion molecules (82). GM-CSF acts both as a hematopoietic growth factor favoring the proliferation and differentiation of myeloid cells into mature cells such as neutrophils and macrophages, and as an enhancer of the antimicrobial functions of those cells. Very recently, our group demonstrated that the inflammatory response of BEAS-2B cells and human primary BECs against *A. fumigatus* could be reprogrammed after the first contact with a microbial ligand, in this case *Pseudomonas aeruginosa* flagellin (81). Pre-stimulation with this TLR5 ligand led to a significantly enhanced release of two proinflammatory cytokines, IL-6 and IL-8, after an *A. fumigatus* challenge. This is comparable to the phenomenon called trained immunity or innate immune memory that has been largely studied using monocytes/macrophages (83–85).

Molecules Potentially Active Against *Aspergillus fumigatus*

Antimicrobial peptides (AMPs) are cationic small-peptide chains that exhibit antimicrobial activity against a variety of pathogens including fungi (86). Although membrane permeabilization is the main mechanism of action of AMPs against pathogens, additional mechanisms have been described including inhibition of macromolecular synthesis (87). Under basal conditions, BECs release a number of AMPs or proteins, some of which exhibit potential antifungal activity (**Figure 2C**).

The defensin family, divided into three classes (α-, β-, and θ-defensins), includes broad-spectrum antimicrobial peptides that are evolutionarily conserved across the living world (88). Human β-defensins are a characteristic of epithelial tissues and present a constitutive expression in primary human BECs (89). Human β-defensin 2 and human β-defensin 9 are reported to be highly expressed by BECs (HBE and 16HBE cells) exposed to different morphotypes of *A. fumigatus* (29, 90). Lactoferrin is a protein synthesized and released by 16HBE cells (91). Interestingly, according to Lupetti et al., a synthetic peptide based on the human lactoferrin sequence but containing only the first cationic domain is one of the most potent antimicrobial peptides against *A. fumigatus* hyphae and conidia *in vitro* (92). Lactoferrin can also act by reducing the toxic effect on host cells (cytotoxicity, oxidation level, and DNA damage) of aflatoxin, a mycotoxin synthesized by *Aspergillus* (93). Secretory leukocyte proteinase inhibitor (SLPI), also called anti-leukoprotease or mucus proteinase inhibitor, and elastase-specific inhibitor (or elafin and trappin-2) are two serine proteinase inhibitors constitutively secreted from the airway epithelium (94). Both are secreted by BECs in response to pro-inflammatory cytokines such as IL-1β (95, 96). Different biological functions have been reported for these molecules: protection of the lungs against the damage induced by neutrophil serine proteases and also antimicrobial activity notably against fungi like *A. fumigatus* (97, 98). However, these proteins are not considered as AMPs because their size, 11.7 and 9.9 kDa for SPLI and elastase-specific inhibitor respectively, are too large to be classified as peptides.

Interestingly, BEAS-2B and HBE cells are also able to produce ROS (29, 99). In their study on BECs challenged with *A. fumigatus*, Sun et al. showed that conidia induced ROS generation in a Dectin-1-dependent manner after 6 h of infection (29). ROS are known to be produced by neutrophils thanks to nicotinamide adenine dinucleotide phosphate oxidase produced in response to germinating *A. fumigatus* challenge (100, 101). They are released from granules either into phagosomes or into the extracellular environment, inducing damage to *Aspergillus* (6). But until now, the precise role of ROS produced by BECs in antifungal activity remains undetermined.

The study of the secretome of BEAS-2B cells infected with *A. fumigatus* also gave more insight into the role of some molecules that may possibly act against the fungal infection (102). Among the most significant results, Fekkar et al. found the release of lysosomal enzymes such as N-Acetyl-β-d-glucosaminidase, cathepsin B, and cathepsin D. N-Acetyl-β-d-glucosaminidase is responsible for the hydrolysis of glycosidic bonds. Cathepsin B and D, members of the lysosomal cysteine protease family, are known to acidify the phagosome of macrophages but their role in BECs has not been determined yet. Chitinase, a member of N-Acetyl-β-d-glucosaminidase, degrades the major fungal-wall component chitin and as such could play a role in the control of *Aspergillus* infection (103, 104).

At this time, not all the active molecules may have been described. Recently, Richard et al. reported that BEAS-2B cells prevent the germination of conidia without internalization but failed to demonstrate the role of any soluble compound present in the supernatants of infected cells (32). This antifungal activity of BECs is fungistatic and occurs via a mechanism that is phosphoinositide 3-kinase dependent. The same kind

of observation was made by Clark et al. who found that after infecting BEAS-2B cells with *A. fumigatus* conidia, a large subset of conidia is rendered metabolically inactive, as measured with the metabolic marker FUN-1, while not being internalized by the cells (71).

CONCLUSION

The evidence of BECs playing an important role in the innate immunity-based defense mechanism against *Aspergillus fumigatus* is now growing. Recent studies have been able to demonstrate that, in addition to the production of mucus and ciliary beating that allow the clearance of the conidia, BECs are directly involved in an immune response against *A. fumigatus* through the recognition of fungal cell-wall components, mainly polysaccharides, by cellular ligands such as Dectin-1 or TLRs. There is a consensus for a pro-inflammatory response by BECs stimulated by *A. fumigatus*. However, additional studies are needed to better decipher BECs response. Studies focused on the internalization of the conidia by BECs reported divergent results according to the study model. This warrants further studies to clarify this point such as the investigation of the fate of the internalized conidia that could play a role in the future development of invasive aspergillosis. Different types of AMPs are also produced by BECs, some of them being active directly or indirectly against *Aspergillus*, making these molecules appealing for new therapeutic approaches. Overall, the bronchial epithelium appears as a suitable target for novel therapeutic strategies aiming to restore barrier integrity and to enhance defenses against inhaled pathogens.

AUTHOR CONTRIBUTIONS

JB, VB, and CH: drafting of the manuscript. JB, VB, CH, LG, JG, MR, and HC: revision of the manuscript.

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MINI REVIEW
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Mechanisms of Virus-Induced Airway Immunity Dysfunction in the Pathogenesis of COPD Disease, Progression, and Exacerbation

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Chronic obstructive pulmonary disease (COPD) is the integrated form of chronic obstructive bronchitis and pulmonary emphysema, characterized by persistent small airway inflammation and progressive irreversible airflow limitation. COPD is characterized by acute pulmonary exacerbations and associated accelerated lung function decline, hospitalization, readmission and an increased risk of mortality, leading to huge social-economic burdens. Recent evidence suggests ∼50% of COPD acute exacerbations are connected with a range of respiratory viral infections. Nevertheless, respiratory viral infections have been linked to the severity and frequency of exacerbations and virus-induced secondary bacterial infections often result in a synergistic decline of lung function and longer hospitalization. Here, we review current advances in understanding the cellular and molecular mechanisms underlying the pathogenesis of COPD and the increased susceptibility to virus-induced exacerbations and associated immune dysfunction in patients with COPD. The multiple immune regulators and inflammatory signaling pathways known to be involved in host-virus responses are discussed. As respiratory viruses primarily target airway epithelial cells, virus-induced inflammatory responses in airway epithelium are of particular focus. Targeting virus-induced inflammatory pathways in airway epithelial cells such as Toll like receptors (TLRs), interferons, inflammasomes, or direct blockade of virus entry and replication may represent attractive future therapeutic targets with improved efficacy. Elucidation of the cellular and molecular mechanisms of virus infections in COPD pathogenesis will undoubtedly facilitate the development of these potential novel therapies that may attenuate the relentless progression of this heterogeneous and complex disease and reduce morbidity and mortality.

Keywords: chronic obstructive pulmonary disease, virus, inflammation, infection, lung damage, acute pulmonary exacerbation

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the umbrella term for chronic obstructive bronchitis and pulmonary emphysema, and is characterized by persistent small airway inflammation and progressive irreversible airflow limitation (1–5). COPD is associated with acute pulmonary exacerbations, accelerated lung function decline and increased risk of mortality (6, 7). As a common global epidemic, COPD affects 10% of the population and is the third leading cause of death worldwide (3). Viral and bacterial infections are key elements in the pathogenesis of exacerbations (5–9). Recent evidence suggests respiratory viral infections cause ∼50% of COPD acute exacerbations (5, 6, 10). Secondary bacterial infections often ensue with pronounced illness (6).

However, the underlying mechanisms of how viruses subvert host immune defense systems in COPD exacerbations are not completely understood. Herein, we review current advances in understanding the cellular and molecular mechanisms associated with the increased susceptibility to virus infections. As respiratory viruses preferentially infect airway epithelial cells, we focus on virus-induced inflammatory responses in airway epithelium. Understanding these pathogenic pathways may facilitate the development of potential novel therapies to attenuate the relentless progression of the disease.

COPD PATHOGENESIS

Cigarette smoking is the predominant etiologic factor in the development of COPD (3–5). Other risk factors include host genetic factors, which is most evident in alpha-1 antitrypsin (AAT) deficiency (11–13). Recently, childhood respiratory viral infections have been postulated as an independent risk factor associated with COPD later in life (14). Other environmental factors such as pollutant and occupational exposure to dusts or fumes, particularly organic dusts are strongly associated with COPD (4, 13, 15, 16). Social deprivation is also a factor in the development of COPD (6, 17, 18).

Cigarette smoke and other inhale noxious gases induce an abnormal inflammatory response, that is further amplified by protease and oxidative stress, which are central to COPD pathogenesis (8, 11). Persistent small airway inflammation and the resulting destruction of the lung architecture leads to emphysema and loss of lung elastic recoil, chronic bronchitis induced mucus hypersecretion and airflow obstruction, as well as peribronchial fibrosis (11, 19, 20). Excessive neutrophilic infiltration and associated proteolytic enzymes including neutrophil elastase are hallmark features of smoke-induced inflammation (19, 21–25). Consequently, the protease/antiprotease imbalance contributes to the pathogenesis of emphysema due to the increased breakdown of elastin and loss of elastic recoil in the lung parenchyma (19, 21–24). Diminished activity of protein phosphatase 2A (PP2A), a regulator of the inflammatory response in the airways, has been demonstrated in COPD and upregulation of PP2A activity can ameliorate inflammation in a cigarette smoke model of COPD by reducing activity of the cysteine protease, cathepsin S (26). Recent research has proposed a role for formylated peptides and formyl peptide receptor (FPR) receptor signaling in the initiation and progression of lung disease in current and former smokers (27, 28). These peptides are present in tobacco leaves and are actively secreted by bacteria or passively released from dead and dying host cells after tissue injury (29). FPR1 and FPR2 activation may play a role in neutrophil migration, degranulation, reactive oxygen species (ROS) production, and phagocytosis (29, 30). A novel cross-talk mechanism was identified in neutrophils, by which signals generated by the purinergic receptor for ATP ($P2Y_2$) reactivate ligand-bound inactive FPRs, which resume signaling (31). Furthermore, a role for purinergic receptors in the pathophysiology of COPD has been demonstrated in human and experimental models (32–35), however, further work is needed to elucidate its role in the immune dysfunction associated with COPD (36). Excessive production of ROS results in an oxidant-antioxidant imbalance leading to oxidative stress and is a major predisposing feature in the development of the disease (37–41). Therefore, a vicious cycle is created in which inflammation drives a protease-antiprotease and oxidant-antioxidant imbalance, as well as multiple intracellular cell signaling mechanisms, which potentiate inflammation, goblet cell hyperplasia and mucus hypersecretion (8, 40).

Chronic low-grade respiratory syncytial virus (RSV) infection has also been implicated in COPD pathogenesis (42–45). However, the detection of RSV infection in stable COPD remains controversial (46, 47). Hogg and colleagues showed that the E1A region of the adenovirus may contribute to COPD pathogenesis by enhancing soluble ICAM-1 expression and inflammatory cells infiltration (48). In contrast, another study failed to demonstrate the persistent presence of adenovirus V or E1A (49). Polosukhin at al. detected Epstein Barr Virus (EBV) positive cells in COPD lung tissue sections by immunochemistry staining (50). Consistent with this finding, we have demonstrated that EBV DNA is frequently present in COPD sputum compared with unaffected smokers (51). Latent viral infections and cigarette smoke may synergistically contribute to the chronic inflammation in COPD (52). COPD is a heterogeneous disease with a complex etiology, however, acute and chronic lower respiratory tract infections occur with increased frequency in patients with COPD. Whatever the cause, it is clear that a defective host response plays an important role and improving our understanding of the mechanisms involved is essential to improving prevention and treatment strategies.

AIRWAY EPITHELIUM DYSFUNCTION IN COPD

Normal airway epithelial cells play a pivotal role in innate immune defense. They act as a barrier to pathogens and noxious stimuli and produce mediators and enzymes to orchestrate and maintain proper functioning of the innate and adaptive immune responses (24, 53, 54). As illustrated in **Figure 1**, the COPD airway epithelium responds to cigarette smoke by secreting inflammatory mediators and recruiting immune cells to the site of damage to orchestrate the inflammatory response. A robust

infiltration of macrophages and $CD8^+$ T cells, and to a lesser extent $CD4^+$ T cells, in the airway mucosa as well as elevated neutrophils in the airway lumen are the hallmark features of COPD inflammation, the degree of which correlates to disease severity (46, 55). Increased levels of epithelial-derived CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) and their receptor CXCR3 has been demonstrated to contribute, in part, to the mechanism of $CD8^+$ cellular accumulation (40, 53, 54). $CD8^+$ T cells release IP-10, TNF-α, IFN-γ, perforins, and granzyme, and have been associated with alveolar epithelial cell apoptosis (19, 37, 56, 57). As COPD progresses, elevated numbers of dendritic cells and B lymphocytes also appear in the airways and alveolar walls. $CD8^+$ T cells and B cells organize into lymphoid follicles and may contribute to increased "immune surveillance" in COPD (19, 37, 39). The airway epithelium also releases a cascade of secondary mediators including cytokines, lipid mediators, growth factors, proteases, antiproteases and ROS to escalate COPD inflammation (53, 54, 58). Cigarette smoke and other irritants also activate epithelial cells and macrophages to release neutrophil and macrophage chemoattractants, such as LTB4, IL-8, and related CXC chemokines (MCP-1, GRO-α and GM-CSF), which contribute to the development of emphysema (39, 46, 59, 60).

The mechanism of neutrophilic inflammation has been linked to CD11b/CD18 on neutrophils binding to ICAM-1 on bronchial epithelium, which is up-regulated in COPD (54, 61–63). Neutrophils migrate to the respiratory tract and release serine proteases, matrix metalloproteinases (MMPs) and oxidants (24, 40, 46). Neutrophil serine proteases are associated with emphysema, mucus hypersecretion, increased risk of exacerbation and accelerated forced expiratory volume in 1 s (FEV_1) decline (64–66). Subsequently, these proteases degrade extracellular matrix components leading to the destruction of the alveolar wall, epithelial barrier dysfunction, reduction in mucociliary clearance, mucus hypersecretion and goblet cell metaplasia through activation of the epidermal growth factor receptor (EGFR) (37, 59, 64). Moreover, alveolar epithelial cells also secrete transforming growth factor-β (TGF-β) which may contribute to small airway fibrosis and emphysema (67).

MOLECULAR MECHANISMS ASSOCIATED WITH VIRAL-INDUCED COPD EXACERBATIONS

Viral-Induced COPD Exacerbations

Acute exacerbations of COPD are characterized by a sudden decline in lung function, hospitalization and high mortality (7, 9, 46). The complicated interaction between the host and viral or bacterial infections or co-infection, as well as environmental factors, precipitate the onset of exacerbations. These factors amplify the inflammatory burden in the small airway, overpowering host anti-inflammatory mechanisms leading to profound airway obstruction in COPD (46, 68–70). Severe virus-associated exacerbations also induce elevated levels of $CD8^+$ T cells, neutrophils, eosinophils, TNF-α and IL-6 in the sputum of COPD patients (68–70).

Exacerbations often occur seasonally accompanied by common cold-like symptoms implicating respiratory viral infections rather than hitherto suspected bacterial infection (43, 44). Respiratory virus infection, including human rhinovirus (HRV), influenza virus (IAV), coronavirus, RSV, human parainfluenza, metapneumovirus (hMPV) and adenovirus initiate nearly 50% of COPD exacerbations often with more severe symptoms (69–73). Viruses have developed a myriad of aversion strategies to subvert and manipulate host immune responses and these have been recently reviewed elsewhere (74, 75). Most respiratory viruses target airway epithelial cells leading to epithelial barrier destruction, microvascular dilatation, oedema and immune cell infiltration (58, 70–72). These viruses are associated with small airway secondary bacterial infection, thus magnifying the inflammatory response in COPD leading to a synergistic deterioration in lung function and prolonged hospitalization (42, 44, 71).

As detailed below, recent research has focused on immune regulators and inflammatory signaling pathways orchestrating the underlying mechanisms of increased susceptibility to virus-associated exacerbation and the exaggerated inflammatory response in COPD airways and potential therapeutic inventions.

T Cell Exhaustion

Although accumulated $CD8^+$ T cells are present in greater numbers in severe COPD, a diminished $CD8^+$ T cell antiviral response, worsened airflow limitation and respiratory symptoms have been reported in IAV and HRV-induced COPD exacerbations (68, 71, 76, 77). As a result, $CD8^+$ cells potentially amplify airway epithelium destruction and promote tissue injury through mechanisms including direct cytotoxic effects, pro-inflammatory signaling and recruitment of other immune cells, leading to increased susceptibility to virus infections of airway epithelium (42–44, 69).

In COPD, prolonged receptor–ligand interaction during T cell activation may be linked to T cell exhaustion. McKendry and colleagues investigated increased $CD8^+$ activation through the programmed cell death protein (PD)-1 exhaustion pathway as a potential mechanism of viral-induced COPD exacerbations (76). Dysregulation of T-cell cytotoxicity was associated with elevated levels of PD-1, which further increased following influenza infection in COPD patients (76). In contrast, infection-induced expression of the ligand PD-L1 on COPD macrophages was diminished, with a concomitant increase in IFN-γ release. These synergistic effects may cause excessive T-cell inflammation in response to virus infection.

The NF-κB Pathway

The NF-κB pathway is consistently activated in COPD macrophages and airway epithelium, in particular, during bacterial or viral infections (78). Upon pathogen stimulation, the canonical pathway is mainly triggered by Toll like receptors (TLRs) and pro-inflammatory cytokines such as TNFα and IL-1 leading to the activation of the RelA containing NF-κB complexes. This initiates the translocation of RelA (p65)/p50 to the nucleus, where it induces the transcriptional response of pro-inflammatory and cell survival genes (78–80). The alternative

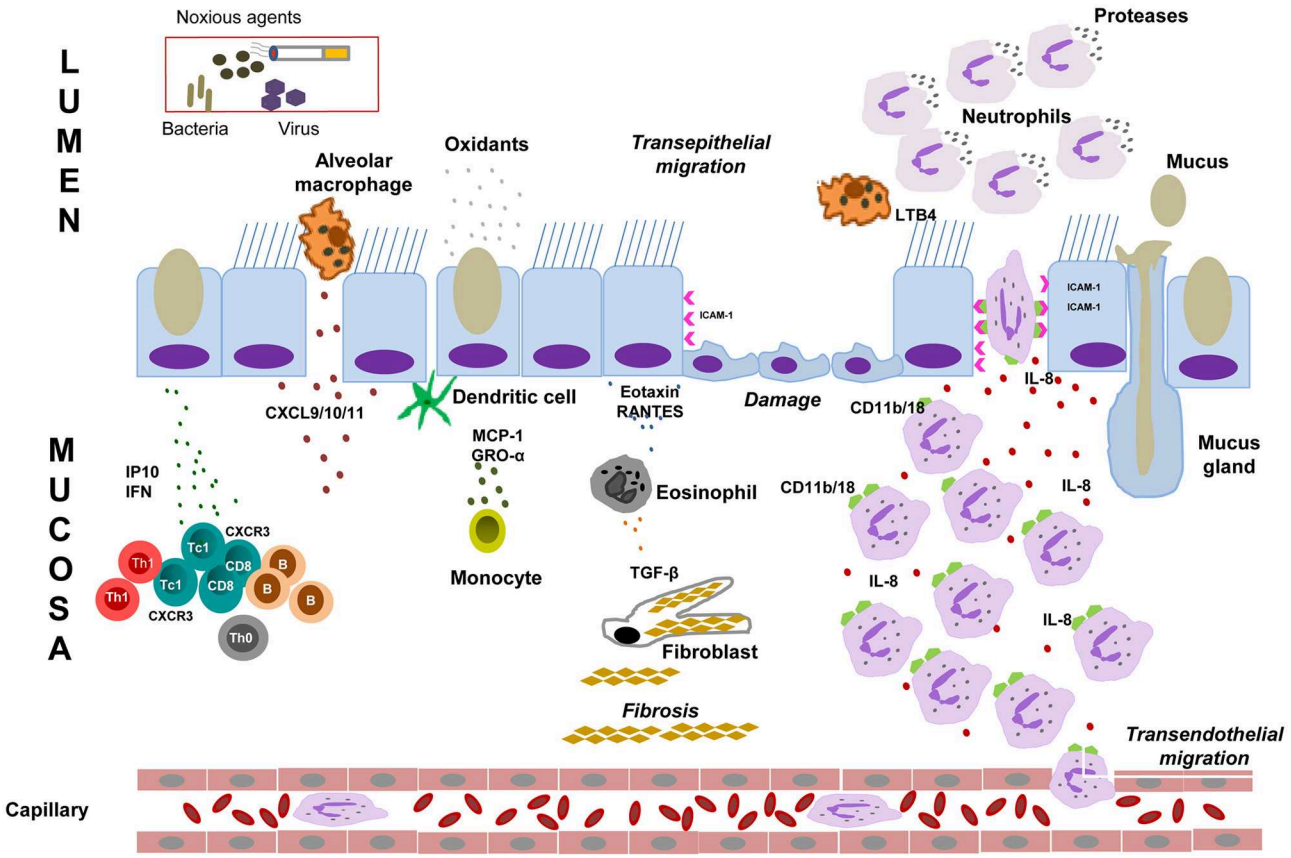

FIGURE 1 | Mechanisms of airway immunity dysfunction in COPD. Cigarette smoke and noxious agents activate epithelial cells and macrophages to release chemotactic factors such as CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (I-TAC), which increase CD8+ T cells, dendritic cells, B lymphocytes and eosinophil infiltration into the airway mucosa. These inflammatory cells together with macrophages and epithelial cells initiate an inflammatory cascade that triggers the release of inflammatory mediators such as TNF-α, IFN-γ, proteases (such as MMPs), inflammatory cytokines and chemokines (IL-1, IL-6, IL-8) and growth factors. These inflammatory mediators sustain the airway mucosal inflammatory process in COPD, which cause elastin degradation and emphysema. Epithelial cells and macrophages also release TGF-β, which stimulates fibroblast proliferation resulting in small airway fibrosis. During exacerbation, the inflammatory burden in the small airways over-powers host anti-inflammatory mechanisms leading to profound alveolar damage and inflammation. Cigarette smoke and other irritants activate epithelial cells and macrophages to release neutrophil chemoattractants, such as LTB4, IL-8, TNFα, CXC chemokines (MCP-1, GRO-α, and GM-CSF). CXC chemokines also act as chemoattractants for monocytes. Cigarette smoke causes increased level of ROS produced in the airways is reflected by increased markers of oxidative stress. Oxidative stress is involved in several events in the pathogenesis of COPD including oxidative inactivation of anti-proteases and surfactants, mucus hypersecretion, alveolar epithelial injury, remodeling of extracellular matrix and apoptosis. Neutrophils bind to ICAM-1, the level of which has been found to upregulated in bronchial epithelial cells in COPD. Neutrophils then migrate to the respiratory tract under the control of IL8/LTB4 chemotactic gradient. These cells then release proteases that break down connective tissue in the lung parenchyma, resulting in emphysema. Neutrophil elastase release in airway induces epithelial barrier dysfunction, mucus hypersecretion and reduces mucociliary clearance. GM-CSF, Granulocyte-macrophage colony-stimulating factor: GRO-α, Growth-regulated oncogene-α; ICAM-1, epithelial intercellular adhesion molecule-1; LTB4, leukotriene B4; IL, interleukin; IP10, CXCL10, interferon g-induced protein 10; I-TAC, CXCL11, interferon-inducible T-cell α chemoattractant; MCP-1, monocyte chemoattractant protein-1; MIG, CXCL9, monokine induced by g interferon; MMPs, matrix metalloproteinases; RANTES, regulated on activation, normal T cell expressed and secreted; ROS: reactive oxygen species; TGF, transforming growth factor; TNF-α, Tumor necrosis factor-α; IFN, interferon.

non-canonical NF-κB pathway signals through a subset of receptors to activate the kinase NIK and IKKα complexes and downstream NF-κB2 p100 leading to the p52/RelB nucleus translocation and lymphoid organogenesis and B cell activation (78, 79).

Persistent or prolonged activation of NF-κB may contribute to COPD pathogenesis by switching on the transcriptional response of pro-inflammatory cytokines, chemokines, cell adhesion molecules (CAMs), proteases, and inhibitors of apoptosis to amplify inflammation. Therefore, strategies, which block the activation of NF-κB, offer attractive therapeutic options to regulate COPD inflammation. Several IKK-β inhibitors have been identified to inhibit p65 nuclear translocation and exert anti-inflammatory effects (81, 82). Lung-targeted overexpression of RelB has also been demonstrated to protect against cigarette smoke–induced inflammation by reducing inflammatory mediator production (83). In COPD airway epithelium, influenza virus infection increased microRNA-125a/b, which directly inhibits A20 and mitochondrial antiviral-signaling protein (MAVS) to promote inflammation and impair

antiviral responses in COPD (84). Thus, miR-125a/b may provide a potential therapeutic target for both inflammation and antiviral responses in COPD.

TLR Sensing and EGFR Signaling

Figure 2 illustrates key virus innate recognition signaling pathways in COPD airway epithelium. Briefly, ssRNAs of HRV, RSV, and IAV are recognized by TLR3 in the endosomes which consequently activate IRF-3 via the Toll/IL-1 receptor domain-containing adaptor (TRIF), leading to the induction of IFN-β and IFN-λ1. Other endosomal TLRs (TLR7/8 and TLR7/9) recognize the dsRNAs of IAV and adenovirus through MyD88-dependent pathway to activate NF-κB and IRF-7 to secrete pro-inflammatory mediators and IFNs, respectively. TLR4 expressed on the cell surface senses RSV and IAV, signaling through both the MyD88 and TRIF pathways to activate NF-κB and IRF-7. The airway epithelium may recognize EBV by endosomal TLRs and TLR2 at the cell surface to activate downstream pathways (85, 86). As a risk factor for RSV-induced COPD exacerbations, TLR3 activation has been found to correlate with lung function deterioration during exacerbations highlighting TLR3 blockade as a therapeutic target (87). However, Silkoff et al. showed that TLR3 inhibition was inefficient in attenuating HRV-induced experimental asthma exacerbation (88).

Many TLRs recognize pathogen-associated molecular patterns (PAMPs) to activate airway epithelial EGFR signaling cascades. Aberrant EGFR signaling promotes progressive lung fibrosis and mucus hypersecretion; characteristic features of COPD, asthma and cystic fibrosis pathogenesis (24, 89). The EGFR cascade consists of multiple receptors and extracellular ligands that function via receptor auto-phosphorylation and cytoplasmic protein binding of four downstream complexes including the mitogen-activated protein kinases/extracellular signal–regulated kinases (MEK/ERK), phosphatidylinositol 3-kinases/protein kinase B (PKB) (PI3K/AKT), Just Another Kinase/signal transducer and activator of transcription (JAK/STAT) and mammalian target of rapamycin (mTOR) pathways (89). In a murine COPD model, EGFR activation through PI3K inhibited ciliated cell apoptosis and allowed IL-13 to stimulate the trans-differentiation of ciliated to goblet cell metaplasia (90). HRV infection induced the phosphorylation of PKD, a downstream kinase of PI3K. PKD inhibitors have been reported to effectively block HRV, poliovirus (PV) and foot-and-mouth disease virus (FMDV) replication at an early stage of infection, highlighting the potential of PKD inhibition in anti-HRV therapy in COPD (91). Chronic inflammation can also induce ICAM-1 and its ligand fibrinogen has been shown to promote EGFR-dependent mucin production in the airways of subjects with mucus hypersecretion (92).

EGF and the EGFR ligand, TGF-α, have been reported to directly enhance TNF-α-induced IL-8 secretion in airway inflammation (93). Ganesan et al. found that abnormal EGFR activation contributed to enhanced IL-8 expression in COPD airways via the NF-κB regulator, FoxO3A (94). Interestingly, TLR3 also induced EGFR activation and EGFR ligands (TGF-α and amphiregulin), which in turn promote EGFR-ERK signaling and mucin production through an autocrine/paracrine loop (95). Collectively, TLR antiviral defense mechanisms integrate with the EGFR mediated epithelial proliferation/repair pathways and may play an important role in viral-induced airway remodeling and airway disease exacerbations (93, 95, 96).

Viral infection *per se* also activates EGFR and EGFR signaling to ERK1/2, while STATs control the severity of HRV mediated airway inflammation. *In vitro*, HRV induced goblet cell hyperplasia was demonstrated to function through NF-κB-dependent MMP-mediated TGF-α release, leading to EGFR activation and mucus secretion (97). Interestingly, virus-induced EGFR activation suppressed interferon regulatory factor 1 (IRF1)-dependent IFN-λ airway epithelial antiviral signaling (98, 99). Inhibiting virus-mediated EGFR signaling augmented IRF1, IFN-λ secretion and viral clearance, indicating EGFR pathways as potential therapeutic targets in viral-induced COPD exacerbations (99).

Cytoplasmic-Sensing Pathways

As shown in **Figure 2**, the airway epithelium also detects viral invasion through cytoplasmic pathogen recognition receptors. DNA and RNA viruses release their genomes into cytoplasm, which are detected by the host through cytoplasmic retinoic acid-inducible gene I/melanoma differentiation-associated protein 5-mitochondrial antiviral-signaling protein (RIG-I/MDA5–MAVS) RNA-sensing and the cyclic GMP–AMP synthase- signaling effector stimulator of interferon genes (cGAS–STING) DNA-sensing pathways, respectively (100). Upon ss/dsRNA binding, the RNA helicases, RIG-I and MDA5, interact with the adaptor protein MAVS on the mitochondrial outer membrane to activate the downstream signaling of type I interferon antiviral responses (100, 101). In contrast, the cGAS receptor senses retroviral replication products, dsDNA and RNA/DNA hybrids, to induce the synthesis of cGAMP which binds and activates STING (100). Interferon γ-inducible protein 16 (IFI16), a novel DNA sensor, has been found to recruit STING to activate type I IFN signaling through an unknown molecular mechanism (102). STING and MAVS also stimulate downstream multiple kinase signaling cascades resulting in IRF3 phosphorylation and NF-κB nuclear translocation (101, 102).

The primary consequence of these virus-sensing pathways is the induction of type I/type III IFNs and IFN stimulated genes as well as the production of inflammatory cytokines and chemokines. Attenuation of the IFN response following virus infection could result in uncontrolled viral replication and an escalated inflammatory response, a potential mechanism of virus-induced exacerbations in COPD. IFNα/β deficiency has been demonstrated in bronchial biopsies of asthmatic patients with rhinovirus-induced exacerbations and smoking-induced COPD (103). Farazuddin et al. have demonstrated that quercetin, a potent antioxidant and anti-inflammatory agent with antiviral properties, effectively mitigates rhinovirus-induced COPD exacerbation in a mouse model (104). Elevated ICAM-1 expression on the surface of airway epithelium has been directly linked to the mechanism of increased susceptibility of HRV-induced acute exacerbation. As the receptor of the major group of HRV and a ligand of lymphocyte function-associated antigen 1 (LFA-1) on neutrophils, ICAM-1 over-expression has been

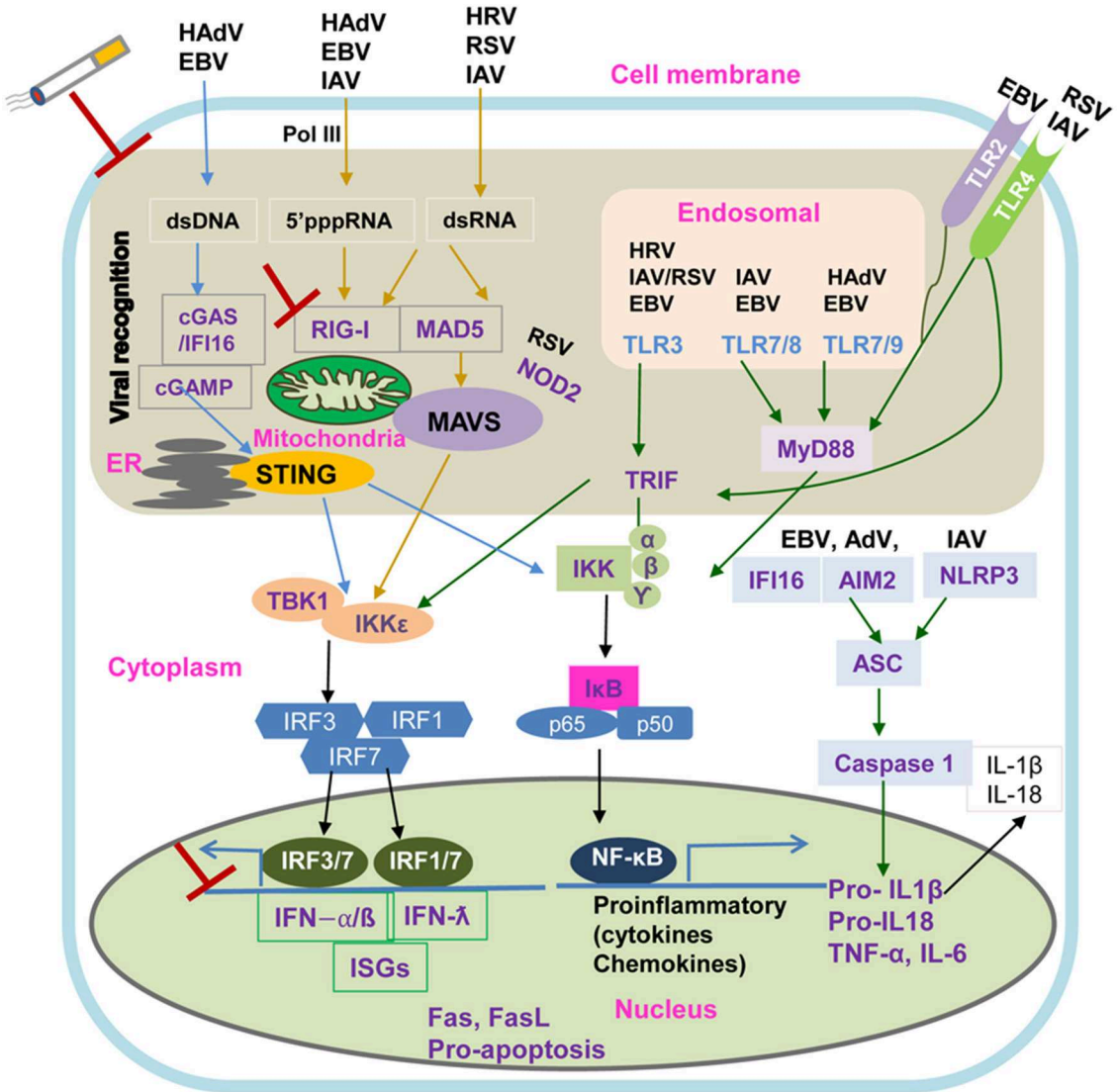

FIGURE 2 | Intracellular Viral Sensing Pathways. DNA and RNA viruses release their genomes in the cytoplasm, where host innate sensors for nucleic acids reside. Upon ss/dsRNA binding, RIG-I engages the adaptor protein MAVS on the mitochondrial outer membrane. The cGAS receptor recognizes dsDNA and the RNA:DNA hybrids generated during retroviral replication and catalyzes the synthesis of cGAMP, which is the primary agonist of the adaptor protein STING. Another sensor, IFI16 can recruit STING in response to cytoplasmic DNA through a molecular mechanism yet to be described. Both STING and MAVS stimulate downstream signaling cascades that involve multiple kinases and finally lead to IRF3 phosphorylation and nuclear translocation. The primary consequence of these virus sensing pathways is the induction of type I IFN and IFN stimulated genes. cGAS, cyclic GMP-AMP synthase; cGAMP, 2′3′guanosine-adenosine monophosphate; IFI16, interferon-g inducible protein 16; IKK, IkB kinase; IRF3, interferon regulatory factor 3; MAVS, mitochondrial antiviral-signaling protein; RIG-I, retinoic acid inducible gene-I; ss/dsRNA, single-stranded/double-stranded RNA; vRNA/DNA, viral RNA/DNA; STING, stimulator of interferon genes; TANK, TRAF-associated NF-kB activator; TBK1, TANK binding kinase 1.

shown on epithelial cells in smokers and patients with COPD (63, 105, 106). Blocking ICAM-1 may also represent as a potential therapeutic option in HRV-induced exacerbations.

Direct Targeting of Viral Binding, Entry, and Replication

Strategies that directly prevent virus binding, entry and replication may provide attractive alternatives in the treatment of COPD exacerbations (107). Capsid binders represent attractive potential inhibitors of HRV entry, however, they are strain-specific and have shown no effect on improving lung function and exacerbation in clinical trials to date (106). Mousnier and colleagues demonstrated that a dual inhibitor of human N-myristoyltransferases NMT1 and NMT2 can inhibit host-cell N-myristoylation and completely prevent rhinoviral replication, highlighting the therapeutic potential of targeting myristoylation in blocking rhinovirus infection in COPD (108). Short palate, lung, nasal epithelium clone 1 (SPLUNC1), a multifunctional host defense protein, was demonstrated to inhibit IAV binding and entry into airway epithelial cells,

indicating an antiviral role for this protein in the airways (109). Therefore, in the COPD lung, SPLUNC1 degradation by proteases such as neutrophil elastase and/or inactivation by cigarette smoke may increase susceptibility to viral as well as bacterial infections, in addition to airway dehydration (110, 111). Recent research suggests that, in addition to modulating neutrophil chemotaxis, FPR2 signaling may be an important player in viral replication and IAV pathogenesis (30, 112, 113).

Inflammasome

The inflammasome is a multiprotein pro-inflammatory complex and serves as an important link between the innate and adaptive immune responses. Inflammasomes that are activated by IAV RNA, EBV and adenoviral DNA include the nucleotide binding and oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) protein, absent in melanoma 2 (AIM2) protein and IFI16 protein (114). The inflammasome complexes assemble after recognition of PAMPs or danger-associated molecular patterns (DAMPs) induced by virus-killed cells or tissue damage and interact with apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) via caspase activation and recruitment domains (CARD)-CARD/caspase-1 pathway (115–117). Activation of the inflammasome complex results in the autocatalytic cleavage of caspase-1 and ultimately leads to the production of pro-inflammatory cytokines including IL-1β, IL-18 and pro-IL-33 (116, 117). Upon maturation, these cytokines mediate inflammatory responses by activating lymphocytes and facilitating their infiltration to the site of primary infection and by inducing IFNs and other pro-inflammatory cytokines secretions (116).

CONCLUDING REMARKS

COPD is a heterogeneous and complex disease resulting from the deregulation of multiple immune regulators and inflammatory signaling pathways. Significant progress has been made to elucidate the causative mechanism of COPD pathophysiology including viral infection in disease development, severity and exacerbations. Targeting virus-induced inflammatory pathways such as T cell exhaustion, NF-κB, TLRs, EGFR, interferons and the inflammasome provide attractive future therapeutic options. Understanding the cellular and molecular mechanisms of virus-induced COPD pathogenesis could potentially limit pathogen-mediated disease exacerbations and minimize viral-associated inflammation, tissue destruction and pulmonary function deterioration.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Expression and Roles of Antimicrobial Peptides in Innate Defense of Airway Mucosa: Potential Implication in Cystic Fibrosis

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The treatment of respiratory infections is associated with the dissemination of antibiotic resistance in the community and clinical settings. Development of new antibiotics is notoriously costly and slow; therefore, alternative strategies are needed. Antimicrobial peptides (AMPs), the central effector molecules of the immune system, are being considered as alternatives to conventional antibiotics. Most AMPs are epithelium-derived and play a key role in host defense at mucosal surfaces. They are classified on the basis of their structure and amino acid motifs. These peptides display a range of activities, including not only direct antimicrobial activity, but also immunomodulation and wound repair. In the lung, airway epithelial cells and neutrophils, in particular, contribute to AMP synthesis. The relevance of AMPs for host defense against infection has been demonstrated in animal models and is supported by observations in patient studies, showing altered expression and/or unfavorable circumstances for their action in a variety of lung diseases. Of note, AMPs are active against bacterial strains that are resistant to conventional antibiotics, including multidrug-resistant bacteria. Several strategies have been proposed to use these peptides in the treatment of infections, including direct administration of AMPs. In this review, we focus on studies related to direct bactericidal effects of AMPs and their potential clinical applications with a particular focus on cystic fibrosis.

Keywords: respiratory infections, antibiotic resistance, antimicrobial peptides, antimicrobial effect, immune modulation, cystic fibrosis

HISTORICAL OVERVIEW AND DEFINITION

In the early 1920s, Fleming independently discovered both AMPs and penicillin. In 1922, he identified, in his nasal discharge, an antimicrobial substance, later named lysozyme, which was able to kill certain bacteria in few minutes. Seven years later, penicillin was carried forward for clinical application (1). After that, several AMPs were isolated and identified as having activity against both Gram-positive and Gram-negative bacteria. In 1939, gramicidin was the first natural peptide-based drug to be introduced in the market. It was isolated from *Bacillus brevis* and was active against a wide range of Gram-positive and some Gram-negative bacteria but was not devoid

of toxicity (2). The real explosion of therapeutic potential of AMPs began in the early 1980s when Hans Boman isolated and characterized AMPs, known as cecropins, from the hemolymph of silk moth (*Hyalophora cecropia*) (3). Later in 1987, the significance of AMPs was increased when Zasloff discovered magainins in frog skin (*Xenopus laevis*) (4) and showed for the first time that AMPs are present not only in lower invertebrates but also in higher vertebrates (5). Antimicrobial activities in fluids such as blood, saliva, plasma, sweat, leucocytes secretions, and granule extracts were discovered at that period, suggesting the natural production of AMPs in humans (6). Since then, more than 3,000 naturally occurring AMPs have been isolated from different kingdoms (bacteria, archea, protists, fungi, plants, animals, and humans) and were registered in the AMP database (http://aps.unmc.edu/AP/main.php). Thus, AMPs were discovered at the same time as antibiotics (ATBs) but were eclipsed by the success of those drugs. Now that the emergence of ATB resistance is a major threat to human health, global voices are calling for solutions. Among the existing research lines for alternatives to conventional ATBs, AMPs, both natural and synthetic, seem to be promising candidates (7).

AMPs, also referred to as host defense peptides, are biologically active molecules with a rapid and broad spectrum of activity against bacteria, yeast, viruses, and fungi in addition to immunomodulatory activities, wound healing, and cytotoxic effects on cancer cells (8, 9). To date, the large majority of identified AMPs are antibacterial peptides representing 83% of all AMPs (10). AMPs, evolutionarily conserved in the genome, are produced by most living organisms as an essential component of their innate immune system, representing an ancient host defense mechanism to eliminate invading pathogens and boost immune response. In mammals, the primary site at which a host encounters a pathogen is classically the skin or the mucosal surface, such as the respiratory tract, the gastrointestinal tract, and the urogenital tract (11). Infections at these sites are prevented by the innate host defense responses intended to maintain host integrity (12). AMPs, being an important component of the innate immune system, constitute one of the early, rapid, nonspecific mechanisms by which the host immune system provides protection against infections (13). Studies using knockout mice and transgenic (Tg) expression systems have confirmed that AMPs play a major role in limiting microbial proliferation to skin and mucosal surfaces, therefore preventing spread to the deep tissues where serious infection may occur (14). AMPs are produced by epithelial cells of vertebrates as a first line of defense against microbial pathogens.

Abbreviations: AMPs, Antimicrobial peptides; ARDS, Acute respiratory distress syndrome; ASL, Airways surface liquid; ATBs, Antibiotics; cDNA, complimentary DNA; CF, Cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; EGR1, Early growth response gene-1; GBS, Group B Streptococcus; HBD, Human β-defensin; HIV, Human immunodeficiency virus; HNP, Human neutrophil defensin; HSV, Herpes simplex virus; IL, Interleukin; KLF, Krüppel-like transcription factor; LPS, Lipopolysaccharides; MCP, Monocyte chemoattractant protein; MIC, Minimum Inhibitory Concentration; MOA, Mechanism of action; MRSA, methicillin-resistant *Staphylococcus aureus*; PAMP, Pathogen-associated molecular pattern; sPLA2, secreted phospholipase A2; sPLA2-IIA, Type-IIA secreted phospholipase A_2; TNF-α, Tumor necrosis factor alfa; Tg, Transgenic; VRE, vancomycin-resistant *Enterococci;* WT, Wild-type.

AMPs: STRUCTURE AND CLASSIFICATION

Despite their extreme diversity in terms of composition and length, AMPs share several common structural characteristics (15). The most studied AMPs are short polypeptides of fewer than 50 amino acids, cationic with an average net charge of +3, and having a hydrophobic content of 42% on average. Both the net positive charge and the hydrophobicity of these AMPs generate the observed amphipathic structure. This structure determines their conformational flexibility, enables electrostatic attraction between these cationic peptides and the anionic bacterial membranes, and allows penetration into bacterial cells inducing membrane lysis. Cationic AMPs, however, do not affect the neutrally charged mammalian cells; this chemical property favors their use as future drugs (7, 15). The differences in composition between bacterial cell membranes rich in phosphatidylglycerols and human cell membranes dominated by zwitterionic phospholipids is believed to be the major reason of the selectivity of AMPs (**Figure 1**) (7).

Based on structural features, AMPs can be classified into three subgroups: α-helical, β-sheet, and extended AMPs (16–18). These structures are highly correlated with the functional specificity of each peptide. Some of these peptides demonstrate no secondary structure in aqueous solution but become structured when exposed to a lipid, such as the bacterial cell membrane (19). In addition to that, some peptides might have mixed α-helical and β-sheet structures; classification is then based on the predominant one (20).

The first subgroup contains AMPs that form α-helical structures and are predominately found in the extracellular matrix of frogs and insects in addition to the extensively studied human AMP LL-37, which is a member of the cathelicidins. Cathelicidins, originally isolated from granule extracts of bovine neutrophils (21), are among the most diverse AMPs of vertebrates; they can adopt a variety of structures and play, in addition to their antimicrobial activity, an important immunomodulatory role (22). Magainins, which are active against a broad spectrum of microbial agents, present another example of AMPs with an α-helical structure. They have been extensively studied and are among the first ones to have been tested clinically (23). Cecropin is a prototype of this group and is active against Gram-negative bacteria. Other cecropins, which can act synergistically against both Gram-negative and Gram-positive bacteria, have been recently identified (24). Another final example of the α-helical AMPs is the aureins that are secreted from the granular dorsal glands of the Australian Green and Golden Bell Frog *Litoria aurea* and the southern Bell Frog *L. raniformis*. The aurein family is mostly active against Gram-positive bacteria, such as *Staphylococcus aureus* and *S. epidermidis*, and have anti-cancer activities (20).

The second subgroup includes cyclic AMPs that adopt a β-sheet structure, such as protegrins, defensins, and tachyplesins. Although they have antifungal properties in some cases, they are often considered to be antibacterial peptides (19). Defensins, the largest group of AMPs produced by mammals, were first discovered in human neutrophils as small cationic molecules. They have been found later in mammals, insects, plants,

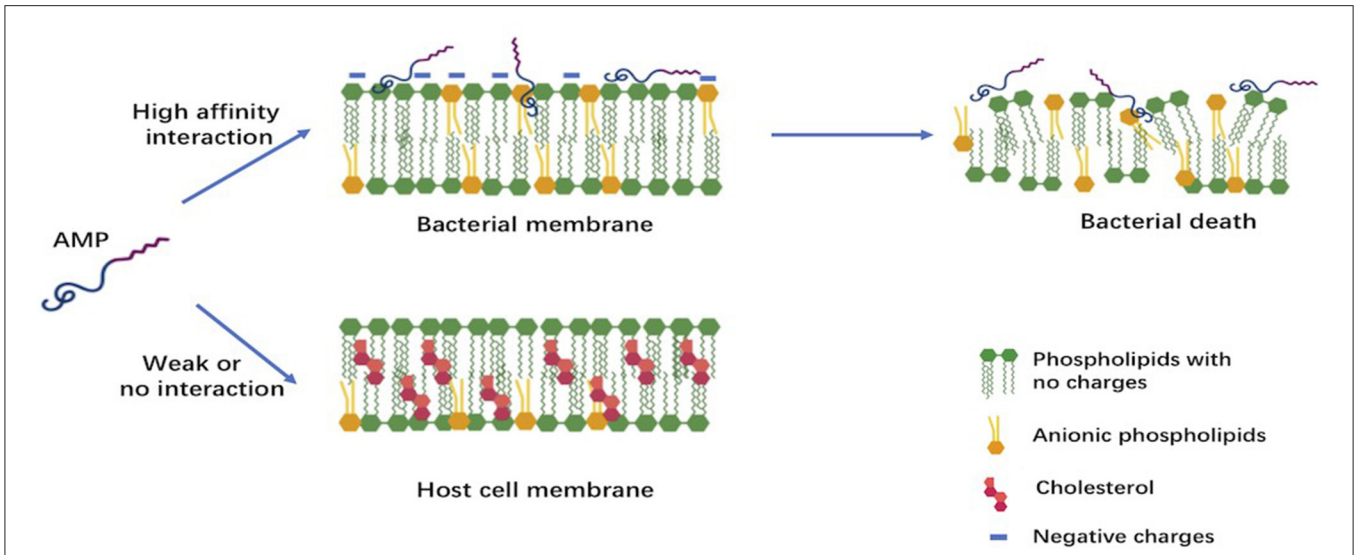

FIGURE 1 | Early interactions of cationic antimicrobial peptides with bacterial or host cell membrane. The anionic molecules in the membranes of Gram-negative and Gram-positive bacteria attract cationic AMPs via electrostatic and hydrophobic interactions. In contrast to bacteria, the cytoplasmic membrane of host cells with a neutral net charge connects with cationic AMPs via hydrophobic interactions, which are relatively weak.

parasites, and fungi. Defensins are also involved in immune and inflammation responses (25). Although most defensins lose much of their antimicrobial activity at the physiological concentrations of Na^+, Mg^{2+}, or Ca^{2+}, they have been shown to exhibit broad-spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses *in vitro*. Of note, electrolytes may have a more complex effect on peptide-induced antimicrobial effects (25). Another example of β-sheet AMPs are tachyplesins, isolated from hemocytes of horseshoe crabs (20).

The third and last subgroup comprises peptides with a unique extended/random coil structure. In this category, most of the AMPs are from the cathelicidin family, which are known to have linear structure rather than secondary structure due to the presence of proline residues. One of the best studied peptides in this subgroup is indolicidin, which is produced by bovine leucocytes and consists of only 13 amino acids (17, 20).

Sources of some AMPs, their classes, and chemical structures are shown in **Table 1**.

TABLE 1 | Classification of some antimicrobial peptides along with their chemical structure and origin.

Classification	AMP	Origin	Chemical structure
α-helix	LL-37	Human	GIGAVLKVLTTGLPALISWIKRKRQQ
	Melittin	Honey bee	GIGKFLHSAGKFGKAFVGEIMKS
	Dermaseptin-S1	Frog	LLGDFFRKSKEIGEFKRIVQRIKDFLR NLVPRTES
β-helix	Protegrin-1	Pig	RGGRLC[1]YC[2]RRRFC[2]VC[1]VGR
	HNP-1	Human	AC[1]YC[2]RIPAC[3]IAGGRRYGTC[2]I
	HBD-1	Human	YGGRKWAFC[3]C[1] DHYNC[1]VSSGGQC[2]LYASC[3]PIF TKIQGTC[2]YRGKAKC[1]C[3]K
Extended structure	PR-39	Pig	RRRPRPPYLPRRPRPPFFPPLRLPPR
	Indolicidin	Cow	IPPGFPPRFPPRPFP
	Tritrpticin	Pig	ILPWKWPWWPWRR VRRFPWWWPFLRR

AMP, antimicrobial peptide; HBD-1, human β-defensin 1; HNP, human neutrophil defensing.

MODE OF ACTION OF AMPs

Enhanced understanding of the mechanism of action (MOA) of AMPs is of great importance to facilitate further development of peptide-based drugs as therapeutic agents. The MOA can be divided into two major classes: direct antimicrobial activity and immune modulation (16). Although it has been thought for many years that membrane destabilization was the sole direct MOA of AMPs against bacteria, additional mechanisms have been described. These MOA embrace non-membrane targeting mechanisms, including inhibition of the cell wall synthesis, intracellular translocation of AMPs, inhibition of protein/nucleic acid synthesis, and disruption of enzymatic/protein activity (20, 26). In both cases, electrostatic interaction is the key factor for the direct antimicrobial activity of cationic AMPs with the negatively charged molecules of the bacterial membrane, enabling further intrusion of the peptides into the inner part of the cell membrane (27). These interactions occur with the anionic phospholipids and phosphate groups of lipopolysaccharides (LPS) in case of Gram-negative bacteria as well with teichoic acids and lipoteichoic acids in case of Gram-positive bacteria (15, 24).

Direct Antibacterial Activity

AMPs exert their direct antibacterial activity by either disrupting bacterial membranes or interfering with intracellular processes following to translocation. The direct antibacterial mechanism of AMPs is schematized in **Figure 2**.

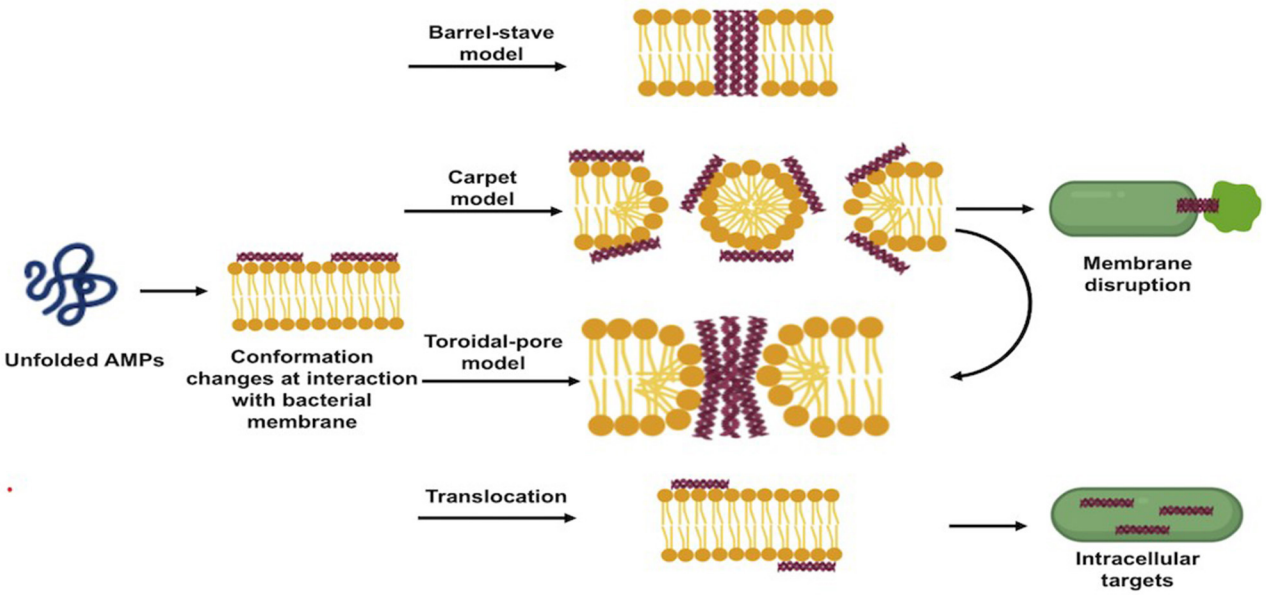

FIGURE 2 | Schematic illustration of direct antibacterial mode of actions of antimicrobial peptides.

Membrane Disruption Mechanism of Action

Upon adsorption into the membrane surface, the AMPs form, if not already present, an amphipathic secondary structure essential for interaction with the cell membrane (28). At this stage, several models have been proposed to describe the next events occurring at the bacterial cytoplasmic membrane, which ultimately lead to a remarkable dose-dependent membrane disruption (26). The three most popular models are the "barrel-stave pore model," "toroidal-pore model," and "carpet model" (10, 20).

In the barrel-stave pore model, when a threshold concentration of the peptides is reached, AMPs insert perpendicularly into the lipid bilayer forming transmembrane pores within the hydrophobic membrane core, in a manner similar to that of membrane protein ion channels. This model is consistent with the MOA of alamethicin, pardaxin, and protegrins (20, 26).

In the toroidal-pore model, once the minimum threshold concentration is reached, the peptides are perpendicularly incorporated into the bilayer membranes, enabling the lipid monolayers to curve around the pore. Consequently, the hydrophobic residues of peptides interact with the hydrophobic region of the membrane, forming pores that are partially bordered by the peptides and partially by the phospholipid head group, allowing the water core to be lined. Magainins and LL-37 adopt this MOA (10, 21).

In the carpet model, AMPs adsorb parallel to the lipid bilayer and cover the surface of the target membrane. Once their concentrations reach a certain threshold, AMPs exert detergent-like effects, which eventually disintegrate the membrane via the formation of micelles and pores. This model explains the MOA of cecropins and some magainins (10, 28). The formed pores act as non-selective channels for ions, toxins, and metabolites, thus preventing the microbe from maintaining vital homeostasis and leading eventually to microbial death (16, 27).

TABLE 2 | Classification of different antimicrobial peptides according to their membrane targeting mechanism of action.

Pore-forming models	Example of AMP	Origin
Barrel-Stave	Ceratotoxin	*Ceratitis capitate* (Mediterranean fruit fly)
	Alamethicin	*Trichoderma viride* (fungus)
	Amphotricin B	*Streptomyces nodosu* (bacteria)
Toroidal	Melittin	*Xenopus Laevis* (African clawed frog)
	LL-37	*Homo sapiens*
	Piscidin	*Morone Saxtilis* (Atlantic striped bass)
	Pardaxin	*Pardarchirus marmoratus* (Finless sole fish)
Carpet-like	Magainin 2	*Xenopus Laevis* (African clawed frog)
	RL-37	*Macaca mulatta* (Rhesus macaque)
	Cecropins	*Hyalophora cecropia* (North American moth)
	Dermaseptins	*Phyllomedusa spp.* (Frogs genus)
	Ovispirin	*Ovis aries* (Sheep)
	Mastoparan X	*Vespa xanthoptera* (Japanese yellow hornet)

AMP, antimicrobial peptide.

The carpet-like model is also called the "detergent-like model," and the toroidal model is called the "wormhole model." **Table 2** shows different AMPs classified based on their membrane disruption mode of action.

Intracellular Targeting Mechanism of Action

Apart from the membrane-targeting MOA, some AMPs may exert other MOA, including the inhibition of extracellular wall synthesis and may have intracellular targets, thus disrupting intracellular processes (26). It has been shown that membrane permeabilization results in AMP translocation into the cytoplasm

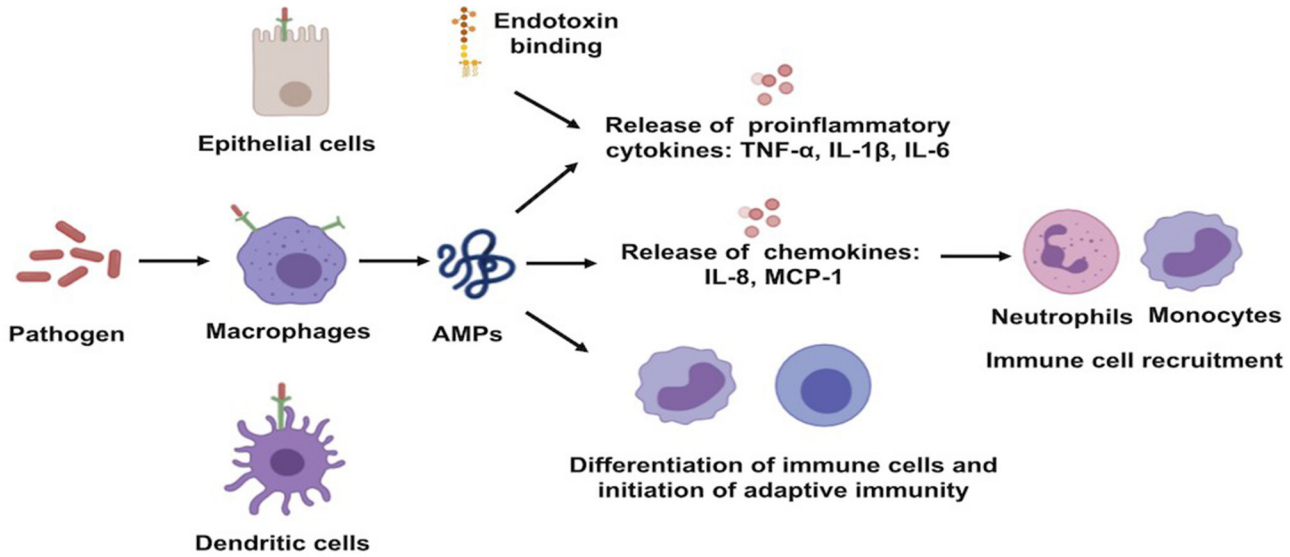

FIGURE 3 | Schematic illustration of immune-regulatory functions of antimicrobial peptides. AMPs, antimicrobial peptides; IL, interleukin; MCP, monocyte chemoattractant protein; TNF-a, tumor necrosis factor alfa.

without disruption of its integrity, allowing binding to the anionic charge present in nucleic acids (DNA/RNA), some intracellular enzymes, and other targets (18, 26). For instance, AMPs, such as defensins, often confer antibacterial activity by interacting with various precursor molecules that are required for cell wall synthesis, such as the highly conserved lipid II (20). Other AMPs, such as indolicidin, interfere with protein synthesis, whereas papiliocin induces the production of oxygen free radicals, which damages both DNA and the cell membrane. Others can inhibit the activity of a few intracellular enzymes crucial for metabolism and proliferation of pathogens (26). Remarkably, it is suggested that AMPs may cause bacterial death via multiple and complementary actions known as a multi-hit mechanism, serving in increasing the efficiency of AMPs and evading resistance development (28).

Immune Modulation

Well-characterized for their antimicrobial activities, AMPs are also known for their immuno-regulatory functions. The expression of these AMPs can be constitutive or can be inducible by infectious and/or inflammatory stimuli, such as proinflammatory cytokines, bacteria, or bacterial molecules that induce innate immunity (29). AMP production constitutes one of the early mechanisms by which the host immune system provides protection against invaders (13). They can recruit and activate immune cells, resulting in enhanced bactericidal activity and/or control of inflammation (20, 28). They act as effective inflammatory modulators by stimulating chemotaxis and angiogenesis, modulation of immune cell differentiation, and initiation of adaptive immunity. The broad range of mechanisms of action exerted by AMPs also includes toxin neutralization in an extremely rapid manner (**Figure 3**) (30). As examples, human neutrophil defensin (HNP)-1, HNP-2, and HNP-3 have been shown to upregulate the production of tumor necrosis factor alfa (TNF-α) and interleukin (IL)-1 by human monocyte activated upon bacterial infection, which, in turn, produces pro-inflammatory cytokines to attract immune cells to fight off the pathogens (31). In addition to that, HBD-2 and HBD-3 promote bacterial clearance of *Pseudomonas aeruginosa* by suppressing macrophage autophagy through downregulation of early growth response gene-1 (EGR1) and proto-oncogene c-FOS (32). Moreover, it has been demonstrated that cathelicidin exerts a direct chemoattractant action on monocytes, neutrophils, and T cells (33) and induces the transcription and release of IL-8 and monocyte chemoattractant protein (MCP)-1 and MCP-3, resulting in the recruitment of different immune cells requisite to remove the invading pathogen (34). LL-37, in addition to its direct MOA, neutralizes the activity of LPS and, thus, helps to protect the tissues from its harmful effects. In addition, it maintains a balance between pro- and anti-inflammatory mediators in the presence of LPS.

ANTIMICROBIAL SPECTRUM OF ACTIVITY

AMPs have broad-spectrum antibacterial activity and may exhibit their effects at minimum inhibitory concentrations (MICs) as low as 1–4 μg/ml (10). In addition to their potent antibacterial impact, some AMPs possess antiviral, antifungal, antiparasitic, and insecticidal properties. For instance, LL-37, the sole human cathelicidin, possesses a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, such as *S. aureus*, *Enterococcus faecalis*, Group A *Streptococcus*, *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Prevotella intermedia* among others, including antibiotic-resistant strains containing methicillin-resistant

TABLE 3 | Example of peptides with their spectrum of activity.

Targeted microbes/PAMP	Examples of AMP
Gram-negative and –positive bacteria	IB-367, protegrin, MSI-78, gramicidin S, indolicidin, CEMA
Gram-negative bacteria	Polymyxin B
Gram-positive bacteria	HNP1, sPLA2-IIA
Fungi	Protegrin, indolicidin, gramicidin S, CEMA, polyphemusin, sPLA2-V
Virus	Indolicidin, protegrin, polyphemusin
Parasite	Magainin II, indolicidin
Endotoxin*	CEMA, polyphemusin variants

AMP, Antimicrobial peptide; PAMP, Pathogen-associated molecular pattern.
**Endotoxin named also LPS, is a PAMP present in the cell wall of Gram-negative bacteria.*

Staphylococcus aureus (MRSA) and vancomycin-resistant *Enterococci* (VRE) (35–37). It has also a preventive action against *S. aureus* biofilm formation (38, 39) and can kill, *in vitro* and *in vivo*, both enveloped and non-enveloped viruses (40). Moreover, this peptide shows toxicity to tripomastigotes of the protozoan parasite *Trypanosama cruzi* at micromolar concentrations (35). On the other hand, magainins exhibit a broad spectrum of antimicrobial activity that includes Gram-positive and Gram-negative bacteria [*E. coli* (41) and *P. aeruginosa* (42)] and fungi, such as *Candida albicans* (43) at concentrations in the range of 1–10 μg/ml (44). The Type-IIA secreted phospholipase A_2 (sPLA2-IIA) kills selectively Gram-positive bacteria (see below) while sPLA2-V contributes to the innate immune response against *C. albicans* by regulating phagocytosis and killing through a mechanism that is likely dependent on phagolysosome fusion (45). Defensins are also active against bacteria, fungi, and some viruses at low concentrations under optimal conditions (37). The antimicrobial activity of defensins is inhibited in the presence of increasing concentrations of salts and plasma proteins (44). Their spectrum of activity includes sexually transmitted infections causing pathogens, such as *Treponema pallidum*, *Chlamydia trachomatis*, human immunodeficiency virus (HIV)-1, and herpes simplex virus (HSV)-2 (43); fungal infections, such as candida species (43); skin infections due to *S. aureus* and *P. aeruginosa*; and other important bacterial pathogens, such as *Salmonella* and *Haemophilus influenzae* (46). Examples of peptides with their spectrum of activity are presented in **Table 3**.

AMPs are generally capable of killing microbes independently. However, they often show enhanced antimicrobial activity when tested in combination with either other AMPs or conventional antibiotics (7, 35). Many previous studies have shown that the use of antibacterial agents in a therapeutic cocktail can reduce the dose of each drug in the combination, limiting the development of resistance *in vitro* (18). For instance, LL-37 and HNP-1 were shown to work synergistically together with a significant enhancement of both their antimicrobial activities and membrane permeabilization effects (35). It has been also demonstrated that the efficacy of conventional antibiotics could be further boosted through combination with AMPs, and some studies revealed synergistic relationships between antibiotics and AMPs (47, 48). For example, Dosler and Mataraci reported the synergistic effect of indolicidin combined to conventional antibiotics daptomycin, teicoplanin, and ciprofloxacin against MRSA biofilm (48). Furthermore, our recent studies showed that the AMP LL-37 potentiated the bactericidal effects of the antibiotics colistin and imipenem on both antibiotic susceptible and multidrug resistant strains of *P. aeruginosa* (49).

TYPE-IIA SECRETED PHOSPHOLIPASE A_2: A PARTICULAR HOST ANTIMICROBIAL PEPTIDE

The type-IIA secreted phospholipase A_2 (sPLA2-IIA) is a member of the super-family of enzymes called sPLA2 originally defined by their ability to catalyze the hydrolysis of phospholipids from both eukaryotic and prokaryotic cell membranes at the *sn*-2 position leading to the generation of lysophospholipids and free fatty acids (50, 51). The sPLA2-IIA can be classified as a member of the AMP family although it kills bacteria via a different MOA (see below) and is larger than most AMPs (120 amino acids). The classifications of $sPLA_2$ in different types is based on the number and position of their disulfide bridges (50, 51). The encoding sequences of some $sPLA_2$ complimentary DNA (cDNA) predicted the presence of the putative signal peptide, thus indicating that these types of $sPLA_2$ are secreted proteins. To date, 10 distinct members of $sPLA_2$s have been identified so far in mammals with around 50% homology among them (50, 51). It becomes clear now that sPLA2-IIA is a major actor in host defense against invading bacteria and is produced by host cells at sufficient levels to ensure this role (52, 53).

Discovery of the Bactericidal Functions of sPLA2-IIA

$sPLA_2$-IIA, the most studied enzyme of the sPLA2 group, is the most abundant in human and animal biological fluids, and it has been initially proposed to play a role in the pathogenesis of various inflammatory diseases (50, 51). However, this notion evolved progressively, and it is now accepted that bacterial killing represents the most physiologically relevant and recognized function of sPLA2-IIA (52, 53). The group of J. Weiss reported for the first time that the potent antistaphylococcal activity present in the inflammatory peritoneal exudate can be attributed mostly to sPLA2-IIA (54). This bactericidal effect is due to the ability of $sPLA_2$-IIA to bind and penetrate the cell wall of Gram-positive bacteria with greater efficiency compared to its Gram-negative effect (52, 53, 55). Subsequent studies report that mouse and human $sPLA_2$ exhibit various bactericidal activities toward two Gram-positive bacteria, *Listeria monocytogenes* and *S. aureus*, and that $sPLA_2$-IIA is, by far, the most bactericidal $sPLA_2$. The concentrations of $sPLA_2$-IIA in biological fluids are sufficient to kill all Gram-positive bacteria that may infect mammals (52, 53). Whereas, the concentrations of $sPLA_2$-IIA in the normal human tear exceed 30 μg/ml, only 1.1 ng/ml of the enzyme is sufficient to achieve the killing of *L. monocytogenes* (56). Concentrations at 15–80 ng/ml of sPLA2-IIA are necessary for *S. aureus* killing. The $sPLA_2$-IIA efficiently kills Gram-positive

bacteria due to the high net positive charge of this enzyme compared to that of other $sPLA_2$s, allowing rapid and highly efficient binding of $sPLA_2$-IIA to the negatively charged surface of these bacteria (52, 53). The cell wall bacterial component lipoteichoic acid has been reported to play a key role in the tight binding of sPLA2-IIA to Gram-positive bacteria, such as *S. aureus* (57).

The contribution of sPLA2-IIA to antibacterial host defense is supported by *in vivo* experiments using sPLA2-IIA Tg mice (52, 53). sPLA2-IIA Tg mice were generated in the C57Bl/6 background. This mouse strain contains an inactivating point mutation in murine sPLA2-IIA, making them natural knockouts (58, 59). Therefore, expression of sPLA2-IIA in this strain background is not confounded by the co-expression of murine sPLA2-IIA. Using these mice, it has been established that sPLA2-IIA protects from lethal infections of *S. aureus*, *Bacillus anthracis*, and *Streptococcus pyogenes* (60–64).

AMPs AND DISEASES

The skin or the mucosal surface, such as the respiratory tract, the gastrointestinal tract, and the urogenital tract (11), are classically considered as the primary sites at which a host encounters a pathogen. At these sites, infections are controlled by the innate defense responses that allow the host to maintain its integrity (12). Knockout mice and Tg expression systems have confirmed that AMPs play a central role in limiting microbial proliferation in various host sites, thus preventing spread to the deep tissues where serious infection may occur (14). AMPs are produced by epithelial cells of vertebrates as a first line of defense against microbial invaders and tend to exhibit intrinsic specificity for the encountered pathogens. For instance, HNPs are expressed at high levels in lesions of superficial folliculitis due to skin infection by *S. aureus* (11). As an initial part of the inflammatory response, AMPs are produced by inflammatory cells, such as neutrophils and tissue phagocytes, including macrophages (31). For example, HBD is upregulated in monocytes exposed to bacteria, LPS, or IFNɤ (65). Furthermore, the immunomodulatory activities of AMPs enable the activation of adaptive immune responses. LL-37 represents a classical example of AMPs that binds to LPS leading to repressed LPS-induced responses and targeting the NF-κB pathway. Moreover, studies have shown that a downregulation of AMP expression is associated to an increase in susceptibility to infections by viruses and other microorganisms (13).

AMPs play an integral role in a large number of respiratory diseases [for example, tuberculosis, cystic fibrosis (CF), rhinitis, etc.], gastrointestinal diseases (shigellosis, inflammatory bowel disease, etc.), and cutaneous diseases (atopic dermatitis, psoriasis, wound healing, and rosacea) among others (13, 66). Group B Streptococcus (GBS) is killed by human serum from patients with GBS-related infections in an sPLA2-IIA-mediated manner (63). In healthy patients, sPLA2-IIA is the only sPLA2 isoform that is constitutively present at low ng/ml concentrations in the circulation (67–69). Increased levels of sPLA2-IIA have been observed in biological fluids in various inflammatory and infectious diseases, such as allergic rhinitis, rheumatoid arthritis, pancreatitis, septic shock, acute respiratory distress syndrome (ARDS), or CF, and correlated to symptom severity of these diseases (50, 70). However, it remains unclear whether upregulation of sPLA2-IIA expression is the cause and/or the consequence of inflammation (e.g., increased cytokine production) in these diseases. Elevated sPLA2-IIA levels have also been observed in arterial plasma and in bronchoalveolar lavage fluids of patients with septic shock. These levels have a prognostic value and correlated with the development of pulmonary failure (50). We focus in more detail in the following paragraph, the potential relevance of AMPs in CF.

Cystic Fibrosis

Patients with disruptions in lung immunity or mucosal clearance, such as patients with CF, suffer from bacterial infections that typically don't resolve even with antibiotic treatment (71). CF is a well-characterized, lethal, autosomal, recessive, inherited disorder found predominantly in Caucasians due to mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, characterized by chronic lung bacterial infections (72). These infections are major causes of morbidity and mortality of CF patients. Ultimately, 80 to 95% of patients with CF succumb to respiratory failure brought on by these chronic bacterial infections associated with airway inflammation (73). *P. aeruginosa* is arguably the major colonizing infection for people with CF (74).

The main AMPs detected in lung tissues and secretions of CF patients are neutrophil α-defensins/HNPs, HBDs, LL-37, and sPLA2-IIA that play a major role in lung immunity and protect them against infection with harmful microorganisms (75). The persistence of lung bacterial infection may be partly explained by an acidification of the airway surface liquid (ASL) within the CF lung that exhibits reduced bacterial killing due to the compromised function of AMPs (72, 76). Our recent studies showed that ASL was significantly more acidic in CF than in wild-type (WT) respiratory cells. This was consistent with a defect in bicarbonate secretion involving CFTR and SLC26A4 (pendrin) and a persistent proton secretion by ATP12A. This was associated to a defect in *S. aureus* clearance, which was improved by pH normalization (72).

Abnormal salinity of ASL has also been suggested to impair the bactericidal activity of AMPs, which can form bacterial proliferation within CF airways (77). We recently showed that the defensin BigDef1 from the oyster *Crassostrea gigas* exhibits natural salt-stable and broad-range bactericidal activity against various bacterial species. We took advantage of this salt-stability, due to an evolutionary adaptation of oyster defensins to sea environment, to treat bacteria from CF patients. We showed that BigDef1 efficiently kills multidrug-resistant clinical isolates of *S. aureus* from CF patients even at high salt concentrations (78).

In the early stages of CF, the airways are mainly colonized by *S. aureus*, whereas in later stages, *P. aeruginosa* is the major pathogen (46). This shift in infection is a characteristic feature of CF. Once it colonizes the CF airways, *P. aeruginosa* induces a robust expression and secretion of sPLA2-IIA by airways epithelial cells via a Krüppel-like transcription factor (KLF)-2-dependent pathway, leading to subsequent and selective

killing of *S. aureus* by sPLA2-IIA, a process contributing to the infection shift (16). A similar phenomenon has been reported during periodontal diseases caused by *Porphyromonas gingivalis*. The latter induces sPLA2-IIA production and secretion by oral epithelial cells via activation of the Notch-1 receptor (45). The sPLA2-IIA concentrations reach levels leading to the killing of other oral bacteria much more susceptible to this enzyme sPLA2-IIA compared to *P. gingivalis* (45). This process is a potential cause of dysbiosis associated with periodontal disease. Thus, it is of great importance to examine the role of individual bacterial species within the microbiome in the induction or inhibition of sPLA2-IIA expression at mucosal sites and whether this may contribute to occurrence of dysbiosis at mucosal surfaces in diseases characterized by polymicrobial infections.

ADVANTAGES OF AMPs AND CHALLENGES

As the emergence of super-bacteria is causing a serious concern across the globe, researchers are working on the development of new anti-infective therapies. Among the alternatives to combat antimicrobial resistance, AMPs have garnered much attention over the years (79). AMPs, which are widely expressed in all kind of living organisms and have been preserved in the long evolutionary process, are with no doubt effective natural immunologically active molecules (80). AMPs have excellent *in vitro* antimicrobial activity against a wide range of microbes and, therefore, represent a promising alternative to combat resistance (18). The rapid bactericidal activity of AMPs constitutes a strong advantage to the future of peptide-based antibacterial therapy. In addition, AMPs are active against multidrug-resistant bacteria (49, 81). Furthermore, AMPs possess concomitant broad anti-inflammatory and immunomodulatory activities. Besides, AMPs exhibit synergistic or additive effects upon co-administration with conventional ATBs to treat both susceptible and multidrug-resistant bacteria at non-toxic concentrations (70, 71).

Due to the overlapping MOA of AMPs involving multiple low-affinity targets, unlike the MOA of conventional ATBs characterized by one defined, high-affinity target, the development of bacterial resistance toward AMPs has generally been considered to be improbable (28, 82). In particular, given that the bacterial cell membrane is the primary target of AMPs, it is challenging for microbes to preserve the cell membrane functional and structural integrity while at the same time avoiding the membrane disruption activity of AMPs (28). Because the AMP is composed of amino acids with no specific primary sequence signature, the microbe is unable to synthesize a protease that can cleave the AMP but not its own proteins. Furthermore, our recent study showed that the AMPs LL-37 and CAMA, a derivative of cecropin, were associated with only transient and low levels of induced resistance compared to the induced resistance by the antibiotic gentamicin (49). However, it appears somehow that some bacteria, such as *Serratia marcescens*, present natural resistance to AMPs (83). Moreover, some bacteria exposed to AMPs may evolve under selective pressures to develop resistance mechanisms. Even though the existence of these selective pressures are, evolutionarily speaking, quite old, human AMPs still possess a broad spectrum of effective activity against a diverse range of microorganisms (14).

In the last 30 years, various pharmaceutical companies have tried to develop AMPs as clinically useful antimicrobials. To date, several AMPs are currently undergoing laboratory testing, and a few have already reached clinical trials (19). The review (18) shows a number of AMPs and AMP derivates already at the preclinical stage and in clinical trial.

Although AMPs have very attractive qualities, the challenges for successful development for clinical application are considerable (84). One of the biggest restraints in the large scale of development and commercialization of AMPs may be their high production costs estimated around US$300–$500 per gram, which is several hundred times more expensive than the production of conventional ATBs (17). In addition to that, the excellent antimicrobial activity *in vitro* is rarely translated *in vivo* (41). In most studies in the field, the killing effects of AMPs on bacteria have been examined *in vitro* and in the absence of host cells, which do not reflect real life. Indeed, in human and animal infectious diseases, infecting bacteria multiply within biological fluids and/or in contact with host cells, which may interfere with AMP bactericidal activity. This led us to compare the bactericidal effects of LL-37 on *P. aeruginosa* in a cell-free system and when this strain was added to a bronchial epithelial cell line IB3, isolated from a CF patient, prior to addition of LL-37. These studies show that the presence of IB3 cells markedly reduces the bactericidal effects of LL-37 on *P. aeruginosa*. Although the mechanisms involved in this alteration are still under investigation, we hypothesized that degradation of LL-37 by a protease produced by IB3 cells upon infection by *P. aeruginosa* may explain the alteration of LL-37 bactericidal activity (unpublished data). Thus, most peptides have relatively short circulating plasma half-lives and are cleared primarily by proteolytic degradation and by renal filtration, generally leading to suboptimal pharmacokinetic properties (84). Indeed, the most obvious cause of poor or incomplete *in vivo* activity of AMPs is the lack of stability due to the peptide susceptibility to protease degradation if they are ingested. In regard to drug delivery, oral bioavailability of peptides is often no more than 2% (79). Thus, oral administration of AMPs can lead to proteolytic digestion by enzymes in the digestive tract, such as trypsin and pepsin, making intravenous or subcutaneous injections the only viable routes of administration to treat people. Moreover, systemic administration outcomes with short-half lives *in vivo*, protease degradation, and cytotoxic profiles in blood (20). In addition, the direct antibacterial activity of some of these AMPs is certainly prevented due to the affinity of these AMPs to polyvalent anions, such as glycosaminoglycans (29). AMPs can also bind avidly to host cells, which may reduce their availability to bind to and kill bacteria (unpublished data).

Another key factor to consider is the potential of these peptides to elicit an immunogenic response that can significantly reduce their efficacy and alter their pharmacokinetic profile (84). An additional challenge to overcome is the differences in pH, salt, and serum concentrations *in vivo*, resulting in decreased antimicrobial activity (10). Hence, a number of AMPs have failed

approval by the FDA after reaching phase II clinical trials due to their short-half life and their poor physical-chemical properties (17). However, even with limitations, AMPs still possess a broad spectrum of potent antimicrobial activity (14). Another potential issue includes the cytotoxicity to mammalian cells when bactericidal concentrations are high. However, there are very few studies of AMP cytotoxicity on human cells (10).

Methods to overcome these challenges have been evaluated. Scientists and pharmaceutical companies have invested in research and development to overcome the barriers limiting the practical application of AMPs. To circumvent proteolysis, sequence modifications, and half-life, advances in peptide formulation have ended in the development of improved formulas with sufficient plasma exposure using a delivery system (for example, a lipid self-assembly system, inorganic systems, nanoparticles, etc.), chemical modifications of AMPs, and altering structure to have cyclic peptides with strained peptide bonds displaying a resistant profile (41, 85). Another approach is to identify possible molecular cleavage sites followed by substitution of the relevant amino acids (86). The recognized route of administration for therapeutic peptides remains parenteral, in which AMPs pierce the membrane barriers where they are poorly absorbed. Nevertheless, other challenges remain, pre- and post-administration, in achieving both the desired pharmacokinetic profile and high patient compliance (84). Specific cell-penetrating peptide sequences have been identified and can be used to transport AMPs across membranes (79). More tools to increase AMP activity include modifications in charge and hydrophobicity. Among the various methods for peptide optimization, quantitative structure-activity relationship, and the introduction of fluorine atoms or triflurometyl groups have been recently used (25). Besides, recent studies have focused on designing a sequence of AMP analogs with modified yet improved antibacterial, cytotoxic, and hemolytic activities. Thus, synthetic peptides have been designed to mimic the structure, function, and mode of action of AMPs with enhanced properties, resulting in low cytotoxicity and high resistance to proteolytic degradation, resulting in prolonged half-lives and cost-effective molecules (18). Furthermore, the progress in designing non-immunogenic peptides is rapid, resulting in disarming the immunogenic response, which should increase clinical success (84).

CONCLUSIONS AND PERSPECTIVES

AMPs, owing to their broad spectrum of antibacterial activity and their effectiveness against multidrug-resistant bacteria, are a promising replacement for conventional ATBs, invoking a multi-hit mechanism that cannot be easily overcome by bacteria. However, the future of peptide-based anti-infective drugs is still uncertain. The major barriers that hinder their clinical use are mainly their stability *in vivo*, their non-well-studied toxicity, and their high production costs. Thus, the development of optimal formulations of AMPs at a reasonable cost, finding the preferred route of their administration, and evaluating their cytotoxicity remain the main interest to scientists. Regardless of the field of applications, AMPs constitute the most promising drug candidate in a foreseeable future in overcoming the alarming rise of bacterial resistance.

AUTHOR CONTRIBUTIONS

RG and CM wrote the article. ZX did the figures and tables. LT and DK revised and corrected the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Impact of the Local Inflammatory Environment on Mucosal Vitamin D Metabolism and Signaling in Chronic Inflammatory Lung Diseases

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Vitamin D plays an active role in the modulation of innate and adaptive immune responses as well as in the protection against respiratory pathogens. Evidence for this immunomodulatory and protective role is derived from observational studies showing an association between vitamin D deficiency, chronic airway diseases and respiratory infections, and is supported by a range of experimental studies using cell culture and animal models. Furthermore, recent intervention studies have now shown that vitamin D supplementation reduces exacerbation rates in vitamin D-deficient patients with chronic obstructive pulmonary disease (COPD) or asthma and decreases the incidence of acute respiratory tract infections. The active vitamin D metabolite, 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$), is known to contribute to the integrity of the mucosal barrier, promote killing of pathogens (via the induction of antimicrobial peptides), and to modulate inflammation and immune responses. These mechanisms may partly explain its protective role against infections and exacerbations in COPD and asthma patients. The respiratory mucosa is an important site of local $1,25(OH)_2D$ synthesis, degradation and signaling, a process that can be affected by exposure to inflammatory mediators. As a consequence, mucosal inflammation and other disease-associated factors, as observed in e.g., COPD and asthma, may modulate the protective actions of $1,25(OH)_2D$. Here, we discuss the potential consequences of various disease-associated processes such as inflammation and exposure to pathogens and inhaled toxicants on vitamin D metabolism and local responses to $1,25(OH)_2D$ in both immune- and epithelial cells. We furthermore discuss potential consequences of disturbed local levels of 25(OH)D and $1,25(OH)_2D$ for chronic lung diseases. Additional insight into the relationship between disease-associated mechanisms and local effects of $1,25(OH)_2D$ is expected to contribute to the design of future strategies aimed at improving local levels of $1,25(OH)_2D$ and signaling in chronic inflammatory lung diseases.

Keywords: inflammation, airway mucosa, vitamin D, vitamin D metabolism, host defense, COPD exacerbations

INTRODUCTION

Vitamin D is a pleiotropic hormone that is well-known for its role in the regulation of calcium and phosphate homeostasis and bone mineralization. The vitamin D receptor (VDR) acts as the receptor for the active form of vitamin D, i.e., 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D], and is expressed in nearly all tissues and cell-types and regulates a large number of genes (∼0.8–5% of the total genome) (1, 2). As a result, vitamin D affects many additional processes including cell proliferation and differentiation, apoptosis, DNA repair, ion transport, metabolism, cell adhesion, and oxidative stress responses (1, 3). Vitamin D deficiency [serum 25-hydroxy-vitamin D [25(OH)D] < 50 nmol/L; 25(OH)D is the main circulating form of vitamin D and its levels are used to assess vitamin D status in the clinic (4, 5) affects more than 30% of the children and adults worldwide and is a major cause of bone diseases such as rickets and osteoporosis (6). Increasing evidence has indicated that vitamin D deficiency is also associated with various other diseases such as cancer, cardiovascular disease, Alzheimer's disease and muscle myopathy, as well as several immune-related diseases such as type 1 diabetes, multiple sclerosis, inflammatory bowel disease (IBD), psoriasis and chronic inflammatory lung diseases including asthma, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD) (6–9).

Several studies have now shown that vitamin D deficiency is prevalent in COPD patients and inversely correlated with lung function and severity of the disease (8–12). It is currently unknown whether vitamin D deficiency is a cause or consequence of COPD, since many COPD patients have low physical activity levels and spend most time indoors (13). There are however studies suggesting that low 25(OH)D levels are associated with development of COPD, based on observed associations between polymorphisms in the vitamin D binding protein (VDBP), 25(OH)D serum levels and COPD severity (8, 10, 11, 14). In addition, one study in mice showed that maternal vitamin D deficiency can impair lung -development, -structure and -function in the offspring and suggests that even before birth, maternal 25(OH)D serum levels are important for a healthy lung development (15). This might be relevant, since associations have been found between lower childhood lung function and development of COPD later in life (16). The link between maternal 25(OH)D status and asthma development is however much clearer, since two recent randomized controlled trials (RCTs) have shown that maternal vitamin D supplementation reduces the risk of childhood asthma/recurrent wheeze (17). This might be explained by the fact that multiple vitamin D-regulated genes are transcriptionally active during alveolar maturation and a number of these genes are differentially expressed in asthma (18). Additionally, this protective effect was linked to the GG-genotype of the 17q21 functional SNP rs12936231, which is associated with lower expression of *ORMDL3* and increased sphingolipid metabolism (19). Moreover, maternal circulating 25(OH)D levels affect the gut microbiota and can therefore indirectly modulate immune responses in the lung via the gut-lung-axis (20). Also later in life, optimal 25(OH)D levels remain crucial for keeping the lungs healthy. For example, Heulens et al. showed that subacute and chronic cigarette smoke (CS) exposure decreased lung function and promoted early signs of emphysema and airway inflammation in vitamin D-deficient mice compared to vitamin D-sufficient animals (21). Similarly in an elastase-induced COPD mouse model, topical administration of vitamin D in the lungs counteracted alveolar damage and improved lung function (22). Yet in humans, it is still unclear whether vitamin D status influences COPD development and disease progression. Taken together, these observations suggest an important role for vitamin D during fetal and childhood lung maturation, and indicate that sufficient 25(OH)D levels might contribute to protection against development of childhood asthma and possibly COPD at older age.

Systemic levels of biologically active 1,25$(OH)_2$D are tightly regulated to preserve sufficient levels of calcium (Ca^{2+}) and phosphate (PO_4^{2-}) for optimal bone mineralization, whereas in mucosal tissues locally produced (autocrine) 1,25$(OH)_2$D levels and signaling can be elevated or decreased upon exposure to inflammatory mediators, pathogens or inhaled toxicants (6). This could be important, since the inflamed airway mucosa of patients suffering from chronic inflammatory lung diseases is constantly exposed to these disease-associated factors (8, 23, 24). Impaired local levels of 1,25$(OH)_2$D and VDR signaling might have consequences for disease pathogenesis and progression. Dysregulated host defenses as found in patients with chronic inflammatory airway diseases include aberrant immune responses, altered microbiome composition, impaired epithelial barrier function, and aberrant secretion of host defense molecules (25–27). Adequate 1,25$(OH)_2$D levels may provide protection against these dysregulated processes by maintaining the integrity of the mucosal barrier and promotion of killing of pathogens (e.g., via the induction of the antimicrobial peptide [AMP] hCAP18/LL-37) and via the modulation of both innate and adaptive immune responses (7, 28, 29).

In this review, we first discuss the effects of these disease-associated factors on local synthesis and availability of 1,25$(OH)_2$D and 1,25$(OH)_2$D-induced responses in the lung mucosa. In the second part of the review we will describe the mechanistic links between vitamin D deficiency and the pathogenesis of chronic inflammatory lung diseases such as asthma, CF and COPD, and discuss recent evidence related to the protective effects of vitamin D on COPD and on COPD exacerbations.

MUCOSAL VITAMIN D METABOLISM IN HEALTH

Vitamin D enters the circulation either via food intake (plant-based: vitamin D_2/animal-based: vitamin D_3) or as a result of its synthesis in the skin by UVB radiation. It subsequently binds to the VDBP (30, 31), after which this complex is transported to the liver where it is converted by vitamin D-25-hydroxylases (CYP2RI and CYP27A1) into 25(OH)D. However, recent studies showed that also other cell types such as airway epithelial cells, keratinocytes, intestinal epithelial cells, and monocytes/macrophages express CYP2RI and CYP27A1, and

thus are able to (locally) convert vitamin D_3 into 25(OH)D_3 (32, 33). This inactive 25(OH)D needs to be converted into the active 1,25(OH)$_2$D by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney and in other cells, including several immune- and epithelial cells (34–40). 1,25(OH)$_2$D regulates expression of several genes by binding the nuclear VDR, which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of these genes (1, 2). VDR is most abundantly expressed in intestinal enterocytes, pancreatic islets, renal distal tubules and osteoblasts, but is also present at lower levels in most other tissues and several other epithelial- and immune cells (41–45). Expression of VDR is classically regulated by 1,25(OH)$_2$D, growth factors and hormones such as FGF-23 and PTH, respectively, circulating calcium levels, bile acids, transcriptional co-activators/repressors, and genetic- and epigenetic modifications, which is tissue specific (46–49). 1,25(OH)$_2$D regulates its own negative feedback by several mechanisms, including induction of expression of the catabolic enzymes 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) and CYP3A4 (50, 51). CYP24A1 is expressed in most tissues and converts both 25(OH)D and 1,25(OH)$_2$D into 23,25(OH)$_2$D or 24,25(OH)$_2$D and 1,23,25(OH)$_3$D or 1,24,25(OH)$_3$D, respectively (dependent on whether CYP24A1 hydroxylates at C-23 or at C-24). These are further converted into metabolites that have been found to be excreted into the bile (summarized in **Figure 1**) (50, 52, 56). CYP3A4 is mainly expressed in the liver and small intestines and contributes to the metabolic clearance of 25(OH)D and 1,25(OH)$_2$D by converting 25(OH)D into 4β,25(OH)$_2$D, and 1,25(OH)$_2$D into

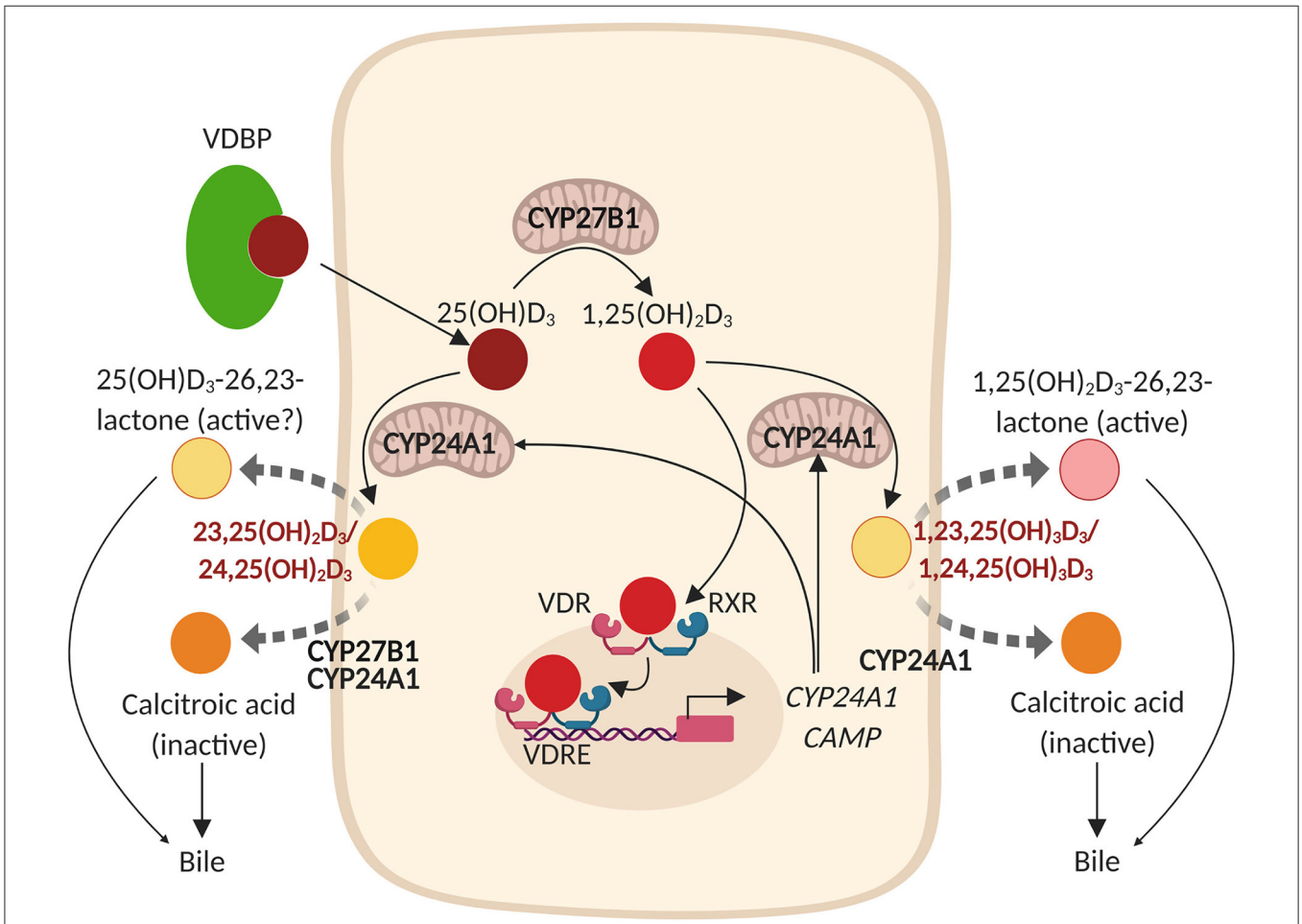

FIGURE 1 | Vitamin D metabolism and expression of hCAP18/LL-37 (*CAMP*) in epithelial cells. The vitamin D binding protein (VDBP)-25(OH)D_3 complex enters the epithelial cell from the circulation and 25(OH)D_3^* is subsequently released from the complex. In the cytoplasm, 25(OH)D_3 is hydroxylated by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1; localized to the inner mitochondrial membrane) into the active metabolite 1,25(OH)$_2D_3$. 1,25(OH)$_2D_3$ subsequently binds to the nuclear vitamin D receptor (VDR) which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of numerous genes, including *CAMP* (hCAP18/LL-37) and *CYP24A1* (25-hydroxyvitamin D-24-hydroxylase). 1,25(OH)$_2D_3$ thereby regulates its own negative feedback via direct induction of CYP24A1 that hydroxylates both 25(OH)D_3 and 1,25(OH)$_2D_3$ either at the C-23 or at the C-24 to 23,25(OH)$_2D_3$ or 24,25(OH)$_2D_3$ and 1,23,25(OH)$_3D_3$ or 1,24,25(OH)$_3D_3$, respectively. These metabolites are further converted by CYP27B1 [that first converts 24,25(OH)$_2D_3$ into 1,24,25(OH)$_3D_3$] and CYP24A1 into 25(OH)D_3-26,23-lactone and 1,25(OH)$_2D_3$-26,23-lactone or into calcitroic acid, metabolites that are excreted in the bile (50, 52–55). *The metabolism of 25(OH)D_3 is shown in this figure, since there is more consensus regarding the metabolism of 25(OH)D_3 and 1,25(OH)2D_3 in literature.

1,23R,25$(OH)_2$D or 1,24S,25$(OH)_2$D (51). Expression of both CYP27B1 and CYP24A1 in the kidneys is tightly regulated to maintain optimal Ca^{2+}- and PO_4^{2-} levels in the circulation, which are important for bone mineralization (57). In short, in response to low Ca^{2+} levels, parathyroid hormone (PTH) is secreted by the pituitary glands, which in turn reduces Ca^{2+} excretion and reabsorption of PO_4^{2-} (57). PTH further induces expression of CYP27B1 and represses expression of CYP24A1 in the kidneys (57). This will increase the levels of 1,25$(OH)_2$D in the circulation, which promotes intestinal Ca^{2+} and PO_4^{2-} absorption (57). These elevated circulating Ca^{2+} and PO_4^{2-} levels will subsequently induce expression of fibroblast growth factor 23 (FGF-23) in osteocytes and osteoblasts and impair secretion of parathyroid hormone (PTH) by the parathyroid glands (3). In the kidneys, FGF-23 suppresses expression of CYP27B1 and induces expression of CYP24A1, thereby inhibiting the synthesis and promoting degradation of 1,25$(OH)_2$D (3). These complex mechanisms that explain how vitamin D and its metabolic enzymes maintain sufficient Ca^{2+} and PO_4^{2-} levels in the circulation are more extensively discussed by Quarles et al. (57). In summary, it has become increasingly evident that the effects of vitamin D are not limited to homeostasis of Ca^{2+} and PO_4^{2-} and bone mineralization, because several extra-renal cells such as airway epithelial cells and immune cells express the VDR and are capable of converting circulating 25(OH)D into the active 1,25$(OH)_2$D metabolite.

MUCOSAL VITAMIN D METABOLISM AND VITAMIN D SIGNALING IN CHRONIC INFLAMMATORY AIRWAY DISEASES

Local levels and activity of 1,25$(OH)_2$D are in part determined by expression of VDR and the equilibrium between the vitamin D metabolic enzymes CYP27B1 and CYP24A1. It is important to realize that mucosal expression of CYP24A1, CYP27B1 and also VDR can be affected by several disease-associated inflammatory mediators, toxicants and pathogens, summarized in **Table 1**. As a consequence of this, the local availability of 1,25$(OH)_2$D and/or VDR signaling in tissues such as the inflamed airways of patients that suffer from chronic inflammatory airway diseases might be reduced.

Epithelial Cells

Chronic lung diseases are characterized by airway inflammation and impaired respiratory host defense, which is illustrated by the increased susceptibility for respiratory infections and exacerbations (25, 80, 81). Furthermore, exposure to inhaled toxicants such as cigarette smoke and air pollutants are associated with disease pathogenesis and exacerbations in COPD, CF and in asthma patients (82–84). It would therefore be of great interest to investigate these effects on local 1,25$(OH)_2$D levels and on 1,25$(OH)_2$D-mediated respiratory host defense in the airway mucosa. Studies in airway epithelial cells have shown that exposure to UV-inactivated non-typeable *Haemophilus influenzae* (NTHi) increased expression of the catabolic enzyme CYP24A1, whereas exposure to viral double stranded-RNA analog polyinosinic:polycytidylic acid (Poly[I:C]) increased expression of CYP27B1 and thereby conversion of 25(OH)D into 1,25$(OH)_2$D, the active metabolite (38, 61). On the

TABLE 1 | Effects of inflammatory mediators on the expression of VDR, CYP24A1, and CYP27B1 in immune cells and epithelial cells.

Cell/tissue type	Stimulus	Effect	References
Primary airway epithelial cells	Poly(I:C); RSV; IL-13; IL-4; PM	CYP27B1 ↑	(38, 58–60)
	TNF-α; IL-1β; IL-17A; TGF-β1; NTHi	CYP24A1 ↑	(61, 62)
	CSE	CYP27B1 ↓	(63, 64)
	A. fumigatus; HRV; RSV	VDR ↓	(58, 65)
BEAS-2B (bronchial epithelial cell line)	HRV; RSV	VDR ↓	(58)
	PM	VDR ↑	(59)
16HBE (bronchial epithelial cell line)	*A. fumigatus*	VDR ↑	(21)
	TGF-β1	CYP27B1 ↑	(60)
	A. fumigatus	CYP27B1 ↑	(21)
A549 (lung carcinoma cell line)	CSE	VDR translocation ↓	(66)
HCT116 (colon cancer epithelial cell line)	LPS; TNF-α	CYP27B1 ↑	(67)
	LPS; TNF-α	VDR ↓	(67, 68)
	LPS	CYP24A1 ↓	(67)
COGA-1A (colon cancer epithelial cell line)	TNF-α ± IL-6	CYP27B1 ↓	(69)
Trophoblasts	TNF-α; IL-1β; IL-6	CYP24A1 ↑	(70)
	IFN-γ	CYP27B1 ↑	(70)
Macrophages	ss-RNA	CYP27B1 ↑ VDR ↑	(71)
Macrophages (derived from THP-1)	CSE	VDR ↑	(72)
Macrophages (derived from THP-1)	BaP	CYP24A1 ↑	(73)
Monocytes	TLR2/1L ± IFN-γ; LPS; IL-15	CYP27B1 ↑ VDR ↑	(39, 74–76)
	IL-4 ± TLR2/1L	CYP24A1 ↑	(39)
Neutrophils	IFN-γ *S. pneumoniae* T4R	CYP27B1 ↑ VDR ↑	(77)
T cells	T cell activators (anti-CD3/anti-CD28; PHA; PMA/ionomycin)	CYP27B1 ↑ VDR ↑	(78)
B cells	B cell activators (anti-IgM/anti-CD40/IL-21)	CYP27B1 ↑ VDR ↑	(79)

Poly(I:C), Polyinosinic:polycytidylic acid; PM, Particulate matter; NTHi, nontypeable Haemophilus influenzae; A. fumigatus, Aspergillus fumigatus; CSE, Cigarette smoke extract; HRV, Human rhinovirus; RSV, Respiratory syncytial virus; ssRNA, Single stranded RNA; BaP, Benzo[a]pyrene; TLR2/1L, Toll like receptor 2/1 Ligand; PHA, Phytohemagglutinin; PMA, Phorbol 12-myristate 13-acetate.

other hand, in the bronchial cell line BEAS-2B expression of VDR was decreased after infection with respiratory viruses such as human rhinovirus (HRV) and respiratory syncytial virus (RSV) (58). Collectively, these studies have shown in airway epithelial cells that respiratory viral- and bacterial infections can either promote or impair $1,25(OH)_2D$ synthesis and responses.

A local airway inflammatory milieu can also exert differential effects on $1,25(OH)_2D$ synthesis and signaling, dependent on the type of inflammatory mediators that are predominantly present. We have shown in differentiated primary airway epithelial cells that Th2 cytokines such as IL-4 and IL-13, enhance expression of CYP27B1 and expression of hCAP18/LL-37 upon 25(OH)D3 treatment, which suggests that a Th2-inflammatory environment, as found in allergic airway inflammation, increases the conversion of 25(OH)D into the active $1,25(OH)_2D$ (83, 85). The observation that levels of both $1,25(OH)_2D$ and hCAP18/LL-37 were increased in bronchoalveolar lavage (BAL) after allergen challenge is in line with this proposed mechanism (86). This effect of Th2 cytokines was in contrast to the effects (chronic) exposures to the proinflammatory cytokines IL-1β, TNF-α and IL-17A that strongly increased the expression of the 25(OH)D- and $1,25(OH)_2D$-degrading CYP24A1, even in absence of its inducer $1,25(OH)_2D$ (61). Furthermore, short-term exposures to TGF-β1, a pleiotropic growth factor which is elevated in the lungs of COPD, CF and asthma patients, also increases the expression of CYP24A1 (62). As a consequence, $1,25(OH)_2D$-mediated expression of the AMP hCAP18/LL-37 was impaired, which was likely the result of the enhanced degradation of both 25(OH)D and $1,25(OH)_2D$ by this enzyme (61, 62). In addition to pathogens and cytokines, exposure to inhaled toxicants such as cigarette smoke (CS) and particulate matter (PM) may also alter expression or activity of VDR and CYP27B1. Studies have demonstrated that cigarette smoking or exposure to CS extract (CSE) decreases expression of CYP27B1 and inhibited membrane bound (m)VDR translocation to the cell membrane in airway epithelial cells and A549 cells (an alveolar tumor cell line), respectively (63, 64, 66). This inhibition reduces the conversion of 25(OH)D to $1,25(OH)_2D$ and $1,25(OH)_2D$-mediated gene expression as well as non-genomic actions of $1,25(OH)_2D$-membrane associated, rapid response steroid-binding (MARRS)- signaling (63, 64, 66). This adverse effect of cigarette smoking on the synthesis and effects of $1,25(OH)_2D$ in airway epithelial cells was recently confirmed *in vivo* by Vargas Buonfiglio et al. who demonstrated that vitamin D supplementation increased antimicrobial activity in apical surface liquid (ASL) in the airway of healthy non-smokers, but not in smokers (64). On the other hand, exposure to PM increases the expression of both CYP27B1 and VDR in airway epithelial cells, thereby possibly promoting the synthesis and effects of $1,25(OH)_2D$ (59). It is however important to consider that several retrospective and observational studies have demonstrated that air pollution is an independent risk factor for developing vitamin D deficiency (87). In conclusion, exposure to CS, TGF-β1 and presence of a proinflammatory milieu appeared to most strongly decrease local presence and signaling of $1,25(OH)_2D$ in airway epithelial cells.

Immune Cells

Whereas, various studies show that exposure to proinflammatory stimuli most likely affects local 25(OH)D and $1,25(OH)_2D$-levels and reduces the effects of 25(OH)D and $1,25(OH)_2D$ in (airway) epithelial cells, the opposite appears to be the case for immune cells. In monocytes, macrophages and neutrophils, effects on $1,25(OH)_2D$ synthesis and antimicrobial responses upon 25(OH)D treatment were generally enhanced by these proinflammatory stimuli as illustrated by increased expression of both VDR and CYP27B1 (39, 71, 74–77). It is therefore tempting to speculate that this apparent increase in antimicrobial responses upon 25(OH)D treatment in immune cells in an inflammatory environment may serve as a second line of defense and compensate for the enhanced epithelial degradation of 25(OH)D and $1,25(OH)_2D$ during inflammation. Inhaled toxicants may also affect $1,25(OH)_2D$ availability and responsiveness of immune cells. This is illustrated by two recent studies studying the effects of cigarette smoke on the human monocyte/macrophage-like cell line THP-1. One study showed that treatment with cigarette smoke extract (CSE) increased the expression of VDR without enhancing $1,25(OH)_2D$ responses (72), while the other study -that focused on the effects of Benzo[a]pyrene (BaP) (a component produced by cigarette combustion)- demonstrated that $1,25(OH)_2D$ -mediated CYP24A1 expression was induced, which was found to further enhance degradation of $1,25(OH)_2D$ (73). In summary, proinflammatory stimuli generally increased the effect of 25(OH)D and $1,25(OH)_2D$ on immune cells, whereas more studies are needed to fully determine the impact of exposure to cigarette smoke and other inhaled toxicants.

Lung Mucosa

Whereas, these studies provide evidence that inflammation and inhaled toxicants may affect 25(OH)D and $1,25(OH)_2D$ metabolism and responsiveness in epithelial cells and immune cells, it is not clear whether this has an impact on these events in lung tissue of patients with chronic lung diseases. Although evidence is limited, we can speculate that levels of $1,25(OH)_2D$ and responses are also affected by disease-associated factors in mesenchymal cells that are present in the lung mucosa. One study that showed in a bleomycin fibrosis model and in primary lung mouse fibroblasts that TGF-β1 reduced expression of the VDR might support this assumption (88). It is currently insufficiently studied whether exposures to disease-associated factors promote or impair levels of $1,25(OH)_2D$ and responses in immune-, mesenchymal and epithelial cells combined to give a better reflection of the *in vivo* situation. Interestingly, one study did already show that nasal CYP27B1- and $1,25(OH)_2D$-levels are both reduced in chronic rhinosinusitis (CRS) patients with nasal polyps as compared to CRS-patients without nasal polyps, whereas no difference was found in circulating $1,25(OH)_2D$-levels (89). Since most other studies were performed *in vitro* using monocultures of epithelial cells or immune cells, more complex models are needed to delineate this. Therefore, animal models or preferably more complex animal-free cell culture models using co-cultures or organs-on-chips models of primary fully differentiated epithelial cells, airway-derived fibroblasts or

smooth muscle cells and immune cells could be considered in future studies.

PROTECTIVE EFFECTS OF VITAMIN D ON MUCOSAL HOMEOSTASIS

After discussing altered 25(OH)D and $1,25(OH)_2D$ metabolism and responsiveness in the inflamed airway mucosa, it is important to consider the possible consequences of these inflammation-induced changes in the airway mucosa keeping in mind the pleotropic effects of $1,25(OH)_2D$ that were introduced earlier. In several cells, tissues and organs, $1,25(OH)_2D$ regulates multiple cellular processes that affect normal and malignant cell growth and differentiation (90, 91). $1,25(OH)_2D$ displays furthermore protective effects on mucosal host defense by maintaining the integrity of the epithelial barrier, inhibition of epithelial-to-mesenchymal transition (EMT), stimulating production of AMPs and modulating both innate- and adaptive immune functions (7, 29, 92). In addition, $1,25(OH)_2D$ maintains both energetic and survival homeostasis in the mucosal epithelium through the modulation of stress and damage responses, including clearance of disturbing and stressful agents (3, 93) (**Figure 2**).

Epithelial Barrier Function

In chronic inflammatory lung diseases, epithelial barrier function is impaired, and as a consequence the susceptibility toward respiratory infections is increased (94). There is increasing evidence that $1,25(OH)_2D$ promotes epithelial barrier integrity or protects against epithelial barrier destruction. In cells of the bronchial epithelial cell line 16HBE, $1,25(OH)_2D$ inhibited CSE-mediated reduction of the epithelial barrier and expression of

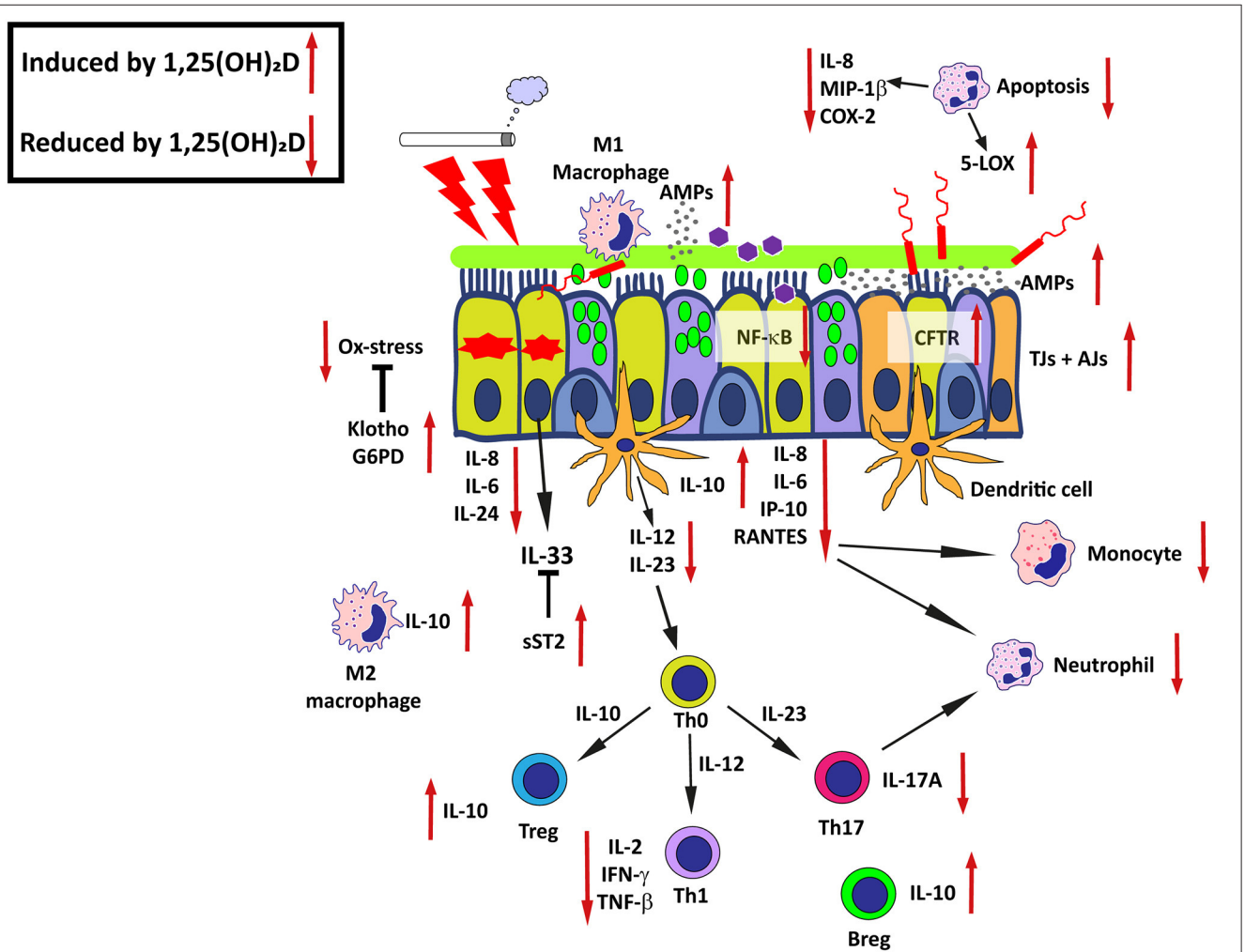

FIGURE 2 | Effects of active $1,25(OH)_2D$ on airway epithelial host defense-mechanisms in chronic airway disease. The promoting or inhibitory effects of $1,25(OH)_2D$ are indicated by the red arrows. AMPs, Antimicrobial peptides; CFTR, Cystic fibrosis transmembrane conductance regulator; Ox-stress, Oxidative stress; SOCS, Suppressor of cytokine signaling proteins; TJs, Tight junctions; AJs, Adherens junctions; G6PD, Glucose-6-phosphate dehydrogenase; sST2, Soluble suppression of tumorigenicity 2; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; Th0, Naieve T cell; Treg, Regulatory T cell; Th1, T helper type 1 cell; Th2, T helper type 2 cell; Th17, T helper type 17 cell; Breg, Regulatory B cell. See text for details and references.

E-cadherin and β-catenin (95). Recently, two murine studies were published that investigated the effects of vitamin D on pulmonary epithelial barrier function. Shi et al. showed that vitamin D-supplementation alleviated lung injury in LPS-treated mice through maintenance of the pulmonary barrier by inducing expression of Zonula occludens (ZO)-1 and occludin in whole lung homogenates (96), whereas Gorman et al. showed in healthy mice, fed with a vitamin D-poor diet, that vitamin D supplementation had little effect on epithelial integrity (97). Only the first study that used a more severe mouse model with higher levels of inflammation and edema found an effect of vitamin D on epithelial barrier function. Since inflammation is detrimental for epithelial barrier integrity (98), it cannot be excluded that the main protective effects of $1,25(OH)_2D$ on the epithelial barrier in the first study by Shi et al. were in fact exerted through inhibition of inflammation rather than via direct induction of cell junction proteins. $1,25(OH)_2D$ might also promote epithelial barrier function through its ability to increase expression of cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells (32). CFTR maintains optimal ASL- and mucus hydration, volume and pH that support mucociliary clearance and activity of AMPs (99). Moreover, CFTR is also affected in the airways of smokers and COPD patients (100). In summary, these studies indicate that $1,25(OH)_2D$ promotes both the integrity and function of the epithelial barrier and might additionally protect against epithelial damage by dampening inflammatory responses.

Anti-fibrotic Effects of Vitamin D

The loss of epithelial barrier function with a decrease in epithelial polarization and cell-junction proteins and a gain of expression of mesenchymal markers is a hallmark of EMT (94). EMT is primarily involved in development, wound healing and stem cell differentiation, and TGF-β signaling plays a major role in this process (101). Elevated TGF-β1 levels are found in the lungs of patients with chronic inflammatory lung diseases and this was associated with cigarette smoking, inflammation and fibrosis (80, 102). There are indications that $1,25(OH)_2D$ counteracts various pathways leading to EMT. In mouse models and in airway epithelial cell lines, vitamin D supplementation and $1,25(OH)_2D$, respectively, has been shown to inhibit EMT and fibrosis, in particular when this process is induced by TGF-β1 (88, 103–106).

Effects of Vitamin D on Epithelial Antimicrobial Responses

In addition to maintenance of the epithelial barrier and inhibition of fibrosis as discussed in the previous paragraphs, vitamin D is also actively involved in respiratory host defense by a variety of mechanisms (3, 29). $1,25(OH)_2D$ is an important inducer of AMPs, which are mostly cationic peptides that have a broad-spectrum antimicrobial activity, the ability to modulate immune responses and to promote epithelial wound repair and angiogenesis (107). hCAP18/LL-37 is likely to be the most prominent AMP that is induced by $1,25(OH)_2D$ and is expressed in several types of mucosal epithelial cells and immune cells such as monocytes and neutrophils (38, 77, 108). In macrophages and intestinal epithelial cells, $1,25(OH)_2D$ also increases expression of human β-defensin-2 (hBD-2), whereas in keratinocytes expression of both hBD-2 and human β-defensin-3 (hBD-3) is increased by $1,25(OH)_2D$ (109–112). Collectively these data show that AMPs are modulated by $1,25(OH)_2D$ in mucosal tissues, which could have impact on susceptibility to both bacterial and viral infections and on the composition of the microbiota, which will be discussed in the next section.

Effects of Vitamin D on Innate and Adaptive Immune Responses

Diseases such as COPD and asthma are characterized by chronic inflammation, a low-grade and prolonged inflammation that may result in destruction and aberrant repair of surrounding tissue by growth factors, proteases and cytokines that are released at the site of inflammation (113–115). Cumulative data suggest that vitamin D exerts anti-inflammatory effects via its actions on both innate and adaptive immune responses. Upon viral infection or exposure of pro-inflammatory stimuli such as Poly(I:C) or PM, $1,25(OH)_2D$ attenuates induced expression of cytokines and chemokines e.g., via inhibition of nuclear factor (NF)-κB or oxidative stress, respectively, in (airway) epithelial cells (38, 59, 116). Furthermore, $1,25(OH)_2D$ increases expression of the soluble decoy receptor for IL-33 (sST2) by airway epithelial cells, which in turn inhibits the actions of the type 2 alarmin IL-33 (117). Further effects of $1,25(OH)_2D$ on local innate and adaptive immune responses in the epithelial mucosa are mediated through its actions on immune and structural cells and have been reviewed by Heulens et al. (29) Vanherwegen et al. (118), and Pfeffer et al. (119).

Taken together, these findings suggest that on the one hand $1,25(OH)_2D$ protects against infections by enhancing epithelial barrier function and production of AMPs, and on the other hand $1,25(OH)_2D$ induces tolerance and dampens proinflammatory responses in various cell types of the airway mucosa. Thereby, $1,25(OH)_2D$ may prevent exaggerated inflammatory responses and further damage to the mucosal tissue, qualities that are very relevant in the context of chronic inflammatory (lung) diseases (**Figure 2**).

Effects of Vitamin D on Epithelial Oxidative Stress and Aging

COPD is considered to be a disease of accelerated aging lungs, underscored by markers of aging being increased in these patients partly as a result of oxidative stress (120). Evidence that $1,25(OH)_2D$ may protect epithelial cells from oxidative stress was provided by Pfeffer et al. who demonstrated that $1,25(OH)_2D$ increased expression of the antioxidant gene *G6PD* in airway epithelial cells. Furthermore, $1,25(OH)_2D$ increased the ratio of reduced to oxidized glutathione and decreased the formation of 8-isoprostane after exposure to PM (59). The induction of klotho by $1,25(OH)_2D$ might be another $1,25(OH)_2D$-mediated anti-aging mechanism (121). Klotho is an anti-aging protein that is mainly expressed in the kidney, brain and in the lung by airway epithelial cells and exerts its protective effects through the inhibition of inflammation, insulin/IGF-1 signaling and activation of forkhead transcription factor (FoxO) signaling, which enables removal of reactive oxygen species

(ROS) (122–124). Expression of klotho is impaired in the airways of smokers and further decreased in the airways of COPD patients and in cultures of the bronchial epithelial cell line 16HBE after CSE exposure (124). These studies suggest that $1,25(OH)_2D$ may protect against aging via inhibition of oxidative stress and possibly via its ability to restore klotho expression (**Figure 2**). However, direct evidence showing that $1,25(OH)_2D$ indeed increases expression of klotho in airway epithelial cells is currently lacking.

Effects of Vitamin D on Epithelial Autophagy and Apoptosis

In addition to providing protection against oxidative stress and aging, data from studies using intestinal epithelial cells suggest that $1,25(OH)_2D$ may also promote cellular survival via the induction of autophagy and reduction of apoptosis (125, 126). In chronic inflammatory lung diseases, aberrant activation of autophagy plays a role in disease pathogenesis (127). A recent study showed that club cells and autophagy-related proteins were both decreased in COPD patients and that these proteins were important for club cell structure and function in airways (128). However, the effects of $1,25(OH)_2D$ on autophagy in the airway mucosa of chronic inflammatory lung diseases are still unclear and need to be further evaluated (127).

ROLE OF VITAMIN D IN THE TREATMENT OF CHRONIC AIRWAY DISEASES

Clearly vitamin D has pivotal actions in host defense that are relevant in the context of chronic inflammatory lung diseases, in which vitamin D deficiency may be prevalent. Strategies to promote local levels of $1,25(OH)_2D$ or use it as a treatment itself could be therefore of interest. Here, we will discuss the latest clinical evidence accompanied with functional *in vitro* and animal studies that may explain the effects of vitamin D supplementation on typical hallmarks of chronic airway diseases.

Effect of Vitamin D on Inhaled Corticosteroid Responsiveness in Chronic Airway Diseases

Currently, inhaled corticosteroid (ICS)-use with or without long acting bronchodilators is the most frequently used treatment for COPD and asthma patients[1]. However, the response to corticosteroids is not always effective in many COPD patients and in patients with steroid resistant (SR)-asthma (129). There are several complex mechanisms that underlie the resistance to corticosteroids in both COPD and SR-asthma that include but are not limited to genetic background, impaired glucocorticoid receptor binding, T helper type 17 cell (Th17)-inflammation and oxidative stress (e.g., from air pollution or smoking) and decreased numbers of IL-10 secreting regulator T cells (Tregs), which normally prevent skewing toward Th17-inflammation (129). Direct evidence of the ability of $1,25(OH)_2D$ to reverse SR was provided by a study showing that *ex-vivo* stimulation with $1,25(OH)_2D$ promoted generation of IL-10–secreting Tregs which restored sensitivity toward corticosteroids in CD4+ T cells that were derived from SR-asthma patients (130). A further potential treatment role of $1,25(OH)_2D$ was elegantly illustrated by studies that showed that vitamin D deficiency is associated with decreased steroid responsiveness in asthmatics and by the fact that several potential underlying mechanisms of SR such as oxidative stress and Th17-mediated inflammatory responses could be reversed by vitamin D treatment (59, 131–136). Interestingly, the corticosteroid dexamethasone was shown to increase expression of the 25(OH)D and $1,25(OH)_2D$ degrading enzyme CYP24A1 in renal cells and osteoblasts (137), which suggests a bidirectional interaction between corticosteroids and $1,25(OH)_2D$ and could further limit $1,25(OH)_2D$ levels for patients. Additional research is needed to determine if vitamin D may also improve corticosteroid responsiveness in COPD.

Vitamin D and Exacerbations in COPD

Exacerbations are a major burden for COPD patients, they accelerate decline in lung function and frequently result into hospital admissions (138, 139). Exacerbations are often triggered by pollutants or by bacterial- and/or viral infections (82, 140, 141). COPD patients generally have lower serum 25(OH)D levels than age- and smoking-matched controls, which is associated with more and more severe exacerbations (8, 10). Several *in vivo* and *in vitro* studies have provided evidence that explain the protective effects of vitamin D on exacerbations in COPD patients and this will be discussed accordingly.

Air Pollution

First of all, Pfeffer et al. showed that 25(OH)D and $1,25(OH)_2D$ reduce the production of proinflammatory cytokines in part via the ability to enhance antioxidant responses in airway epithelial cells that were exposed to PM (59). This was also demonstrated in human DCs that were matured in presence of PM, where treatment with $1,25(OH)_2D$ counteracted the expansion of proinflammatory IL-$17A^+$ and IFN-γ^+ Th17.1 cells (134). In line with this, Bolcas et al., showed that vitamin D supplementation counteracted the development of airway hyperresponsiveness and accumulation of Th2/Th17 cells in mice that had been repeatedly exposed to both diesel exhaust and house dust mite allergens (142). Vitamin D could therefore exert a protective role in air pollution-triggered exacerbations.

Respiratory Viral Infections

In addition to its protective effects against pollutants, there is also increasing evidence that $1,25(OH)_2D$ may enhance clearance of respiratory viral infections that account for 30–50% as underlying cause of exacerbations in COPD patients (143). Infections with respiratory viruses such as HRV, coronaviruses and to a lesser extend respiratory syncytial virus (RSV) and (para)influenza virus are present during exacerbations and may predispose the host toward secondary bacterial infections that can eventually lead to uncontrolled bacterial outgrowth, more severe exacerbations and neutrophilic inflammation (143, 144). Two recent *in vitro* studies showed that acute exposure to relatively high doses (100–1000 nM) of $1,25(OH)_2D$ reduced

[1]https://goldcopd.org (2019).

HRV-infection in undifferentiated cultures of airway epithelial cells (58, 145). In those models, $1,25(OH)_2D$ most likely interfered with viral replication by increasing expression of interferon-stimulated genes and expression of hCAP18/LL-37, which has been shown to have direct antiviral activity (58, 145, 146). In fully differentiated airway epithelial cells, treatment with lower concentrations of $1,25(OH)_2D$ (10 nM) during epithelial differentiation had no effect on acute HRV infection (147). As for other viruses than HRV, both Hansdottir et al. and Telcian et al. showed that $1,25(OH)_2D$ did not decrease RSV infection in airway epithelial cells, but did reduce virus-induced inflammatory responses (58, 116). In addition, two other studies reported in influenza (H9N2 and H1N1)-infected A549 cells comparable findings (148, 149). Moreover, inhibitory effects of $1,25(OH)_2D$ on poly(I:C)-induced inflammatory responses were furthermore confirmed in primary airway epithelial cells Hansdottir et al. and by our group (38, 85). Up to now, the afore mentioned studies suggest that higher doses of $1,25(OH)_2D$ might be protective against HRV-infections in undifferentiated airway epithelial cells only, whereas for other respiratory viral infections $1,25(OH)_2D$ mainly reduces inflammatory responses without affecting viral clearance. However, more studies are needed, especially in differentiated airway epithelial cells using multiple HRV-serotypes that use different receptors for infection to verify if $1,25(OH)_2D$ indeed is capable of promoting HRV-clearance. There is more consensus about $1,25(OH)_2D$ reducing virus-induced inflammatory responses and this may certainly help to alleviate the burden of exacerbations in COPD (38, 85).

Bacterial Infections

In addition to viral infections, also bacterial infections are associated with COPD exacerbations and account for ∼50% of all exacerbations (150). Due to improved study design and sampling techniques from the lower airways using bronchoscopy in recent decades, the causative role of bacteria in COPD-related exacerbations has become clear (150). This was additionally supported by Sethi et al., who found that acquisition of a new strain of pathogenic bacterial species into the airways was linked to COPD exacerbations (151). Recent developments in assessing the airway microbiota using 16S rRNA sequencing techniques further demonstrated that during exacerbations, the relative abundance of Haemophilus, Pseudomonas, and Moraxella was increased and the microbial composition was shifted toward the Proteobacteria phylum (141). The ability of $1,25(OH)_2D$ to promote antibacterial activity was recently demonstrated in cultures of airway epithelial cells. In differentiated airway epithelial cells, we have shown that both 25(OH)D and $1,25(OH)_2D$ treatment enhances epithelial expression of hCAP18/LL-37 and antibacterial activity against NTHi, a Gram-negative bacterium, which is associated with COPD exacerbations (61, 152). In addition, Yim et al. demonstrated that $1,25(OH)_2D$ treatment increased expression of the AMP hCAP18/LL-37 and killing of *Pseudomonas aeruginosa* and *Bordetella bronchiseptica*, which are both Gram-negative bacteria (153). These observed antibacterial effects of $1,25(OH)_2D$ on airway epithelium *in vitro* were recently confirmed *in vivo* by Vargas Buonfiglio et al. The authors demonstrated that vitamin D supplementation increased antimicrobial activity against the Gram-positive *Staphylococcus aureus* in ASL in healthy non-smokers and was dependent on presence of hCAP18/LL-37 (64).

In murine airways, studies showed no effects of $1,25(OH)_2D$ on the expression of *Defb4* or *mCramp* (the murine homolog for *CAMP*) (154). This can be explained by the fact that both the promotors of *mCramp* and *Defb4* lack VDREs, suggesting that mice might not be suitable for studying the role of $1,25(OH)_2D$ in AMP-mediated host defense in infection (155). Indeed, Niederstrasser et al. showed no effects of vitamin D deficiency on the susceptibility of mice to pulmonary infection with *Streptococcus pneumoniae* or *Pseudomonas aeruginosa* (156). However, in a recently developed mouse model by Lowry et al., who transfected *mCramp* knockout mice with the human *CAMP* gene, topical vitamin D_3 treatment increased expression of *CAMP* and promoted antibacterial effects on the mucosa of the skin (157). There are also multiple other murine studies that demonstrate protective effects of vitamin D on bacterial infections in the gut, indicating that $1,25(OH)_2D$ - mediated antibacterial effects are additional modulated by other mechanisms such as via enhancement of epithelial barrier integrity (67, 158). In conclusion, these observations show that $1,25(OH)_2D$ promotes protection against pollutants and enhances clearance of viral– and bacterial infections (both Gram-positive and negative bacteria) in combination with a dampening effect on exaggerated immune responses and these features might explain why vitamin D (deficiency) is linked to COPD exacerbations.

Modulation of Microbiota by Vitamin D

There are strong indications that modulation of immune responses and antibacterial activities by $1,25(OH)_2D$ and/or $1,25(OH)_2D$-regulated AMPs as well as autophagy have implications for the composition of the microbiota at the epithelial mucosa of the airways and the gut (159). Evidence for a role of AMPs in regulating the composition of the microbiota in the gut came from a variety of studies, including those showing that Paneth cell-derived defensins may modulate the composition of the microbiome (160). This notion is further supported by observations showing that many commensal gut bacteria are protected from killing by AMPs such as the $1,25(OH)_2D$-inducible hCAP18/LL-37 and hBD-2, whereas pathogens are in general more sensitive (161). Alterations in the gut microbiota have been linked to many diseases of the gut such as IBD but also with diseases affecting the lungs such as COPD and asthma, implicating an important role for the so-called gut–lung axis (162, 163). The mechanisms that explain how gut microbiota affect lung health and disease are complex and include the production of short chain fatty acids (SCFAs). SCFA have a wide range of effects on both immune and structural cells, and the effect of SCFA produced in the intestine on lung immunity may in part be explained by modulation of myeloid cells in the bone marrow, which subsequently migrate to the airways and modulate local immune responses (163). Microbiota that are diverse, rich and contain a higher abundance of SCFA-producing species within these populations are considered to be

associated with health (164). In the gut there is strong evidence that both vitamin D deficiency and/or supplementation affect composition of the adult and infant microbiota (164, 165), specifically in relation to disease (166). However, due to the limited number of RCTs and small sample sizes, the precise effects on the microbiota and the mechanisms involved in this are still unclear (164). Alterations in the lung microbiota are also observed in COPD and asthma patients and are likely the result of environmental exposures, airway remodeling, infections and treatments such as the use of antibiotics. This may contribute to disease pathogenesis through altered epithelial innate and adaptive immune responses that damages the airway epithelial barrier and provokes further changes in the lung microbiome that accumulates with increasing disease severity (167, 168). To date only 2 studies describe a possible influence of vitamin D on composition of the microbiota in the airways (169, 170). Toivonen et al. showed an association between low serum 25(OH)D levels and reduced richness of the nasopharyngeal microbiota and bronchiolitis severity in patients with low 25(OH)D levels (169), whereas in another study vitamin D supplementation decreased the abundance of *Staphylococcus aureus*, *Staphylococcus epidermidis* and Corynebacterium species in sputum samples in vitamin D-deficient CF patients compared to sufficient CF patients (170). In summary, there is evidence that alterations in the airway or gut microbiota can affect chronic airway disease and that these changes could be related to both vitamin D deficiency and/or supplementation. However, due to the limited number of RCTs and small sample sizes more RCTs are needed in larger patient populations.

Effect of Vitamin D Supplementation on Chronic Airway Diseases

COPD

The above described protective and therapeutic possibilities of vitamin D, together with observations that many COPD patients are vitamin D deficient, suggest that COPD patients might benefit from vitamin D supplementation. As discussed elsewhere in this review, the link between circulating 25(OH)D levels and the number of exacerbations has been extensively studied (8). So far however, only 4 RCTs have investigated the effect of vitamin D supplementation in the context of COPD: only 2 out of 4 RCTs showed that vitamin D supplementation reduces the number of exacerbations (171–174). However, in a *post-hoc* analysis, selecting those patients that were vitamin D deficient, exacerbations were indeed reduced after vitamin D supplementation. Jolliffe et al. summarized these 4 RCTs and performed a recent individual participant data meta-analysis and concluded that vitamin D supplementation is only protective against exacerbations in COPD patients with baseline serum 25(OH)D levels < 25 nmol/L (175). These important findings suggest that exacerbations in this specific subset of COPD patients are connected to vitamin D deficiency and this part can be resolved with supplementation. In summary, the protective effects of vitamin D in patients suffering from COPD are most prominent in those with vitamin D deficiency and this would indicate that serum levels 25(OH)D in these patients should always be determined before considering using vitamin D supplementation. Since only 4 RCTs with relatively small patient populations have been conducted in both vitamin D-sufficient and -deficient COPD patients, more RCTs are needed, especially in vitamin D-deficient patients. Currently, a multicenter RCT is being conducted by Rafiq et al. in a group of vitamin-deficient COPD patients (25(OH)D < 50 nmol/L), which may reveal whether vitamin D supplementation is indeed protective against exacerbations in this group (176).

Vitamin D Supplementation in Asthma, Cystic Fibrosis and Acute Respiratory Tract Infections

In addition to the effects of vitamin D supplementation in COPD patients, the effects of vitamin D supplementation has also been extensively investigated in other lung diseases (which have associations with vitamin D deficiency) such as asthma, cystic fibrosis, upper respiratory tract infections. Most RCTs that investigated the effects of vitamin D supplementation were performed in acute respiratory tract infections (ARTIs) and asthma. A recent meta-analysis that assessed the effects of vitamin D supplementation in 25 RCTs (11,321 participants) showed that indeed vitamin D supplementation was protective against ATRIs and this effect was again more profound in patients with vitamin D deficiency 25(OH)D < 25 nmol/L at baseline (177). A recent meta-analysis in asthma that included a total of 14 randomized controlled trials (1,421 participants), indicated that vitamin D supplementation reduced the rate of asthma exacerbations and increased lung function, especially in patients with vitamin D insufficiency (25(OH)D < 75 nmol/L) (178). Interestingly, in asthma patients that were supplemented with vitamin D, the frequency of respiratory infections was reduced, and this effect was related to the increase of hCAP18/LL-37 (179). CF patients with vitamin D deficiency had a higher rate of exacerbations as compared to patients with sufficient 25(OH)D levels (180). However, only one recent multicenter RCT was conducted and indicated that vitamin D supplementation did not affect the number of exacerbations in CF patients with serum 25(OH)D concentrations between 25 and 137.5 nmol/L (181). In summary, the protective effects of vitamin D supplementation in patients suffering from COPD, asthma or ARTIs are most prominent in those with vitamin D deficiency and this would indicate the importance of establishing serum levels 25(OH)D in these patients as supplementation could reduce unnecessary aggravated disease pathology as a result of this deficiency.

CONCLUSION AND PERSPECTIVES

Many drivers of COPD pathogenesis such as chronic exposure to noxious particles and gases, which are present in CS and air pollution, proteolytic enzymes, cytokines and chemokines that are released by infiltrating inflammatory cells, are known to harm the epithelial barrier and cause aberrant remodeling of the airway epithelium with important functional consequences for e.g., host defense. A dysfunctional epithelial barrier increases the susceptibility toward bacterial and viral infections, which are important triggers of COPD exacerbations and these exacerbations contribute importantly to disease

progression. Sufficient local levels of $1,25(OH)_2D$ may provide partial protection against these effects by reducing the effects of oxidative stress induced by exposure to inhaled oxidants or those derived from recruited inflammatory cells. $1,25(OH)_2D$ furthermore protects against impairment of epithelial barrier function by promoting the integrity of the epithelial barrier, and by modulating both innate and adaptive immune responses. Protection against the detrimental effects of both bacterial and viral infections is provided by the ability of $1,25(OH)_2D$ to promote of antiviral responses, induce expression of AMPs and modulate of inflammatory responses. Taken together, these activities suggest that $1,25(OH)_2D$ may provide protection against development and progression of COPD, and against disease exacerbations.

In addition, the local inflammatory milieu as well as the chronic exposure to noxious particles and gases, which are present in CS and air pollution, may negatively affect synthesis and signaling of $1,25(OH)_2D$. Here we discussed recent *in vitro* studies that demonstrated that disease-associated factors such as inflammation and exposure to CS and air pollution could interfere with $1,25(OH)_2D$ signaling and its degradation and activation by affecting expression of VDR, CYP24A1 and CYP27B1, respectively. These findings indicate that $1,25(OH)_2D$ levels and its activities on airway mucosa might be impaired especially in patients with COPD with exposures to cigarette smoke and cytokines such as TNF-α, IL-1β, IL-17A and TGF-β1. This suggests that even in patients with sufficient 25(OH)D serum levels the local activity of $1,25(OH)_2D$ in the lungs can be improved. We have to start generating more information on both systemic and local $1,25(OH)_2D$ levels and gene expression signatures related to 25(OH)D and $1,25(OH)_2D$ metabolism or responses in COPD (and other chronic inflammatory diseases that are related to vitamin D deficiency), both at baseline and after vitamin D supplementation. This information could lead to improved treatment strategies that enhance local efficacy of $1,25(OH)_2D$, using e.g., specific CYP24A1-inhibitors such as VID400 (182). Alternatively, degradation by CYP24A1 could be prevented by using $1,25(OH)_2D$ analogs that are insensitive to CYP24A1-mediated degradation, such as sulfone and sulfoximine derivatives, that also act as a VDR agonist (183). A third option is to entail the use of combination treatment with vitamin D and anti-inflammatory or certain anti-fibrotic drugs that target cytokines/proteins that are known to potentially decrease local levels and signaling of $1,25(OH)_2D$ by inducing expression of CYP24A1 (48, 184, 185). When considering such strategies, it should be noted that these may enhance the calcemic side effects and lead to unwanted inhibition of the immune system. We therefore need to carefully analyze the preclinical *in vivo* and *in vitro* studies and balance the pros and cons of the different strategies. In conclusion, future studies in COPD and but also in other chronic inflammatory diseases that are related to vitamin D deficiency, should be designed with more focus on assessing and improving local levels of $1,25(OH)_2D$. These new insights may lead to the development of new treatment strategies, such as those targeting CYP24A1 to enhance local $1,25(OH)_2D$ resulting in improved homeostasis and protection of the airway mucosa in patients with chronic inflammatory lung diseases.

AUTHOR CONTRIBUTIONS

JS, AD, and PH: Conception and design. JS: Analyzing literature and drafting the manuscript. AD and PH: Revision of the manuscript. All authors: reviewed the manuscript and agree with its submission.

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Rhinovirus Infection Drives Complex Host Airway Molecular Responses in Children With Cystic Fibrosis

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Early-life viral infections are responsible for pulmonary exacerbations that can contribute to disease progression in young children with cystic fibrosis (CF). The most common respiratory viruses detected in the CF airway are human rhinoviruses (RV), and augmented airway inflammation in CF has been attributed to dysregulated airway epithelial responses although evidence has been conflicting. Here, we exposed airway epithelial cells from children with and without CF to RV *in vitro*. Using RNA-Seq, we profiled the transcriptomic differences of CF and non-CF airway epithelial cells at baseline and in response to RV. There were only modest differences between CF and non-CF cells at baseline. In response to RV, there were 1,442 and 896 differentially expressed genes in CF and non-CF airway epithelial cells, respectively. The core antiviral responses in CF and non-CF airway epithelial cells were mediated through interferon signaling although type 1 and 3 interferon signaling, when measured, were reduced in CF airway epithelial cells following viral challenge consistent with previous reports. The transcriptional responses in CF airway epithelial cells were more complex than in non-CF airway epithelial cells with diverse over-represented biological pathways, such as cytokine signaling and metabolic and biosynthetic pathways. Network analysis highlighted that the differentially expressed genes of CF airway epithelial cells' transcriptional responses were highly interconnected and formed a more complex network than observed in non-CF airway epithelial cells. We corroborate observations in fully differentiated air–liquid interface (ALI) cultures, identifying genes involved in IL-1 signaling and mucin glycosylation that are only dysregulated in the

CF airway epithelial response to RV infection. These data provide novel insights into the CF airway epithelial cells' responses to RV infection and highlight potential pathways that could be targeted to improve antiviral and anti-inflammatory responses in CF.

Keywords: cystic fibrosis, RV, airway epithelial cells, transcriptomic, innate immune response

INTRODUCTION

Lung disease is the major cause of morbidity and mortality in cystic fibrosis (CF) (1). Progressive lung damage is associated with mucus obstruction, neutrophilic inflammation, and chronic airway infection and is already evident in the first years of life (2–6). Intermittent pulmonary exacerbations occur in individuals with CF who experience increased respiratory symptoms and reduction in pulmonary function that are responsive to therapy with antibiotics (7). Moreover, the frequency of exacerbations is a predictor of long-term morbidity and irreversible loss of lung function (8, 9). The triggers for these pulmonary exacerbations are not fully understood although it is recognized that lower respiratory infections caused by viruses are likely to play a significant role (10–14).

The most common virus detected in the airway of adults and children with CF is human rhinovirus (RV) (15–19). The clinical impact of RV includes reduction of lung function/FEV_1 (15, 20, 21), hospitalization (22), and increased requirement for intravenous antibiotic treatment (11, 14). Recent longitudinal data suggest that RV infection persists for a longer period in individuals with CF compared to non-CF controls (14), a finding consistent with *in vitro* observations that suggest a defective innate response of epithelial cells to RV (23, 24). The nature of any intrinsic deficiency still remains unclear although some explanations are now emerging (25).

In this study, we hypothesized that the antiviral responses of primary airway epithelial cells (AEC) from children with CF are dysregulated following RV infection. We utilized transcriptome sequencing (RNA-Seq) to assess the gene expression of CF (ΔPhe508del homozygous) and non-CF primary AEC pre- and post-RV infection. Differential expression analysis was carried out to compare the antiviral responses between CF and non-CF AEC. Functional analyses identified diverse biological pathways and complex networks in response to RV infection in CF AEC that were less apparent in non-CF AEC. We performed additional work to validate some of these unique biological pathways using primary differentiated AEC culture models, and data corroborates observations made from the RNA-Seq analysis. Overall, this study provides insights into the global transcriptomic response by non-CF and CF AEC to RV infection and has identified potential therapeutic targets that could reduce the harmful contribution of RV to progressive lung disease in individuals with CF.

MATERIALS AND METHODS

Patient Recruitment and Establishment of Primary Bronchial Epithelial Cells

The study was approved by the St. John of Gods Human Ethics Committee (SJOG#901) and Perth Children's Hospital Ethics Committee (#1762), and written informed consent was obtained from parents or guardians. Children without CF were recruited prior to undergoing elective surgery for non-respiratory-related conditions. Children with CF and homozygous for the Phe508del mutation were recruited during annual early surveillance visits (2, 3, 23). Subject demographic data for RNA-Seq analysis are provided in **Table 1**. Samples were obtained by brushing of the tracheal mucosa of children using a cytology brush as previously described (23, 26). Submerged monolayer primary airway epithelial (AEC) cultures from non-CF children and those with CF were then established, expanded in Bronchial Epithelial Basal Medium (BEBM®; LONZA™), supplemented with growth additives and 2% (v/v) Ultroser G (Pall Corporation) (23, 26–28), and used for experimentation. Subject demographic data for the validation experiments are provided in **Table 2**. Here, primary AECs were differentiated into ciliated pseudostratified AECs as described previously (29). Briefly, AECs were initially seeded on 0.4-μm polyester membrane culture inserts grown to confluence (Corning, NY, USA) and ALI cultures established. These were maintained for 28 days, and both beating cilia and mucus production were well-established. Prior to ALI validation experiments, inserts were confirmed to have a transepithelial electrical resistance (TEER) measurement >800 Ω/cm^2.

Human RV Infection and RNA Extraction

To emulate an acute RV infection episode *in vitro*, we exposed AEC with RV1b (courtesy of P. Wark, University of Newcastle) at MOI 12.5 (23, 30, 31). After 24 h, culture supernatant was collected for cytokine measurement and cell pellets for RNA extraction. RNA was extracted using a PureLink® RNA (Life Technologies) mini kit as per manufacturer instructions. Total RNA was eluted with 30 μL RNase free water with the addition

TABLE 1 | Patient demographic for subjects used for RNA sequencing analysis including five non-CF children and seven children with CF.

	Non-CF control[#]	Cystic fibrosis
Number of participants	5	7
Mean Age ± sd (yr)	3.5 ± 1.4	2.8 ± 2.3
Age range (yrs)	(1.7–5.4)	(0.2–5.6)
Male (%)	40	57
Genotype	Healthy non-CF	p. Phe508del/ p. Phe508del
NE Activity (%)	NA	43
IL-8 Detected in BALs (%)	NA	100
Microorganisms detected in BALf (%)	NA	14 (*Pseudomonas aeruginosa*)
PRAGMA Disease (%)	NA	3.44(2.24–4.16)

[#]*Non-CF control were children who underwent elective surgery for non-respiratory-related conditions.*

TABLE 2 | Patient demographics for subjects used for validation work including six non-CF children and six children with CF.

	Non-CF control[#]	Cystic fibrosis
Number of participants	6	6
Mean Age ± sd (yr)	3.3 ± 0.65	2.3 ± 2.3
Age range (yrs)	(2.4-4.0)	(0.2–5.9)
Male (%)	50	83
Genotype	Healthy non-CF	p. Phe508del/ p. Phe508del
NE Activity (%)	NA	50
IL-8 Detected in BALs (%)	NA	100
Microorganisms detected in BALf (%)	NA	50
PRAGMA Disease (%)	NA	3.53

[#]*Non-CF control were children who underwent elective surgery for non-respiratory related conditions.*

of 1 μL of RNase Inhibitor (Life Technologies). RNA purity and yield were determined using a NanoDrop, and integrity was assessed using an Agilent RNA 6000 Nanochip on an Agilent Bioanalyser.

RNA Sequencing (RNA-Seq) and Analysis

Samples identified with high purity (1.8–2.0 range A260/280) and quality (RIN > 8.0) were then processed for library preparation. Here, the KAPA Stranded mRNA-Seq kit (KAPABiosystems) was used for mRNA capture and fragmentation (∼200–300 bp fragments). RNA fragments were then subsequently reverse transcribed into cDNA strands, followed by adapter ligation and library amplification. Sequencing of these libraries (100 bp, single-end) was performed on the Illumina HiSeq 2500 platform at an average depth of 5.08 ± 1.17 million reads (**Figure S1A**) per. The quality and quantity of the FASTQ sequence reads were assessed using FastQC (v0.11.3) (32), followed by mapping to the reference genome (Homo sapiens hg19/GRCh37 – Ensembl) using "hisat" (v0.1.6-beta) (33). Gene-level quantification (counts) of hisat alignments was performed using SummarizeOverlaps and, finally, post-alignment QC using Samstat (v1.5.2.) (34). Mapping rates to the human genome were within the expected rate for all samples at 88.2–91.2% (35), and post-alignment quality control using SAMStat 1.5.2 reported an average high quality (mapping quality score of thirty) mapping rate of 89.98% ± 0.67 (**Figure S1B**).

Bioinformatics and Statistical Analysis

Bioinformatics and statistical analyses were performed on five non-CF and seven CF samples. Statistical analysis was conducted in PRISM 8 (v8.1.2; GraphPad Software Inc., California, USA) and included the Mann–Whitney test to compare the statistical variance between genotype, and the Wilcoxon test was used to compare the statistical difference between paired samples. All subsequent bioinformatic analyses post-alignment were performed in R (v3.4.1) (36). To remove low-abundance genes, only those that had a minimum of 10 counts per sample in at least five or more samples were included, resulting in a total of 12,757 genes analyzed. The R package RUVseq (1.10.0) (37) was applied to normalize RNA-Seq read counts between samples to remove the unwanted variance. Differential gene expression was determined using DESeq2(v1.16.1) (38) after calculating variance-stabilizing transformation (VST) from the fitted dispersion mean relation to yield count data with constant variance along the range of mean values. We determined those genes with an adjusted p-value ≤ 0.05 and ± 1.5-fold change as statistically and biologically significant, respectively. To visualize the variance between samples, a principal component analysis plot was generated using the plotPCA function in DESeq2 and visualized using ggplot2 (v3.1.0) (39). Next, we identified non-infected baseline non-CF and CF enriched gene ontology (GO) terms from the biological process (BP) using Metascape (http://metascape.org) (40). Visualization of GO term analysis was performed using the GOPlot (v1.0.2) (41). The GoCircle function was used to highlight gene expression changes within each of the selected terms. The value of the z-score from GOPlot is calculated as $\text{zscore} = (\text{up - down}) \div \sqrt{\text{count}}$, where up and down were the number of up- and down-regulated genes respectively.

Pathway Analysis and Protein–Protein Interaction Network-Based Enrichment Analysis

Pathway analysis based upon Reactome repositories was performed using Signature Over-Representation Analysis (SIGORA) version 2.0.1. The pathway enrichment by SIGORA was identified according to statistically over-represented Pathway Gene-Pair Signatures (Pathway-GPS) (42). To expose the interactive associations among the DEGs at the protein level, genes obtained from both non-CF and CF responses were mapped using protein–protein interactions (PPI) via NetworkAnalyst (http://www.networkanalyst.ca/). Network Analyst (43, 44) and was based upon IMEX Interactome, a comprehensive, high-quality protein–protein interaction database curated from InnateDB (45) to characterize the relationships and interactions of input genes. The network was built by limiting the original seed proteins only and picking zero order interactions.

ELISA

Cytokine production of interleukin 8 (IL-8) (Becton Dickinson, Biosciences, San Diego, CA), interferon lambda 1, 2, 3 (IFNλ1, λ2, λ3), RANTES (CCL5), interleukin (IL)-1B, Interferon gamma-induced protein 10 (IP-10) (R&D, MN, Minneapolis) in culture supernatant was measured by ELISA. Production of interleukin 6 IL-6 was measured using a time-resolved fluorometry detection system (PerkinElmer, Waltham, MA). Expression of interferon beta (IFNβ) was measured using an AlphaLISA® bead-based assay (PerkinElmer).

Corroboration of RNA-Seq Observations in Fully Differentiated Cultures

Experiments were then performed to assess whether unique pathways identified from the initial RNA-Seq analysis were evident in fully differentiated 3-D cultures. Primary ALI cultures were established and, upon TEER confirmation, were rinsed three times with sterile room temperature 1× phosphate-buffered saline for 10 min at 37°C. Cultures were then subsequently infected with RV1b at MOI 0.1 in 200 μL for 24 h. An MOI

of 0.1 was chosen based on the lowest infection dose from the literature with no reported cytopathic effects or major disruption to the epithelium (46–48) as this prevents assessment of mucin-related enzymes and other downstream analyses. After 24 h, inserts were harvested in RNA lysis buffer for RNA extraction using the PureLink® RNA (Life Technologies) mini kit as per manufacturer instructions. Total RNA was eluted with 30 μL RNase free water. Genes were chosen from at least two independent pathways identified to be uniquely expressed by AEC in children with CF in response to RV infection and included *IL1R2*, *STS8SIA4*, *ST6GALNAC2*, *MAN1A1*, and *B3GNT8*. Gene expression was determined via real-time qRT-PCR (refer to **Supplementary Materials** and Methods 1.1 and 1.2) using TaqMan® pre-designed primer/probes (ThermoFisher Scientific). Details on all primer probes are listed in **Table S8**.

RNA sequencing was performed on all samples as summarized in the workflow diagram (**Figure 1A**). A sample elimination process was carried out to exclude unqualified samples (detailed in **Figure S3**). Samples that were run on a different sequencer did not pass rigorous quality control for RNA sequencing (mapping quality score >30, $n = 3$), and those with sequencing depth of less than one million reads ($n = 9$) were excluded from analysis. Finally, RNA sequencing samples from a total of seven CF and five non-CF children were included for the differential expression analysis by applying a fold change cutoff of ≥1.5-fold. Only one child with CF had detectable microorganisms in bronchioalveolar lavage fluid (BALf) during the time of AEC sampling. The PRAGMA CT score presented as percentage of disease was also conducted to demonstrate the quantitative measurement of disease progression during the time of sampling in children with CF.

RESULTS

Patient Demographics

The demographic information of children participating in this study is shown in **Table 1** (RNA sequencing) and **Table S1** (entire sample sets, including those additional samples used for ELISA). Non-CF controls were children who underwent elective surgery for non-respiratory-related conditions and did not possess existing lung disease. RNA samples of primary AECs obtained from these children ($n = 32$, 16 non-CF and 16 CF) were originally collected, both pre- and post-infection with RV *in vitro*.

Distinct Transcriptional Changes of AEC in Response to RV Infection

The normalized read counts matrix was used to build a non-supervised principal component analysis to visualize the major contributors to transcriptional variation within this data set (**Figure 1B**). The first principal component (PC1, 69% of the variance) completely separated RV-infected and non-infected AEC, and separation of uninfected or infected CF and non-CF AEC was observed on PC2 (8% of the variance), indicating that

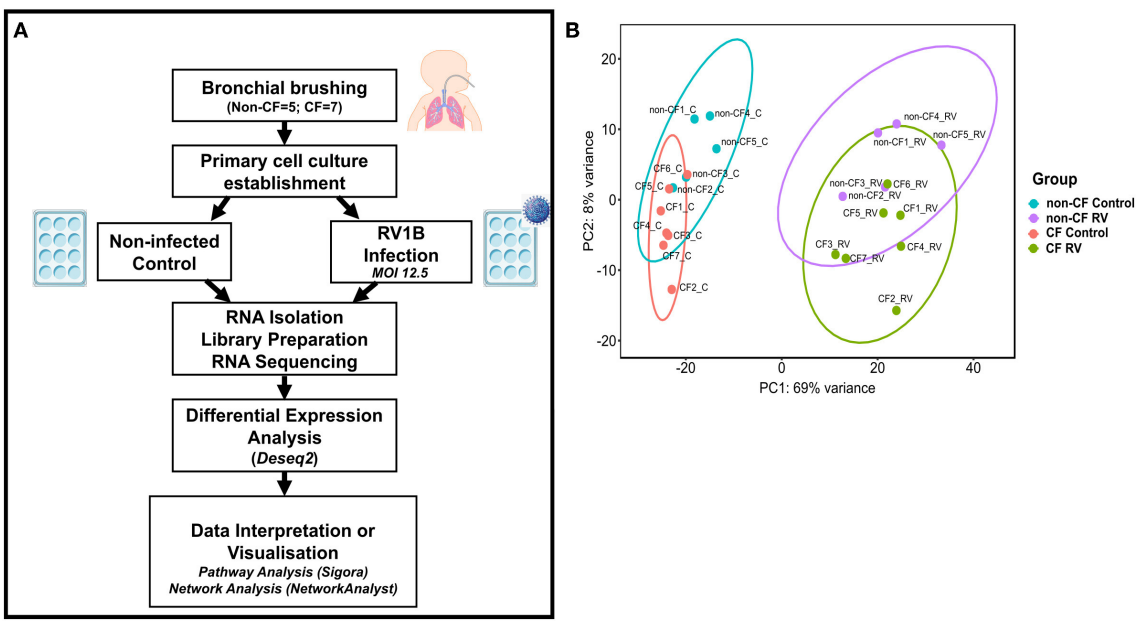

FIGURE 1 | Analytical methods. **(A)** Schematic workflow describing experimental procedure and transcriptomic analysis. Primary airway epithelial cells (AECs) from non-CF children ($n = 5$) and children with CF ($n = 7$) were obtained from bronchial brushings. AECs were established for infection with human rhinovirus 1B (RV1B) at MOI 12.5. At 24 h post-infection, RNA was isolated and processed for library preparation. Sample libraries were sequenced on the Illumina HiSeq 2500 platform as described in the methods section **(B)** Principal component analysis (PCA). Components 1 (PC1) and 2 (PC2) highlight distinct clustering of samples. PC1 shows the highest percentage of variance (69%) for all samples and completely separates the control and RV-infected samples. PC2 shows the second highest variance (8%) and separates non-CF and CF samples. Data points represent individual samples for non-CF controls (turquoise), non-CF infected (purple), CF control (coral), and CF infected (green).

patient genotype is the second largest source of variation within the data set.

Modest Transcriptional Differences Between Uninfected CF and Non-CF AEC

To determine whether the AECs transcriptional profiles from children with CF are intrinsically differed from non-CF controls, non-infected baseline CF and non-CF AECs were analyzed for differential gene expression. We observed a total of 162 DEGs with absolute fold change $\geq$1.5 between non-infected baseline CF and non-CF AECs. Among those, 92 genes were significantly downregulated, and 70 genes were significantly upregulated in CF AEC compared to non-CF AEC. To identify in which biological processes the 162 DEGs were involved, we performed gene ontology (GO) term enrichment analysis (41). The predominant enriched GO term in CF AEC is depicted by a circle plot (**Figure 2A**). The circle plot highlights the overall gene expression change by showing increased expression in red and decreased expression in blue. The *p*-value of the GO terms is represented by the height of the inner rectangle, which is also colored by *z*-score based on GOPlot formula (zscore = ([number of up-regulated genes] − [number of down-regulated genes]) $\div\sqrt{}$[gene count]). Analysis identified the cytokine-mediated signaling pathway and type 1 interferon signaling pathway as the top enriched GO terms with decreased *z*-score and extracellular matrix as the GO term with an increased *z*-score. The full list of the top upregulated and downregulated genes is summarized in **Table S2**. The top DEG from differential expression analysis comparing non-infected baseline CF and non-CF AEC was *HLA-DQB1* (HLA Class II GWAS genes). We also identified the top 20 genes with the highest fold change between non-infected CF and non-CF AEC (**Figure 2B**). These genes were found to be involved in biological processes including type 1 interferon signaling pathway (*AIM2*, *BST2*, *IFI27*), keratin (*KRT14*), DNA methylation (*H19*), cell cycle (*BEX1*), extracellular matrix (*COL1A2*, *COL5A1*, *COL6A1*, *COL6A2*), cell–cell interaction (*LGALS7*), signal transduction (*FST*, *LRCH2*, *LRRN1*), calcium ion binding (*PCDH20*), potassium channel (*KCNJ5*), transferase activity (*NEURL3*), and phosphatase activity (*PTPRZ1*).

CF AEC Have More Transcriptional Changes in Response to RV Infection Than Non-CF AEC

We next analyzed the RNA-Seq data to assess the transcriptomic response of CF and non-CF AECs collected after infection with RV. Comparative analysis of response profiles indicates that AECs from both CF and non-CF differentially modulated the expression of several genes related to the innate antiviral immune response in response to RV infection. The Venn diagram (**Figure 3A**) was used to compare genes that were uniquely and commonly modulated between CF response (RV-infected CF AEC vs. uninfected CF AEC) and non-CF response (RV-infected non-CF AEC vs. uninfected non-CF AEC) to RV infection. A total of 896 (652 upregulated, 244 downregulated) DEGs were observed in the non-CF response to RV and 1442 DEGs (884 upregulated, 558 downregulated) in the CF response (**Figures 3A,B**). Candidate genes were ranked according to their extent of differential expression when compared to uninfected samples. Although there was considerable overlap between the groups (778 common DEGs, **Figure 3C**), there were significantly more unique DEGs (**Figures 3D,E**) specific to the CF response (664) compared with the non-CF response (118). A majority of overlapping DEGs were involved in the core immune response to RV infection, including interferon signaling, interferon regulation, cytokine signaling, cell death, and metabolism. The unique DEGs for both CF and non-CF AEC in response to RV infection are summarized (**Tables S3**, **S4**, respectively). The top unique DEG for the non-CF response was *CX3CL1*, which is an important chemoattractant to attract other immune cells, such as dendritic cells. Other top unique genes for the non-CF response were found to be associated with the cellular component *(FAXDC2*, *ARMCX4*, *RAB17*, *TMEM17*), DNA repair (*BRCA2*, *RMI2*), and cellular metabolism (*CBR3*, *B4GALNT3*, *HS3ST3B1*, *GIPR*). Nevertheless, 46% (664 out of 1442) of DEGs for the CF AEC response to RV infection were found to be unique with the *IL-1R2* gene, the IL-1 signaling decoy receptor, being the top unique DEG (4.8-fold change). Other unique genes for the CF AEC response were found to be associated with growth factor (*PTN*), immune response (*NOD2*, *CCRL2*, *HMOX1*, *SLC7A2*, *SERPINB4*), cellular metabolism *(MDGA1*, *ANGPT1*), cytoskeletal regulation (*LRCH2*), signal transduction (*MAPK8IP2*, *STK32A*), and transcription regulation (*SPDEF*, *ZNF488*).

RV Infection Drives Common Epithelium-Induced Innate Antiviral Response in CF and Non-CF AEC

Genes that were commonly modulated in CF and non-CF AECs (**Table S5**) were found to be key drivers of core epithelium-induced innate antiviral response to RV infection. Specifically, RV infection triggered a significant upregulation of type I and III interferons (*IFNB1*, *IFNL1*, *IFNL2*, *IFNL3*) in both CF and non-CF AECs (**Figure 3C**). However, it was evident that the fold changes (Log_2FC) of *IFNB1* (5.8-fold), *IFNL1* (5.8-fold), *IFNL2* (5.1-fold), and *IFNL3* (6.1-fold) in gene expression in response to RV infection were lower in the CF AEC response compared to the non-CF AEC response (*IFNB1*: 6.9-fold, *IFNL1*: 7.2-fold, *IFNL2*: 7-fold, *IFNL3*: 7.5-fold). Interferon signaling also triggered the induction of a variety of interferon-stimulated genes (ISGs), including *Mx1*; viperin (*RSDA2*); and the IFITM, IFIT, and OAS family in both CF and non-CF AECs (**Figure 3C**).

We extended our analysis to identify the biological pathways corresponding to all DEGs in CF and non-CF AECs in response to RV infection. The full list of enriched biological pathways for CF and non-CF AECs' antiviral responses are provided in **Tables S6**, **S7**, respectively. SIGORA pathway analysis was then performed using gene-pair signature pathway analysis, which only accounts for statistically significant gene pairs unique to the over-represented pathways. This analysis identified 52 and 31 biological pathways responsible for CF and non-CF AEC host responses to RV infection, respectively. Comparing the two, we identified 26 common significantly enriched biological

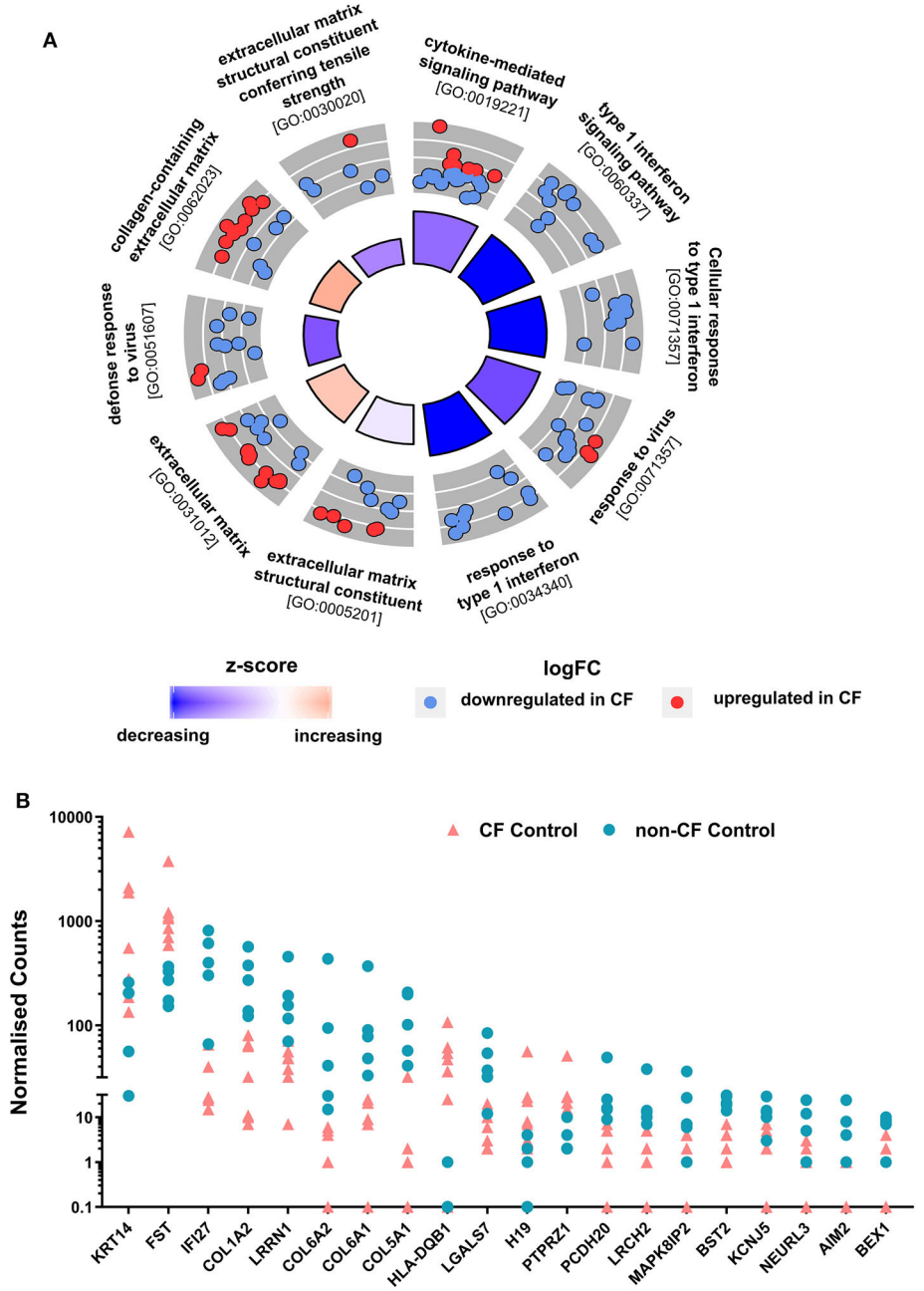

FIGURE 2 | Comparison of non-CF and CF non-infected baseline control. **(A)** Circular visualization of gene-annotation enrichment analysis of non-infected baseline samples. Statistically significant differentially expressed genes (DEGs) between non-infected CF and non-CF samples were annotated using gene ontology (GO). The circular plot combines gene expression and gene-annotation enrichment data. The outer circle shows a scatterplot for each enriched GO term of the Log2FC of the assigned genes. Red dots indicate upregulation, and blue dots indicate downregulation in CF non-infected control compared to non-CF. The inner ring is a bar plot where the height of the bar indicates the significance of GO terms (log10-adjusted *p*-value), and color corresponds to the *z*-score: blue, decreased; red, increased; and white, unchanged. **(B)** Normalized gene counts of the top 20 DEGs between CF and non-CF non-infected baseline samples with the highest fold change, data points represent individual samples for non-CF controls (turquoise; circle) and CF control (coral; triangle).

pathways (**Figure 4**), which are mainly categorized into five main functions, including (1) cytokine signaling in the immune system, (2) presentation to the adaptive immune system, (3) innate immune system, (4) metabolism or biosynthetic, and (5) signal transduction. Consistently, the core antiviral response was demonstrated by type I and III interferon and other antiviral factors as reported earlier with interferon-α/β signaling, interferon-γ signaling, and interferon signaling being the top

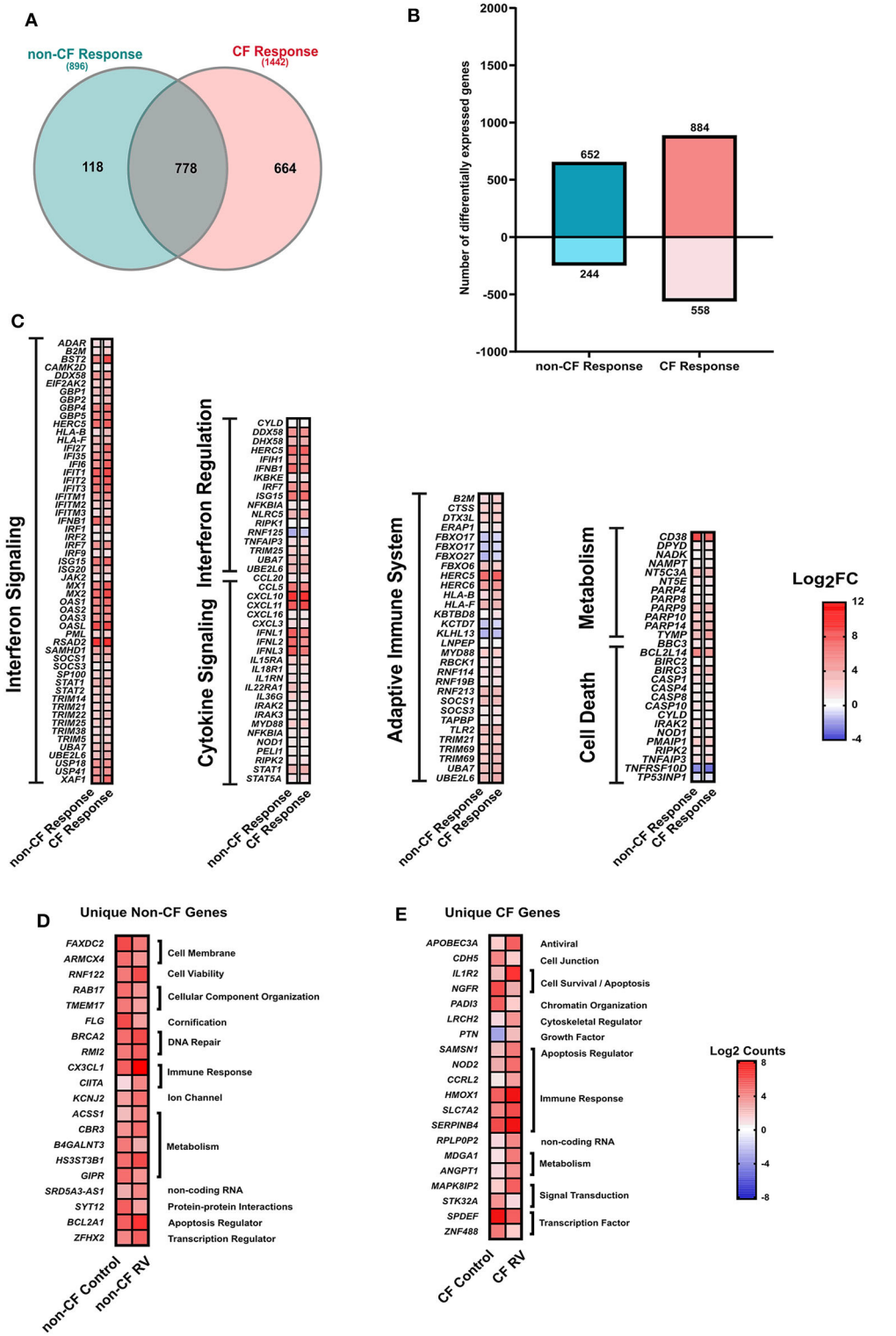

FIGURE 3 | Response to rhinovirus infection. **(A)** Venn diagram comparing the differentially expressed (DEGs) genes between non-CF (teal; *non-CF RV-infected* vs. *non-CF non-infected control*) and CF (pink; *CF RV-infected* vs. *CF non-infected control*) response to RV1B infection. A total of 778 DEGs were common between non-CF and CF response to RV1B infection. **(B)** The number (*y*-axis) and direction of change (upregulated = positive *y*-axis, downregulated = negative *y*-axis) of DEGs ($|Log_2FC|1.5$, adjusted p-value < 0.05) of non-CF and CF response to rhinovirus infection (*x*-axis). **(C)** The relative expression genes that are commonly differentially expressed ($|Log_2FC|>1.5$, adjusted p-value < 0.05) in airway epithelial cells (AECs) from non-CF and CF individuals when infected with rhinovirus. These

(Continued)

FIGURE 3 | genes are associated with immune response, including interferon signaling, cytokine signaling, adaptive immune system, cell death, and metabolism. **(D)** The normalized counts (Log_2Counts) of the top 20 genes that are uniquely differentially in AECs from non-CF children when infected with rhinovirus, including genes associated with cellular component, DNA repsir, immune response, ion channel and activity, cellular metabolism, protein–protein interactions and regulation of apoptotic process. **(E)** The normalized counts (Log_2Counts) of the top 20 genes that are uniquely differentially expressed in AECs from children with CF when infected with rhinovirus, these genes are involved in the apoptotic process, cell–cell junction, chromatin organization, cytoskeletal regulator, growth factor, immune response, cellular metabolism, signal transduction, and transcription regulation.

three most enriched pathways associated with cytokine signaling. Other common cytokine responses, such as interleukin 20 family signaling (**Figure 4A**, **Table S6**), was over-represented with upregulation of *IL22RA1*, STAT family (*STAT1, 2, 3*, and *5A*), JAK family (*JAK1*, *JAK2*), and the negative regulator of IFN signaling *SOCS3*. Infection with RV has also significantly increased gene expression of chemokines such as *CXCL10*, *CXCL11*, *CXCL3*, *CXCL16*, *CCL2*, *CCL5*, and *CCL20* in both CF and non-CF AECs. Additionally, we also detected transcriptional changes in pathogen recognition receptors, such as *TLR3*, *DDX58 (RIG-I)* and *IFIH (MDA5)*, and other key genes that regulate innate immune signaling, including *IKBKE*, *IRF7*, *ISG15*, *NFKBIA*, *UBE2L6*, *UBA7*, and *DDX58*. Genes involved in the over-represented pathway Class I MHC-mediated antigen processing and presentation, such as the gene set of F-box protein, TRIM, and the HERC family were the common DEGs in response to RV infection. The PARP protein family, including *PARP4*, *PARP8*, *PARP9*, *PARP10*, and *PARP14*, responsible for the regulation of nicotinamide metabolism and salvaging of cellular redox reactions, were also upregulated in response to RV infection. The changes of genes involved in nucleotide biosynthesis pathway pyrimidine catabolism were observed, including *NT5C3A*, *DPYD*, *TYMP*, and *NT5E*. Changes in gene expression of caspases (*CASP1*, *4*, *8*, *10*), which provide pivotal links in cell regulatory networks controlling inflammation and cell death, were also observed in both CF and non-CF AEC post-RV infection.

CF AEC Transcriptome Reveals More Biological Pathways and a More Complex Network in Response to RV Infection Than for Non-CF AEC

In addition to the common over-represented pathways induced by RV infection, we observed an additional 26 enriched pathways specific to the CF response (**Figure 4A**). In addition to the five functions mentioned above, the unique over-represented pathways also fall under another two functions, including extracellular matrix organization and vesicle-mediated transport or transport of small molecules. Additional pathways categorized in cytokine signaling in the immune system, such as interleukin 1, 2, 7, 10, and 15 signaling pathways, were the unique enriched pathways specific to CF response. Genes associated with interleukin 1 family signaling–driven proinflammatory activity are *IL36G*; receptor antagonist *IL36RN*; *IL1R2*; *IL1RN*; receptor *IL18R1*; protein phosphatase *PTPN12*; pellino proteins *PELI1*, *PELI3*, and IRAK kinase *IRAK2*, *IRAK3*; and key immune and inflammatory response regulator *S100A12*. Other cytokines with essential immunomodulatory functions, including IL-7, IL-10, IL-15, and IL-2 family signaling, were the significantly over-represented pathways unique for CF response to RV infection. Furthermore, we observed a significant upregulation of the chemotactic factors for neutrophils *CXCL1* and *CXCL2* in the CF AEC response to RV infection. Downregulation of genes encoding E3 ubiquitin ligases, such as *TRIM45* (regulator of TNFα-induced NF-κB-mediated transcriptional activity) and *RNF128* (inhibitor of cytokine gene transcription), were also only observed in the CF response. The transcriptional change of the *HSPA5* gene was also observed in the CF response as part of major histocompatibility complex (MHC) class I molecules mediated adaptive immune regulation.

Several metabolism/biosynthetic pathways of notable interest to CF airway disease include nucleobase catabolism, inositol phosphate metabolism, synthesis of IP3 and IP4 in the cytosol and tryptophan catabolism, which were all altered in CF response to RV infection (**Figure 4A**). We observed transcriptional changes of ectonucleotidases in the nucleobase catabolism pathways, particularly ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases) *ENTPD3* (downregulated) and *ENTPD6* (upregulated). The inositol phosphate metabolism pathway was also found to be altered in CF AECs, namely the downregulation of genes encoding phosphohydrolases *NUDT11*, phospholipase *PLCH2* and *PLCD4*, kinase *ITPKB*, and phosphatase *INPP4B*. We also observed a group of upregulated genes, including *KYNU*, *KMO*, *IDO1*, *AADAT*, and *CCBL1*, which are associated with the key biosynthetic process of tryptophan catabolism. Biological pathways regulating metabolism of proteins, notably mucin metabolism (O-linked Glycosylation of mucins and sialic acid metabolism), were also over-represented pathways for the CF response. Additionally, RV infection in CF AEC triggered transcriptional changes of transport of small molecules (including cellular hexose transport, metal ion SLC transporters, transport of amino acids, and SLC-mediated transmembrane transport). We noted transcriptional changes for genes involved in extracellular matrix organization, such as integrin α5 and β6 (*ITGA5, ITGA6*) and cell adhesion molecule *ICAM1*.

To better understand the potential functional interaction of DEGs, we also visualized expression and investigated the underlying molecular interactions between genes by generating zero-order PPI subnetworks (**Figures S2A,B**). The main CF and non-CF PPI subnetwork consisted of functionally enriched pathways that play imperative roles in the host antiviral response to RV infection. The non-CF AEC response subnetwork identified associations of 254 nodes and 565 edges (**Figure S2A**). We observed 172 genes with a degree more than one interactor, where 27 nodes were observed with ≥10 connections with other nodes. Key hub genes regulating the antiviral response

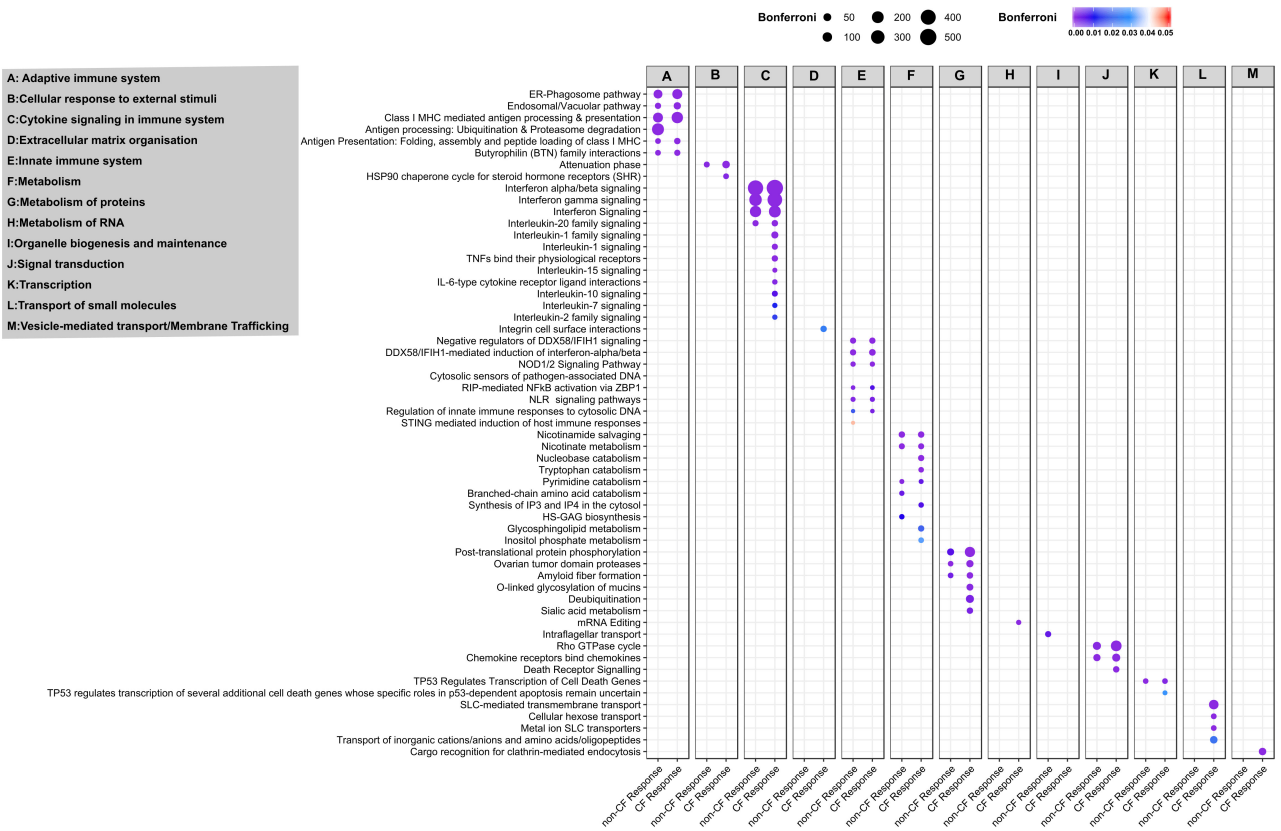

FIGURE 4 | Pathway and network analysis of non-CF and CF AECs response to rhinovirus infection. Pathway enrichment analysis results from 896 and 1,442 differentially expressed genes in non-CF and CF response to rhinovirus infection, respectively. Figure depicting the association of common and unique enriched pathways using Reactome database with the differentially expressed gene lists based on SIGORA successes metric (circle size) and the color bar depicting the significance of the association (Bonferroni < 0.05). The enriched biological pathways are categorized according to functions **(A)**, adaptive immuno system, **(B)** cellular response to external stimuli, **(C)** cytokine signaling in immune system, **(D)** extracellular matric organization, **(E)** innate immune system, **(F)** metabolism, **(G)** metabolism of proteins, **(H)** metabolism of RNA, **(I)** organelle biogenesis and maintenance, **(J)** signal transduction, **(K)** transcription, **(L)** transport of small molecules and **(M)** vesicle-mediated transport/membrane trafficking.

found included *STAT1*, *STAT2*, *IRF2*, *IRF1*, *ISG15*, *DDX58*, *IRF7*, *RIPK1*, *IKBKE*, and *CASP8*. Conversely, a more complex CF AEC response subnetwork projected the associations of 493 nodes and 1156 edges (**Figure S2B**). We observed 320 genes with a degree more than one, where 66 nodes were observed with $\geq$10 connections with other nodes. The key hub genes regulating the CF AEC response subnetwork included *IRF1*, *ISG15*, *STAT1*, *STAT3*, *HSAP1B*, *CASP8*, *TBK1*, *IKBKE*, *TRAF2*, and *CASP8*. The key hub genes of both CF and non-CF subnetworks are represented by key regulators related to the innate immune system and cytokine signaling.

Aberrant Cytokine Production of CF AECs to RV Infection

In order to validate the transcriptional changes of the enriched cytokine signaling pathways, we measured the levels of key innate and inflammatory cytokine production at 24 h post-RV infection (**Figure 5**). Although *IFNB1* was significantly induced upon RV infection in both cohorts, this is not reflected at the protein level with significantly lower levels (average 10.8-fold) of IFNβ1 (type 1 interferon) released by CF AEC (668.3 ± 576.2 pg/ml; $p < 0.05$) compared to non-CF AEC (7,265 ± 6,558 pg/ml). As shown in **Figure 3C**, all type 3 IFN genes (*IFNL1*, *IFNL2*, *IFNL2*) were upregulated post RV infection. Cytokine levels of the type III interferons IFNλ1, IFNλ2, and IFNλ3 were also significantly elevated in both CF and non-CF AEC infected with rhinovirus. However, levels of IFNλ1 (296.4 ± 293.3 pg/ml) and IFNλ2 (334.6 ± 642.8 pg/ml) produced by CF AEC in response to RV infection were significantly (3.5- to 5-fold) lower when compared to non-CF AEC (1,059 ± 1,170 pg/ml and 1,665 ± 1,932 pg/ml, respectively; $p < 0.05$). IFNλ3 produced by CF AEC (285.3 ± 287.3 pg/ml) following RV infection was somewhat but nonsignificantly lower compared to that produced by non-CF subject AEC (928.6 ± 997.9 pg/ml). Similar cytokine levels of antiviral chemokines CCL5 (RANTES) and IP10 and pro-inflammatory cytokines, including IL6 were detected in non-infected CF and non-CF AECs, and similar increases in these proteins occurred in response to RV infection. However, IL-8 and IL-1β cytokine production is significantly elevated in non-CF AECs in response to RV infection compared to CF AECs.

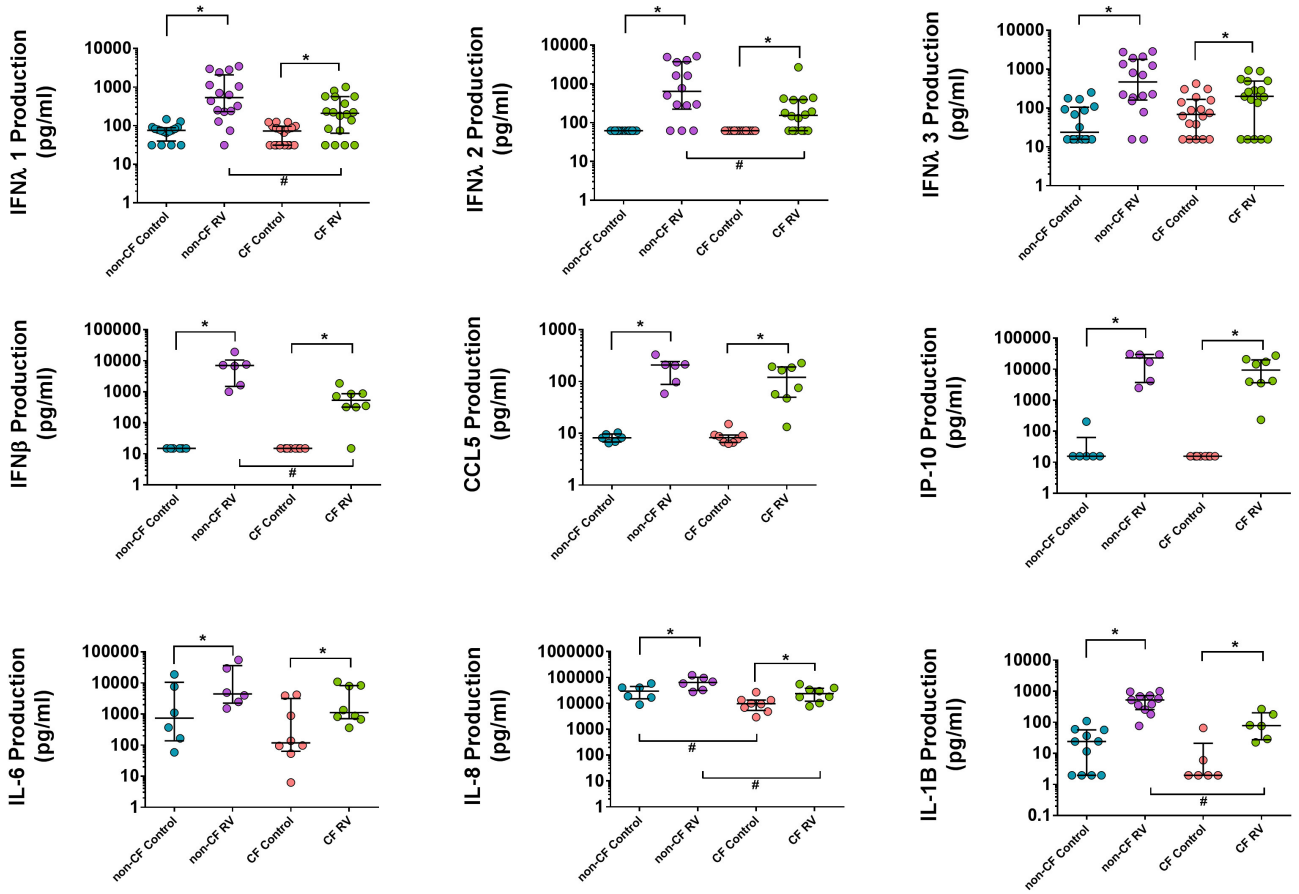

FIGURE 5 | Cytokine production in the supernatant AEC of non-CF individuals and children with CF following RV infection. Cytokine release was measured in cell culture supernatants using commercial ELISA kits and an in-house time-resolved fluorometry detection system. Type 1 and III interferons (IFN-β, IFN-λ1, and IFN-λ2) were significantly higher in non-CF AEC post-RV infection compared to CF AEC. Inflammatory cytokines IL-6, IL-8, and IL-1B were significantly increased in both CF and non-CF RV-infected samples with significantly higher IL-8 and IL-1B levels produced by non-CF RV-infected samples compared to CF RV-infected samples. RANTES (CCL5) and IP-10 were significantly elevated in CF and non-CF RV-infected samples but not significant between phenotype. Note: $n = 9$–11 for non-CF and 6–12 for CF. The data were represented as median ± IQR, symbols show statistical significance in RV-infected samples relative to paired non-infected control samples; $^{*}p < 0.05$, determined using Wilcoxon test. Statistically significant for comparison between CF and non-CF non-infected samples determined using unpaired *t*-test or Mann–Whitney depending on Gaussian distribution, $^{\#}p < 0.05$.

Corroboration of Unique Gene Expression Patterns in Response to RV Infection in CF ALI Cultures

To validate results generated from the RNA-Seq in a model that better represents the airway, we assessed the expression of some unique DEGs identified in submerged CF cultures post- RV infection by challenging ALI cultures with the same RV and again assessing gene expression at 24 h (**Figure 6**). Expression of the top unique DEG for the CF response, *IL1R2*, was validated with a consistent increase in CF ALI post RV infection (9.4-fold over uninfected, $p < 0.05$; **Figure 6**). Upregulation of *IL1R2* appeared bimodal in non-CF ALI and was not significant ($p = 0.30$). Furthermore, expression of genes involved in glycosylation of mucins and sialic acid metabolisms, namely sialyltransferase *ST8SIA4*, *ST6GALNAC2*, mannosidase *MAN1A1*, and acetylglucosaminyltransferase *B3GNT8*, was also validated as unique to CF (**Figure 6**). A significantly higher level of sialyltransferase *ST8SIA4* expression (2.2-fold, $p < 0.05$) was observed in RV-infected CF ALI cultures while *ST6GALNAC2* was significantly downregulated (−1.4-fold, $p < 0.05$). The mannosidase *MAN1A1* were dramatically upregulated by 16.3-fold ($p < 0.05$) in CF ALI at 24 h post RV infection. *B3GNT8*, an acetylglucosaminyltransferase that adds N-acetylglucosamine (GlcNAc) to N-glycans was also increased by 1.9-fold ($p < 0.05$) in CF ALI in response to RV infection. Expressional changes of these genes were all consistent with the RNA-Seq data from submerged cultures. We observed that these genes did not change expression in non-CF ALI cultures upon infection with RV; *ST8SIA4* (3.6-fold, $p = 0.14$); *ST6GALNAC2* (−1.3-fold, $p = 0.09$); *MAN1A1* (5.4-fold; $p = 0.08$); *B3GNT8* (1-fold, $p = 0.28$).

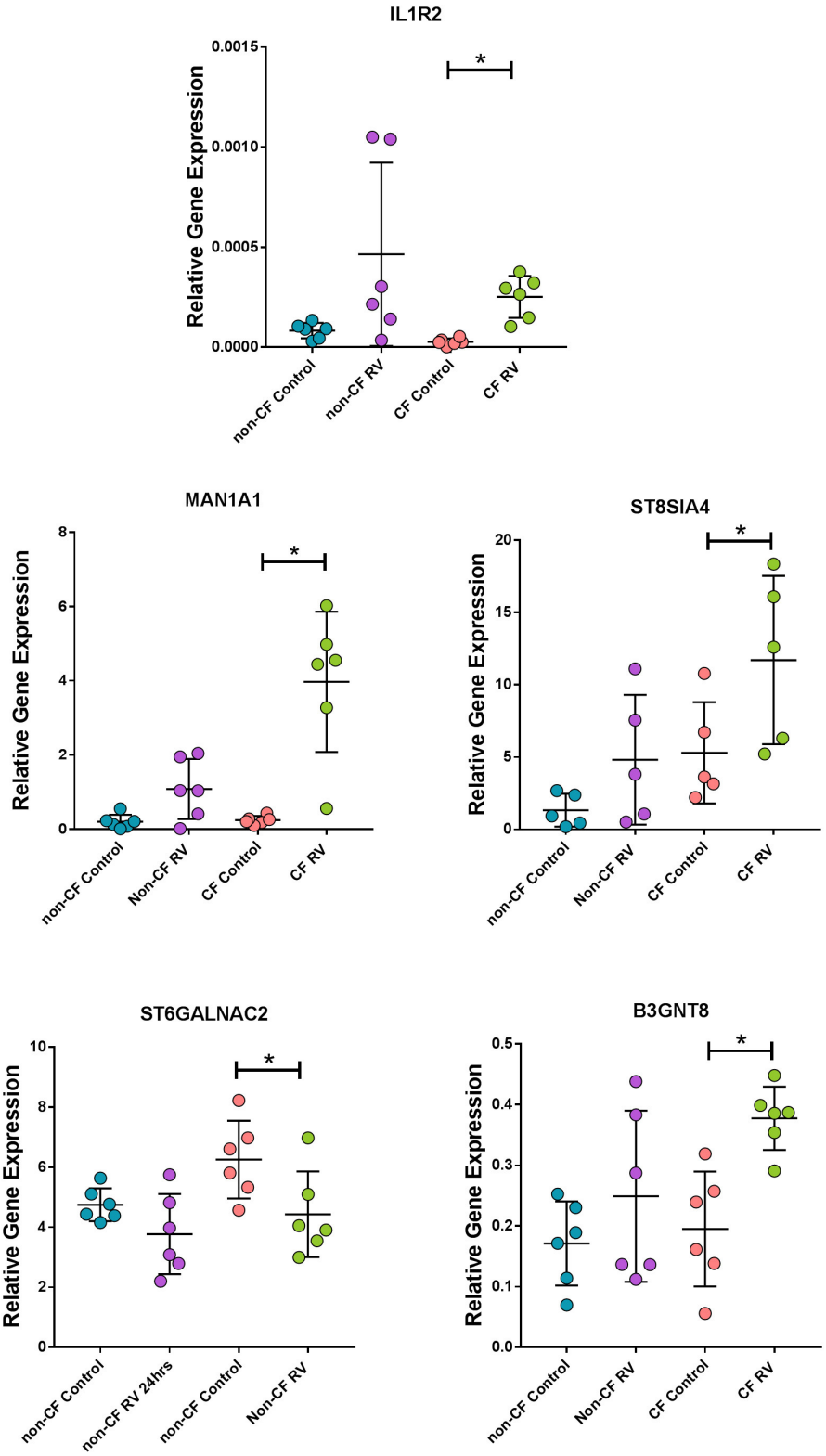

FIGURE 6 | Corroboration of uniquely expressed genes by CF AEC in fully differentiated cultures. Gene expression of the top unique gene *IL1R2* as well as mannosidase (*MAN1A1*), sialyltransferases (*ST8SIA4* and *ST6GALNAC2*), and acetylglucosaminyltransferase (*B3GNT8*), were uniquely altered in CF air–liquid interface cultures when infected with rhinovirus (RV) for 24 h. Data points represent individual samples for non-CF controls (turquoise), non-CF infected (purple), CF control (coral), and CF infected (green). Expression of all genes were normalized to the housekeeping gene PPIA (49). *Indicates $p < 0.05$ by one-way ANOVA following normal distribution test.

DISCUSSION

To improve knowledge of the underlying epithelial transcriptional responses during infection with rhinovirus, a major respiratory pathogen, we performed RNA sequencing on primary AEC from children with CF and non-CF controls *in vitro* at baseline and post-RV infection. There are five important findings from this study: (i) There were only modest baseline transcriptional differences between non-infected CF and non-CF AECs prior to exposure to RV, (ii) there was conservation in certain core antiviral responses (e.g., IFN signaling) of CF and non-CF AECs at the transcriptomic level but not the protein level, (iii) CF AECs elicited a larger and more complex transcriptional response compared to non-CF AECs with multiple unique biological pathways represented, (iv) key among these biological pathways are cytokine signaling and biosynthetic pathways (e.g., O-linked glycosylation of mucins) as they are highly relevant to CF lung pathology, (v) we corroborated observations made from the RNA-Seq analysis in fully differentiated cultures and identified genes involved in IL-1 signaling and mucin glycosylation that are only dysregulated in the CF airway epithelial response to RV infection. Collectively, these results identify potential biological pathways and processes that could be contributing to the adverse outcomes typically seen in people with CF during virus infection.

There were only modest baseline transcriptional differences between non-CF and CF AECs. This is most likely reflective of the very early and mild lung disease in the CF cohort. Minimal baseline differences also provide confidence that any difference in the antiviral transcriptional changes that we observed were due to infection. Nevertheless, the top differentially expressed baseline was HLA-DQB1, previously identified in a GWAS study with a high association signal to CF lung disease severity [reviewed in (50)]. Interestingly, the major enriched GO terms for the differentially expressed genes in non-infected baseline samples were denoted by the cytokine-mediated signaling pathway and type 1 interferon signaling pathway. Among these, *AIM2* inflammasome (associated with induction of pyroptosis, activation of pro-inflammatory cytokines, and viral suppression) (51, 52) *IFI27* (also known as *ISG12a*) contributes to IFN-dependent perturbation of normal mitochondrial function and enhanced cellular apoptosis (53), and the IFN-dependent antiviral factor *BST2* were all significantly downregulated in CF AECs. In response to RV infection, several common responses were found, including interferon signaling. However, the induction of type 1 and 3 interferon genes was lower in CF AEC. This was mirrored by reduced type 1 (IFNβ1) and type 3 interferon (IFNλ1 and IFNλ2) protein in supernatant. The reduction of type 1 and 3 interferon production of CF AECs in response to RV infection could be associated with the negative regulation of interferon signaling by the unique key gene, such as STAT3 (54, 55); however, this requires further characterization. Conversely, the IL-1 family signaling pathway was unique to the CF AECs response to RV infection, but in this case, IL-1β protein was significantly lower in CF supernatant compared to non-CF. This unusual observation could be, in part, mediated by negative regulators of IL-1 signaling expressed in CF AEC, including *IL1R2* and *IL1RN*, pellino protein genes *PELI1* and *PELI3*, together with interleukin 1 receptor-associated kinase *IRAK2* and *IRAK3*. We then assessed the expression of *IL1R2* pre- and post-RV infection in a differentiated culture model and made similar observations to those obtained using submerged cultures.

The IL-1 signaling pathway has been suggested as a link between hypoxic cell death and sterile neutrophilic inflammation in CF (56). Both IL-1α and IL-1β were detectable in bronchioalveolar lavage fluids (BALs) of young children with CF in the absence of bacterial infection, highlighting potential for inflammation of the CF airway under sterile inflammation (57). Since *S100A12* (key regulator of inflammatory process) is also part of the IL-1 family signaling pathway in CF response to RV infection, we postulate that the CF AECs could be directing from pro-inflammatory IL-1 signaling under sterile inflammation to a hyperinflammatory condition characterized by NF-κB signaling cascades during RV infection. Other evidence suggests the alteration of the inflammatory response with abundant cytokine signaling pathways (interleukin 1, 2, 7, 10, and 15 signaling) in CF AEC post-RV infection could be explained by downregulation of *RNF128* genes, which functions as an inhibitor of cytokine gene transcription and could interact with TBK1 (key hub of CF AEC response in our study here) kinase activity to enhance antiviral immunity. We also observed an elevated IL-8 production in both CF and non-CF AECs post-RV infection with higher amounts produced by non-CF AECs compared to CF AECs. Our IL-8 results agree with a previous study that also utilized primary AEC cultures in a similar infection setting (58) but contrasts with another that observed elevated inflammatory mediator release by the CF AECs (23). Overall, the over-represented cytokine signaling pathways suggest a unique and prominent role in regulating inflammation in CF AECs when infected with RV. However, with conflicting observations in this area, elucidating the complexity of the inflammatory response with associated cell death in CF AECs warrants further investigation.

We also identified over-represented metabolic pathways in CF AEC in response to RV infection specifically involved in the regulation of immunity, including inositol phosphate metabolism and synthesis of IP3 and IP4 in the cytosol, suggesting an altered CF airway microenvironment after RV infection. The induction of inositol phosphate has previously been related to endoplasmic reticulum expansion and Ca^{2+} storage, resulting in Ca^{2+}-dependent transcriptional activity of inflammatory mediators (59), which could contribute to hyperinflammatory responses seen in the CF AEC to viral infection (23). Upregulation of extracellular ectonucleotidase in the inositol phosphate metabolism pathway was found to cause depletion of ATP concentrations, reduction of air–surface liquid volume, ASL collapse, and failure in mucociliary clearance may trigger CF lung disease exacerbations as shown previously in a model of respiratory syncytial virus infection (60). Another metabolic pathway, trytophan catabolism, was also one of the over-represented pathways in the CF AECs following RV infection. Tryptophan metabolism has been previously found to be dysregulated in CF AEC (61) and has been implicated in *Pseudomonas aeruginosa* infection, oxidative stress, and Th17 hyperinflammation (62, 63). Alteration of

tryptophan metabolism results in accumulation of kynurenine and anthranilate, which could subsequently disrupt the homeostatic balance of the host's innate immune system and reduce the antimicrobial activity of airway epithelium.

Other identified biosynthetic pathways associated with RV infection in CF include sialic acid metabolism and O-linked glycosylation of mucins. Sialic acids are a family of negatively charged monosaccharides that are commonly expressed as the terminal residues in glycans of the glycoconjugates on the epithelial cell surface lining the airways and are also major components of secreted mucins in the airway. Previous studies have identified increased fucoslyation and decreased sialylation in cultured AEC while a contrary observation was reported in CF sputum (64–67). As a key player that contributes to the rheological properties of mucus, aberrant sialic acid metabolism may worsen the pathological conditions of CF. O-linked glycosylation is a post-translational modification process and occurs within the endoplasmic reticulum (ER) and Golgi complex. The enzymes in ER and Golgi complex regulate glycosylation of N-glycans and O-glycans by successively adding to and then remodeling mucin oligosaccharides prior to transport to cell membranes for tethering or secretion. Here, alteration of genes encoding glycosyltransferases, such as N-acetylgalactosaminyltransferases, N-acetlyglucosaminyltransferase, and galactosyltransferases, were reported from our RNA-Seq analysis. We corroborated a number of these as unique to the AEC response to RV in children with CF. Changes in these glycosyltransferases could potentially alter the O-glycans on cell surfaces and, thus, affect interactions with airway pathogens and irritant exposures. Emerging evidence suggests alteration of mucin glycosylation is a response to infection and inflammation and might induce extended conformational changes to prevent damage from proteolytic enzymes (68). Although the impact that *CFTR* mutations has on mucin biomolecules is unknown, our results suggest that RV infection could be a potential mechanism that contributes to changes in mucin glycosylation that are exclusive to CF and that might influence mucosal barrier function. A previous investigation has demonstrated that a surplus of unfolded proteins that results from blocked glycosylation leads to prolonged ER stress and activation of the unfolded protein response (UPR) causing cell death (69). Previous *in vitro* work using an immortalized cell line discovered a pronounced reprogramming of host cell metabolism toward an anabolic state, including upregulation of glucose uptake, glycogenolysis, nucleotide synthesis, and lipogenesis (70). Considering most of the metabolic changes found in this study occur post-RV infection, future studies integrating the transcriptomic signature patterns with analyses of the metabolites produced by CF AEC in response to RV infection will provide significant insight into the exact metabolic changes that occur during infection.

Interestingly, RV infection in CF AECs results in the upregulation of a group of SLC transporter genes, including upregulation of *CP* (ferroxidase), *SLC41A2* (magnesium transporter), *SLC30A1* (zinc transporter), and *SLC39A8* (zinc transporter) and downregulation of *SLC39A10* (zinc transporter) and *SLC40A1* (iron-regulated transporter). Increasing total iron and zinc has previously been associated with airway inflammation in CF (71). These results suggest that RV infection in the CF airway is associated with the presence of redox active biometals. A previous study (72) has suggested that the dysregulation of iron homeostasis is accompanied by a respiratory virus infection, which, in turn, facilitates pseudomonas biofilm growth. Understanding the mechanistic link of virus infection to the alteration of the cellular microenvironment and instigation of secondary infection might aid in development of new treatment.

We acknowledge some limitations in the experimental design. First, we only analyzed transcriptomics at the 24-h time point, primarily due to the limited number and expansion of primary cells established from each patient. However, early optimization of our infection model did assess the transcriptional changes earlier (data not shown), and the greatest transcriptional change identified occurred at the 24-h time point. Although methodologies now exist to assist with primary AEC expansion *in vitro* (29), its effects at the transcriptomic level remain unknown and, thus, the use of unaltered primary airway cells remains a significant strength of this study. Future investigations could possibly include additional time points to better appreciate the transcriptional signature changes over the full course of RV infection as well as the long-term consequence of viral infection on CF AECs. Second, this study utilized a laboratory strain of rhinovirus (RV1b), which might exert differential effects on CF AEC compared to clinically derived isolates known to cause exacerbations in this cohort. With different RV serotypes causing infection in CF airways (10), future studies may identify whether innate immune responses may be serotype-specific. Similarly, comparison studies to other viruses (respiratory syncytial virus, influenza) would also assist in our understanding of the contribution of early-life viruses to CF disease progression. Finally, the simplified monolayer cell culture model of basal CF AECs may be regarded as a limitation, but basal cells are the primary target of RV (73). While monolayer cultures may oversimplify the multicellular interactions of epithelial (ciliated, goblet, basal, secretory cells) and immune cells (dendritic cells, neutrophils), it is an important, repeatable model with low methodological variation, and we were able to validate genes in differentiated AEC. Overall, we are highly confident that limitations are minor and that our results provide new insight into new therapeutic targets for treating acute viral infections in CF that can be validated in future transcriptomic studies assessing differentiated AEC models.

In conclusion, this study shows that, at the transcriptomic level, CF AECs induce a complex and unique set of responses when infected with RV *in vitro* that have implications for lung disease progression in CF. Despite type 1, II, and III interferon signaling being involved in the core CF antiviral response, IFNs protein levels were lower in CF AEC when compared to non-CF AEC. Metabolic and biosynthetic pathways were unexpectedly integrated with the core CF antiviral response, and multiple key regulatory molecules of antiviral response were dysregulated in CF AEC, revealing new potential to modulate CF AEC innate immunity to RV infection. Future work will explore whether these regulatory molecules are potential targets for therapy

unique to RV and may be leveraged to reduce the impact viral infections have on lung disease progression CF.

DATA AVAILABILITY STATEMENT

Raw datasets have been uploaded to GEO, with accession number GSE138167.

ETHICS STATEMENT

The study was approved by the St. John of Gods Human Ethics Committee (SJOG#901) and Perth Children's Hospital Ethics Committee (#1762) and written informed consent was obtained from parents or guardians.

AUTHOR CONTRIBUTIONS

Conceptualization: SS and AK. Funding acquisition: SS, AK, RH, and ST. Methodology: K-ML, EG, AL, TL, PA-R, TI, and TR. Supervision: SS, AK, TL, and LG. Validation: K-ML, ES, and LG. Manuscript writing: K-ML, LG, and AK. Manuscript review: SS, RH, TL, PA-R, ES, ST, TI, and EG. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01327/full#supplementary-material

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ORIGINAL RESEARCH
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Epithelial-Mesenchymal Transition in Asthma Airway Remodeling Is Regulated by the IL-33/CD146 Axis

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Epithelial-mesenchymal transition (EMT) is essential in asthma airway remodeling. IL-33 from epithelial cells is involved in pulmonary fibrosis. CD146 has been extensively explored in cancer-associated EMT. Whether IL-33 regulates CD146 in the EMT process associated with asthma airway remodeling is still largely unknown. We hypothesized that EMT in airway remodeling was regulated by the IL-33/CD146 axis. House dust mite (HDM) extract increased the expression of IL-33 and CD146 in epithelial cells. Increased expression of CD146 in HDM-treated epithelial cells could be blocked with an ST2-neutralizing antibody. Moreover, HDM-induced EMT was dependent on the CD146 and TGF-β/SMAD-3 signaling pathways. IL-33 deficiency decreased CD146 expression and alleviated asthma severity. Similarly, CD146 deficiency mitigated EMT and airway remodeling in a murine model of chronic allergic airway inflammation. Furthermore, CD146 expression was significantly elevated in asthma patients. We concluded that IL-33 from HDM extract-treated alveolar epithelial cells stimulated CD146 expression, promoting EMT in airway remodeling in chronic allergic inflammation.

Keywords: epithelial-mesenchymal transition, IL-33, CD146, asthma, allergy

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INTRODUCTION

Asthma is a disease that is characterized by airway inflammation, airway remodeling, and airway hyperresponsiveness (1). Airway remodeling is described as a change in the composition, thickness or volume of airway walls, including subepithelial fibrosis, and increased smooth muscle composition, in asthmatic patients compared to normal individuals (2). Epithelial-mesenchymal transition (EMT) is a pathophysiological process induced by multiple signaling pathways centered on TGF-β and refers to the loss of function of epithelial cells and their transformation to mesenchymal cells, including a decrease in E-cadherin, and an increase in N-cadherin expression (3–5). An increasing number of studies have demonstrated that increased EMT plays an important role in airway remodeling in asthma (5, 6).

CD146 was originally acknowledged as a tumor marker for melanoma (MCAM). As a multifunctional molecule (7), CD146 plays diverse biological roles in tumors, atherosclerosis, systemic sclerosis, and other diseases (8–10). CD146 in macrophages promotes cell adhesion and foam cell formation (8). CD146 in $CD4^+$ T cells is associated with Th17 differentiation in systemic sclerosis (9). CD146 is also associated with pulmonary infections, in which it promotes the adherence of bacteria or viruses to airway epithelial cells (11–14). Increased expression ofCD146 in gastric cancer leads to decreased expression of E-cadherin and increased expression of N-catenin and vimentin (15). CD146 also regulates the EMT process in hepatocellular carcinoma

via the MAPK1 signaling pathway, which exacerbates the invasion and metastasis of hepatocellular carcinoma (16). These studies suggest that the EMT process is associated with cancer progression. Increased expression of CD146 in the airway epithelial cells of asthma patients was recently discovered, and IL-13 (a type 2 inflammatory cytokine) regulates the expression and function of CD146 in airway epithelial cells (11, 12). Although the regulation of EMT by CD146 has been extensively reported in studies of tumor metastasis (17), the roles of CD146 in asthma EMT and airway remodeling have not been explored.

Interleukin-33 (IL-33) is a member of the IL-1 cytokine family and is expressed in fibroblasts, endothelial cells, epithelial cells, and other cell types (18, 19). Once bound with the membrane receptor ST2, IL-33 activates the MyD88/NF-κB signaling pathway (20) and induces the type 2 response in $CD4^+$ T cells, which release IL-4, IL-5, and IL-13 (19). Serum IL-33 and the soluble form of ST2 are closely associated with asthma disease progression (21) and exacerbation (22). Moreover, recent studies have shown that IL-33 is involved in asthma airway collagen deposition, suggesting that IL-33 may be involved in the EMT process in the lung (23–25). The regulation of IL-33 signaling related to CD146 expression and the EMT process in asthma, however, remains largely elusive. In the present study, we demonstrated that IL-33 increased the expression of CD146, which promoted the EMT process in asthma.

MATERIALS AND METHODS

Animals and a Murine Model of Asthma

Specific pathogen-free (SPF) female C57BL/J mice aged 6–8 weeks were obtained from the Laboratory Animal Center, Nanjing Medical University (Nanjing, China). CD146 knockout (KO) mice on a C57BL/J background were obtained from Cyagen, Suzhou, China. IL-33 KO mice on a C57BL/J background were obtained from Dr. Hong Zhou (Department of Immunology, Nanjing Medical University). All animal treatments were approved by the Nanjing Medical University Ethics Committee (IACUC 1709011).

To establish a murine model of asthma, the mice were intranasally administered house dust mite (HDM, Greer Laboratories, Lenoir, NC, USA) extract (25 μg of HDM extract dissolved in 40 μL of phosphate-buffered saline) 5 days/week for 5 weeks. All mice were treated with HDM extract under isoflurane anesthesia and were ultimately sacrificed (26).

Cell Culture

The mouse pulmonary epithelial cell lines MLE-12 and A549 were obtained from ATCC (VA, USA) and cultured in DMEM containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. MLE-12 or A549 cells were seeded in 6-well plates or 24-well plates overnight and then treated with HDM extract or the cytokine IL-33 for the indicated durations. Primary alveolar epithelial cells from mice were purified using 0.1% collagenase, 0.25% trypsin, and DNase I and were selected with mouse IgG (36111ES60, Yeasen, China) as previously described (27). To exclude the potential effects of lipopolysaccharide (LPS) contamination, HDM extract was treated with the ToxinEraser™ endotoxin removal Kit (L00338, Genscript, China). The purified product was the major constituent of HDM.

TABLE 1 | Antibodies in the study.

Antibody	Brand name	Product code	Source	Dilutability
Anti-CD146 antibody	Abcam	ab75769	Cambridge, UK	1:1000
Anti-IL-33 antibody	Abcam	ab54385	Cambridge, UK	1:1000
Anti-SPD antibody	Abcam	ab220422	Cambridge, UK	1:1000
Anti- E-cadherin antibody	Abcam	ab76055	Cambridge, UK	1:1000
Anti-N-cadherin antibody	Abcam	ab76011	Cambridge, UK	1:5000
Anti-α-SMA antibody	Abcam	ab7817	Cambridge, UK	1:200
AntiTGF-β antibody	Abcam	ab170874	Cambridge, UK	1:1000
Anti- fibronectin antibody	Proteintech	15613-1-AP	Wuhan, Hubei, China	1:1000
Anti-beta-actin antibody	Cell signaling technology	#4970	Beverly, MA	1:1000
Anti-P38 antibody	Cell signaling technology	#8690	Beverly, MA	1:1000
Anti-P38 (phospho-Thr180/Tyr182) antibody	Cell signaling technology	#4511	Beverly, MA	1:1000
Anti-P44/42 antibody	Cell signaling technology	#4695	Beverly, MA	1:1000
Anti- P44/42(phospho-Thr202/Tyr204) antibody	Cell signaling technology	#4370	Beverly, MA	1:1000
Anti-P65 antibody	Cell signaling technology	#8242	Beverly, MA	1:1000
Anti- P65 (phospho-Ser536) antibody	Cell signaling technology	#3033	Beverly, MA	1:1000
Anti-JNK antibody	Cell signaling technology	#9252	Beverly, MA	1:1000
Anti-JNK(phospho-Thr183/Tyr185) antibody	Cell signaling technology	#4668	Beverly, MA	1:1000
Anti-STAT3 antibody	Cell signaling technology	#4904	Beverly, MA	1:1000
Anti-STAT3 (phospho-Tyr705) antibody	Cell signaling technology	#9145	Beverly, MA	1:1000
Anti-SMAD3 antibody	Cell signaling technology	#9523	Beverly, MA	1:1000
Anti-SMAD3 (phospho-Ser423/425) antibody	Cell signaling technology	#9520	Beverly, MA	1:1000

Cell Transfection

MLE-12 cells were seeded and incubated overnight before transfection. The CD146 expression plasmid, an siRNA plasmid, or blank vehicles (Abmgood, China) were mixed with Lipofectamine 2000 (Invitrogen, USA) in DMEM without FBS, penicillin or streptomycin for 25 min and were then transfected into MLE-12 cells at 60–80% density in DMEM for 48 h. The cells were treated with HDM extract or PBS for 24 h before total protein extraction.

Western Blotting

Total protein from the cells or tissues was lysed with RIPA buffer (89900, Thermo, USA) containing protease and phosphatase inhibitors (78443, Thermo, USA) on ice for 20 min. Then, the samples were centrifuged for 10 min, and the supernatants were collected and transferred into new EP tubes. The protein concentrations were measured by a BCA assay (P0012S, Beyotime, China). The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes at 300 mA. The PVDF membranes were blocked with 5% skim milk powder for 1 h at room temperature and were then incubated with primary antibodies (**Table 1**) at 4°C overnight. The PVDF membranes were washed with TBST 4 times for 5 min each and were then incubated with goat anti-rabbit HRP IgG (EarthOx Life Sciences) or goat anti-mouse HRP IgG (EarthOx Life Sciences) for 1 h at room temperature. The PVDF membranes were washed with TBST 4 times for 7 min each. The specific antibody-bound proteins were visualized with the Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) and the G:Box gel doc system (Syngene, UK).

Quantitative Real-Time PCR

Total RNA was extracted from cells using the TaKaRa Universal Total RNA Extraction Kit (Dalian, China) and was then used to synthesize cDNA using PrimeScript RT master mix (TaKaRa). The expression of specific RNAs was quantified by using SYBR Green Universal PCR master mix (TaKaRa) in a StepOnePlus Real-Time PCR System (ABI, USA). The primer sequences used for real-time PCR were synthesized by Genescript. The primer sequences are as follows: CD146 forward, 5′- GGACCTTGAGTTTGAGTGG−3′; CD146 reverse, 5′- CAGTGGTTTGGCTGGAGT−3′; β-actin forward, 5′- GAGAAGCTGTGCTATGTTGCT−3′; and β-actin reverse, 5′- CTCCAGGGAGGAAGAGGATG -3’.

Immunofluorescence

After treatment with HDM extract or PBS for 24 h, the culture medium was removed, and the MLE-12 cells were washed in PBS 3 times. The cells were then fixed in 4% paraformaldehyde at 4°C for 15 min, followed by 3 washes with PBS. Afterwards, the cells were blocked with 5% goat serum for 1 h at room temperature and were incubated with mouse anti-E-cadherin, rabbit anti- N-cadherin or rabbit anti-SPD primary antibody at 4°C overnight. The cells were washed with PBS 3 times and

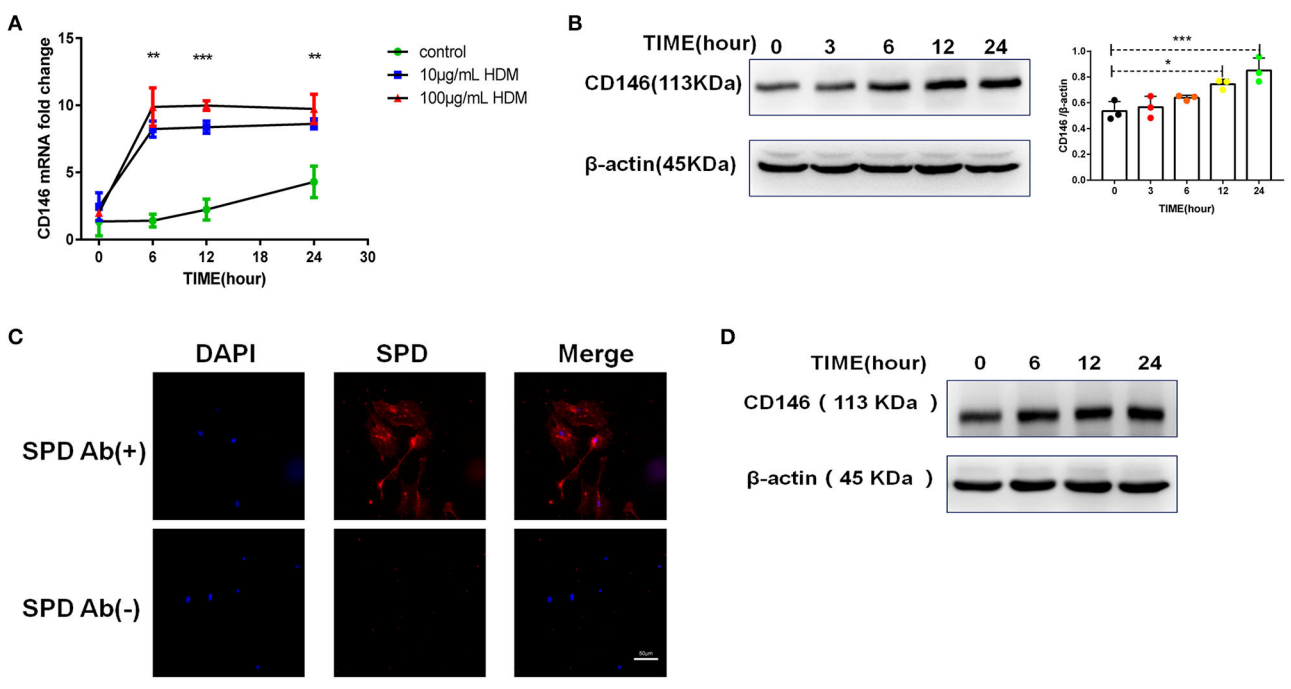

FIGURE 1 | HDM promoted CD146 expression in alveolar epithelial cells. **(A)** CD146 mRNA in HDM-treated MLE-12 cells was measured by qRT-PCR. **(B)** Western blot analysis of CD146 expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(C)** The level of SPD in primary alveolar epithelial cells was measured by immunofluorescence. Ab(+): stained with anti-SPD antibody; Ab(–): stained with isotype antibody **(D)** Western blot analysis of CD146 expression in primary alveolar epithelial cells treated with HDM extract (100 μg/ml). *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

incubated with Alexa Fluor 555 donkey anti-mouse IgG (H+ L) or Alexa Fluor 647 donkey anti-rabbit IgG (H+L) at 37°C for 1 h in the dark. Next, the cells were washed with PBS and stained with DAPI (4′,6-diamidino-2-phenylindole; Yeasen, China) at 37°C for 10 min in the dark. Images were visualized with a ZEISS LSM710 confocal fluorescence microscope or an Olympus IX73 fluorescence microscope.

Airway Responsiveness

The FinePointe RC System (Buxco Research Systems, Wilmington, NC) was used to measured airway responsiveness. Mice were challenged with aerosolized PBS and methacholine to measure lung resistance. The airway resistance values were recorded for 3 min after each challenge. Then, we calculated the average airway resistance (28).

Differential Counts of Inflammatory Cells in BALF

The bronchoalveolar lavage fluid (BALF) was collected from mice and centrifuged to separate the supernatant and sediment. The sediment was resuspended in PBS and measured with a blood cell analyzer (ADVIA 2120i).

Histological Staining

Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were stained with H&E, PAS, and Sirius red. Images were visualized with a Zeiss Axio Examiner microscope.

Immunohistochemistry

Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were blocked with 5% goat serum for 30 min at 37°C and incubated with mouse anti-E-cadherin at 4°C overnight. Tissue sections were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. 3,3-Diaminobenzidine (DAB) was used as a color developer, and hematoxylin was used for counterstaining. Images were visualized with a Zeiss Axio Examiner microscope.

ELISA

Mouse blood was collected and centrifuged to extract the serum. Total IgE was measured with an ELISA kit (432401, Biolegend, USA). The lungs of mice were ground and centrifuged to extract the supernatant. The cytokines IL-4

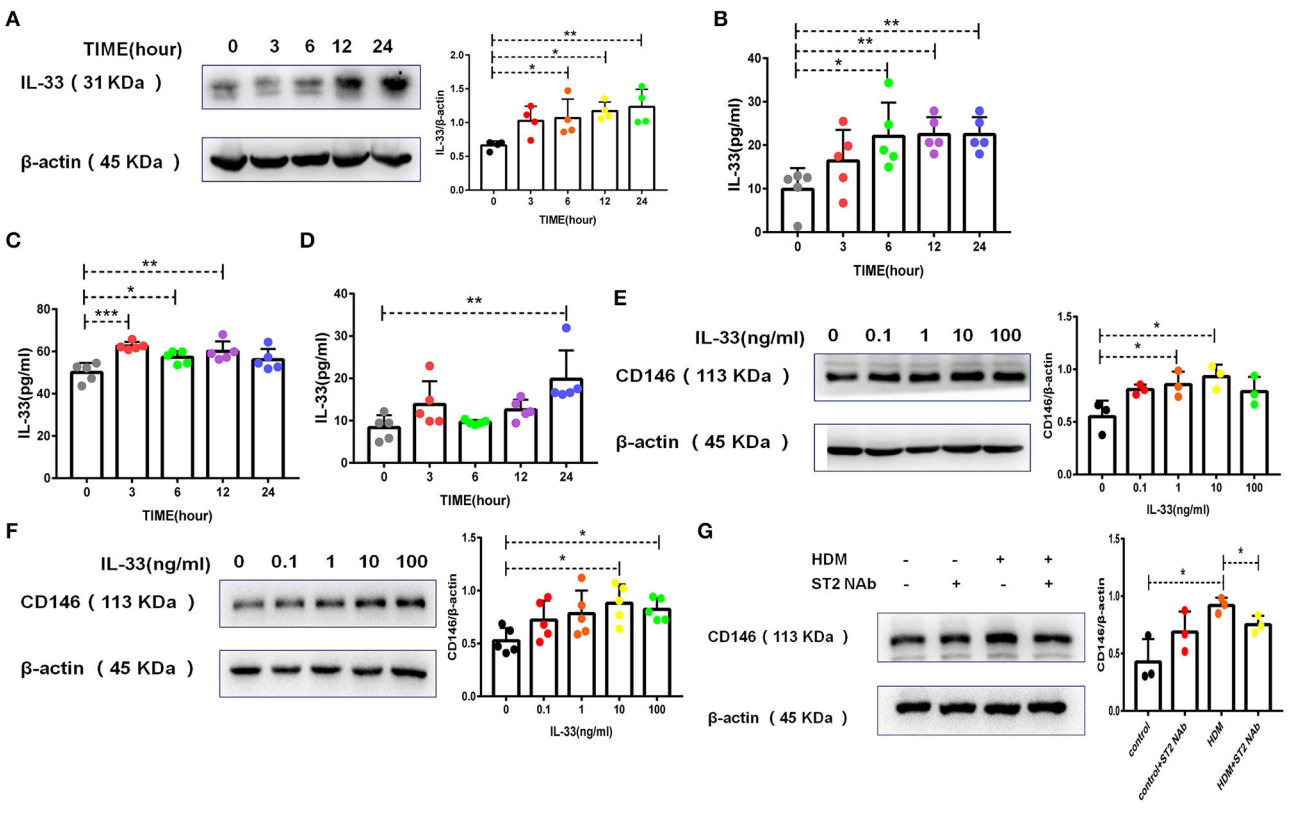

FIGURE 2 | HDM promoted CD146 expression in alveolar epithelial cells via IL-33/ST2 signaling. **(A)** Western blot analysis of IL-33 expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(B)** ELISA analysis of IL-33 levels in the cell culture supernatant of MLE-12 cells treated with HDM extract (100 μg/ml). **(C)** ELISA analysis of IL-33 levels in the cell lysates of MLE-12 cells treated with Derp1 (extracted HDM without LPS). **(D)** ELISA analysis of IL-33 levels in the cell culture supernatant of MLE-12 cells treated with Derp1 (extracted HDM without LPS). **(E)** Western blot analysis of CD146 expression in MLE-12 cells treated with IL-33 for 24 h. **(F)** Western blot analysis of CD146 expression in A549 cells treated with IL-33 for 24 h. **(G)** Western blot analysis of CD146 expression in MLE-12 cells treated with HDM extract (100 μg/ml) with or without an ST2-neutralizing antibody (5 μg/ml). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(431104, Biolegend, USA), IL-5 (431204, Biolegend, USA), IL-13 (900-K207, PeproTech, USA), IL-33 (88-7333-88, Invitrogen, USA) and IFN-γ (430804, Biolegend, USA) in the supernatants of lung homogenates were measured using an ELISA kit. Collagen I in the lung homogenates was measured using an ELISA kit (E-EL-M0325c, Elabscience, China). Soluble CD146 in human plasma (E-EL-H2403c, Elabscience, China) was measured using commercial ELISA kits. All ELISA experiments were performed according to the instructions provided by the manufacturers.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA), and the data are displayed as the means ± SEM. Images from the Western blotting or immunofluorescence results were analyzed with ImageJ. Student's *t*-test or one-way ANOVA was applied to assess the statistical significance. A value of $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $\#P > 0.1$).

RESULTS

HDM Promoted CD146 Expression in Alveolar Epithelial Cells via IL-33/ST2 Signaling

Once inhaled into the respiratory tract, HDMs may directly stimulate alveolar epithelial cells. As shown in **Figure 1A**, HDM extract challenge increased CD146 transcripts in the mouse alveolar epithelial cell line MLE-12, which was further validated in the immunoblotting assay (**Figure 1B**). Primary alveolar epithelial cells purified from the lung were subjected to SPD staining (**Figure 1C**). Similarly, HDM extract increased CD146 expression in primary alveolar epithelial cells (**Figure 1D**). In agreement with a previous study that showed that IL-33 was increased in asthma (29), HDM extract increased IL-33 expression (**Figure 2A**) and secretion (**Figure 2B**) in alveolar epithelial cells. To explore whether HDM-mediated IL-33 induction was associated with the major HDM component Derp or LPS contamination, we removed endotoxin from HDM extract and treated epithelial cells with treated HDM that lacked LPS.

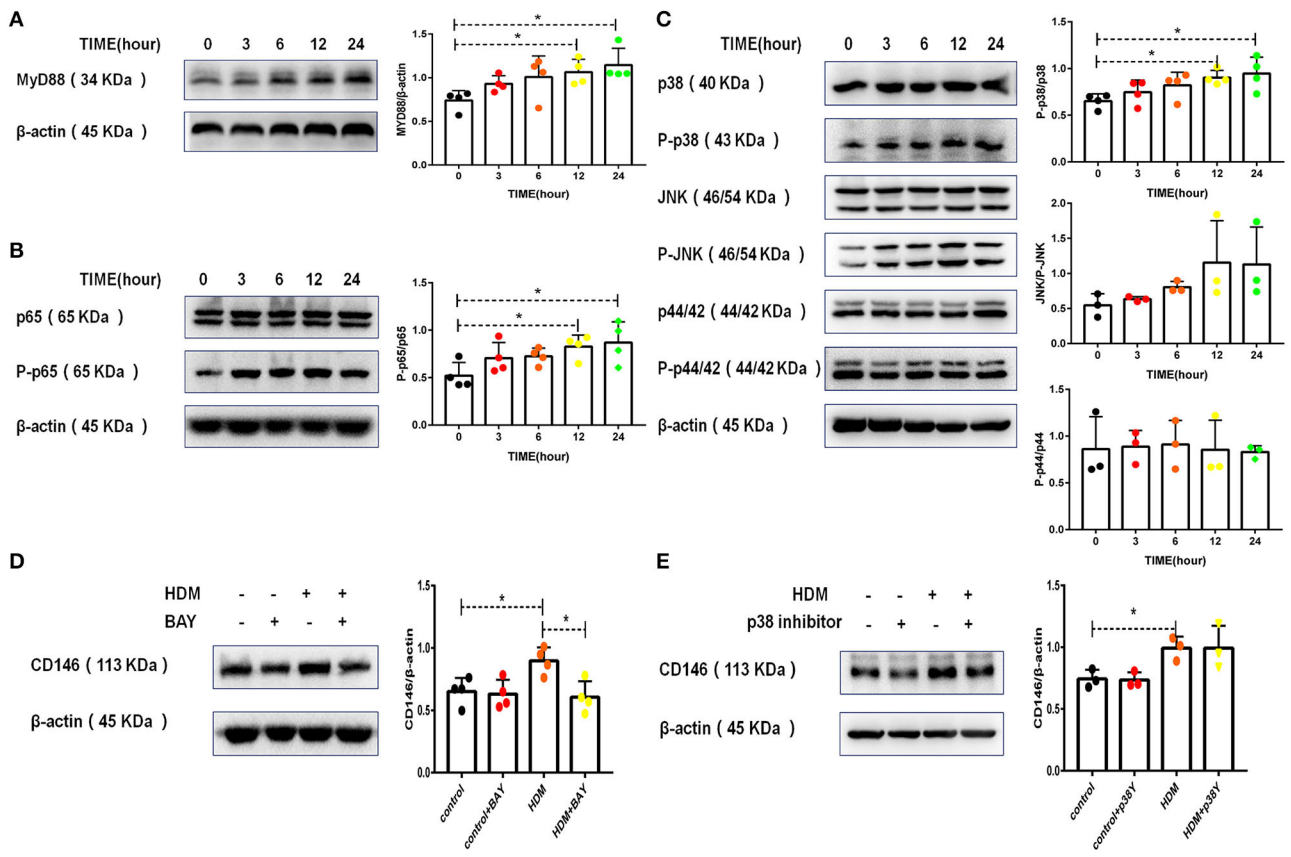

FIGURE 3 | CD146 expression in alveolar epithelial cells was dependent on p65. **(A)** Western blot analysis of MyD88 expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(B)** Western blot analysis of NF-κB p65 expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(C)** Western blot analysis of MAPK expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(D)** Western blot analysis of CD146 expression in MLE-12 cells treated with HDM extract (100 μg/ml) and a p65 inhibitor (BAY, 10 μm) for 24 h. **(E)** Western blot analysis of CD146 expression in MLE-12 cells treated with HDM extract and a p38 inhibitor (SB203580, 10 μm) for 24 h. $^{*}P < 0.05$.

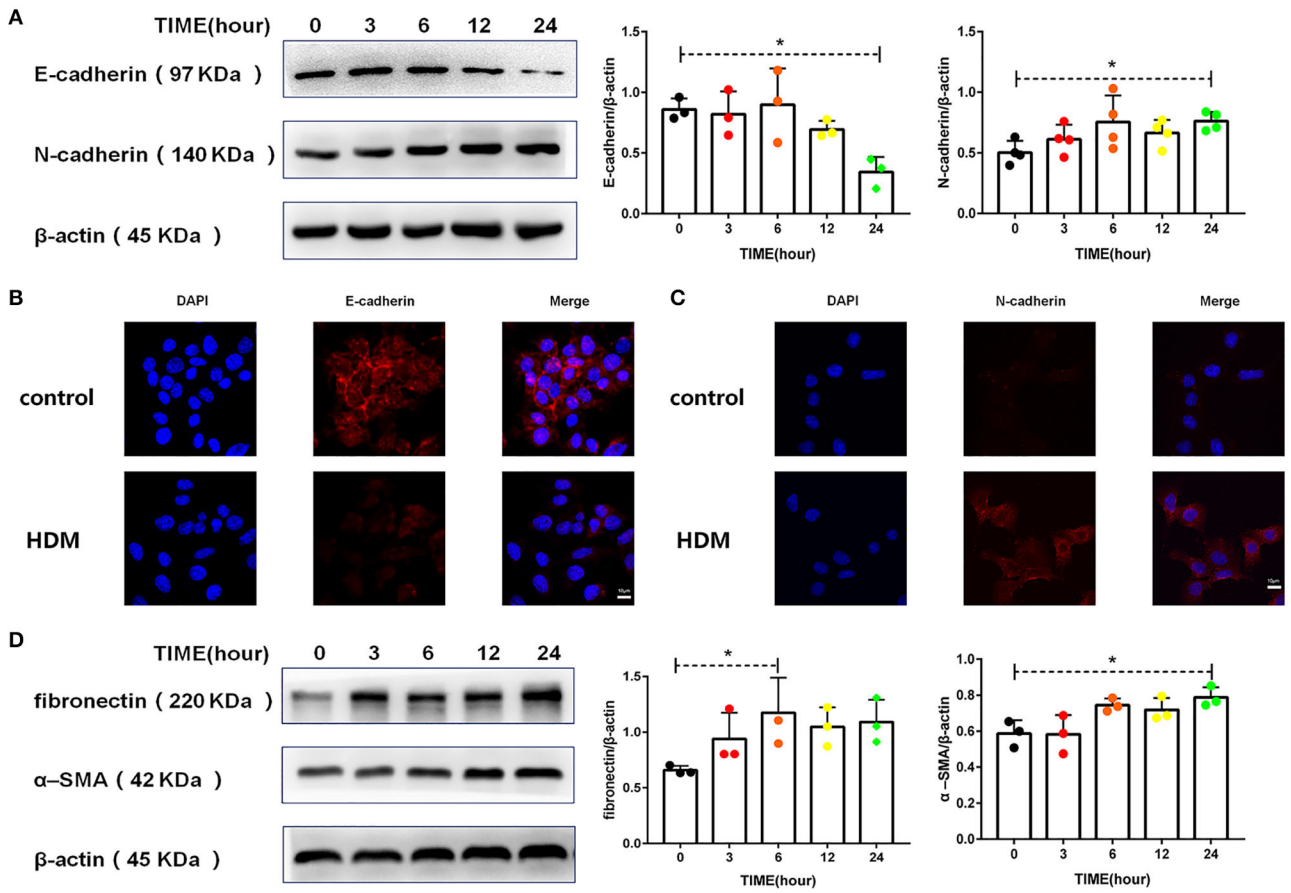

FIGURE 4 | HDM promoted EMT in alveolar epithelial cells. **(A)** Western blot analysis of E-cadherin and N-cadherin expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(B)** Immunofluorescence analysis of E-cadherin expression in MLE-12 cells treated with HDM extract (100 μg/ml) for 24 h. **(C)** Immunofluorescence analysis of N-cadherin expression in MLE-12 cells treated with HDM extract (100 μg/ml) for 24 h. **(D)** Western blot analysis of fibronectin and α-SMA expression in MLE-12 cells treated with HDM extract (100 μg/ml). *$P < 0.05$.

Again, IL-33 was increased in the cell lysate (**Figure 2C**) or culture supernatant (**Figure 2D**) was increased.

To explore whether IL-33 is involved in CD146 expression, we stimulated epithelial cells with IL-33 and found that IL-33 directly promoted CD146 expression in mouse alveolar epithelial MLE-12 cells (**Figure 2E**) and human alveolar epithelial A549 cells (**Figure 2F**). The ST2-neutralizing antibody decreased CD146 expression (**Figure 2G**), suggesting that IL-33/ST2 was required for CD146 expression in HDM-treated epithelial cells. In summary, HDM extract increased the expression of CD146 in alveolar epithelial cells, which was mediated by IL-33 and its receptor ST2.

CD146 Expression in Alveolar Epithelial Cells Was Dependent on p65

IL-33 binding to ST2 on epithelial cells may activate a series of downstream signaling pathways, including the MyD88, NF-κB, and MAPK pathways (30). As shown in **Figure 3A**, HDM extract activated MyD88 in MLE-12 cells. Similarly, HDM extract increased the phosphorylation of NF-κB p65 (**Figure 3B**). In the MAPK signaling pathway, p38 but not JNK, and p42 was activated in MLE-12 cells treated with HDM extract (**Figure 3C**). More importantly, the p65 inhibitor antagonized the HDM-induced upregulation of CD146 (**Figure 3D**), highlighting the importance of NF-κB in CD146 expression. In contrast with the results observed with the p65 inhibitor, the p38 inhibitor showed insignificant effects on the expression of CD146 in MLE-12 cells treated with HDM extract (**Figure 3E**). Therefore, CD146 in HDM-treated alveolar epithelial cells was regulated by NF-κB p65.

HDM Promoted EMT in Alveolar Epithelial Cells via CD146

There is now evidence that asthma patients have more EMT than normal individuals (5, 31). The cadherin switch, which is a fundamental event in EMT, was induced in MLE-12 cells treated with HDM extract. As shown in **Figures 4A–C**, HDM extract decreased the expression of E-cadherin and increased N-cadherin expression. In addition, HDM extract increased fibronectin and α-SMA levels in MLE-12 cells (**Figure 4D**), suggesting that HDM extract promoted EMT in alveolar epithelial cells.

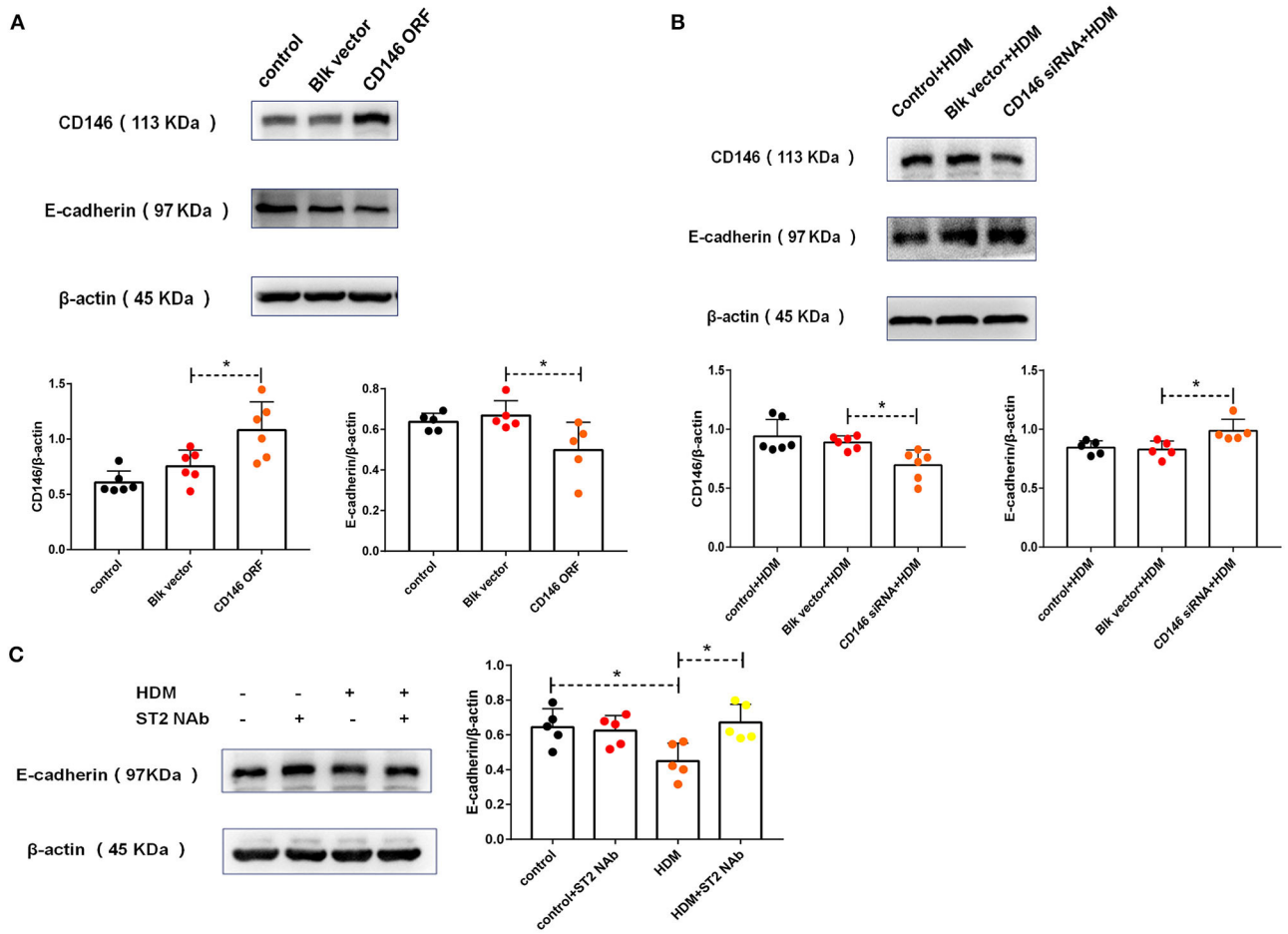

FIGURE 5 | HDM promoted EMT in alveolar epithelial cells via CD146. **(A)** Western blot analysis of CD146 and E-cadherin expression in MLE-12 cells treated with a CD146 expression plasmid (CD146 open reading frame, CD146 ORF) or blank vehicle (Blk vector). **(B)** Western blot analysis of CD146 and E-cadherin expression in MLE-12 cells treated with a CD146 siRNA plasmid and HDM extract (100 μg/ml). **(C)** Western blot analysis of E-cadherin expression in MLE-12 cells treated with HDM extract (100 μg/ml) and an ST2-neutralizing antibody (5 μg/ml). *$P < 0.05$.

To explore the roles of CD146 in HMD-induced EMT, CD146 was either overexpressed via an expression plasmid or silenced with a siRNA plasmid in MLE-12 cells. Accompanied by CD146 elevation, E-cadherin was significantly decreased (**Figure 5A**). In contrast, CD146 silencing caused the increased expression of E-cadherin in epithelial cells (**Figure 5B**). E-cadherin expression was inversely correlated with CD146 expression, suggesting that CD146 may positively regulate EMT in alveolar epithelial cells. Moreover, the ST2-neutralizing antibody rescued E-cadherin expression in epithelial cells treated with HDM extract (**Figure 5C**). Considering that the ST2-neutralizing antibody decreased CD146 expression in epithelial cells treated with HDM extract (**Figure 2G**), we concluded that IL-33/ST2 contributed to CD146-mediated EMT in alveolar epithelial cells treated with HDM extract.

TGF-β and SMAD3 Played Dominant Roles in EMT in Alveolar Epithelial Cells Treated With HDM Extract

TGF-β has been shown to be the most common EMT inducer in asthma (5, 32). Accordingly, HMD extract increased TGF-β levels in alveolar epithelial cells (**Figure 6A**). STAT3 and SMAD3 are downstream molecules of the TGF-β signaling pathway in the EMT process. Administration of HDM extract contributed minimally to STAT3 activation (**Figure 6B**) but resulted in the phosphorylation of SMAD3 (**Figure 6C**) in alveolar epithelial cells. More importantly, a SMAD3 inhibitor (SIS3) partially but significantly increased E-cadherin expression in MLE-12 cells treated with HDM extract (**Figure 6D**), suggesting that TGF-β and SMAD3 regulate EMT in HDM-treated alveolar epithelial cells.

IL-33 Was Essential for CD146 Expression in a Mouse Model of Asthma

To demonstrate the significance of IL-33/ST2 in CD146 expression, we developed an asthma model in wild-type mice and IL-33 KO mice (**Figure 7A**). IL-33 deficiency reduced lung resistance in the murine model of asthma (**Figure 7B**). The number of total cells and eosinophils in BALF were decreased in the IL-33 KO mice treated with HDM extract (**Figures 7C,D**). Similarly, pulmonary tissue sections stained with H&E exhibited more inflammatory infiltration in WT mice than in IL-33 KO

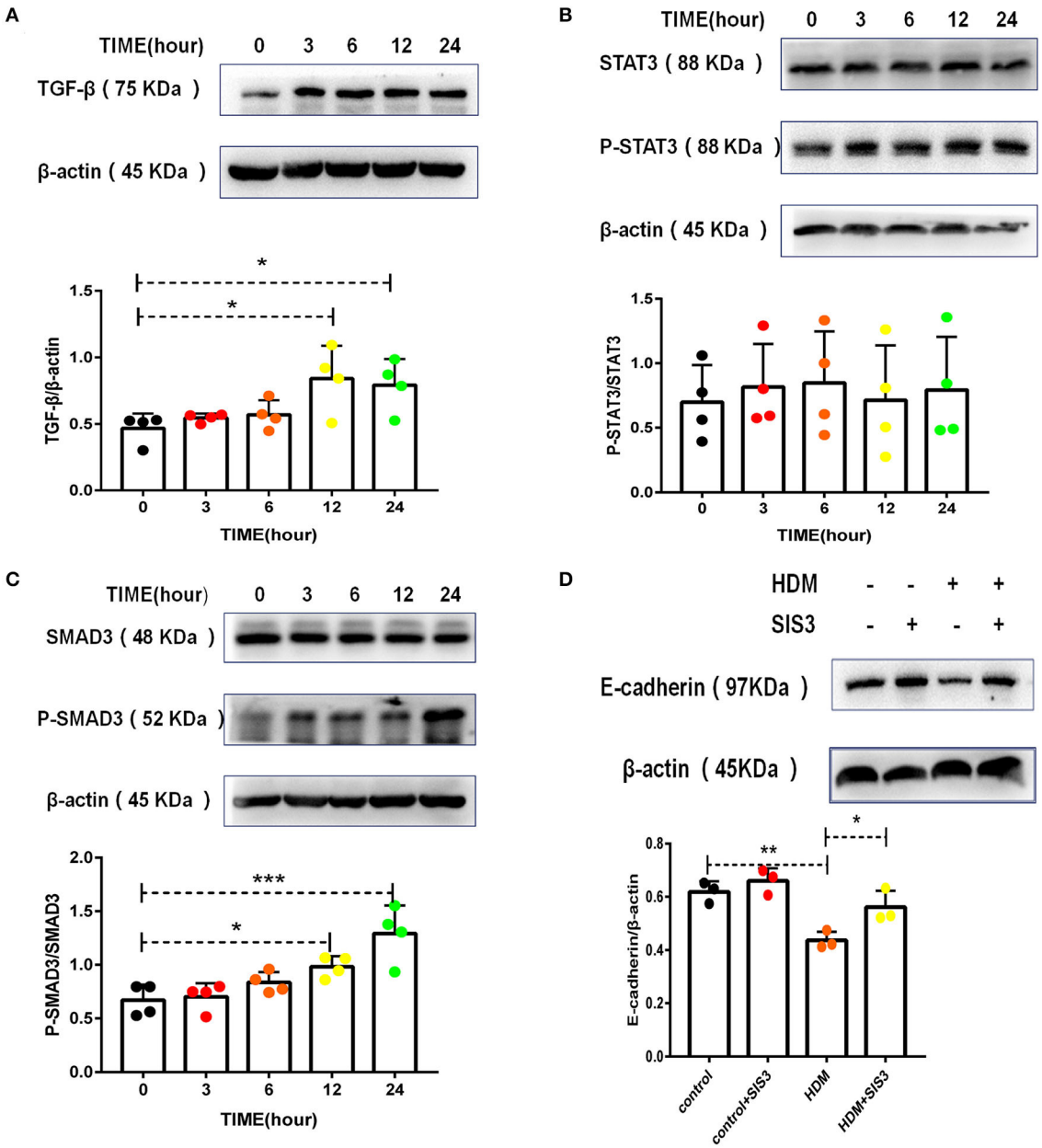

FIGURE 6 | TGF-β and SMAD3 played dominant roles in HDM-treated alveolar epithelial cell EMT. **(A)** Western blot analysis of TGF-β expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(B)** Western blot analysis of STAT3 expression in MLE-12 cells treated with HDM extract (100 μg/ml). **(C)** Western blot analysis of SMAD3 expression in MLE-12 cells treated with HDM extract. **(D)** Western blot analysis of E-cadherin expression in MLE-12 cells treated with HDM extract (100 μg/ml) and a SMAD3 inhibitor (SIS3, 10 μm) for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$.

mice (**Figure 7E**). Total IgE in sera was significantly elevated in the HDM-treated mice; however, the IgE concentration was comparable in WT and IL-33 KO mice challenged with HDM extract (**Figure 7F**). The expression of type 2 cytokines, including IL-4, IL-5, and IL-13, was increased in the HDM extract-treated mice. IL-33 deficiency reduced IL-4, IL-5, IL-13, and IFN-γ levels in the lung tissue of the HMD-treated mice (**Figure 7G**). These results suggest that IL-33 deficiency may alleviate asthma disease severity.

To further explore EMT in asthma, collagen I in pulmonary tissue was quantified, and the results showed that the level of collagen I was decreased in IL-33 KO mice compared to WT mice treated with HDM extract (**Figure 8A**). As expected, pulmonary tissue sections stained with PAS (**Figure 8B**) or Sirius red (**Figure 8C**) revealed that collagen deposition and glycogen storage were more pronounced in WT mice than in IL-33 KO mice. Consistent with the previous *in vitro* observations, decreased CD146 (**Figure 8D**) and elevated

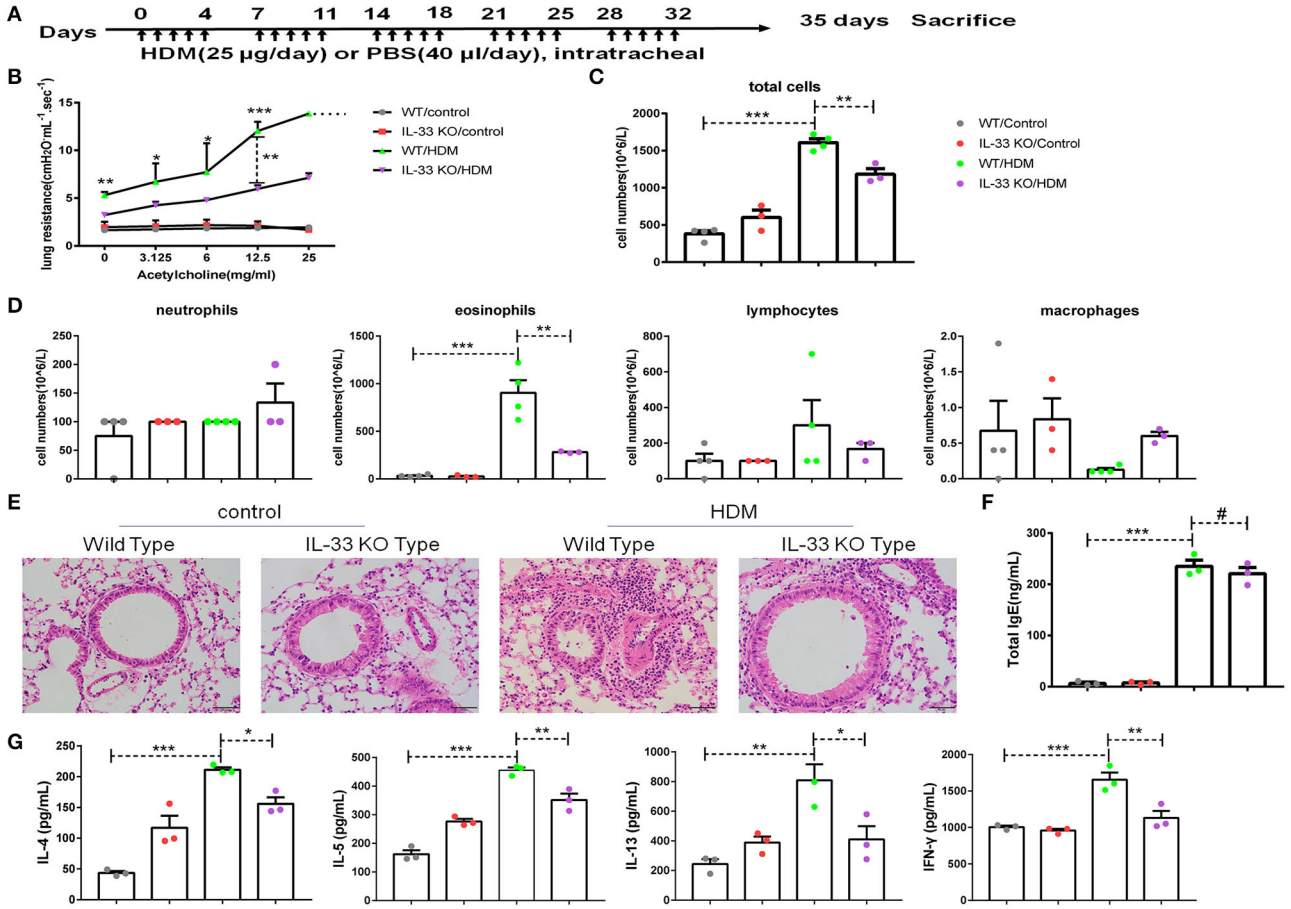

FIGURE 7 | IL-33 played an important role in a mouse model of asthma. **(A)** Flow chart showing the chronic asthma model (5 days/week for 5 weeks). **(B)** Lung resistance in mice was measured with the FinePointe RC System. **(C,D)** The numbers of total cells, eosinophils, neutrophils, and lymphocytes in BALF were quantified. **(E)** Representative images of lung sections stained with H&E. **(F)** ELISA analysis of total IgE levels in sera. **(G)** ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ levels in the supernatants of lung homogenates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P > 0.1$.

E-cadherin (**Figures 8D,E**) levels were observed in IL-33 KO mice compared to WT type mice after HDM treatment. As observed *in vitro*, Myd88, NF-κB, and p38 may be involved in EMT in the mouse model of asthma (**Supplementary Figure 1**). In summary, IL-33 deficiency alleviated disease severity and decreased CD146 expression and EMT in asthma.

CD146 Deficiency Decreased EMT in a Mouse Model of Asthma

To further evaluate the roles of CD146 in the asthma-associated EMT process, we established an asthma model in WT mice and CD146 KO mice. As shown in **Figure 9A**, lung resistance was reduced in CD146 KO mice compared to WT mice treated with HDM extract. The IgE level in the asthmatic WT mice and CD146-deficient mice was comparable (**Figure 9B**). In pulmonary tissues stained with H&E, the inflammatory response was decreased in the CD146 KO murine model of asthma (**Figure 9C**). Pulmonary cytokines, including IL-4, IL-5, IL-13, and IFN-γ, were decreased in CD146 KO mice compared to WT mice after HDM treatment (**Figure 9D**). Of note, IL-33 levels in asthmatic WT or CD146-deficient mice were comparable (**Figure 9E**). Because CD146 regulated EMT in alveolar epithelial cells, the level of collagen I was significantly decreased in the mouse model of asthma with a CD146 KO background (**Figure 10A**). Similarly, collagen deposition and glycogen storage in asthmatic CD146 KO mice were decreased, as evidenced by PAS (**Figure 10B**) and Sirius red staining (**Figure 10C**), respectively. Furthermore, CD146 deficiency caused an increase in E-cadherin in the asthma model (**Figures 10D,E**), suggesting that CD146 may orchestrate EMT in asthma.

Soluble CD146 Was Elevated in the Plasma of Asthma Patients

We demonstrated that CD146 contributed to asthma pathogenesis in a mouse model. CD146 is not only expressed on the cell membrane but could also be released into circulation (33). To demonstrate the clinical significance of the study, we

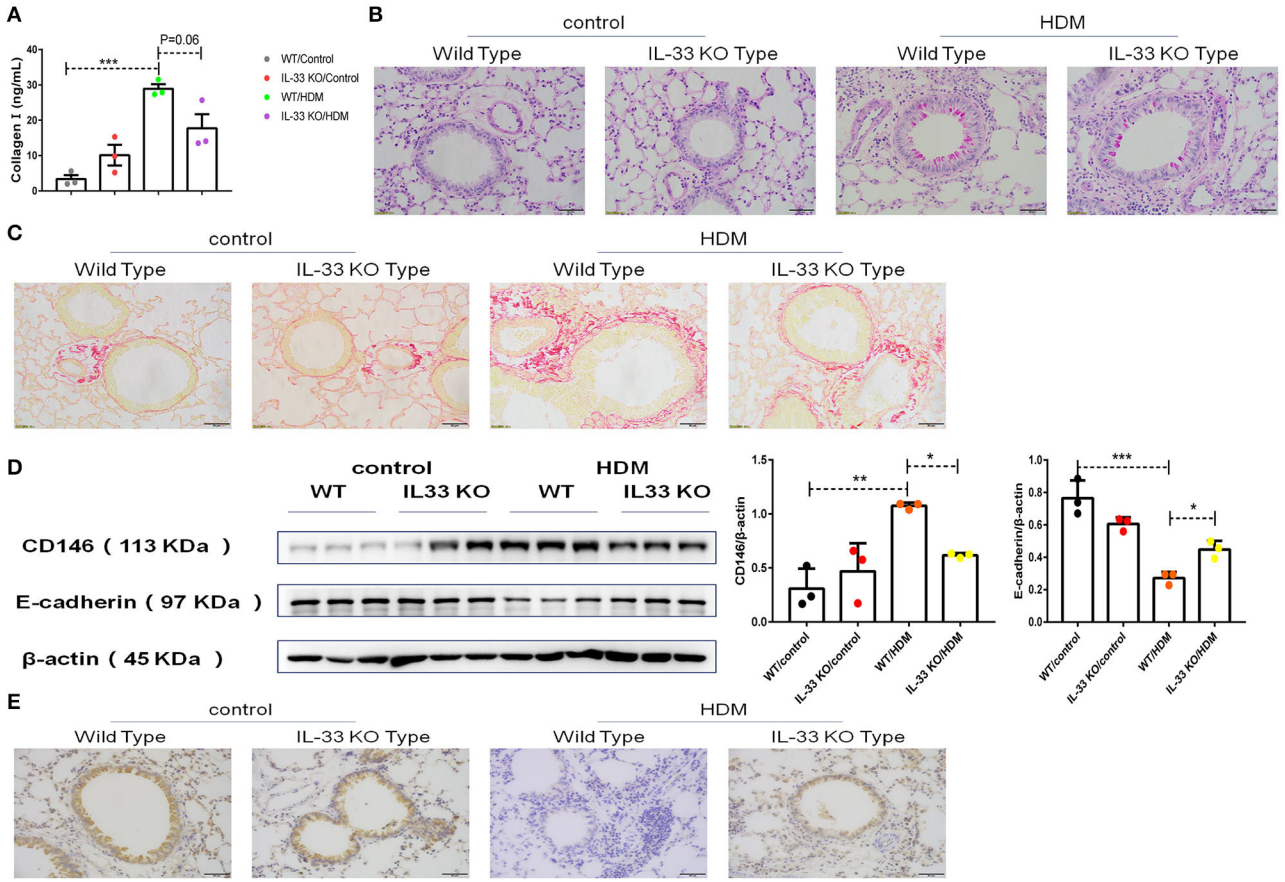

FIGURE 8 | IL-33 was essential for CD146 expression and EMT in a mouse model of asthma. **(A)** ELISA analysis of collagen-I levels in the lung homogenates of mice. **(B)** Representative images of lung sections stained with PAS. **(C)** Representative images of lung sections stained with Sirius red. **(D)** Western blot analysis of CD146 and E-cadherin expression in lung tissues. **(E)** Representative images of immunohistochemical analysis of E-cadherin expression in lung tissues. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

measured soluble CD146 (sCD146) levels in the plasma of asthma patients. As shown in **Figure 11**, the level of sCD146 was significantly increased in asthma patients compared to healthy controls. Considering that CD146 was increased in the airway epithelial cells of asthma patients (11, 12), we hypothesized that CD146 may be important in asthma.

DISCUSSION

In the present study, we first demonstrated that HDM extract promoted CD146 expression in alveolar epithelial cells via IL-33, and this effect was blocked with an antibody against the IL-33 receptor ST2. CD146, which was upregulated with an expression plasmid or downregulated with an siRNA plasmid, was found to play essential roles in E-cadherin expression in alveolar epithelial cells, suggesting that CD146 may mediate EMT in asthma. In a chronic asthma model in IL-33-deficient mice, CD146 expression was decreased in the pulmonary tissues, accompanied by increased E-cadherin expression, suggesting that IL-33 is essential in the CD146 expression and airway remodeling observed in asthma. Accordingly, CD146 deficiency in this chronic asthma model caused elevated E-cadherin expression, suggesting that CD146 deficiency reduced EMT in asthma. Moreover, we found that the level of soluble CD146 was increased in asthma patients. Therefore, we hypothesized that CD146 may mediate airway remodeling in chronic asthma in a manner that was dependent on the IL-33 signaling pathway.

In pulmonary epithelial cells, HDM extract stimulated CD146 expression and IL-33 production. As an alarmin molecule (34) and mucosal response amplifier (35), IL-33 binding with its receptor ST2 promoted CD146 expression. It has been demonstrated that IL-33 receptor knockout decreases the airway inflammatory response but induces the persistence of IL-5^{+} IL-13^{+} type 2 innate lymphocytes to maintain certain characteristics of asthma (36). Consistent with the above observation, we observed that IgE levels were comparable in the WT and IL-33 KO murine asthma models. In HDM-treated CD146 KO mice, the IL-33 concentration was similar to that in HDM-treated WT mice and was accompanied by comparable IgE levels in

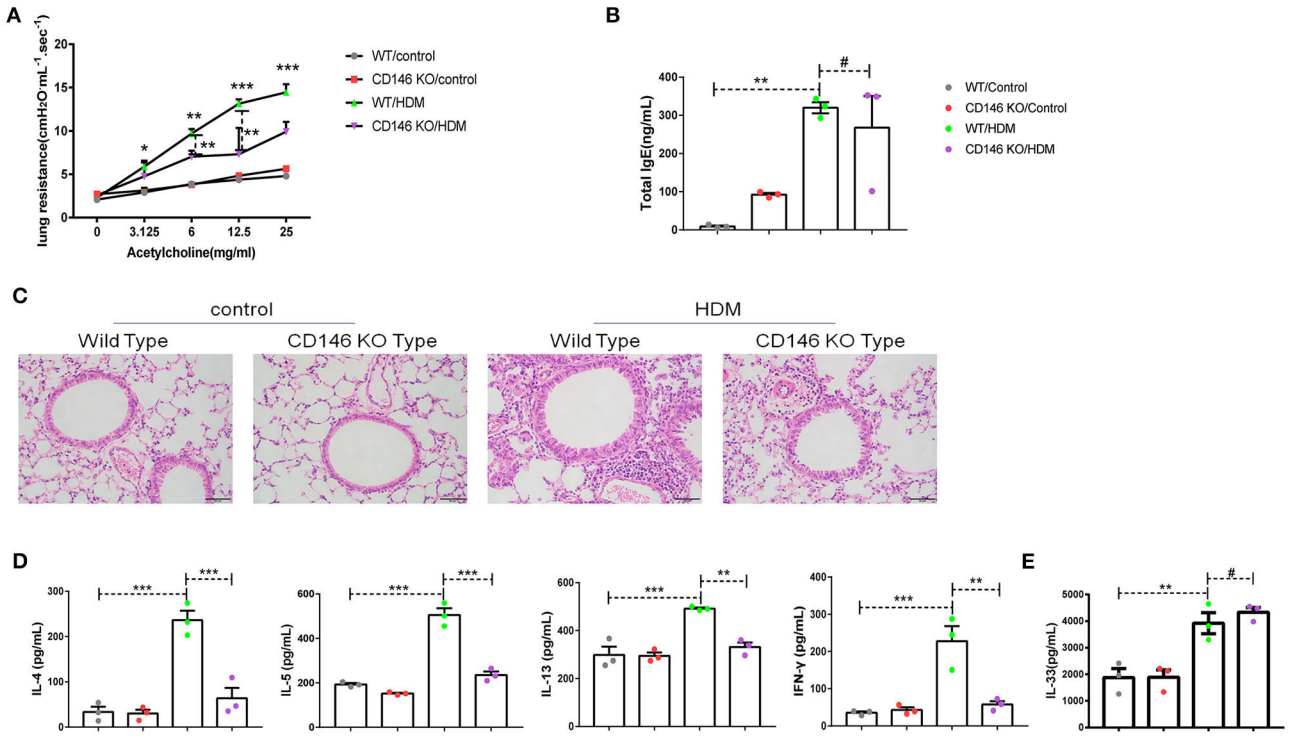

FIGURE 9 | CD146 deficiency mitigated disease severity in a mouse model of asthma. **(A)** Lung resistance in mice was measured with the FinePointe RC System. **(B)** Representative images of lung sections stained with H&E. **(C)** ELISA analysis of total IgE levels in sera. **(D)** ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ levels in the supernatants of lung homogenates. **(E)** ELISA analysis of IL-33 levels in the supernatants of lung homogenates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P > 0.05$.

WT and CD146 KO mice treated with HDM extract. These results suggest that IL-33 was not indispensable for IgE induction in asthma.

EMT has been reported to be intricately involved in airway remodeling in asthma (37, 38). In contrast, inhibition of the EMT process may slow airway remodeling in asthma (39). The increased expression of IL-33 in airway epithelial cells is closely related to the severity of asthma (40), and IL-33 has been shown to not only exacerbate airway inflammation (41) but also promote airway remodeling in asthma (42–44). Downstream signaling molecules of ST2, including MyD88, NF-κB p65, and MAPK, are then activated. However, only NF-κB p65 was indispensable for the CD146 expression observed in alveolar epithelial cells after stimulation with HDM extract. Because CD146 dimerization may activate NF-κB p65 (45), the reciprocal regulatory mechanisms between CD146 and NF-κB p65 warrant further study.

CD146 has been shown to be expressed by diverse cell types with multiple functions (7). In mouse tracheal epithelial cells, CD146 expression was accompanied by IL-13-mediated eotaxin-3 expression, suggesting that CD146 is an enhancer of the IL-13 response (46). In human primary nasal airway epithelial cells stimulated with TLR agonists, the absence of CD146 decreased expression of the inflammatory chemokine IL-8 (47), suggesting that CD146 may amplify inflammation. Consistent with the roles of CD146 in the inflammatory response, IL-4, IL-5, IL-1, and IFN-γ levels were significantly reduced in CD146-deficient mice with chronic asthma. Moreover, CD146 was directly linked to EMT in alveolar epithelial cells, and this relationship was dependent on the TGF-β/Smad-3 signaling pathway.

CD146 has been shown to be expressed on not only epithelial cells but also other cells, including endothelial cells (48), subpopulations of T cells (49), and mesenchymal stromal cells (MSCs) (50). All of these cell types may be involved in asthma pathogenesis and tissue remodeling (51, 52). In addition to epithelial cells, the roles of other $CD146^{+}$ cells in EMT and airway remodeling in asthma need to be elucidated in the future. Moreover, CD146 is shed from the cell membrane via MMP-3 activity (53). Elevated sCD146 levels in the plasma of asthma patients may enhance the production of vascular endothelial growth factor receptor (VEGFR) and VEGF2 (54). Therefore, we hypothesized that CD146 also regulated neovascularization, which is closely associated with EMT in asthma (55).

In summary, we expanded the role of CD146 in the EMT process from cancer metastasis to airway remodeling in asthma. We proposed that the binding of IL-33 to ST2 on HDM-stimulated airway epithelial cells promoted CD146 expression, which further amplified the inflammatory response, EMT and airway remodeling.

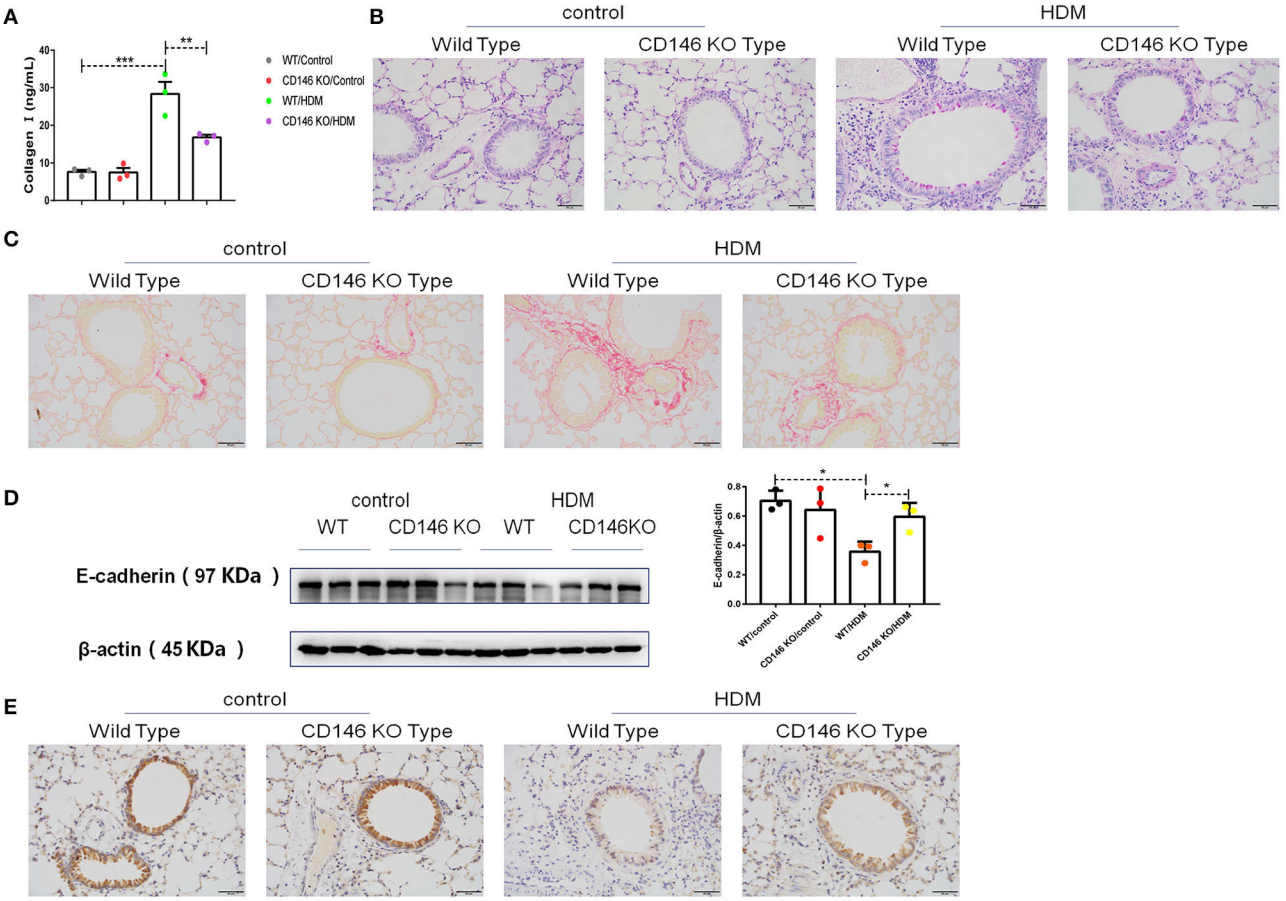

FIGURE 10 | CD146 deficiency decreased EMT in a mouse model of asthma. **(A)** ELISA analysis of collagen-I levels in the supernatants of lung homogenates of WT mice and CD146 KO mice. **(B,C)** Representative images of PAS- or Sirius red-stained lung sections from WT mice and CD146 KO mice. **(D)** Western blot analysis of E-cadherin expression in lung tissues from WT mice and CD146 KO mice. **(E)** Representative images of immunohistochemical analysis of E-cadherin expression in lung tissues from WT mice and CD146 KO mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

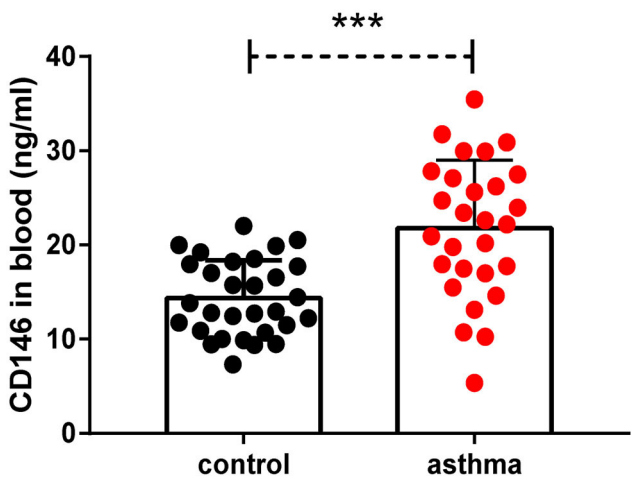

FIGURE 11 | Soluble CD146 was elevated in the plasma of asthma patients. The level of soluble CD146 in the plasma of asthmatic patients and healthy people was measured by ELISA. $^{***}P < 0.001$.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the First Affiliated Hospital of Nanjing Medical University (2017-SR-298). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Use Committee of Nanjing Medical University (IRB: 1709011).

AUTHOR CONTRIBUTIONS

ZS, MH, and MZ designed the experiments. ZS, QM, RZ, ZC, ZW, and FH performed the experiments and analyzed the data. NJ and CW collected and characterized the clinical samples.

MH and MZ conceived and supervised the project. ZS and MZ wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01598/full#supplementary-material

Supplementary Figure 1 | Western blot analysis of MyD88, p38, and p65 expression in lung tissues. $^{**}P < 0.01$; $\#P > 0.1$.

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Gestational Exposure to Cigarette Smoke Suppresses the Gasotransmitter H_2S Biogenesis and the Effects Are Transmitted Transgenerationally

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Rationale: Gestational cigarette smoke (CS) impairs lung angiogenesis and alveolarization, promoting transgenerational development of asthma and bronchopulmonary dysplasia (BPD). Hydrogen sulfide (H_2S), a proangiogenic, pro-alveolarization, and anti-asthmatic gasotransmitter is synthesized by cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and *3*-mercaptopyruvate sulfur transferase (3MST).

Objective: Determine if gestational CS exposure affected the expression of H_2S synthesizing enzymes in the mouse lung and human placenta.

Methods: Mice were exposed throughout gestational period to secondhand CS (SS) at approximating the dose of CS received by a pregnant woman sitting in a smoking bar for 3 h/days during pregnancy. Lungs from 7-days old control and SS-exposed pups and human placenta from mothers who were either non-smokers or smokers during pregnancy were analyzed for expression of the enzymes.

Measurements: Mouse lungs and human placentas were examined for the expression of CSE, CBS, and 3MST by immunohistochemical staining, qRT-PCR and/or Western blot (WB) analyses.

Results: Compared to controls, mouse lung exposed gestationally to SS had significantly lower levels of CSE, CBS, and 3MST. Moreover, the SS-induced suppression of CSE and CBS in F1 lungs was transmitted to the F2 generation without significant change in the magnitude of the suppression. These changes were associated with impaired epithelial-mesenchymal transition (EMT)—a process required for normal lung angiogenesis and alveolarization. Additionally, the placentas from mothers who smoked during pregnancy, expressed significantly lower levels of CSE, CBS, and 3MST, and the effects were partially moderated by quitting smoking during the first trimester.

Conclusions: Lung H_2S synthesizing enzymes are downregulated by gestational CS and the effects are transmitted to F2 progeny. Smoking during pregnancy decreases H_2S synthesizing enzymes is human placentas, which may correlate with the increased risk of asthma/BPD in children.

Keywords: gestational cigarette smoke, H_2S biogenesis, human placenta, lungs, transgenerational effect

INTRODUCTION

Maternal smoking during pregnancy remains relatively common (1, 2) and about 1/4th of mothers, who smoke during pregnancy, misreport as quitters (3). Epidemiological data and animal studies suggest that exposure to CS, including secondhand CS (SS) during pregnancy increases the risk of allergic asthma (AA) and BPD in the progeny (4–7); the latter encompasses alveolar simplification (8). Gestational exposure of mice to CS/SS impairs angiogenesis, exacerbates AA, and induces BPD-like alveolar simplification through downregulation of HIF-1α; this phenotype is transmitted to the F2 progeny (9–11). The mechanisms by which gestation CS promotes AA and BPD are unclear. H_2S is the newest member of gasotransmitter that affects many physiological systems (12). H_2S is an anti-inflammatory that promotes angiogenesis/vascularization and wound healing (7, 13). In the lung, H_2S attenuates lipopolysaccharide-induced acute lung injury (14), confers protection against ventilation-induced pulmonary inflammation and injury (15), promotes alveolarization and airway development (16), and protects against asthma and allergic inflammation (17, 18).

In mammals, H_2S is mainly produced from L-cysteine by three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfur transferase (3MST) (19, 20). The distribution of these enzymes in various tissue is somewhat uncertain. It is generally believed that CSE and CBS are the two most prominent H_2S synthesizing enzymes, where CBS is primarily localized to the brain and CSE in non-neuronal tissues (21, 22). However, this is not an inflexible rule. For example, adult rat lung expresses CSE and 3MST, but insignificant levels of CBS (23), but CBS has been reported in airway vasculature and lung epithelial cells, and CSE is present in the lung parenchyma (16). All three H_2S synthesizing enzymes (CSE, CBS, and 3MST) are present in the lungs of cows and sea lions (24) and lung biopsies from non-small cell lung cancer patients (25) and the lung epithelial cell line A549 (26) also express all the three enzymes. In a recent report, 3MST was shown to be upregulated in the lung adenocarcinoma (27). Similarly, while the portal vein and thoracic aorta contain CSE, ileum expresses both CSE and CBS (28). Thus, the expression of H_2S enzymes depends on the tissue type and the state of cell differentiation.

Epithelial mesenchymal transition (EMT) is an important process for cell differentiation during development, organogenesis, and carcinogenesis (29, 30). While dysregulated EMT in the adult lung promotes multiple respiratory diseases, it is indispensable for the development of lung epithelium (31), where the TGF-β/Smad pathway plays a key role (31, 32). Although, H_2S has been shown to inhibit EMT in lung cancers through Wnt/Catenin signaling and the activation of HIF-1α (25, 33), HIF-1α is dramatically downregulated by gestational CS in the 7-days old mouse lung (11, 34) and, in some lung injuries, H_2S promotes EMT and lung repair (35, 36). Moreover, HIF-1α mediates cellular differentiation through TGF-β (37, 38)—a key participant in EMT (39, 40). Thus, EMT is important in lung development and organogenesis, and requires H2S-induced HIF-1α/TGF-β.

In this communication we demonstrate that gestational SS suppresses TGF-β, EMT, and anti-asthmatic factors, and the 7-days old mouse lung and human placentas contains all the three H_2S synthesizing enzymes. Gestational exposure to CS suppresses the expression of these enzymes in the mouse lung and human placentas from mothers' who smoke during pregnancy. The latter prompts the possibility that the placental levels of H_2S synthesizing enzymes may correlate with the risk of AA and BPD in children.

Abbreviations: AA, allergic asthma; BPD, bronchopulmonary dysplasia; CBS, cystathionine-β-synthase; CCSP, Clara-cell secretory protein; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, cystathionine-γ-lyase; CS, cigarette smoke; EMT, epithelial mesenchymal transition; FA, filtered air; GesCS, gestational cigarette smoke; GesCS1/3, mothers who quit smoking during the 1st trimester; HIF-1α, hypoxia Inducible factor-1α; H_2S, Hydrogen sulfide; 3MST, 3-mercaptopyruvate sulfur transferase; MFI, mean fluorescence intensity; NaHS, sodium hydrosulfide; SS, secondhand CS; SP-C, surfactant protein-C; TGF-β, transforming growth factor beta; ZO-1, zonula occludens-1.

MATERIALS AND METHODS

Animals

Pathogen-free BALB/c mice were purchased from the FCR Facility (Frederick, MD). The animals were housed at the Animal Facility of Lovelace Respiratory Research Institute, Albuquerque, NM in accordance with the Guidelines from the Association for the Assessment and Accreditation for Laboratory Animal Care International. Animals were kept in exposure chambers maintained at 26 ± 2°C with 12-h light/dark cycle. Food and water were provided *ad libitum*.

Study Approval

All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with the Guide for Laboratory Animal Practice under the Association for the Assessment and Accreditation for Laboratory Animal Care International.

Gestational Exposure to Sidestream Cigarette Smoke (SS)

Adult (3–4 months old) male and female mice (BALB/c) were separately acclimatized to SS or filtered air (FA) for 2 weeks before being paired for mating under the same exposure conditions. Briefly, mice were exposed to whole-body SS or FA for 6 h/days, 7 days/weeks (total particulate matter 1.52 ± 0.41 mg/m^3) using Type 1,300 smoking machine (AMESA Electronics, Geneva, Switzerland) that generated two 70 cm^3 puffs/min from 2R1 cigarettes as described previously (9, 10). The dose of SS was approximately equivalent to the amount of SS a pregnant woman would receive by sitting in a smoking bar for 3 h/days throughout the gestational period (10). After pregnancy was established, male mice were removed and the pregnant mice continued to receive SS or FA until the pups were born. Immediately after the birth of pups the exposures were stopped. On the postnatal day 7, some animals were sacrificed by an intraperitoneal injection of 0.2 ml Euthasol. Some adult F1 mice from FA and SS groups were mated to obtain the F2 progeny as described previously (10). Representative results are presented using animal from two different sets of SS-exposure. At least 15 animals per group were used; each analysis used 5 mice/group and the analysis was repeated twice. Specific details are given under figure legends.

Human Placenta Samples

Placentas were collected at the University of New Mexico Hospital (UNMH), Albuquerque, NM according to protocol #17-064 approved by the University of New Mexico Medical Center Institutional Review Board and Human Research Protection Office in accordance with the NIH guidelines. All donors had agreed to donate the tissues. We were able to collect 10 placentas in a span of 7 months (by Dr. A. Sheybani and Dr. V. Exil, both from UNMH) representing three controls (mothers who did not smoke during the pregnancy), 4 CS-exposed (mothers who smoked throughout the pregnancy), two first-trimester quitters (mothers who stopped smoking during the first trimester of pregnancy), one false-control (mother who claimed to have quitted smoking during pregnancy, but the placenta had high level of cotinine). A 3 cm^3 section of each placenta was dissected and frozen immediately for RNA and protein assays. Rest of the placentas were kept at -80°Cuntil use. Tissue slides (5 μm) were prepared by the institutional Pathology Core.

Determination of Cotinine Levels in Placentas

The smoking status of the mothers was confirmed by determining the cotinine levels in the placental tissues using the cotinine ELISA kit (Calbiotech Inc., CA) with a sensitivity of 5 ng/ml. Immunoblots were developed using placental homogenates.

Assays for H_2S Synthesizing Enzymes

The expression of CSE, CBS, and 3MST was determined by WB analysis, IF-IHC, and/or qPCR. Assay details are given under relevant figure legends.

Immunostaining and Immunofluorescent Imaging

For immunohistochemical (IHC) staining, deparaffinized and hydrated lung and placental tissue sections were washed in 0.05% v Brij-35 in PBS (pH 7.4) and immunostained for antigen expression as described previously (41). Briefly, the antigens were unmasked by steaming the sections in 10 mM Citrate buffer (pH 6.0) followed by incubation in a blocking solution containing 3% BSA, 1% Gelatin and 1% normal donkey serum with 0.1% Triton X-100 and 0.1% Saponin. Serial sections were stained with antibodies to Vimentin, E-cadherin, and ZO-1 (Invitrogen Inc., Carlsbad, CA), or isotype control antibodies. The immunolabelled tissues were detected using respective secondary antibodies conjugated with fluorescent dyes (Jackson ImmunoResearch Lab Inc., West Grove, PA). Where indicated, the sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) containing Fluormount-G (SouthernBiotech, Birmingham, AL) to visualize nuclei. Immunofluorescent images were captured with BZX700 Microscopy system (Keyence, Tokyo, Japan). Specific details are given under appropriate figure legends.

Western Blot Analysis

Western blot (WB) analysis of mouse lung and human placenta homogenates was carried out as described previously (10). Briefly, lung or placental tissues were homogenized in RIPA buffer and the protein content of the extracts was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL). The homogenates were run on SDS-PAGE on 10% precast polyacrylamide gels (BioRad Lab, Hercules CA). The gels were transferred electrophoretically to nitrocellulose membranes (BioRad Lab). The blots were incubated with the respective antibodies. The mouse anti-β actin antibody (Santa Cruz Biotech) was used as the house-keeping protein. After incubating with an appropriate secondary antibody, the blots were developed with Amersham ECL Western Blotting Detection Reagent (GE Healthcare Bio-Science Corp. Piscataway, NJ) and the images were captured by Fujiform LAS-4000 luminescent image analyzer (FUJIFILM Corporation, Tokyo). Densitometry was used to quantitate the expression of specific proteins and expressed as the protein/β-actin band ratio.

Quantitative Real-Time PCR (qPCR)

Total RNA was extracted by using a ToTALLY RNA kit (catalog number AM1910; Ambion, Austin, TX, USA). RNA samples were quantified by using a NanoDrop spectrophotometer and quality was analyzed on an RNA Nano-drop by using the Agilent 2100 bioanalyzer (Agilent Technologies). Synthesis of cDNA used 1 μg of total RNA in a 20 μl reaction mixture and TaqMan Reverse Transcription Reagents kit from ABI (catalog number N8080234; Applied Biosystems). qPCR amplification (performed in triplicate) used 1 μl of cDNA in a total volume of 25 μl of Faststart Universal SYBR green master mix (Roche Applied Science #04913850001). The mRNA sequences for CSE, and CBS for mouse and human reported under GenBank accession numbers NM145953 (CSE mouse), NM144855.3 (CBS mouse), NM_001902 (CSE human), and NM000071 (CBS (human)

and were used to design primers for qRT-PCR assay (42–44). Expression of 3MST mRNA was performed using total RNA from lung and placental tissues by qPCR analysis and One-Step Real-Time PCR MasterMix containing TaqMan probes and a specific-labeled primer/probe set (Applied Biosystems). 18S RNA was used as housekeeping gene for normalization. PCR assays were run in the ABI Prism 7500 Sequence Detection System. Triplicate cycle threshold (C_T) values were analyzed using the comparative C_T ($\Delta\Delta C_T$) method as per manufacturer's instructions (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalization to the endogenous reference (18S) sample. RNA isolation, primer design, and qRT-PCR assays were performed using the Molecular Genomic Core, UTMB, Galveston, TX.

Statistical Analysis

Grouped results were expressed as mean ± SD and $p \leq 0.05$ were considered significant. The data were normalized via natural log transformations and when the data was normally distributed, statistical significance among the groups was determined by one-way ANOVA with Bonferroni correction with multiple pairwise comparisons. When the data was not normally distributed, we used Kruskal-Wallis assessment on ranks followed by Dunn's multiple comparison tests. Student's *t*-test was employed for comparison between two groups at 95% confidence interval using Prizm software (GraphPad Software Inc., San Diego, CA). $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Gestational SS Inhibits EMT in the F1-Progeny Lung

Gestational exposure to SS impairs alveolarization and promotes BPD in the progeny, and these effects are transmitted to the F2 progeny and associated with suppressed levels of HIF-1α (11, 34). EMT is a biological process that allows epithelial cells to assume mesenchymal phenotype, which is critical for normal alveolarization (45) and regulated by HIF-1α, TGF-β, and VEGF (38, 39, 46, 47). VEGF promotes angiogenesis that stimulates EMT and alveolarization (48, 49) and intratracheal transplantation of mesenchymal stem cells attenuate lung injury in newborn mice (50). During embryogenesis and organ development, epithelial markers such as E-cadherin and ZO-1 are decreased and mesenchymal markers such as vimentin are increased (48, 51). The transcription factor HIF-1α promotes synthesis of TGF-β–the most potent inducer of EMT (52, 53) and HIF-1α is potently reduced in gestationally SS-exposed lungs (11). To determine whether gestational SS affected EMT, we determined the lung levels of epithelial (E-cadherin and ZO-1) and mesenchymal (vimentin) cell markers by IHC and Western blot analysis in 7-days old lungs from control and gestationally SS-exposed mice. Compared to control lungs, levels of E-cadherin (**Figure 1A**) and ZO-1 (**Figure 1B**) were significantly higher than those of vimentin (**Figures 1A,C**) in gestationally SS-exposed lungs. Moreover, the concentrations of TGF-β by Western blot analysis (**Figure 1D**) and of the anti-asthmatic factor SOX2 by IHC (**Figure 1E**), were significantly lower in gestationally SS-exposed lungs. SOX2 is a pluripotent transcription factor in bronchoalveolar progenitors, which promotes the Club cells to express Clara-cell secretory protein (CCSP) and surfactant protein-C (SP-C) (54). CCSP and SP-C are suppressed by gestational SS (10) and reduced SOX2 and CCSP levels are associated with higher risk of asthma (55); humans and mice deficient in CCSP, exhibit airway hyperresponsiveness (56). Together, these results suggest that gestational exposure to CS inhibits EMT and is associated with decreased numbers of SOX2-positive Clara cell progenitors.

Gestational SS Suppresses CSE and CBS in the F1 and F2 Progeny Lungs

H_2S is required for normal angiogenesis and alveolarization (16, 25) and produced in the periphery mainly by CSE and CBS (12). H_2S attenuates lung injury (15, 57) and CSE deficiency exacerbates airway hyperreactivity (44) and impairs alveolarization. Impaired angiogenesis and alveolarization in CSE- and CBS-deficient mice are partially restored by H_2S donor compounds (16). Moreover, H_2S levels are lower in the exhaled air from asthma and COPD patients and correlates with lower FEV_1 (58). Expression level of H_2S enzymes is reported to be tissue specific. Thus, the brain and the vascular endothelium have a strong expression of CBS and CSE, respectively; however, both tissues also express 3MST (22). The situation in the lung is somewhat confusing. Lungs were reported to primarily express CSE (21); however, human lung cell lines such as A549 (26) and the lungs from cow and sea lions express all the three H_2S synthesizing enzymes (24).

To ascertain whether gestational SS affected H_2S production in the lung, we determined the mRNA levels of CSE (**Figure 2A**) and CBS (**Figure 2B**) and 3MST (**Figure 2C**) by qPCR in 7-day-old lungs from control and SS-exposed mice. Gestational CS inhibited mRNA levels of CSE, CBS, and 3MST, which would decrease the level of H2S in the lung and increase the risk of inflammatory lung diseases in these animals.

Gestational CS/SS/nicotine increases the risk of asthma and/or BPD transgenerationally in humans and animals (10, 59–61). To determine whether the transgenerational pro-asthmatic/pro-BDP effects of gestational SS were related to changes in H_2S, 7-days old lungs from control and gestationally SS-exposed F1 and F2 mice were analyzed for CSE (**Figure 2D**) and CBS (**Figure 2E**) levels by IHC staining. Results showed that control lungs contained about equal numbers (∼25%of total cells) of CSE- and CBS-positive cells, and gestational exposure to SS significantly reduced the number CSE/CBS-positive cells in both F1 and F2 animals. Thus, as reported for HIF-1α, angiogenesis, and alveolar volumes (11), gestational SS suppresses the levels of CSE and CBS in F1 progeny and the effects are transmitted to F2. Given the relationship between HIF-1α, TGF-β, EMT, angiogenesis, alveolarization, BPD, AA, and H_2S, it is likely that the CS-induced proinflammatory lung responses in F1 and F2 progenies are related to

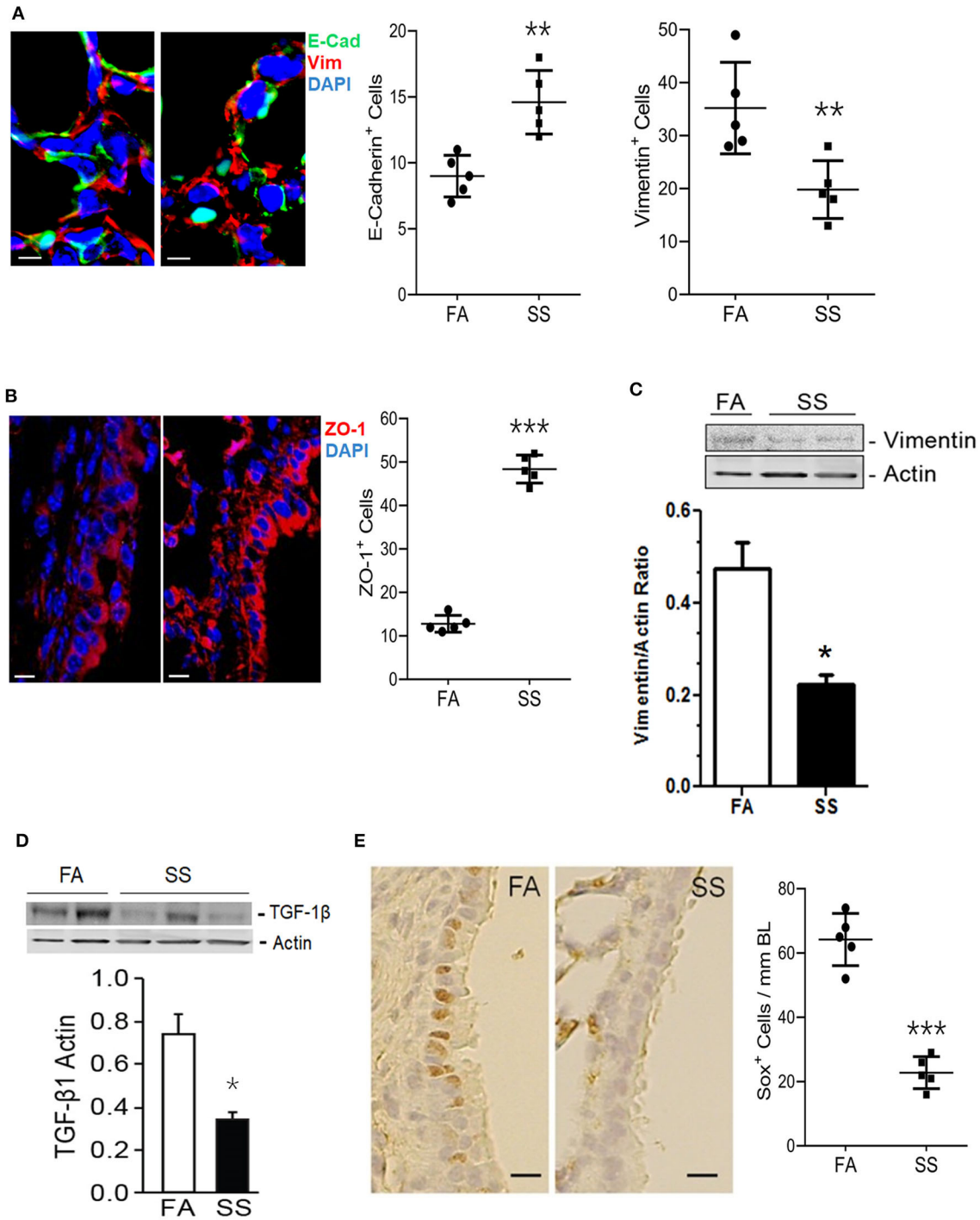

FIGURE 1 | Gestational exposure to SS inhibits EMT in the mouse F1 lung. **(A)** Representative micrographs of lung sections from mice exposed gestationally to filtered-air (FA) or side-stream cigarette smoke (SS) and co-stained with vimentin (red) and cadherin (green); DAPI-stained nuclei (blue). E-Cadherin$^+$ and Vimentin$^+$ cells per unit area (18,000 μm^2) were counted blind using NDP View on a Nanozoomer (Hamamatsu Photonics Inc.). **(B)** Representative micrographs of lung sections from gestationally FA or SS-exposed mice and stained with ZO-1 (red). ZO-1$^+$ cells (12,400 μm^2; NDP scanner). **(C)** Representative Western blot of lung tissue homogenates (70 μg) from FA or SS-exposed mice and probed with anti-vimentin antibody. Lower panel is the densitometry of the blot and expressed as Vimentin/Actin ratio. **(D)** Western blot analysis of lung tissue homogenates (70 μg) from FA or SS-exposed lungs probed with anti-TGF-β1 antibody (Cat# ab92486, Abcam). Lower panel is densitometry of the blot presented as TGF-β1/Actin ratio. **(E)** Representative image of the lung sections (5 μm) stained with anti-Sox2 antibody and detected by immunohistochemical staining. Right panel shows Sox$^+$ cells (17,000 μm^2; NDP scanner) counted blind. Data shown as mean±SD ($n = 5$/gp; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

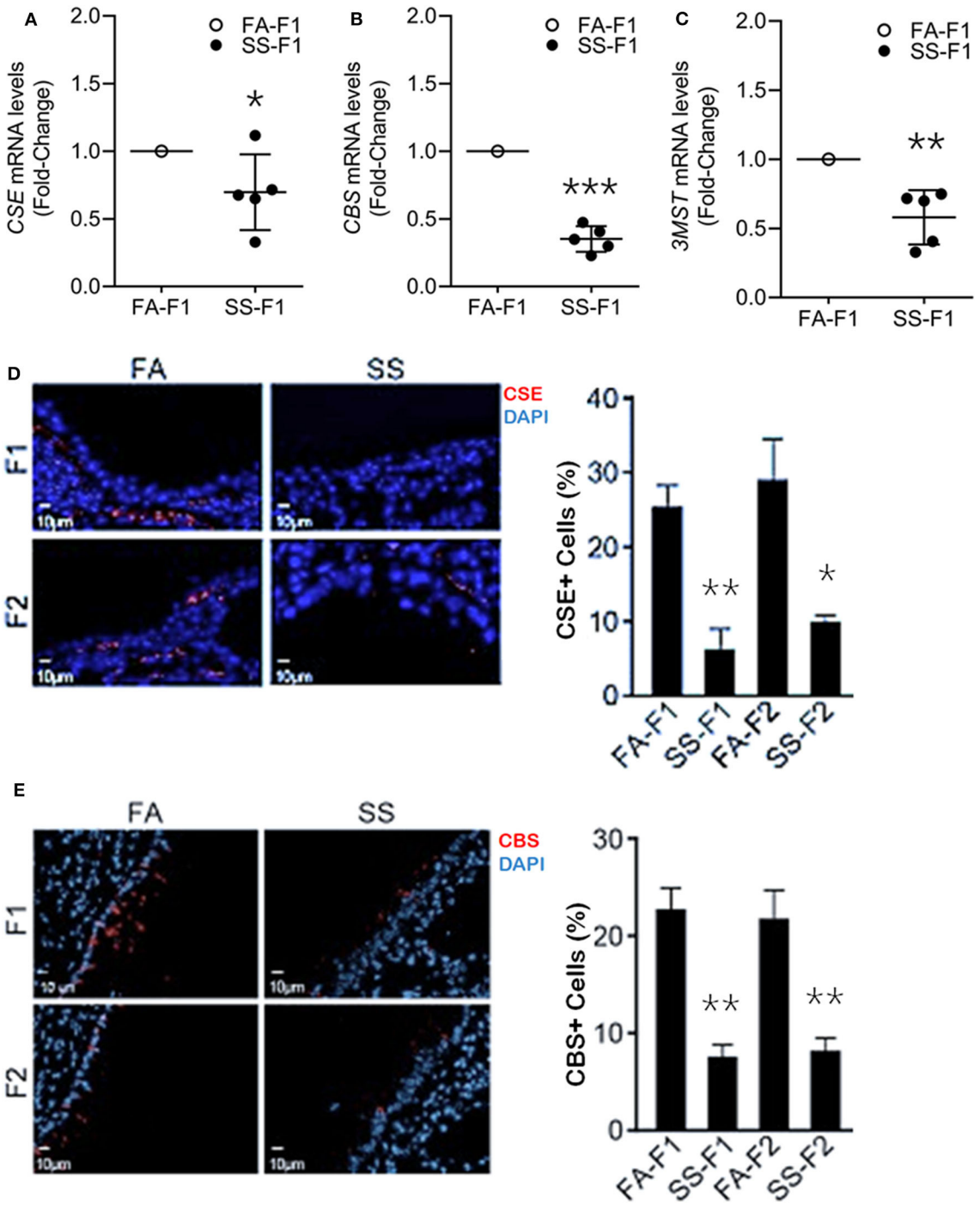

FIGURE 2 | Gestational SS suppresses H2S biogenesis enzymes, CSE, CBS and 3MST in 7d old F1 mouse lung. Relative mRNA levels of *CSE* **(A)**, *CBS* **(B)**, and *3MST* **(C)** in F1 lungs exposed gestationally to FA or SS. **(D)** Representative micrographs of lung sections from F1 and F2 progenies exposed gestationally to SS or FA. Sections were stained for CSE (red) and DAPI (blue). Right pane is quantitation of CSE^+ cells (%) in each group. **(E)** Micrographs of lung sections from F1 and F2 stained for CBS (red) and DAPI (blue). Right panel shows quantitation of CBS^+ cells (%) in each group. Data shown as mean ± SD ($n = 5$/gp; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

changes in H_2S levels regulated by H_2S synthesizing enzymes. These data suggest that mouse lungs contain all three H_2S synthesizing enzymes and gestational exposure to CS suppresses their expression. Reduced levels of these enzymes has the potential to promote lung diseases such as asthma and BPD.

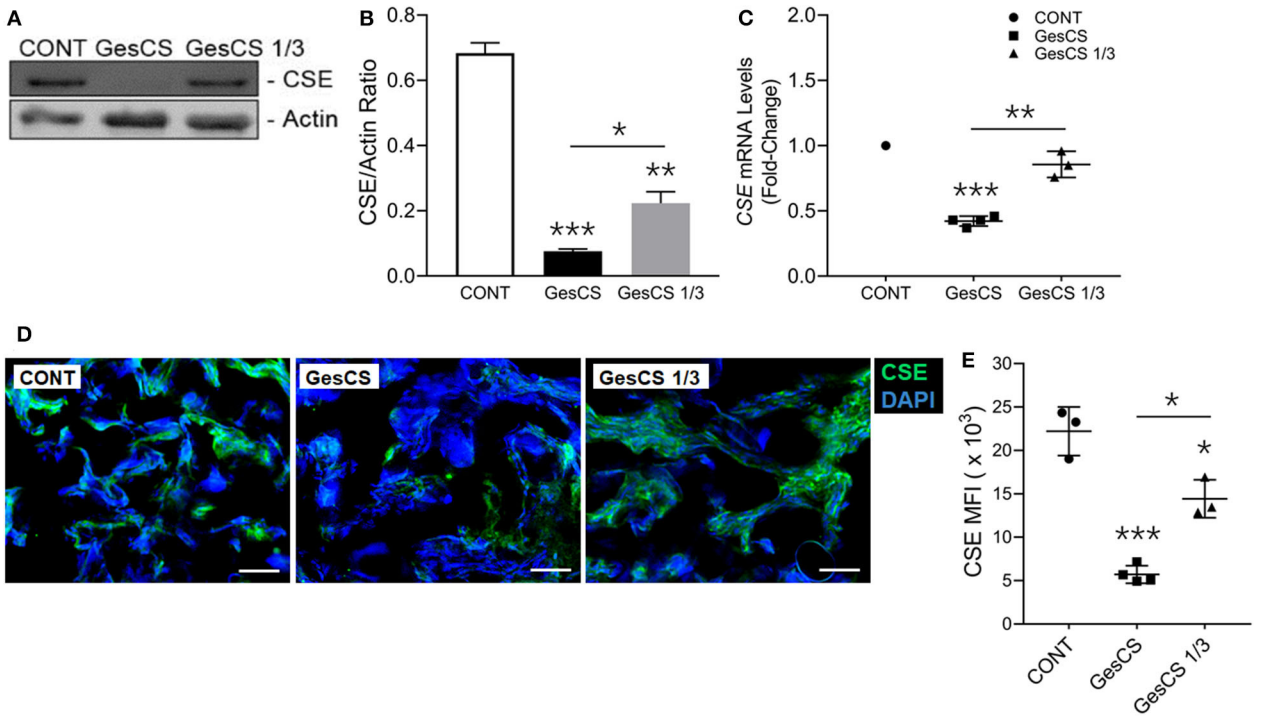

FIGURE 3 | Gestational exposure to CS suppresses H2S biosynthetic enzyme CSE in human placenta. Placentas were analyzed for CSE expression using WB, qPCR and IF-IHC staining. **(A)** Western blot analysis of the placental tissue homogenate (150 μg protein) with anti-CSE antibody (Abcam, MA, USA). **(B)** Densitometry of CBS normalized to β-actin levels. **(C)** *CSE* mRNA detection by qPCR and expressed relative to CONT group. **(D)** Representative micrographs showing placental CSE (green) along with DAPI-stained nuclei (blue), scale−10 μ. **(E)** Quantification of CSE expression by MFIs (mean fluorescence intensity). CONT, control non-smoker; GesCS, cigarette smoking during whole pregnancy; GesCS1/3, CS exposure during first trimester; Data shown as mean ± SD (n = 3–4/gp; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Placentas From Women Who Smoked During Pregnancy Express Low Levels of H_2S Synthesizing Enzymes

Children from women who smoke during pregnancy have increased risk of AA and BPD (4–7) and herein our results suggest that the increased susceptibility may correlate with decreased levels of H_2S synthesizing enzymes. Thus, the lung levels of H_2S enzymes at birth may predict the risk of AA and BPD in children; however, it is unrealistic to obtain lung samples from newborn babies. Because, H_2S synthesizing enzymes are present in most tissues (12, 62), we ascertained whether the enzymes were present in human placentas and, if so, whether smoking during pregnancy affected their expression. We were able to obtain 10 human placentas representing 3 controls (mothers who did not smoke during the pregnancy), four CS-exposed (mothers who smoked throughout the pregnancy), two first-trimester quitters (mothers who stopped smoking during the first trimester of pregnancy), one false-control (mother who claimed to have quitted smoking during pregnancy, but the placental showed high cotinine). Cotinine was determined on all placentas by ELISA to ensure that the tissues were from smoking/non-smoking mothers. CSE expression was determined by WB analysis, qPCR, and IF-IHC; CBS by WB and IF-IHC, and 3MST by qPCR analysis.

Immunoblot analysis of the placental homogenates from mothers who smoked throughout the pregnancy (GesCS) showed very low expression of CSE as compared to control non-smokers (CONT) or the mothers who quit during the 1st trimester (GesCS1/3) (**Figures 3A,B**). Similarly, as determined by qPCR analysis, CSE-specific mRNA content of GesCS placentas was significantly lower than CONT and GesCS1/3 (**Figure 3C**). Although the protein content of CSE in GesCS1/3 was higher than GesCS, it was still significantly lower than CONT (**Figure 3B**), suggesting that quitting smoking during the first trimester may be beneficial; however, the effects are not totally reversible and may persists after the birth. CSE expression was further confirmed by immunostaining of placental sections showing a 4-fold lower expression of CSE in GesCS than CONT; CSE expression in GesCS1/3 placentas was intermediate between CONT and GesCS (**Figures 3D,E**).

WB analysis also indicated that the expression of CBS was lower in GesCS than CONT or GesCS1/3 (**Figures 4A,B**). Furthermore, IHC analysis of CBS in placentas showed the expression was 3-fold lower in GesCS than CONT; however, the difference between CONT and GesCS 1/3 groups was not statistically significant (**Figures 4C,D**). We also examined the status of 3MST mRNA expression in human placentas by qPCR. Like CSE and CBS, exposure to cigarette smoke significantly

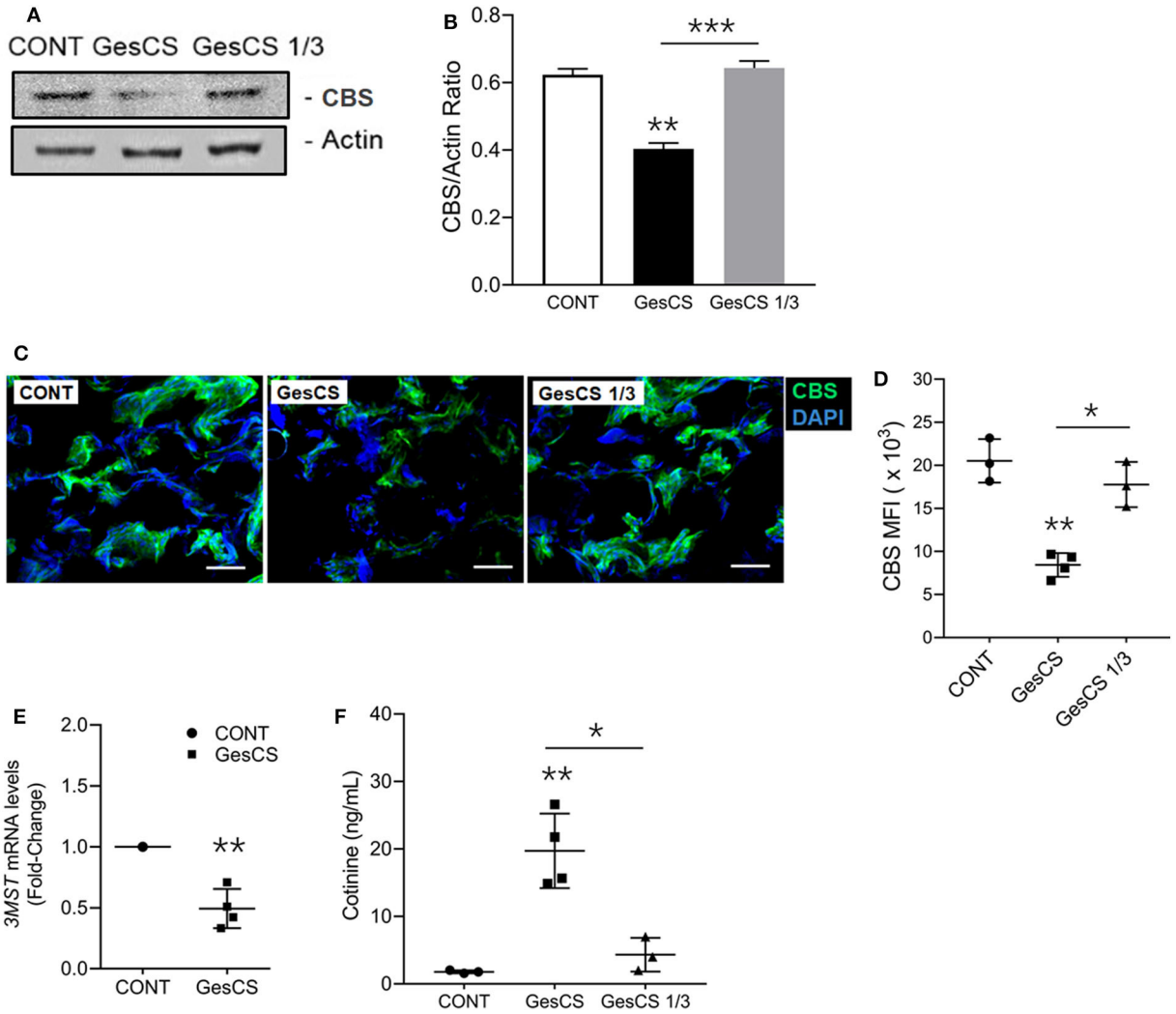

FIGURE 4 | Gestational exposure to CS suppresses CBS and 3MST in human placenta. **(A)** Western blot analysis of placental tissue homogenates (150 μg protein) probed with anti-CBS antibody (Abcam, MA). **(B)** Densitometry of CBS normalized to β-actin. **(C)** Representative micrographs showing placental CBS (green) and DAPI-stained nuclei (blue), scale−10 μ **(D)** Quantification of CBS expression by MFI (mean fluorescence intensity) of CBS-immunoreactive fluorescence. **(E)** Quantitative RT-PCR of *3MST* mRNA expression *(n* = 5). **(F)** The smoking status of mother's was ascertained by the cotinine levels in the placental homogenate using cotinine ELISA kit (Calbiotech Inc., CA) with sensitivity of 5 ng/ml. CONT, non-smoker control; GesCS, cigarette smoker during pregnancy; GesCS1/3, CS exposure during first trimester; Data shown as mean ± SD (*n* = 3–4/gp; **p* < 0.05; ***p* < 0.01; ****p* < 0.001).

inhibited the expression of 3MST (**Figure 4E**) indicating that, like CSE and CBS, gestational CS also downregulates 3MST expression in human placentas.

The smoking status of the mothers who donated the placentas was verified by measuring the cotinine levels (**Figure 4F**) and, in general, corroborated their assertion. However, we observed one outlier, where the WB and qPCR analyses of the placenta indicated very low levels of CSE (data not shown), yet the donor claimed to have quitted smoking during the pregnancy. The placenta contained high cotinine levels and was not included in the analyses. Sadly, it is not uncommon for the mothers to falsely assert quitting smoking during the pregnancy (3, 63).

The current study does not clearly define the stage(s) of pregnancy, where the fetus is completely resistant to the effects of CS on placental H_2S enzymes. While the epidemiological evidence strongly suggests that CS exposure during pregnancy promotes wheeze and asthma in children (64), but the identity of the susceptible stage(s) of the pregnancy is not unequivocal and may vary from first trimester (6) to third trimester (5). Our data with placental levels of H_2S enzymes suggest that the effects of smoking during first trimester are moderate, but not negligible; however, we have not correlated these levels to the actual incidence of asthma in the progeny. Interestingly, perinatal exposure to nicotine induces asthma in rats (61), suggesting that late stages of embryonic development might be more sensitive

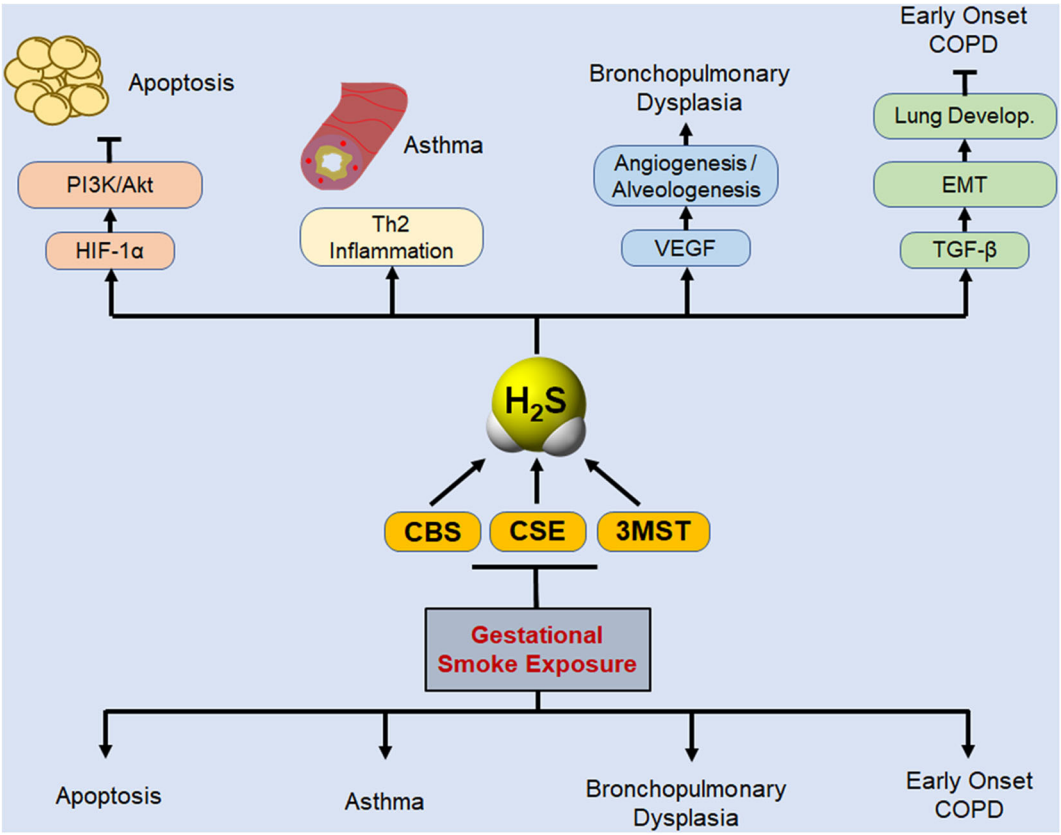

FIGURE 5 | Schematic representation of potential protective role of biosynthesized H_2S in basic lung pathophysiologies that are disrupted by gestational exposure to CS. Gestational exposure to CS is primarily linked to four major pulmonary predicaments: cell apoptosis, asthma, BPD, and susceptibility to early development of COPD. Lung cell apoptosis is associated with allergic inflammation (18) and decreased levels of HIF-1α and PI3K/Akt (34). PI3K/Akt inhibits apoptosis and promotes cell proliferation (67), and activation of Akt protects the neonatal lung against injuries (68). H2S donors inhibit apoptosis, attenuate lung damage, and promote normal lung development (21, 69). Asthma development as a consequence of gestational CS exposure or the deficiency of H_2S enzymes is associated with increased Th2 inflammation (9, 17, 70), and exogenous H_2S was shown to reverse the exacerbated asthma response in CSE-deficient mice (17). Gestation SS suppresses VEGF and angiogenesis, leading to impaired alveolarization and BPD (11, 34), and H_2S stimulates VEGF expression and angiogenesis, (71), and alveolarization (72, 73). Maternal smoking affects lung development and has been linked to early onset of COPD in the progeny (74, 75). TGF-β is critical for EMT and normal lung development (31, 53) and herein we have shown that gestational CS downregulates TGF-β and inhibits EMT. Thus, gestational exposure to CS downregulates H_2S synthesizing enzymes that in turn may increase the susceptibility of children to respiratory diseases associated with gestational exposure to CS.

to gestational CS. Nonetheless, it is highly likely that there is a correlation between placental levels of H_2S enzymes and the risk of asthma/BPD in children and H_2S or H_2S-donor compounds may have therapeutic value to reduce this risk. The manner by which H_2S inhibits allergic asthma is related to its ability to suppress Th2 immune responses (65) and CSE deficient mice have elevated GATA3 nuclear content, higher levels of Th2 cytokines, and exaggerated asthma response; H_2S donors attenuate asthma (17, 66). Thus, gestational exposure to CS downregulates H_2S synthesizing enzymes that in turn may increase the susceptibility of children to respiratory diseases associated with gestational exposure to CS. Taken together, the data presented herein provide a basic outline of the potential interaction between gestational CS exposure, *de novo* synthesis of H_2S, and development of lung developmental diseases as described schematically in **Figure 5**.

RESEARCH IMPACT

Exposure to cigarette smoke (CS) during pregnancy impairs epithelial-mesenchymal transition (EMT) and angiogenesis in the lung, increasing the risk of allergic asthma and bronchopulmonary dysplasia (BPD), transgenerationally. Hydrogen sulfide (H2S), a recently recognized gasotransmitter, promotes angiogenesis and inhibits asthma and alveolar simplification. H_2S is synthesized by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3MST). Results presented herein show that exposure of mice to CS during pregnancy suppressed the lung expression of CSE, CBS, 3-MST, and the CS-induced suppression of CSE and CBS was transmitted to F2. Similarly, smoking during pregnancy downregulated the expression of CSE, CBS, and 3MST

in human placentas; the downregulated expression of the enzymes might be a biomarker for asthma susceptibility in children.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of New Mexico Medical Center's Institutional Review Board and Human Research Protection Office in accordance with the NIH guidelines. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Lovelace Respiratory Research Institute IACUC.

AUTHOR CONTRIBUTIONS

SS performed the experiments, analyzed the data, and wrote the manuscript, DD, MM, AS, and TI analyzed the data, VE, HA, and VR performed the sample analysis and analyzed the data. RG analyzed the data and wrote the manuscript. HC performed the sample analysis, analyzed the data, and wrote the manuscript. MS designed the studies, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript.

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REVIEW
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Role of Cystic Fibrosis Bronchial Epithelium in Neutrophil Chemotaxis

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A hallmark of cystic fibrosis (CF) chronic respiratory disease is an extensive neutrophil infiltrate in the mucosa filling the bronchial lumen, starting early in life for CF infants. The genetic defect of the CF Transmembrane conductance Regulator (CFTR) ion channel promotes dehydration of the airway surface liquid, alters mucus properties, and decreases mucociliary clearance, favoring the onset of recurrent and, ultimately, chronic bacterial infection. Neutrophil infiltrates are unable to clear bacterial infection and, as an adverse effect, contribute to mucosal tissue damage by releasing proteases and reactive oxygen species. Moreover, the rapid cellular turnover of lumenal neutrophils releases nucleic acids that further alter the mucus viscosity. A prominent role in the recruitment of neutrophil in bronchial mucosa is played by CF bronchial epithelial cells carrying the defective CFTR protein and are exposed to whole bacteria and bacterial products, making pharmacological approaches to regulate the exaggerated neutrophil chemotaxis in CF a relevant therapeutic target. Here we revise: (a) the major receptors, kinases, and transcription factors leading to the expression, and release of neutrophil chemokines in bronchial epithelial cells; (b) the role of intracellular calcium homeostasis and, in particular, the calcium crosstalk between endoplasmic reticulum and mitochondria; (c) the epigenetic regulation of the key chemokines; (d) the role of mutant CFTR protein as a co-regulator of chemokines together with the host-pathogen interactions; and (e) different pharmacological strategies to regulate the expression of chemokines in CF bronchial epithelial cells through novel drug discovery and drug repurposing.

Keywords: cystic fibrosis, epithelium, lung, chemotaxis, neutrophil, inflammation

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LUNG PATHOLOGY IN CYSTIC FIBROSIS PATIENTS: AN EARLY EVENT ACCOMPANYING WHOLE LIFE

Autosomal recessive inheritance of mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene, encoding a chloride and bicarbonate transporting protein, is at the basis of the multiorgan Cystic Fibrosis (CF) disease (1–3). CF lung disease, characterized by chronic bacterial airway infection, neutrophilic inflammation, and dilation of bronchioles obstructed by mucus plugs, is presently the main limitation to the quality and expectancy of the life of CF patients. Although lung pathology and the mechanisms of the disease were prioritized for decades in CF research, what is between the *CFTR* gene defects and the overt clinical symptoms of the CF patients has still not been completely defined. Consensus has been reached that lung pathology begins in the

early months of life for the majority of CF infants, often before the onset of clinical symptoms, as demonstrated by the presence of inflammatory cytokines in the bronchoalveolar lavage fluid of CF infants (4–6) and by the lung histopathology of CF infants who die within weeks or months after birth, showing bronchial lumena filled and plugged by neutrophils (7).

Different hypotheses have been proposed to link the chloride and bicarbonate transport defects of mutant CFTR protein and the onset of airway disease. Consensus on the mechanism can be summarized in that altered CFTR protein reduces the hydration, and possibly the pH, of the airway surface liquid (ASL), thus affecting the rate of the mucociliary clearance, the principal innate mechanism involved in the defense against microbial infection (8). ASL dehydration worsens the mucociliary clearance by reducing mucus fluidity in both ASL and in the submucosal glands of the airway mucosa. The precise mechanism(s) favoring the early recurrent infections with *Staphilococcus aureus* and *Haemophilus influenzae,* and the stable chronic bacterial infection with *Pseudomonas aeruginosa* (*P. aeruginosa*) that follows in at least 80% of CF teenagers, are not completely understood (9), as ALS dehydration and increased mucus viscosity are considered early predisposing events in CF lung pathophysiology (10, 11).

Hallmarks of the lung pathology of CF patients include defective mucociliary clearance and chronic bacterial infection (especially *P. aeruginosa*) associated with an exaggerated neutrophil dominated inflammation.

NEUTROPHILS IN CF AIRWAY INFLAMMATION: A DOUBLE-EDGED SWORD

Neutrophils are the predominant immune cells infiltrating the airway mucosa and filling the intralumenal space of bronchioles in CF patients (7). Although the recruitment of neutrophils in CF airways' begins early in life and becomes persistent, neutrophils are unable to solve CF bacterial infection. The inefficacy of neutrophils in clearing bacteria prompted a debate on the presence of a neutrophil dysfunction in CF airways, as has been extensively reviewed elsewhere (12, 13). Different *in vitro* and *in vivo* studies in human and mice models evidenced that defective CFTR expressed in CF neutrophils, which is essential for chloride transport into phagolysosome and production of HOCl, impairs bacterial killing, implicating a specific disadvantage in microbial clearance in CF airways (14–18). As an indirect confirmation of the role of CFTR in neutrophilic function, VX-770 CFTR potentiator and VRT-325 corrector partially restored the impaired bacterial killing function in neutrophils of patients bearing G551D-CFTR or F508del-CFTR mutations, respectively (19, 20).

Although defective in clearing the chronic respiratory infection of these patients, neutrophils in CF airways are exposed to bacteria and become a source of continuous release of proteases, mainly elastases, which further impair their killing ability upon cleavage of the CXCR1 chemokine receptor (21). The relevance of elastases released from neutrophils has become an intense field of investigation due to its multiple adverse effects in CF lung pathology. It has been directly correlated with the onset of bronchiectasis and the severity of lung disease. The imbalance between proteases and anti-proteases in the CF ASL has prompted researchers to consider neutrophil elastase as a relevant molecular target in this disease (22–31). Its role in CF lung tissue damage has been further increased by its effect on degradation of CFTR protein (32), which can potentially reduce the efficacy of novel CFTR modulators, and by the evidence that its expression is upregulated by the pro-inflammatory cytokine TNF-alpha (TNF-α) and the chemokine interleukin (IL)-8 (or CXCL8) in CF lung (33). Finally, it amplifies the autocrine circuitry of inflammation by potentiating the recruitment of elastase-producing neutrophils by inducing the release of the neutrophilic chemokine IL-8, acting with an autocrine mechanism on CXCR1 and with activation of TLR4 and MyD88-dependent signaling (34–36).

A second critical adverse effect of a huge amount of neutrophils is their contribution to increasing the pro-oxidant milieu of the CF ALS, as has been extensively reviewed elsewhere (37). Among the different sources of pro-oxidants in the CF airway milieu, neutrophils contribute by releasing reactive oxygen species (ROS) by mechanisms known as "frustrated phagocytosis" or as a result of continuous activation, being the neutrophil-derived ROS critical effectors of bronchial epithelial damage (38–41).

As a third critical adverse effect, the presence of a large amount of neutrophils in CF brochial lumena implies the release of abundant DNA on the surface of the mucosa, which further reduces the fluidity of the ASL and worsens the bronchial obstruction (42). For a long time, neutrophil-derived DNA was thought to be the result of the turnover of neutrophils ending in hypoxic necrosis and consequent DNA release (43). More recently, the free DNA in CF airways has been found to be derived from the Neutrophil Extracellular Traps (NETs) released by neutrophils instead of the results of hypoxic necrosis (44). NETs are part of the innate defense armamentarium that block bacteria, viruses, and parasites facilitating the phagocytosis by neutrophils. However, in the environment of CF lung infection and inflammation, the benefits of NETs seems to be overcome by the adverse effect of the release of DNA that further reduces ASL fluidity, which impairs the clearance of toxic enzymes, such as neutrophil elastase and myeloperoxidase, damaging the respiratory tissue. The balance between the pros and cons of NETs in CF lung disease is therefore critical (45, 46). A crucial issue in CF lung disease is that neutrophils in the bronchial lumena of CF patients are unable to clear the bacterial infection and are co-responsible, together with bacteria, for the tissue damage, since neutrophils release proteases and ROS and further affect the rheology of CF ASL with abundant DNA. A graphical summary is presented in **Figure 1**.

CHEMOKINES RECRUITING NEUTROPHILS AND THE ROLE OF BRONCHIAL EPITHELIAL CELLS

Elevated concentrations of cytokines have been found early in life in the bronchoalveolar lavage fluid of CF infants, even

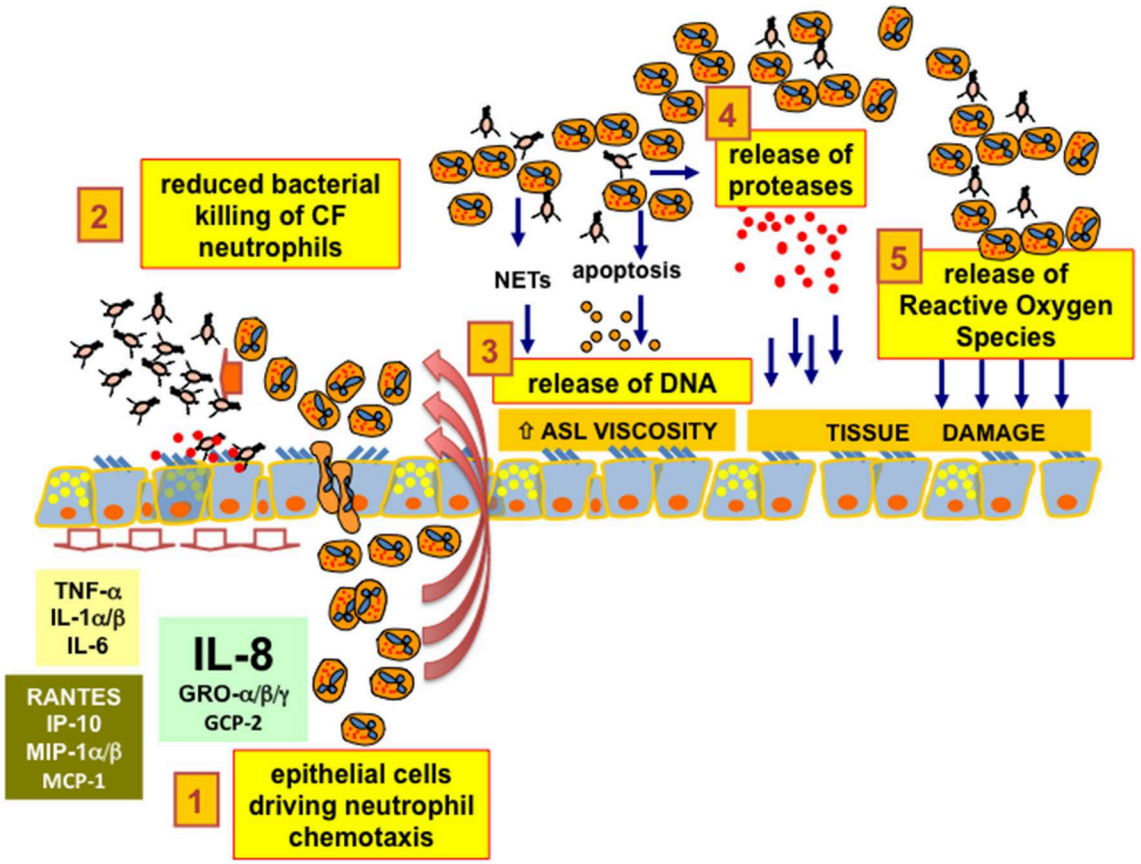

FIGURE 1 | CF bronchial epithelial cells and neutrophil chemotaxis. 1. epithelial cells driving neutrophil chemotaxis: bronchial epithelial cells drive the chemotaxis of neutrophils inside the lumen of bronchi and bronchioles of CF patients, mainly secreting the chemokine IL-8 (CXCL8) expressed upon interaction with bacteria with ASGM1R and TLR2/5 receptors exposed on the apical membrane. 2. reduced bacterial killing of CF neutrophils: CF neutrophils present different degrees of defective killing of the bacteria in the process of phagocytosis, being unable to clear completely the recurrent and, later on, chronic bacterial infection in the airways. 3. release of DNA: CF neutrophils are continuously stimulated in releasing Neutrophil Extracellular Traps. Despite the expected increase in efficiency of bacterial clearing, DNA from NETs increases the viscosity of the Airway Surface Liquid (ASL), further worsening the beat of cilia and the effectiveness of the CF mucociliary clearance. Moreover, CF neutrophils further release DNA as a consequence of apoptosis or necrosis due to the hypoxic environment resulting from mucus plugging of the conductive airways. 4. release of proteases: CF neutrophils exposed to bacteria or bacteria-derived components are prone to exocytosis of granules containing a wide armamentarium of proteases that are usually utilized by neutrophils to disrupt the tissues to allow their migration. In particular, an abundant amount of elastase has been found in CF lungs and its concentration has been related to CF lung disease progression and severity. 5. release of Reactive Oxygen Species: the unbalanced pro-oxidant milieu of the CF ASL due to excessive ROS derives mainly from "frustrated phagocytosis" of CF neutrophils and contributes together with proteases to a neutrophil-dependent airway tissue damage, a clear side effect dependent on the CFTR defect and worsened by an exaggerated neutrophil chemotaxis in CF bronchial lumen.

in the absence of an overt bacterial infection (4–6). Different mechanisms have been proposed to explain the early onset of sterile inflammation in CF lungs, including the role of airway surface mucus and hypoxia (47–49).

Among the soluble mediators of inflammation detected in CF bronchoalveolar lavage fluid, the most potent chemokines recruiting neutrophils have attracted particular interest, both in terms of pathophysiology and therapeutic perspectives, namely the complement system-derived C5a, the leukotriene B4 (LTB4), and the chemokine Interleukin(IL)-8.

C5a activated complement component receptor (C5aR) expressed on neutrophils was found to be critical in the defense against *P. aeruginosa* infection since knock-out mice deficient of C5aR were able to recruit neutrophils but succumbed to pneumonia because of the killing defect (50). Although it was earlier speculated that the C5aR decoy molecule C5L2 could be beneficial in reducing excessive inflammation in several lung diseases, including CF (51), it was later concluded that inactivation of C5aR by cleavage mediated by proteases released from CF neutrophils was at least partly responsible for the reduced killing and clearance of *P. aeruginosa* in CF lungs (52). Due to this critical role of C5aR in host defense, although elevated concentrations of C5a in CF airway fluids have been directly correlated with disease severity, very little effort has been invested in inhibiting the C5a-C5aR axis (53, 54).

LTB4 is released from neutrophils and macrophages in response to different stimuli, and in turn recruits and activates neutrophils (55). LTB4 has been found at elevated concentrations

in different CF respiratory fluids (56–58). Because of its potent neutrophil chemotactic effect, novel drugs, or drug repositioning to inhibit either LTB4 or its receptor have been proposed in CF to reduce inflammatory-dependent tissue damage (59). Particularly relevant was the experience with BIIL 284 BS, a drug acting as an LTB4 receptor antagonist, which was tested in pre-clinical and clinical trials (60, 61). Unfortunately, the clinical trial resulted in major adverse events, as it was apparent that the drug, while effectively reducing the inflammatory response, was untowardly increasing *P. aeruginosa* bacterial load (61, 62), providing a first relevant alert on the delicate balance between the reduction of excessive inflammation by over-inhibiting neutrophil chemotaxis and the mandatory need of preserving a sufficient immune defense. To tackle the crucial issue of anticipating in pre-clinical assays the effect of potential anti-inflammatory molecules modulating neutrophil chemotaxis, an interesting CF *in vitro* model has been set-up, able to simulate closely the transepithelial neutrophil migration and the effect of candidate drugs (63). In spite of inhibiting the LTB4 receptor, such as in the previous unsuccessful experience with BIIL 284 BS (61, 62), acebilistat, a recent drug modulating LTB4 expression, has positively passed pre-clinical assays (63) and is now in clinical trials (64), keeping open the possibility of reducing excessive CF lung inflammation by modulating neutrophil chemotaxis targeting the leukotriene-LTB4 axis.

IL-8/CXCL8 is possibly the neutrophilic chemokine most extensively studied in CF lung pathophysiology. Different cells of the airway tract are known to contribute to the release IL-8; among these, bronchial epithelial cells have been highlighted as a relevant source. To dissect the specific contribution of bronchial epithelial cells in the expression of IL-8 and of several other soluble mediators of inflammation, different *in vitro* experimental models with immortalized cell lines or primary cell cultures have been tested and are currently utilized to investigate molecular mechanisms or novel anti-inflammatory molecules. Key pro-inflammatory challenges able to elicit inflammatory mediators in bronchial epithelial cells are different living bacteria (*S. aureus, P. aeruginosa*), heat-inactivated dead bacteria (Heat-Killed *P. aeruginosa*), flagella-defective or pili-defective recombinant strains of *P. aeruginosa*, single bacterial components (e.g., flagellin), *P. aeruginosa* clinical isolates and bacterial products from patients' airway specimens (e.g., Supernatant of Mucopurulent Material), different pro-inflammatory cytokines (e.g., TNF-α and IL-1), and oxidants such as hydrogen peroxide (65–72). Under these stimuli *in vitro*, bronchial epithelial cells upregulate the basal expression of many pro-inflammatory mediators, such as the cytokines (e.g., TNF-α, IL-1α/β, IL-6), chemokines attracting lympho-monocytes (e.g., IP-10, RANTES, MCP-1, MIP-1α/β), and, as anticipated, IL-8 and other chemokines attracting neutrophils (Gro α/β/γ, GCP-2) (67, 68, 70, 71, 73–81). Among these soluble mediators of inflammation, the neutrophilic chemokine IL-8 (CXCL8) is most strikingly expressed up to two orders of magnitude above the basal release (81). Wide consensus has been reached so far on the role of bronchial epithelial cells as relevant producers of the potent chemokine IL-8, which in turn forwards a strong recruiting soluble signal to neutrophils to reach the lumen of bronchi and bronchioles in the CF mucosa.

HOST-PATHOGEN INTERACTIONS AND INTRACELLULAR SIGNALING MODULATING IL-8/CXCL8 EXPRESSION

A growing series of evidence based on longitudinal clinical investigations of CF patients is building a strong consensus that the inflammatory process could be even more deleterious to CF lung structure and function than the bacterial infection by itself (83) Sterile inflammation in CF lungs has been evidenced based on the presence of elevated concentrations of cytokines in the bronchoalveolar lavage fluid of CF infants of a few months of age (4–6). Airway surface mucus plugging and hypoxia have been proposed to explain the onset of sterile inflammation in CF lungs (47–49). The possible direct contribution of intracellular CFTR protein defects has been proposed (as described later in paragraph 5), together with mechanisms more downstream than the CFTR-dependent ion transport alterations, such as mucus plugging (84). Although inflammation in the absence of detectable bacterial infection has been demonstrated as a likely initiating event, the whole inflammatory process in CF lung is undoubtedly amplified by the occurrence of polybacterial infection. Host-pathogen interactions between bacteria and bronchial epithelial cells in CF have been most extensively studied for *P. aeruginosa*, the pathogen that colonizes CF airways of almost all CF patients. *P. aeruginosa* in a planktonic state interacts through the pili with Asialo-GM1 receptor (AGM1R) on the apical membrane of airway epithelial cells (65). Flagellum-derived flagellin protein also binds to AGM1R, together with Toll-like receptors (TLR) 2 and 5 (85). Flagellin in particular has been found to elicit a strong intracellular pro-inflammatory signaling, as shown by the single purified protein and by recombinant lab strains of *P. aeruginosa*, in different converging studies led by Prince et al. (68, 72, 86, 87). MyD88-dependent signaling downstream TLR2 and TLR5, elicited by *P. aeruginosa*, activates Mitogen-Activated Protein (MAP) Kinases such as MAPK ERK1/2 and MAPK p38, together with ribosomal S6 kinase (RSK)1/2 and heat shock protein (HSP) 27, which are directly involved in inducing the expression of the neutrophilic chemokine IL-8 (88–90). Besides the activation of MyD88-dependent signaling, *P. aeruginosa* is known to also potentiate the expression of IL-8 by a nucleotide-purinergic receptors loop. Interaction of the bacteria with ASGM1R and TLR5 promotes sustained release of ATP as a classical "danger signal" from the apical membrane (85, 91), interacting with purinergic receptors P2Y2R (92). This activates an intracellular calcium signaling (see paragraph 6), in which phospholipase C beta 3 (PLCB3) plays a key role (78, 93).

For transcription factors (TFs) involved in chemokine expression, the promoter elements of IL-8 gene have been widely studied (89, 94, 95). The mapping of the transcription machinery of the IL-8 gene in human bronchial epithelial cells infected with *P. aeruginosa* was studied not only with

the aim of understanding the molecular regulation of IL-8 transcription, but also to propose novel anti-inflammatory approaches and to identify potential pharmacological targets. This issue was addressed by investigating the role of TFs on the transcription of the IL-8 gene in human bronchial epithelial cells. Functional assays were based on the transfection of TF decoy oligodeoxynucleotides, designed to interfere with the interaction of the transcription factors nuclear factor-κB (NF-κB), activating protein (AP-1), CAAT/enhancer-binding protein β (C/EBPβ, also known as NF-IL-6), C/EBP homologous protein (CHOP), and cAMP response element binding protein (CREB) with the corresponding consensus sequences identified in the IL-8 promoter. The treatment of target cells with these decoy oligonucleotides reduced the *P. aeruginosa*-dependent transcription of IL-8, suggesting their participation in the transcriptional machinery (89, 94). These conclusions have been recently confirmed and reviewed (95). On the contrary, IL-8 gene expression is repressed by a combination of molecular events that includes: (a) deacetylation of histones, (b) octamer-1 (Oct-1) binding, and (c) active repression by NF-κB repressing factor (NRF). Histone deacetylase-1 (HDAC-1) activity is involved in IL-8 transcription inhibition, as demonstrated by the fact that HDAC1 inhibition derepresses the expression of IL-8, which involves the recruitment of CREB binding protein (CBP)/p300 to the IL-8 promoter, resulting in hyperacetylation of histones and chromatin remodeling, thus counteracting the repression (96, 97). In terms of Oct-1 activity, it has been demonstrated that Oct-1 (the IL-8 repressor) and CCAAT/enhancer-binding protein (C/EBP) (an IL-8 activator) bind to overlapping elements within the IL-8 promoter. The role of Oct-1 as a transcriptional repressor is sustained by experimental evidence that replacing the Oct-1 repressor with C/EBP induces transcription at the IL-8 promoter (98). Similarly, binding of NRF to a negative regulatory element (NRE) in the IL-8 gene promoter (which incompletely overlaps with the NF-κB response element) also represses IL-8 transcription (99). Interestingly, the transcription factors that have been suggested to regulate IL-8 are also involved in regulating the expression of other pro-inflammatory genes, such as GRO-γ, the intercellular adhesion molecule (ICAM)-1, and the cytokines IL-1β and IL-6 (94).

The possible application of transcription factor(s) targeting (for instance, using decoy oligonucleotides) might be a potential therapeutic intervention, especially in the case delivery issues are solved. In this respect, two recently reported studies have focused on nanomaterial-based delivery systems for overcoming limitations associated with clinical applications of decoy oligonucleotides targeting pro-inflammatory transcription factors (such as those targeting NF-kB) (100, 101).

THE EPIGENETIC REGULATION OF THE KEY CHEMOKINES

The epigenetic regulation of key chemokines in CF occurs at the level of: (a) histone acetyltransferase (HAT)/HDAC balance, (b) histone and DNA methylation, and (c) miRNA-dependent post-transcriptional regulation. It has been recently reported that the transcriptional regulation of IL-8 and other pro-inflammatory genes involves chromatin remodeling through histone acetylation. Interestingly, there is a possible regulatory loop between IL-8 gene transcription and CFTR. In fact, NF-κB facilitates histone acetylation of IL-8 and other pro-inflammatory gene promoters and the histone acetyltransferase (HAT)/HDAC balance is sensitive to CFTR function. This conclusion is supported by the observation that cells with a reduced or absent CFTR function have a decreased HDAC2 protein, resulting in hyperacetylation of the IL-8 promoter and increased IL-8 transcription. In agreement with (a), reduced HDAC2 and HDAC2 activity is observed in cells deficient in CFTR and (b) suppression of HDAC2 expression with HDAC2 shRNA (short hairpin RNA) resulted in enhanced IL-8 expression and promoter acetylation similar to CFTR-deficient cells (102). In conclusion, there is an intrinsic alteration in the HAT/HDAC balance in cells lacking CFTR function *in vitro* and in native CF tissue. This mechanism provides an explanation for the apparent dysregulation of inflammatory mediators seen in the CF airway, as reduced histone deacetylation would potentially influence many genes. IL-8 hypersecretion in CF airway epithelial cells is also caused by the abnormal epigenetic regulation of IL-8 gene involving histone methylation. Under basal conditions, CF cells had increased bromodomain (Brd)3 and Brd4 recruitment and enhanced NF-κB and C/EBPβ binding to the IL-8 promoter compared to non-CF cells due to trimethylation of histone H3 at lysine 4 (H3K4me3) and DNA hypomethylation at CpG6. IL-1β increased NF-κB, C/EBPβ, and Brd4 binding. Furthermore, inhibitors of bromodomain and extra-terminal domain family (BET) proteins reduced IL-8 production in CF cells, suggesting a therapeutic target for the BET pathway (103). Regarding microRNA (miRNA) involved in controlling IL-8 production in the CF lung, three recent studies have determined how miRNAs that are aberrantly expressed in the CF airways may post-transcriptionally regulate IL-8 expression. The first report was focused on miR-155, a miRNA that is highly expressed in CF lung. Bhattacharyya and Coll found that expression of miR-155 was elevated in CF IB3-1 lung epithelial cells in culture, compared with control IB3-1/S9 cells. In addition, clinical evidence indicated that miR-155 was also highly expressed in CF lung epithelial cells and circulating CF neutrophils from CF patients. High levels of miR-155 specifically reduced levels of SHIP1, thereby promoting PI3K/Akt activation and contributing to the pro-inflammatory expression of IL-8 in CF lung epithelial cells (104). The other two studies on this topic have investigated miRNAs that are decreased in the CF lung that directly target IL-8 mRNA. Fabbri et al. identified miR-93 as a miRNA that is decreased in IB3-1 and Cufi-1 cells infected with *P. aeruginosa*. The possible involvement of miR-93 in IL-8 gene regulation was validated using three luciferase vectors, including one carrying the complete 3'-UTR region of the IL-8 mRNA and one carrying the same region with a mutated miR-93 site. Specifically, the results obtained indicate that, in addition to NF-κB-dependent up-regulation of IL-8 gene transcription, IL-8 protein expression is post-transcriptionally regulated by interactions of the IL-8 mRNA with the inhibitory miR-93 (105). The involvement of microRNAs in IL-8 is also supported by the study of Oglesby et al. (106), who measured the expression and function of miRNAs decreased in the CF lung. MicroRNA miR-17 was identified as

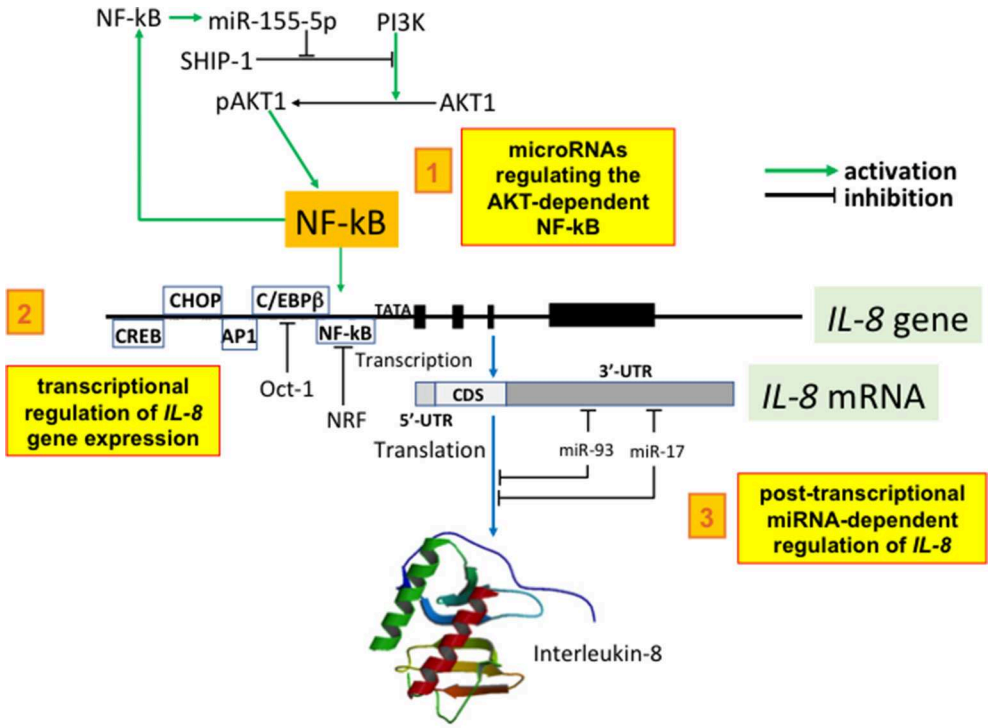

FIGURE 2 | Selected examples of the miRNA network regulating IL-8. 1. microRNAs regulating the AKT-dependent NF-kB: miR-155-5p expression is potentiated by NF-kB and is elevated in CF lung epithelial cells and circulating CF neutrophils biopsied from CF patients. High levels of miR-155 specifically reduce the levels of SHIP1, thereby promoting PI3K/Akt activation. Phospho-Akt levels are therefore elevated in CF lung epithelial cells and can be specifically lowered by either antagomir-155 or elevated expression of SHIP1. Elevated miR-155-5p contributes to the pro-inflammatory expression of IL-8 in CF lung epithelial cells by lowering SHIP1 expression and thereby activating the PI3K/Akt signaling pathway (104). 2. transcriptional regulation of *IL-8* gene expression: *IL-8* promoter is under the control of transcription factors enhancing *IL-8* mRNA production (examples are NF-κB, AP-1, C/EBPβ, CHOP, and CREB); furthermore, IL-8 gene expression is repressed by Oct-1 and by NF-κB repressing factor (NRF). All these transcription factors are regulated by microRNAs. 3. post-transcriptional miRNA-dependent regulation of IL-8: Fabbri et al. identified miR-93 as a miRNA that is decreased in cystic fibrosis IB3-1 and Cufi-1 cells infected with *P. aeruginosa*. The possible involvement of miR-93 in *IL-8* gene regulation was validated using luciferase vectors and the results obtained indicate that IL-8 protein expression is post-transcriptionally regulated by interactions of the *IL-8* mRNA with the inhibitory miR-93 (105). The involvement of microRNAs in IL-8 is also supported by the study of Oglesby et al. (106) who identified miR-17 as a miRNA regulating IL-8. Interestingly, its expression was decreased in adult CF bronchial brushings and bronchial epithelial cells chronically stimulated with *Pseudomonas*-conditioned medium (106). 5′-UTR, 3′-UTR: 5′ and 3′ untranslated region of the IL-8 mRNA; CDS, coding sequences.

a miRNA that regulates IL-8 and its expression was decreased in adult CF bronchial brushings and bronchial epithelial cells chronically stimulated with *Pseudomonas*-conditioned medium (106). Another microRNA involved in inflammation is miR-636, as recently demonstrated by Bardin et al. (107). By analyzing miRNAs in human primary air-liquid interface cell cultures, overexpression of miR-636 in CF patients compared to non-CF controls was shown. Functional studies demonstrated that miR-636 directly interacts with IL1R1 and RANK (two pro-inflammatory cytokine receptors), and IKBKB (which encodes IKKβ, a major protein in the NF-κB pathway). A summary of miRNA regulation is depicted in **Figure 2**.

CFTR PROTEIN DEFECTS COOPERATE TO PRO-INFLAMMATORY INTRACELLULAR SIGNALING

The origin of this abnormal inflammatory response in CF continues to be debated, with researchers unsure whether it is initiated by exogenous stimuli, such as persistent microbial infection, or by intrinsic deficiency of CFTR function, with alterations in signal transduction, or both. Dysregulation of the airway innate immune system is associated with CF, being both airway epithelial cells and immune cells susceptible to intrinsic CFTR-associated alterations in signal transduction.

No differences in TLRs expression have been observed between human CF and non-CF airway epithelial cell lines. However, increased expression of TLR2 and TLR5 on the apical membrane of the polarized human CF airway epithelial cells and in CFTR-knockout vs. WT mice were found (108, 109). These different cellular redistributions of TLRs render the CF airway epithelial cells more susceptible to host-pathogen interactions between bacterial constituents (pili and flagellin) and the receptors involved in the transduction of "danger signal" at apical membrane, favoring the release of cytokines (as described before in section host-pathogen interactions and intracellular signaling modulating IL-8/CXCL8 expression).

An intrinsic defect associated with CFTR deficiency is the susceptibility of CF airway cells to accumulate ROS, particularly

during pathogen infection. Evidence shows increased oxidative stress in CF (37, 110–116). Although several studies suggest that the oxidative stress in CF is a direct consequence of mitochondrial dysfunction due to perturbed CFTR signaling, others suggest that the accumulation of ROS in CF depends on there being a reduced antioxidant capacity (113). Low mitochondrial reduced glutathione (mtGSH) levels were found in CF patient-derived tracheal cells and in CFTR-knockout mice (117, 118). Indeed, in CF patient-derived pancreatic and tracheal cells a reduction of protein expression of Cu/Zn-superoxide dismutase (SOD1) and Mn-SOD (SOD2) and a reduction in the activity of extracellular SOD (119) has been observed. The oxidative stress in CF lung may: (I) affect autophagy, compromising the expression of CFTR channel (120, 121); (II) induce mtDNA oxidation and damage, triggering inflammasome activation, and/or altering the OXPHOS activity, that in turn produces additional ROS (122, 123); and (III) destroy lung tissue, affecting the cell function and/or exacerbating the inflammatory response (37).

An inherent defective CFTR channel leads to transglutaminase (TG2) upregulation, resulting in defective autophagy with consequent accumulation of aggresomes and ROS (121). Lack of autophagy in macrophages and in airway epithelial cells result in a reduced bacterial clearance and in the accumulation of dysfunctional mitochondria, which in turn promotes secretion of pro-inflammatory cytokines, indicating that autophagy may regulate the inflammation responses by suppressing the secretion of immune mediators (124–126). In fact, the rescue of dysfunctional autophagy in CF, mediated by autophagy inducers such as MTOR inhibitor (rapamycin), TG2 inhibitor (cistamine), and/or modulators of Ca^{2+}-dependent signaling (KB-R7943), attenuated the hyperinflammation in CF lung, improving the CFTR transport to PM and reducing ROS production and cytokine release in macrophages and in primary CF airway cells *in vitro* and in CF mouse models *in vivo* (127–132).

The hyperinflammation of the CF airway is sustained by the accumulation of dysfunctional mitochondria, as an indirect consequence of perturbed CFTR signaling, responsible for ROS production and increased inflammasome-dependent IL-1β release. Mitochondrial defects associated with an abnormal oxidative stress and inflammatory response have been found in CF. The first evidence was shown in 1979, demonstrating that mitochondrial Ca^{2+} uptake and oxygen consumption were altered in mitochondria isolated from CF patient-derived fibroblast (133). In the same year, the mitochondrial NADH dehydrogenase (complex-1) was found altered in CF skin fibroblasts (134), while in 1981 a deficiency of 6-phosphate dehydrogenase in CF patients was found (135). Consistent with the first observations, in CF patient-derived tracheal cells a decreased mitochondrial NADH dehydrogenase activity and mitochondrial membrane potential ($\Delta\psi$) were measured, due to the down-regulation of the mt-ND4 gene that codifies for a subunit of the complex-1 essential for its assembly and activity (136). This alteration was rescued through the reintroduction of CFTR-wt, also indicating that mtGSH depletion in CF is responsible for the altered complex-1 activity (118). Recently, it has been observed that the CFTR corrector agent VX-809 and 4,6,4'-trimethylangelicin (TMA) treatment lead to partial restoration of the mitochondrial failure in CF airway cell lines, producing an improvement in complex-1 activity, $\Delta\psi$ generation, ANT-dependent ADP/ATP exchange, and membrane lipid peroxidation (137). These data indicate that the restoration of mitochondrial physiology is linked to the mitigation of the inflammatory response, suggesting that the mitochondrial defects found in CF airway cells contributes to the susceptibility of CF cells to bacterial infection, influencing the innate immune response.

Several pieces of evidence have shown how the hyperinflammation observed in CF lung is sustained by cell defects associated with CFTR deficiency intrinsic to epithelial, but also in inflammatory, cells. In CF patients, the differentiation of T lymphocytes to Th_{17} phenotype is increased (138), while monocyte-derived macrophages do not respond to IL-13/IL-4 and fail to polarize into M2 while the polarization to the M1 phenotype was unaffected (139). CF macrophages serve as a replicative niche for bacteria to avoid host defenses (140), present deficits in bacterial killing (141, 142), and produce excess cytokines (143). In synthesis, although the initiating event of CF lung inflammation between infection-derived exogenous and CFTR-related endogenous components remains a "chicken and egg" debate still without a final consensus, different converging evidence supports the hypothesis that CFTR-specific signal transduction alterations amplify the extent of the response.

In this context, analysis at a single-cell level might be very informative. For instance, Mould et al. (144) investigated inflammatory macrophage heterogeneity during acute lung inflammation in mice and performed single cell RNA sequencing of macrophages isolated from the airspaces during peak inflammation and resolution of inflammation. They found two transcriptionally distinct subdivisions of alveolar macrophages based on proliferative capacity and inflammatory programing. Of course, overcoming technical obstacles (due, for instance, to debris, and apoptotic cells) rendering difficult isolation of individual cells for single cell analyses is a key issue (145). Finally, on-a-chip devices are expected to bring key information at single-cell levels on hyperinflammation in pulmonary diseases, including CF (146–148). As far this key issue is concerned, starting from the notion that cell-to-cell variability in chemokine/cytokine secretion is largely unknown, Ramji et al. developed and validated a microfluidic device to integrate live-cell imaging of fluorescent reporter proteins with a single-cell assay of protein secretion (146). This device was used to image transcription factor dynamics in macrophages in response to LPS, followed by quantification of secretion of TNF, CCL2, CCL3, and CCL5.

INTRACELLULAR CALCIUM HOMEOSTASIS IN THE REGULATION OF THE EXPRESSION OF CHEMOKINES

Ca^{2+} homeostasis is a pivotal element in regulating the immunological and physical barriers of the airway epithelium

in CF. Normally, the exposure to common respiratory bacterial pathogens, such as *P. aeruginosa*, originates in a cytosolic Ca^{2+} transient (about 100 nM) in airway epithelial cell lines necessary to initiate the inflammatory response, inducing the expression, and secretion of pro-inflammatory cytokines (149). A defective CFTR channel leads to the deregulation of Ca^{2+} homeostasis in CF cells, which is detrimental for lung inflammation. Ca^{2+} signaling dysregulations were observed in several human CF airway epithelial cell lines, where the intracellular Ca^{2+} concentration is increased compared to non-CF cells (150) due to: (I) intrinsic defects associated with CFTR deficiency, (II) chronic exposure to bacterial infection, and (III) persistent stimulation by pro-inflammatory mediators.

Intrinsic Defects Associated to CFTR Deficiency

In 1961, Donnell et al. published the first evidence of alterations of Ca^{2+} homeostasis in CF patients (151), which was then confirmed by Feigal et al., through direct measurement of the increased intracellular Ca^{2+} concentration in fibroblasts derived from CF patients (152, 153) and by Cabrini and De Togni as increased cytosolic Ca^{2+} concentration in CF neutrophils (154). In 1982, an increased mitochondrial Ca^{2+} uptake was observed in CF skin fibroblast, due to altered respiratory system activity (155). In 2009, Antigny et al. measured a decreased mitochondrial Ca^{2+} uptake in F508delCFTR airway epithelial cell lines as a consequence of depolarized and fragmented mitochondria (156). The debate is presently lessened because it has been demonstrated that a functional CFTR channel reduces the basal intracellular Ca^{2+} concentration in human airway epithelial cell lines and donor-derived primary airway epithelial cells, influencing the mitochondrial Ca^{2+} signals evoked by physiological and pathological stimuli (132). This abnormal intracellular Ca^{2+} increment in CF epithelial airway cells is in part justified by the reduced activity of Plasma Membrane (PM) Ca^{2+} ATPase (PMCA), which limits the Ca^{2+} efflux through the PM, and increased SERCA activity, which favors the endoplasmic reticulum (ER) Ca^{2+} accumulation (132). This increment in intracellular Ca^{2+} concentration in human epithelial airway cells is normalized by the administration of the corrector agent, VX809 (157). Several other pieces of evidence suggest that the increased intracellular Ca^{2+} concentration observed in CF airway cells depends on multifactorial aspects associated with defective CFTR, involving many Ca^{2+} channels expressed in the PM, including: (I) the Transient Receptor Potential Canonical channel 6 (TRPC6), normally expressed in human primary CF epithelial cells. Its Ca^{2+} influx capacity is enhanced in F508delCFTR and G551D-CFTR cells (158, 159); (II) the Store Operated Ca^{2+} Entry (SOCE) resulted in significantly increased CF airway cell lines and primary cells, due to an enhanced Orai1 channel insertion to PM, with consequent exacerbation of IL-8 secretion (160); and (III) the TRP channel TRPA1, expressed in bronchial columnar epithelial cells. Its direct activation by *P. aeruginosa* increases Ca^{2+} entry, mediating the release of cytokines such as IL-8, IL-1β, and TNF-α (81).

Chronic Exposure to Bacterial Infection and Persistent Stimulation by Pro-inflammatory Mediators

The chronic infection of CF airways amplifies the altered intracellular Ca^{2+} homeostasis of CF epithelial cells, predisposing the airway cells to a hyperinflammatory profile, which contributes to producing an excess of cytokines. Bacterial constituents and pro-inflammatory mediators cooperate, inducing an abnormal intracellular Ca^{2+} signaling in CF airway epithelia due to increased activation of apical G protein-coupled receptors (GPCRs) and a sustained ER Ca^{2+}-release (161). The higher intracellular Ca^{2+} concentration in CF cells contributes to a greater and more prolonged NF-κB activation with consequent effects on the expression and release of pro-inflammatory cytokines, such as IL-8 and IL-1β (82, 161). The persistent NF-κB activation in human CF airway cells is the consequence of the synergistic effects of bacterial components, such as flagellin, where flagellin interacting with asialoGM1 receptor favors the release of ATP from CF airway cell lines, which mediates purinergic receptors and activates downstream intracellular Ca^{2+} signaling that synergizes with the TLR5-dependent signaling to activate NF-κB (see section host-pathogen interactions and intracellular signaling modulating IL-8/CXCL8 expression) (92). The release of nucleotides from bronchial epithelial cells targeting P2Y2 purinergic receptors has been proposed to intervene on different aspects of CFTR regulation and lung pathophysiology (162–165). Bacterial constituents, pili and flagellin, interact with TLRs and TLR-associated glycolipid in airway cells (85, 166). In particular, TLR2 or asialoGM1 linked to TLR2 express both on the apical surface of airway cells and recognize bacterial constituents to induce the pro-inflammatory transcription of CXCL8 or MUC-2 gene via NF-κB activation, through the recruitment of PI3K and phospholipase C gamma (PLCγ), which in turn stimulate the release of Ca^{2+} through Inositol Triphosphate Receptors (IP3R) channels (167, 168). The generation of cytosolic Ca^{2+} transients activates classical Protein Kinase C (PKC) α and β isoforms, which through a phosphorylation cascade mediate the activation of NF-κB (169). In a similar molecular pathway, PLC beta 3 (PLCB3) also plays a relevant role in triggering cytosolic Ca^{2+} transients induced by *P. aeruginosa*, regulating the activation of PKCα and PKCβ to induce an NF-κB-dependent transcription of CXCL8 gene in human airway epithelial cell lines and in patient-derived primary cells (78). β-sitosterol (BSS) was used to inhibit the active form of PKCs involved in the transduction of *P. aeruginosa*-dependent pro-inflammatory Ca^{2+}-dependent signaling in CF patient-derived airway epithelial cells, leading to a significant reduction in expression of IL-8, growth-related oncogene (GRO)-α, and GRO-β (170). The role of PLCB3 in amplifying the expression and release of IL-8 during pathogen infection, through the regulation of intracellular Ca^{2+} transients, is associated with the severity and progression of CF lung disease. Single Nucleotide Polymorphisms (SNP) genetic study with the progression of CF lung disease severity, identify from a panel of 135 genes of immune response the association of c.2534C>T (p.S845L) variant of PLCB3 with a mild progression of pulmonary disease

in CF (93). PLCB3-S845L results in a loss-of-function variant, where defective intracellular Ca^{2+} redistribution and PKCs' activation limited the IL-8, IL-1β, and MUC5 expression in CF patient-derived airway epithelial cells exposed to *P. aeruginosa* or CF patient-derived mucopurulent material. The IP3R-mediated ER Ca^{2+}-release is significantly augmented in CF epithelial cell lines (171), a consequence also of ER Ca^{2+} store expansion observed in CF cells (150). The ER expansion is not dependent on ER retention of misfolded CFTR, but reflects an airway epithelial response acquired following persistent bacterial infection, resulting in ER unfolded protein response (UPR) activation mediated by the IRE1/XBP-1 pathway and in a larger intracellular Ca^{2+} mobilization in response to abnormal GPCRs activation (172).

The sustained ER Ca^{2+}-release in CF airway cells conditions the mitochondria to a direct involvement in the pro-inflammatory response. CF airway cell lines and CF patient-derived airway primary cells are prone to *P. aeruginosa*-dependent mitochondrial perturbations, in which the mitochondrial Ca^{2+} uniporter (MCU) is a signal-integrating organelle that mediates mitochondrial ROS-dependent NLRP3 inflammasome activation and recruitment of both NLRP3 and NLRC4 inflammasome (132). The degree and quality of the inflammatory response in CF airway cells is also sustained by *P. aeruginosa*-dependent mitochondrial perturbations, initiated by flagellin, such as mitochondrial membrane potential loss, ROS production, and mitochondrial fragmentation (132). Rimessi et al. have characterized the role of mitochondria as drivers of the *P. aeruginosa*-triggered inflammatory exacerbation in CF airway cells, demonstrating that mitochondrial Ca^{2+} signaling plays a critical role in inflammasome NLRP3 recruitment and inflammasome-dependent IL-1β and IL-18 release in CF airway cell lines and in CF patient-derived airway primary cells (132). By modulating the MCU-dependent mitochondrial Ca^{2+}-uptake, genetically or mediating pharmacological inhibition with KB-R7943, it is possible to control the pathogen-dependent mitochondrial dysfunction preventing the integration of pro-inflammatory signals from mitochondria into CF patient-derived airway primary cells and *in vivo* mouse models (131, 132).

Although whether the Ca^{2+}-dependent activation of chloride channels in CF bronchial epithelial cells could partially vary the defects of CFTR ion transport is presently under scrutiny (173), the results recalled above support the concept that the up-regulation of intracellular Ca^{2+} signaling is a key amplifier of the inflammatory response and lung pathogenesis in CF, which opens the issue of new potential molecular therapeutic targets.

TARGETING NEUTROPHIL CHEMOTAXIS IN CF: NOVEL MOLECULES AND DRUG REPURPOSING

Preliminary observations in rat lung models (59) suggested the repurposing of ibuprofen, a non-steroideal anti-inflammatory drug used in conditions like osteoarthritis, rheumatoid arthritis, juvenile idiopathic arthritis, and acutely painful musculoskeletal conditions, to clinical use for CF patients (174, 175). Long-term application of this drug, as reported by a Cochrane analysis, has proved the concept that strategies to modulate lung inflammation can be beneficial for people with CF (176). As ibuprofen inhibits prostaglandin synthesis (177), a very broad anti-inflammatory mechanism that is not closely specific to the pathophysiology of CF lung inflammation, innovative approaches to target the adverse effects produced by the huge amount of neutrophils in the CF conductive airways have been recently tested. Some of these approaches have been launched upon the knowledge of the specificity of CF lung inflammation, whereas others were just pure empirical testing. The first attempts were focused on antagonizing neutrophil proteases elastase, one of the main deleterious effect of neutrophil inflammation in CF (28, 29, 178–186). Different elastase inhibitors have also been recently tested in clinical trial, with promising results in terms of safety and tolerability (187, 188), maintaining the high levels of interest in the rationale of targeting neutrophil elastase in CF lung inflammation (186).

Early signaling evoked by bacteria has been tested by inhibition of TLR2 or by inducing extracellular calcium entry through calcium ionophores (81, 189). More downstream the production of IL-8, different antagonists of its receptors that block its action on cell targets have been challenged (190–192). More recently, different molecular approaches targeting the intracellular signaling in bronchial epithelial cells have been developed. In consideration of the key role of NF-κB in the transcriptional regulation of IL-8 and other pro-inflammatory genes, several studies have been focused on pharmacological alteration of NF-κB activity. The transcription factor (TF) decoy strategy was applied by Bezzerri et al., using TF oligodeoxynucleotides (ODNs) to NF-κB able to inhibit transcription of IL-8 in bronchial cells (89). The TF decoy approach was based on the intracellular delivery of double-stranded ODNs causing inhibition of the binding of TF-related proteins (as determined *in vitro* using EMSA assays) to the different consensus sequences in the promoter of specific genes. When CF cells were transfected with double-stranded TF "decoy" ODNs, mimicking different NF-κB consensus sequences, partial inhibition of *P. aeruginosa*-dependent transcription of IL-8 was obtained. In addition, other NF-κB regulated genes were inhibited, such as GRO-gamma and IL-6. In order to demonstrate that TFD against NF-κB interferes with the NF-κB pathway, Finotti et al. demonstrated mediating chromatin immunoprecipitation (ChIP) treatment with TFD oligodeoxyribonucleotides of IB3-1 cells infected with *P. aeruginosa* leads to a decreased occupancy of the IL-8 gene promoter by NF-κB factors (193). Further studies were focused on the development of more stable therapeutic molecules and on the delivery strategy for TFD molecules. Among stable ODN analogs, peptide nucleic acids (PNAs)-based agents were found to be promising for CF. In this respect, PNA-DNA-PNA (PDP) chimeras are molecules of great interest from several points of view: (a) they can be complexed with liposomes and microspheres; (b) they are resistant to DNases, serum, and cytoplasmic extracts; and (c) they are potent decoy molecules (194, 195). By using electrophoretic mobility shift assay and RT-PCR analysis, it was demonstrated that: (a) the effects

of PDP/PDP NF-κB decoy chimera on the accumulation of pro-inflammatory mRNAs in *P. aeruginosa*-infected IB3-1 cells in particular; (b) the PDP/PDP chimera is a strong inhibitor of IL-8 gene expression; and (c) the effect of PDP/PDP chimeras, unlike those of ODN-based decoys, are observed even in the absence of protection with lipofectamine (193–196). In another study, NF-κB decoys were employed with the hypothesis that they may limit lung inflammation in CF. In the study by De Stefano et al. (197), the effects of decoy ODN targeting NF-κB and delivered through biodegradable and respirable poly(D,L-lactide-co-glycolide) large porous particles (LPP) were determined on IL-6 and IL-8 mRNA expression in CF cells stimulated with lipopolysaccharide (LPS) from *P. aeruginosa*. The conclusion was that respirable biodegradable decoy ODN LPP may represent a promising strategy for inhibiting NF-κB transcriptional activity and related gene expression. This treatment, *in vivo*, was expected to reduce lung chronic inflammation in CF patients. Interestingly, De Stefano et al. (198) investigated the effects of NF-kB decoys delivered with inhalable nanoparticles in a rat model of lung inflammation induced by intratracheal aerosolization of LPS from *Pseudomonas aeruginosa*. A single intratracheal insufflation of the decoy ODNs reduced the bronchoalveolar neutrophil infiltration induced by LPS. This reduction was associated with decreased NF-κB/DNA binding activity, and decreased the content of IL-6, IL-8, and mucin-2 in lung homogenates.

In consideration of the involvement of microRNAs in the post-transcriptional regulation of IL-8 and other pro-inflammatory genes, both antagomiR and miRNA replacement approaches have been proposed (199–202). This confirmed that, in addition to relevance for the theoretical point of view, the studies on epigenetic regulation of chemokines (described in Chapter 5) might be important for the development of therapeutic protocols. For instance, transfection of CF cells with miR-93 (105) and miR-636 mimics (107) leads to an IL-8 decrease (105) and to a reduction of NF-κB activity, causing decreased secretion of IL-8 and IL-6 (200, 201). Another miRNA target to be considered is miR-199a-3p, whose expression is inversely correlated with increases in the expression of IKKβ and IL-8 (200). On the other hand, targeting miR-155 with antagomir might also be considered for IL-8 reduction (104). In fact, down-regulation of miR-155 was found to suppress the IL-8-associated pro-inflammatory phenotype in CF cells. In order to reduce the miR-155 levels in CF cells, antagomiR molecules against miR-155 were employed, modified with cholesterol to permit efficient entry into cells. Incubation of IB3-1 cells with antagomir-155 effectively down-regulated miR-155 expression, together with a sharp decrease in IL-8 mRNA and protein levels (104). On the other hand, as expected, miRNAs were also demonstrated to directly target the 3′UTR of IL-8 mRNA, such as miR-93 (105) and miR-17 (105). Therefore, modulating the expression of miRNAs that target IL-8 mRNA in CF bronchial epithelial cells is likely to represent a new therapeutic strategy for CF (199–201).

These studies have conclusively demonstrated that the pro-inflammatory status in CF is under the control of a complex network constituted by transcription factors and non-coding RNAs, responsible for transcriptional and post-transcriptional regulation of the expression of genes, such as IL-8, belonging to the pro-inflammatory CF network. These studies allowed the identification of novel targets for pharmacological interventions based on newly designed therapeutic approaches.

In addition to the development of new experimental approaches, recent efforts have been undertaken on drug repurposing, in order to bring new therapies based on drugs already used for other indications. This is expected to bring to the market several treatments at a lower risk, reduced cost, and less development time when compared to conventional drug development programs (202–204). One of the most interesting classes of molecules are psoralens, extensively studied as molecules to be employed in PUVA (Psoralen and Ultraviolet A)-therapy, a treatment extensively used in a variety of pathological conditions, including eczema, psoriasis, graft-vs.-host disease, vitiligo, mycosis fungoides, large-plaque parapsoriasis, and cutaneous T-cell lymphoma (205). It was in a study that found that 5-methoxypsoralen reduces *P. aeruginosa*-dependent IL-8 transcription in bronchial epithelial cell lines (206). When the analysis was extended to analogs of 5-methoxypsoralen (207, 208), a potent effect was observed with 4,6,4′-trimethyl-angelicin (TMA), which inhibited *P. aeruginosa*-dependent IL-8 transcription at a nanomolar concentration in IB3-1, CuFi-1, CFBE41o-, and Calu-3 bronchial epithelial cell lines. Analysis of phosphoproteins involved in pro-inflammatory transmembrane signaling evidenced that TMA reduces the phosphorylation of ribosomal S6 kinase-1 and AKT2/3, which were found to be involved in *P. aeruginosa*-dependent activation of IL-8 gene transcription (208). In addition, to understand whether the NF-κB pathway should be considered a target of TMA, chromatin immunoprecipitation was performed, demonstrating that TMA (100 nM) preincubated in whole living cells reduced the interaction of NF-κB with the promoter of IL-8 gene. These results suggest that TMA could inhibit IL-8 gene transcription mainly by intervening on driving the recruitment of activated transcription factors on the IL-8 gene promoter, as demonstrated in NF-κB (208). Recently, TMA was also shown to exhibit, in addition to anti-inflammatory activity, potentiation and correction of the CFTR. In conclusion, TMA is a triple-acting compound that reduces excessive IL-8 expression and potentiating/correcting CFTR function (209, 210). Another repurposed drug proposed for possible anti-inflammatory effects is azithromycin (AZM). IL-8 expression and DNA binding activity of two key pro-inflammatory transcription factors, NF-κB and AP-1, were investigated in CF and isogenic non-CF airway epithelial cell lines. AZM reduced both IL-8 mRNA and protein expression in CF cells reaching the levels of non-CF cells. In the presence of AZM reduction of NF-κB and AP-1, DNA binding was also observed (211). Regarding anti-inflammatory approaches, *in vitro* studies have tested the effects of genistein, fluvastatin, and corilagin, amongst others (212–214). The isoflavonoid genistein [5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one] reduces IL-8 production in cultured CF bronchial gland cells by increasing cytosolic IκBα protein levels, thereby inhibiting NF-kB activation (212). The statin fluvastatin [(±)-(3R′,5S′,6E)-7-[3-(4-Fluorophenyl)-1-isopropylindol-2-yl]-3,5-dihydroxy-6-heptenoate] decreased IL-8 production in whole blood in response to *Pseudomonas* or

Aspergillus antigens, by preventing the prenylation of molecules, such as rho-A, ras, or rac, implicated in IL-8 signaling (213). Corilagin [beta-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-d-glucose], a gallotannin identified in several plants, including *Phyllanthus urinaria*, binds to NF-κB, thus inhibiting NF-κB/DNA interactions and decreasing IL-8 gene expression in CF bronchial IB3-1 cells (214).

As for drug repurposing, corilagin, already shown to exhibit versatile medicinal activities, was found to be of potential use as a possible therapeutic molecule for CF. Interestingly, in addition of IL-8 inhibition, corilagin inhibits TNF-α-induced secretion of MCP-1 and RANTES (214).

The possible identification of repurposed drugs was also tackled by alternative approaches, such as connectivity mapping (ssCMap) to predict A20-inducing drugs and their anti-inflammatory action in CF. A20 is a NF-κB down-regulator that is expressed at low levels in CF and it is hypothesized to be a key target to normalize the inflammatory response (215). Publicly available gene array expression data, together with a statistically significant connections' map (sscMap), were employed. The objective was to predict drugs already licensed for therapeutic use in human pathologies to induce A20 mRNA and protein expression and thereby reduce inflammation. Ikarugamycin and quercetin have been identified as possible candidates for anti-inflammatory approaches, analyzing their effects on A20 and NF-κB(p65) expression (mRNA) as well as IL-8 pro-inflammatory cytokine release in the presence and absence of bacterial LPS in bronchial epithelial cells lines and in primary nasal epithelial cells from patients with CF and non-CF controls (215). Despite the very interesting results obtained from studies of drug repurposing in CF, the safety assessment in a new disease indication (CF in this case) is still an important concern in the regulatory process. While the safety assessment is based on drug label information, the drug repurposing approach may involve different formulations, changes in dosage that should be given great attention in the different patient populations considered.

CONCLUDING REMARKS AND PERSPECTIVES

The present review outlines different specific pathophysiological aspects of CF lung inflammation in which the bronchial epithelial cells represent a "crossroad of signaling" in this disease. Particular emphasis has been given to the role of bronchial epithelial cells in driving the process of neutrophil chemotaxis, with special regard to intracellular signaling, that could be considered a therapeutic target to reduce the lung tissue damage dependent on the byproducts released by hyper-activated neutrophils in the CF bronchial mucosa. To translate into therapy, a special focus was placed on innovative molecules or on drug repurposing to target pathways that are specific of the CF lung pathophysiology, instead of testing broad range anti-inflammatory molecules. As CF lung inflammation is a clearly secondary effect of altered CFTR protein defective ion transport, a question arises on whether in the era of CFTR modulators, with increasing efficacy in CFTR rescue, gating potentiation, and PM stabilization, anti-inflammatory drugs maintain a specific therapeutic rationale. It has already been shown that rescue of F508del CFTR in CF in experimental model systems can partly reduce the release of pro-inflammatory mediators, including IL-8 (216–218). However, the CFTR correctors and potentiators are not available for all the classes of CFTR molecular defects, leaving a significant fraction of CF patients without this treatment option. The clinical response has been shown to be variable within patients with the same class of mutations; the advanced inflammatory disease in adolescent and adult CF patients is unlikely to be completely halted using only CFTR correctors and potentiators, leaving the development of novel anti-inflammatory drugs a rational unmet need for CF treatment (219, 220).

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GC, AR, PP, and RG initiated the concept and wrote the manuscript together with MB, IL, and AF. All authors contributed to the article and approved the submitted version.

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Epigenetic Regulation of Airway Epithelium Immune Functions in Asthma

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Asthma is a chronic inflammatory disease of the respiratory tract characterized by recurrent breathing problems resulting from airway obstruction and hyperresponsiveness. Human airway epithelium plays an important role in the initiation and control of the immune responses to different types of environmental factors contributing to asthma pathogenesis. Using pattern recognition receptors airway epithelium senses external stimuli, such as allergens, microbes, or pollutants, and subsequently secretes endogenous danger signaling molecules alarming and activating dendritic cells. Hence, airway epithelial cells not only mediate innate immune responses but also bridge them with adaptive immune responses involving T and B cells that play a crucial role in the pathogenesis of asthma. The effects of environmental factors on the development of asthma are mediated, at least in part, by epigenetic mechanisms. Those comprise classical epigenetics including DNA methylation and histone modifications affecting transcription, as well as microRNAs influencing translation. The common feature of such mechanisms is that they regulate gene expression without affecting the nucleotide sequence of the genomic DNA. Epigenetic mechanisms play a pivotal role in the regulation of different cell populations involved in asthma pathogenesis, with the remarkable example of T cells. Recently, however, there is increasing evidence that epigenetic mechanisms are also crucial for the regulation of airway epithelial cells, especially in the context of epigenetic transfer of environmental effects contributing to asthma pathogenesis. In this review, we summarize the accumulating evidence for this very important aspect of airway epithelial cell pathobiology.

Keywords: airway, allergy, asthma, epigenetic, epithelium, histone, methylation, microRNA (miRNA)

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INTRODUCTION

Asthma is a chronic inflammatory disease of the airways, in which airway obstruction and hyperresponsiveness underlie recurrent breathing problems, with symptoms being especially pronounced during disease exacerbations (1, 2). Respiratory tract epithelium plays an important role in asthma by initiating and controlling immune responses to different types of pathogenic

environmental factors, including allergens, viruses, pollutants, and others. The biology of the airway epithelium in health and its pathobiology in asthma are regulated by epigenetic mechanisms forming the intercellular homeostatic system responding to internal as well as external changing conditions on the level of transcriptional and posttranscriptional regulation of gene expression (3, 4).

AIRWAY EPITHELIUM AND ASTHMA

The airway epithelium is the first structure of the body getting into contact with inhaled air with all its containing environmental components. Initially, it was thought to just constitute a mechanical barrier to enable the bidirectional transfer of air to and from the gas-exchanging alveolar structures. Over the last years, it turned out, however, that the airway epithelium in general and the bronchial epithelium as a major part of it in particular represent a much more complex tissue fulfilling a variety of additional functions such as retrograde transport of inhaled particles, establishment of a biochemical barrier system, and initiation and regulation of innate and adaptive immune mechanisms by release of various cytokines and chemokines. By this, it represents an integrative part of the innate immune system, the coordinated activity of which is essential for maintaining the local tissue and even systemic body integrity (5). To exert these diverse functions, the bronchial epithelium is composed of multiple structurally and/or functionally differing cell types, such as ciliated cells (mucociliary transport), goblet cells (mucus secretion), tuft and M cells (luminal signal sampling and antigen presentation), ionocytes (water regulation), and club cells (mucus and surfactant protein production) (6). All these cell types develop from local stem cell precursors, called basal cells (7). It is quite obvious that the continuous development of the different cell types from such precursors, as well as their concerted action under healthy conditions, requires a high level of control and regulation (8). In asthma, the underlying control mechanisms are disturbed by both external (environmental factors such as allergens, pollen, bacteria, viruses) and internal (i.e., cytokines, chemokines, low-molecular-weight mediators produced by innate and adaptive immune cells) influences, resulting in dysregulated activities of the bronchial epithelium (9). This includes hypersecretion of mucus, release of epithelial-derived cytokines called alarmins [e.g., interleukin 25 (IL-25), IL-33, thymic stromal lymphopoietin], chemokines, and antimicrobial peptides, as well as uncontrolled proliferation and differentiation processes, altogether leading to functional [e.g., airway hyperresponsiveness(AHR)] and structural (e.g., airway remodeling) changes that represent characteristic features of asthma pathology (10). Not unexpectedly, because of the close relation to environmental influences and their changes, epigenetic regulation processes are crucially involved in the appropriate development, maintenance, and functionality of the different components of the airway epithelium (11). Chronic inflammatory processes such as in asthma are expected to interfere with these well-balanced epigenetic mechanisms in the epithelium of the airways. This may happen at the level of the aforementioned finally differentiated cell types and by changing related gene expression patterns that influence their functional behavior. It is also conceivable that epigenetic changes occur already at the level of the basal cells, which would then be inherited to all kinds of cells developing from the affected precursors with multiple functional consequences (12). It needs to be considered that these mechanisms may either lead to further perpetuation of the disease process or, alternatively, represent repair activities initiated to get the complex system back to steady state, that is, healthy conditions.

EPIGENETIC MECHANISMS

Epigenetics comprises molecular mechanisms of inheritable but reversible phenotypic changes that lead to modified gene expression without alterations at the level of the DNA sequence (13). In the human genome, 80% of the DNA is packed into nucleosomes, and the rest forms linkers between nucleosomes. The nucleosomes are further packed into dense three-dimensional structures called chromosomes (14). The core components of the nucleosome are histone proteins, which are accessible to different types of posttranslational modifications (PTMs), including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination. Posttranslational modifications, especially if occurring at important regulatory genomic regions such as enhancers or promoters, are able to change the accessibility of the DNA to the transcriptional machinery, which is associated with active, poised, or silenced status of transcriptional activity. For example, histone acetylations, the changes introduced by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs), are usually associated with transcriptional activation of the gene (15, 16). DNA methylation, in which a methyl group is enzymatically added to the cytosine ring of DNA, is another type of the epigenetic modification. While the methylation reaction is catalyzed by DNA methyltransferases, ten-eleven translocation (TET) methylcytosine dioxygenase family proteins mediate DNA demethylation. DNA methylation is typically associated with gene repression (3, 17). In addition to the classical epigenetic modifications mentioned above, different types of the non-coding RNAs such as microRNAs (miRNAs) and others, for instance, piwi-interacting RNAs or small nucleolar RNAs, are involved in the epigenetic regulation of gene expression. Briefly, miRNAs exert their silencing effects through the binding to the mature mRNA molecules in the cytosol that leads to mRNA degradation or reduction in the translational efficiency of the ribosomes (18, 19) (**Figure 1**).

DNA METHYLATION

DNA methylation is probably the best studied epigenetic modification in general but also in relation to asthma. Although studies conducted so far on the involvement of DNA methylation in asthma have mostly used already available DNA samples and/or DNA extracted from easily available tissues, also

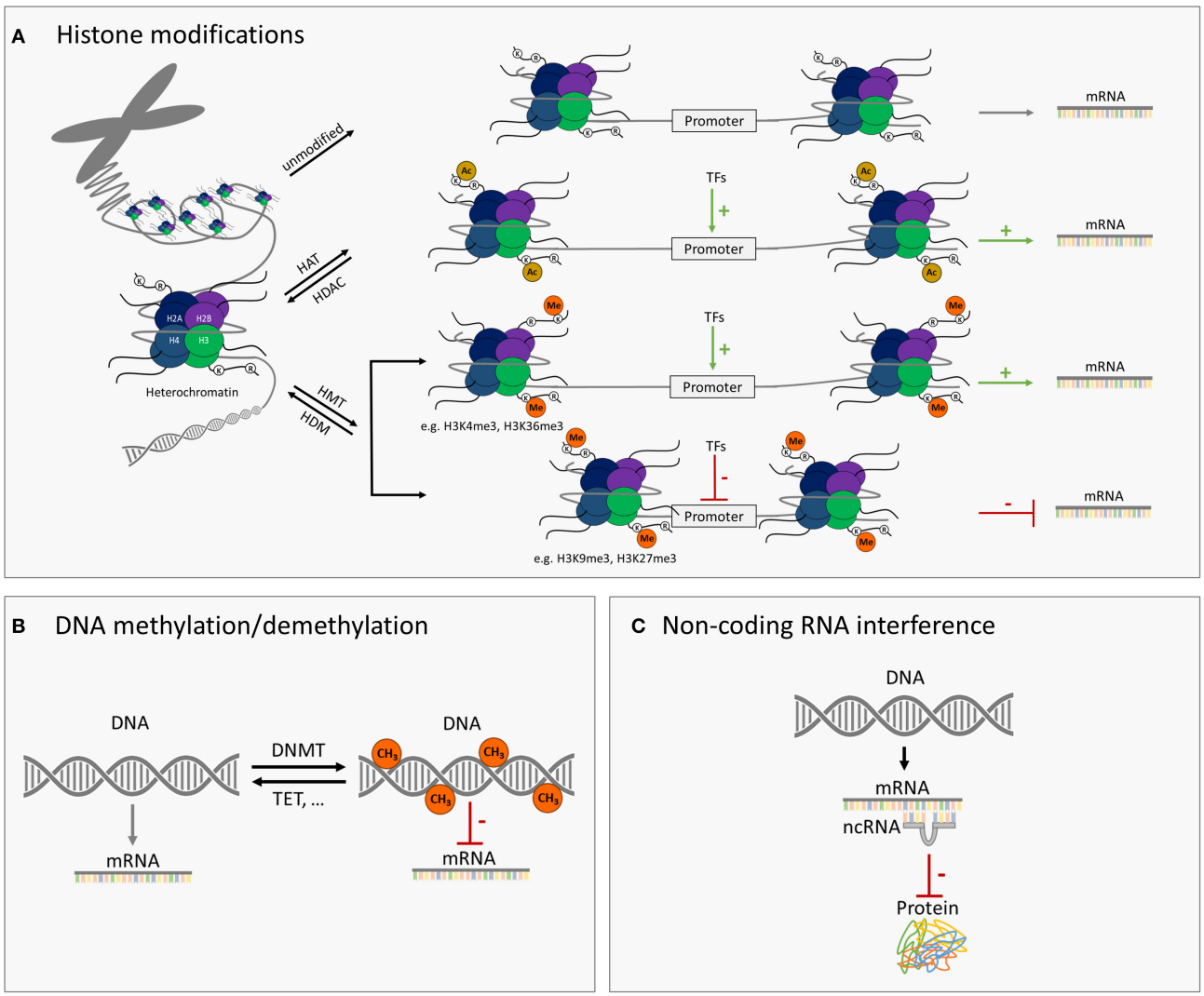

FIGURE 1 | Schematic illustration of major epigenetic modifications. **(A)** Modification of histones such as histone acetylation/deacetylation via histone acetyltransferases (HATs)/histone deacetylases (HDAC) and methylation/demethylation via histone methyltransferases (HMT)/histone demethylases (HDM) can either activate or repress the target gene transcription. Histone acetylation is typically associated with higher expression of the gene. Histone methylation can be related to either higher or lower transcriptional activity, depending on the amino acid residue modified and the number of methyl groups added. **(B)** DNA methylation or demethylation of genomic DNA through DNA methyltransferases (DNMT) or ten-eleven translocation (TET) enzymes and others, respectively. Higher level of DNA methylation is typically associated with lower transcriptional activity of the respective gene. **(C)** MicroRNAs (miRNAs) and further small non-coding RNAs can interfere with gene expression through base pairing with messenger RNAs and thus inhibiting their translation into the encoded protein.

lower airway epithelial cells (AECs) have been investigated (**Figure 2**). Stefanowicz et al. (20) performed a comparative DNA methylation analysis of 807 genes in bronchial AECs and peripheral blood mononuclear cells (PBMCs) obtained from atopics, atopic asthmatics, non-atopic asthmatics, or healthy controls. They identified signature sets of CpG sites differentially methylated between AECs and PBMCs, which were either independent of the disease phenotype or specific to healthy controls, atopics, or asthmatics. Although no differences in the DNA methylation status were found between disease phenotypes in PBMCs, they were observed between asthmatics and atopics in AECs (20). Kim et al. (21) comparatively analyzed genome-wide DNA methylation levels in bronchial mucosa tissues obtained from atopic and non-atopic asthmatics and healthy controls. Although the methylation levels were similar between asthmatics and controls, a set of loci has been identified with significant differences in DNA methylation between atopic and non-atopic asthmatics (21). Clifford et al. (22) investigated in turn the effects of experimental respiratory tract exposure to allergen, diesel exhaust, or both as a coexposure, always observing only minimal resulting changes in the bronchial epithelial DNA methylome of the participating individuals. They found, however, that if any of the two insults occurs in advance of the other (crossover exposure with a 4-week interval), the initial

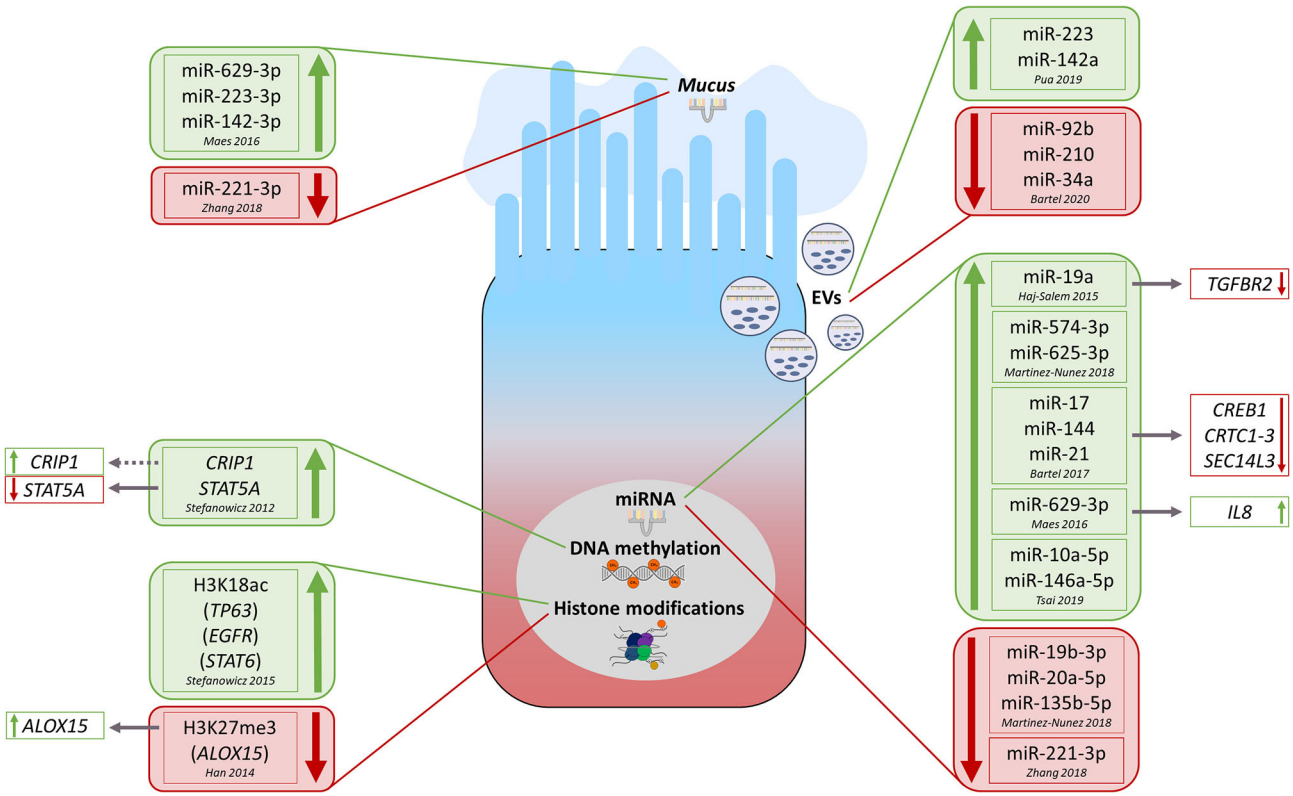

FIGURE 2 | Overview of currently known key epigenetic modifications observed in lower airway epithelial cells from asthma/allergic airway inflammation conditions and—if known—associated functional consequences. The green color always indicates upregulation of the respective modification in asthmatics vs. healthy while red color identifies opposite regulation. EVs, extracellular vesicles; miRNA, microRNA; H3K18ac, histone H3K18 acetylation; H3K27me3, histone H3K27me3 trimethylation.

one primes the bronchial epithelial DNA methylome for the second, resulting in cumulative epigenetic changes with potential biological relevance (22).

In most cases, however, DNA methylation studies conducted in airway tissue in the context of asthma have not been performed in bronchial or lung ACEs but rather in nasal epithelial cells (NECs) due to easier accessibility (23–28). Cardenas et al. (23) conducted an epigenome-wide study on DNA methylation using nasal swabs collected in a large group of early teenagers deriving from a birth cohort. They identified multiple DNA methylation loci associated with asthma, allergies, and related clinical or laboratory parameters (23). In another cohort of adolescents, Forno et al. (24) performed in turn an epigenome-wide analysis of DNA methylation in nasal epithelium. The major findings of this study, replicated in two independent cohorts, comprised the identification of specific DNA methylation profiles associated with atopy and atopic asthma and a nasal methylation panel that could classify children by atopy or atopic asthma (24). Reese et al. (25) sought to identify differential DNA methylation related to pediatric asthma in blood from newborns and school-aged children. Interestingly, they were able to replicate in eosinophils or nasal respiratory epithelium most of the asthma-related differential methylation signatures initially detected in blood (25). Brugha et al. (29) comparatively analyzed DNA methylation in airway and surrogate tissues. They found that the methylation profile in nasal epithelium was most representative of that in the airway epithelium, whereas the profile in buccal cells was moderately and that in blood was least similar (29). In our view, these results clearly suggest that DNA methylation studies performed in the context of asthma as an airway disease should preferentially be conducted using AECs or NECs. Although beyond the scope of this review, we would like to mention that, in our view, sorted specific white blood cell populations would be highly valuable to study systemic adaptive immunity DNA methylation patterns underlying asthma. However, how well those signatures correspond to local lung DNA methylation patterns would need to be assessed in separate studies. Back to NECs, Xiao et al. (27) showed that nasal DNA methylation at the promoter of the vanin 1 gene (*VNN1*) might be a clinically useful biomarker of corticosteroid treatment response in asthmatic children. Another study from the same group demonstrated in turn that DNA methylation at the TET methylcytosine dioxygenase 1 gene (*TET1*) contributes to traffic-related air pollution and asthma (26).

Finally, allelic differences in DNA methylation and thus gene expression in AECs can mediate the effects of certain genetic variants known to be associated with susceptibility to childhood asthma, such as those in chromosome 17q21 (30, 31).

HISTONE MODIFICATIONS

In addition to DNA methylation, also histone modifications participate in epithelial (patho-) mechanisms related to asthma (**Figure 2**). Stefanowicz et al. (32) compared global and gene-specific alveolar epithelial cells histone acetylation and methylation status between asthmatics and healthy subjects. Generally, they observed higher global H3K18ac and H3K9me3 levels in asthmatic subjects. In more detail, they found in asthmatics a higher association of H3K18ac (but not H3K9me3) around the transcription start sites of *TP63* (tumor protein p63, ΔNp63 isoform), *EGFR* (epidermal growth factor receptor), and *STAT6* (signal transducer and activator of transcription 6) genes. Finally, they detected a non-significant increase in protein expression of those three genes in AECs treated with trichostatin A, an HDAC inhibitor (HDACi) (32). In another work, the same group comparatively analyzed the expression of 82 epigenetic modifying enzymes in AECs and bronchial fibroblasts obtained from asthmatics and healthy controls (33). Thirty-nine enzymes were differentially expressed between AECs and bronchial fibroblasts, 24 of which passed the correction for multiple testing. Six histone modifiers turned out to be differentially expressed in AECs between asthmatics and non-asthmatics, however, mostly not significantly when corrected for multiple testing (33).

Beneficial effects of HDACi have been observed in murine models of allergic airway inflammation (AAI) mimicking features of human allergic asthma (34, 35). Application of HDACi in an ovalbumin (OVA)–based model reduced airway inflammation, remodeling, and AHR. In addition, HDACi treatment was associated with lower expression of transforming growth factor β1 (TGF-β1) in AECs and diminished synthesis of contractile proteins by airway smooth muscle cells (34). HDACi treatment in mice subjected to a house dust mite (HDM)–based model was in turn able to prevent them from developing AHR and AAI. Moreover, HDACi restored the integrity of the *ex vivo*–cultured NECs isolated from AR patients (35). Significantly lower H3K27me3 levels at the promoter of the arachidonate 15-lipoxygenase (ALOX15) gene (*ALOX15*) were observed in human lung epithelial A549 cells after the treatment with IL-4, which coincided with higher *ALOX15* mRNA levels (36).

Targeting histone modification—related mechanisms turned out to be effective also in a cockroach allergen extract–induced mouse model of mixed granulocytic (eosinophilic and neutrophilic), T_H2/T_H17-driven asthma (37). Specifically, whereas a bromo- and extraterminal (BET) inhibitor was already alone able to abolish T_H17-driven neutrophilic inflammation, in combination with dexamethasone it completely blocked both T_H2- and T_H17-driven immune responses in the lung, which was associated with reductions in lung eosinophilia and neutrophilia, and mucin secretion. Furthermore, BET inhibition improved cockroach allergen extract– or IL-17A–induced increase in markers of glucocorticoid insensitivity [i.e., decrease in HDAC2 expression (38)] in murine or human AECs, respectively (37). In another study, $Hdac2^{+/-}$ mice subjected to an HDM-induced AAI model demonstrated stronger inflammatory infiltration as well as higher expression of type 2 cytokines and IL-17A in the lung tissue compared to wild-type animals. Additional IL-17A depletion was able to reverse these HDAC2 impairment-induced effects (39). In turn, HDM and IL-17A synergistically reduced HDAC2 expression in human bronchial epithelial cells (BECs) *in vitro*. Besides, silencing the HDAC2-encoding gene further enhanced HDM- and/or IL-17A–induced inflammatory cytokines in human BECs, whereas HDAC2 overexpression or knockdown of the gene encoding IL-17A was able to reduce the release of such inflammatory cytokines (39). Taken together, original findings by Zijlstra et al. (38), who first discovered IL-17A–induced steroid resistance mediated by a reduction of HDAC2 activity, have thus been corroborated and expanded.

MICRORNA

Several recent studies have highlighted the importance of miRNAs in the regulation of epithelial pathobiology in asthma (**Figure 2**). Bartel et al. (40) combined different approaches such as *in vivo* studies in mice with OVA- or HDM-induced AAI, *ex vivo*/*in vitro* experiments including luciferase reporter assay and stimulation–expression analyses, miRNA/mRNA microarrays, and *in silico* approaches. This composed strategy enabled the authors to identify the transcription factor cAMP-responsive element binding protein (*Creb1*) and its transcriptional coactivators (*Crtc1-3*) as targets for miR-17, miR-144, and miR-21, all three deregulated in lungs of mice with AAI. Moreover, they observed downregulation of Sec14-like 3 (*Sec14l3*), a putative target of Creb1, in both AAI models and in primary normal human BECs upon IL-13 treatment suggesting that miRNA-regulated Crtc1-3 and Sec14l3 play a role in early epithelial responses to type 2 stimuli (40). Microarray analysis of miRNA expression in bronchoscopy-isolated human BECs showed in turn an upregulation of miR-19a in samples obtained from severe asthmatic subjects compared to those from mild asthmatics and healthy controls (41). Furthermore, luciferase reporter assay- and Western blot–based functional studies demonstrated miR-19a to enhance proliferation of BECs in severe asthma through targeting TGF-β receptor 2 gene (*TGFBR2*) mRNA (41). Using subcellular fractionation and RNA sequencing (Frac-seq) in human primary BECs from healthy controls and severe asthmatics, Martinez-Nunez et al. (42) assessed paired genome-wide expression of miRNAs along with cytoplasmic (total) and polyribosome-bound (translational) mRNA levels. They identified a hub of six dysregulated miRNAs, displaying preference for polyribosome-bound mRNAs, which accounted for ∼90% of whole miRNA targeting. Interestingly, transfection of such miRNAs into BECs obtained from healthy subjects turned them into cells mimicking features of those obtained from severe asthmatics (42).

Recently, extracellular vesicles (EVs) transferring miRNAs between cells have been identified as a novel mechanism of intercellular communication (3, 43, 44). Of note, the composition of the extracellular miRNA pool in the lung of mice was very similar to that of the airway epithelium, and ∼80% of the detected EVs were of epithelial origin (45). However, following the induction of AAI, the presence of miRNAs preferentially

expressed by immune cells, such as miR-223 and miR-142a, and hematopoietic cell–derived EVs increased also substantially, indicating an importance of the extracellular miRNA pool for the development of local allergic inflammatory processes (45). Gupta et al. (46) focused on EVs secreted by two kinds of human airway cell cultures, that is, primary tracheobronchial cells and a cultured AEC line (Calu-3). Their data suggest that cellular information can be transferred between AECs via miRNA-containing EVs, which may thereby contribute to epithelial biology and remodeling (46). Another study profiled the expression of miRNAs in EVs secreted from the apical and basal sides by normal human BECs treated with IL-13 in order to induce an asthma-like response (47). Significant candidates were then confirmed in EVs isolated from nasal lavages obtained from children with mild to moderate or severe asthma and healthy control subjects. Interestingly, levels of miR-92b, miR-210, and miR-34a turned out to correlate with lung function measures (47).

Two studies investigated miRNAs in asthmatic sputum (48, 49). In two independent cohorts, Maes et al. (48) found a significant upregulation of miR-629-3p, miR-223-3p, and miR-142-3p in sputum of severe asthmatics compared to healthy controls, with the highest levels in patients with neutrophilic asthma. Of those three miRNAs associated with sputum neutrophilia and airway obstruction, miR-629-3p was expressed in BECs. Interestingly, transfection of human BECs with a miR-629-3p mimic induced expression of IL-8, the sputum levels of which were significantly increased and positively correlated with sputum neutrophilia in severe asthmatics (48). Zhang et al. (49) found in turn that epithelial, sputum, and plasma miR-221-3p levels were significantly lower in asthmatics compared to healthy controls. In addition, levels of epithelial and sputum miR-221-3p inversely correlated with airway eosinophilia (49).

Finally, Tsai et al. (50) sought to find the common miRNA-related effects in BECs obtained from subjects with asthma and chronic obstructive pulmonary disease (COPD). First detected with next-generation sequencing, the upregulation of miR-10a-5p and miR-146a-5p in BECs obtained from both asthma and COPD patients was subsequently confirmed by quantitative polymerase chain reaction. Moreover, compared to healthy controls, also serum miR-146a-5p levels were higher in asthma and COPD subjects (50). Further research will establish whether miRNAs mediating intercellular communication can be used for clinical applications as biomarkers or therapeutic targets.

SPECIAL ASPECTS

Airborne viruses, for instance, human rhinoviruses (HRVs), stimulate asthma exacerbations. In addition, repeated early life infections with such viruses can lead to the development of a persistent asthma phenotype, especially in children with atopic susceptibility (3, 51). Interestingly, some studies suggest that the effects of respiratory viral infections are at least partly mediated by epigenetic changes in airway epithelial cells. It has been demonstrated that *ex vivo* experimental HRV infection of NECs obtained from asthmatic children significantly changes patterns of DNA methylation and mRNA expression (52). Moreover, HRV infection in young children has been associated with changes in the airway secretory miRNome, characterized by a highly specific additional appearance of miR-155 in nasal secretion EVs (53). In turn, BECs obtained from asthmatics have been shown to be characterized by dysregulated miR-22 expression after experimental *ex vivo* infection with influenza A virus (IAV) (54). Other epigenetic modifications, specifically histone methylations, also seem to be involved in the regulation of epithelial antiviral responses (55).

Dysregulated epithelial–mesenchymal transition (EMT) is the process driven mostly by TGF-β1, which strongly contributes to the establishment of the persistent asthma phenotype, that is, to disease chronification (56). Epigenetic mechanisms seem to play an important role in EMT. It has been demonstrated in mouse models mimicking allergic asthma that miR-448-5p can inhibit TGF-β1–induced EMT and pulmonary fibrosis (57). Applying an epigenome-wide approach in a human study, McErlean et al. (58) identified in turn multiple loci showing differential H3K27ac enrichment in asthma, which clustered at genes associated with type 2–driven asthma and EMT.

CONCLUSIONS AND PERSPECTIVES

Epigenetic mechanisms play a very important role in the epithelial pathobiology of asthma. While histone modifications seem to be especially interesting as possible therapeutic targets, DNA methylation and miRNAs, also from the easily accessible nasal epithelium, show a substantial diagnostic potential. Although the data gathered by now (for overview, see also **Supplementary Table 1**) already strongly suggest a usefulness of epigenetics in the asthma management, further studies, especially those considering the complex interplay of different epigenetic mechanisms and those focusing on a single-cell type or investigations on the single cell level, are needed.

AUTHOR CONTRIBUTIONS

BA: draft writing and figure drafts. SM: draft writing and final figures. ES: editing and reviewing. DP: conceptualization, draft writing, and reviewing. HG: conceptualization, coordination, draft writing, and reviewing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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