INTRA/EXTRACELLULAR DYNAMICS OF THE RESPIRATORY SYSTEM AND GLOBAL AIRWAY DISEASE

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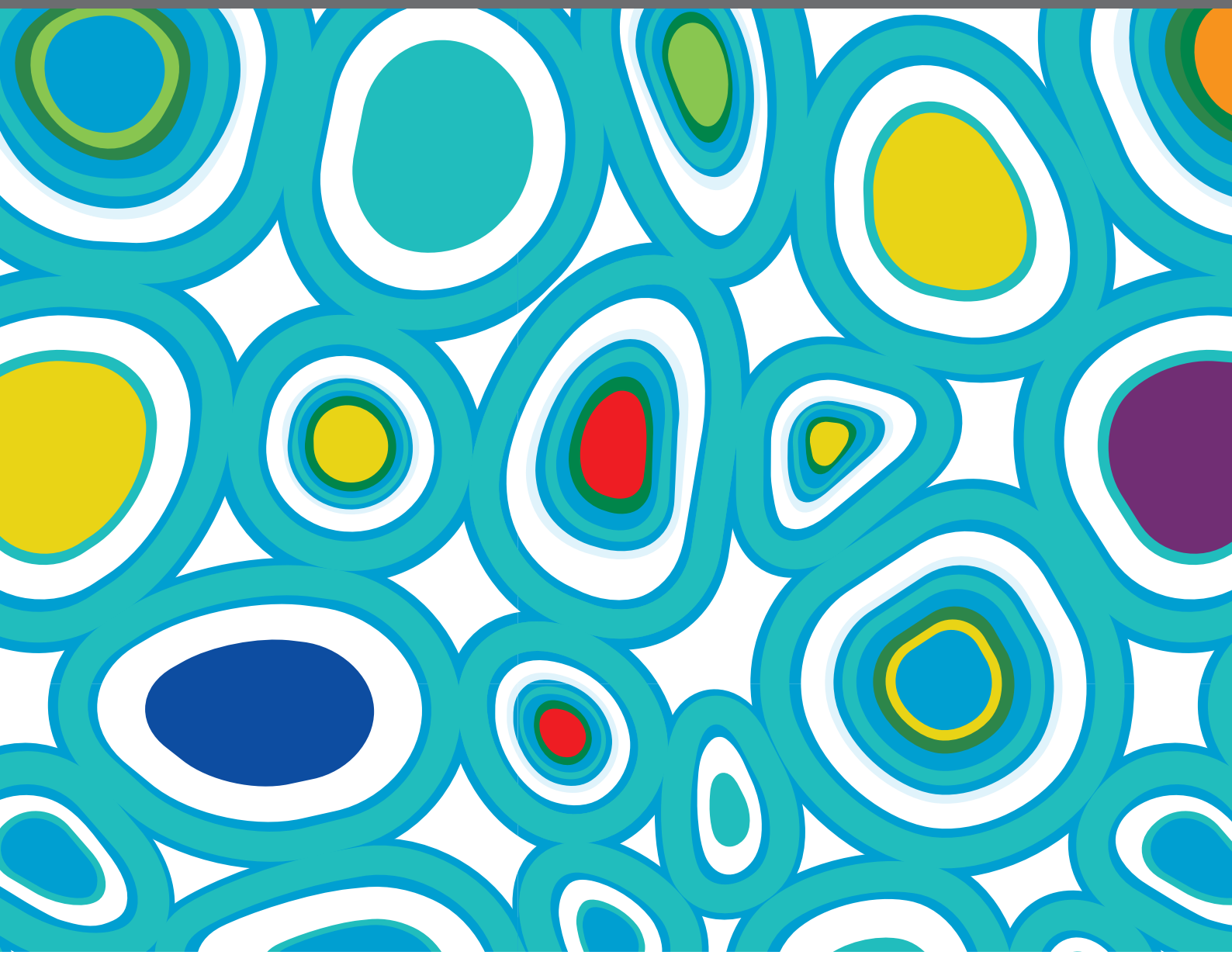

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INTRA/EXTRACELLULAR DYNAMICS OF THE RESPIRATORY SYSTEM AND GLOBAL AIRWAY DISEASE

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EDITORIAL
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Editorial: Intra/Extracellular Dynamics of the Respiratory System and Global Airway Disease

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Editorial on the Research Topic

Intra/Extracellular Dynamics of the Respiratory System and Global Airway Disease

Chronic inflammatory airway diseases are a heterogeneous entity that encompasses an extensive array of allergic (i.e., allergic rhinitis, asthma, eosinophilic bronchitis) and non-allergic diseases (i.e., chronic rhinosinusitis, chronic obstructive pulmonary disease, bronchiectasis, cystic fibrosis). The concept "one airway, one disease" has been increasingly accepted thanks to the accumulating evidence that has pointed to the association between upper and lower airway diseases. Hence, the term "global airway disease" or "united airway disease" might more accurately reflect the co-existing diseases as well as the potential interactions between upper and lower airway diseases (Burgel, 2015; Giavina-Bianchi et al., 2016). Notwithstanding our growing recognition of this concept, there remain many unanswered questions. What are the common triggers of global airway diseases and how do they work? What are the mechanisms underlying these associations? What are the potential therapeutic targets for intervention? In this special issue of *Frontiers in Cell and Developmental Biology*, a series of original research and review articles have sought to unravel some novel mechanistic insights as well as potential therapeutic targets of the global airway diseases.

A number of mechanisms related to the global airway disease have been proposed. Central to these mechanisms is the contribution of the epithelium. Lining both the upper and lower airways, the airway epithelium constitutes a critical layer barrier against the invasion of pathogens, antigens, and other xenobiotics. Thus, disruption of the airway epithelial barrier function has been associated with the pathogenesis of various airway inflammatory diseases. In a review by Laulajainen-Hongisto et al., the authors elegantly summarized that the pathogenic factors contribute to the weakening of airway epithelial barrier function by disrupting the tight junction (i.e., airborne irritants and pollutants) and mucociliary function (i.e., viral infections, aeroallergen inhalation) and shaping the immune responses (i.e., dysbiosis). Respiratory viral infections (in particular, latent infections) disrupt mucociliary clearance, mucus hypersecretion and epithelial cell death, which further readily result in airway dysbiosis, cellular and tissue oxidative stress and the dysregulation of inflammatory responses (Tan K. S. et al.). Similar with viral infections, air pollutants (especially particulate matter) result in decreased mucociliary clearance, increased pathogen adhesion, airway dysbiosis, decreased alveolar macrophage and natural killer cells, and decreased antimicrobial activity—all of which can compromise factors epithelial barrier function (Yang et al.).

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Apart from the defective epithelial barrier function, certain molecules that are constitutively expressed also in the upper and lower airways play key roles in modulating the development of global airway diseases. For instance, cluster of differentiation 151 (CD151) facilitates viral replication via nuclear export signaling and up-regulation of CD151 contributes to airway hyperresponsiveness and smooth muscle contractility (hallmarks of asthma), and lung fibrosis via modulating matrix metalloprotein-7 expression (Wong and Tran). In addition, cell senescence as a consequence of telomere damage, oxidative stress, cellular inflammation, and/or non-selective autophagy, triggered by various pathogenic factors, might also underlie the pathogenesis of global allergic diseases such as asthma (Wang Z.-N. et al.). Moreover, airway smooth muscle cells are vulnerable to inflammation triggers (such as exaggerated release of tumor necrosis factor-α and interleukin-13) which could lead to smooth muscle dysfunction that is related to endoplasmic reticulum stress and mitochondrial dysfunction (Delmotte and Sieck). In another study, the interaction between A-kinase anchoring proteins and phosphokinase A was capable of inducing smooth muscle hypercontractility with concomitant induction of cell proliferation markers (Baarsma et al.). All these novel findings provide further insight into the molecular mechanisms underlying smooth muscle dysfunction that cannot be readily ameliorated by the use of bronchodilators. Collectively, an integrated approach for the disease severity assessment and clinical management is warranted in light of the multifaceted nature of global airway diseases.

Apart from the adverse impacts conferred by direct exposure after birth, maternal exposure to air pollution and cigarette smoke could lead to epigenomic reprogramming leading to altered immune response and aberrant epithelial and mesenchymal cellular function of the fetus that underpin the development of asthma during childhood (Wang B. et al.). The accumulating evidence has pointed to the potential benefits of cessation of cigarette smoking and initiating campaigns to improve air quality to mitigate the risks of allergic diseases such as asthma. Finally, physical factors (such as irradiation) have also been shown to disrupt barrier function by causing cell shape elongation and increasing cell motility—both of which contribute to cell unjamming transition which was dependent on transforming growth factor-β (O'Sullivan et al.). The development of global airway diseases is, therefore, likely to be associated with cellular epigenomic reprogramming and defective epithelial barrier function (i.e., alteration in cell proliferation and motility).

Yet the impact of certain global airway diseases might be more far-reaching. An anecdotal finding of the sequelae of decreased olfactory sensitivity (possibly as a result of allergic rhinitis or chronic rhinosinusitis) was the association with erectile dysfunction. In a prospective study of 102 males, Deng et al. observed a significant correlation between the olfactory function and erectile function. Interestingly, after adjustment for potential confounding factors, erectile dysfunction was associated with the olfactory disorder, rhinology disease, age, and greater visual analog scale (a 5-point scale self-rated by the patients, with higher scores indicating a greater magnitude of symptom burden) for olfactory function (Deng et al.). Therefore, inflammatory upper airway diseases might have more profound systemic implications, although further mechanistic studies are warranted.

We have also had the privilege of spotting some novel targets that would help manage the global airway diseases in the near future. Asthma is one of the most common airway inflammatory diseases which frequently co-exist with allergic rhinitis or chronic rhinosinusitis. Maintaining proper immune balance is crucial to asthma management. Allergen immunotherapy has been effective in a subset of patients with allergic rhinitis or asthma, but the specific mechanisms are not fully elucidated. Feng et al. revealed that an increase in the level of specific immunoglobin G4, but not specific immunoglobin E, against *Dermatophagoides pteronyssinus* correlated with the magnitude of amelioration of basophil activity after subcutaneous allergen immunotherapy. The competence of specific immunoglobin G4 antibodies with specific immunoglobin E binding to the allergens helped interpret why allergen immunotherapy worked at least in certain subpopulations (Feng et al.). These findings represented an opportunity to refine the clinical efficacy of allergen immunotherapy. Therapeutic approaches that could boost the suppression of basophil activation via potent binding with the allergens would be an important adjunct to allergen immunotherapy.

Similar to the vicious cycle seen in airway suppurative diseases, cellular senescence might further result in accelerated aging of the neighboring cells (Wang Z.-N. et al.). Targeted interventions (i.e., caloric restriction, supplementation of antioxidants, senolytic drugs) and replenishment of cells (i.e., stem cell transplantation) might have a role in averting the accelerated senescence of cells and tissues as a consequence of inflammatory insults.

Airway remodeling has been regarded as the hallmark of many chronic airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease. While epithelial membrane thickening and smooth muscle hyperplasia have been identified to be the predominant changes, few studies have linked these changes to the molecular mechanisms. Tan Y. et al. elaborated on the role of fibrocyte growth factor-2 (FGF-2), a potent mitogenic factor, in modulating immunity against viral infections and chronic airway inflammation. Being the "relay player" between airway structural cells (which sensed the insults) and inflammatory cells (usually the effector cells), FGF-2 could promote neutrophil recruitment, activate monocytes, induce smooth muscle cell hyperplasia, and promote its release of cytokines. These have rendered FGF-2 a potentially promising target for future therapeutic interventions that aim at ameliorating airway remodeling.

Bronchiectasis is a chronic airway suppurative disease characterized by irreversible dilatation of bronchi and bronchioles. Through immunofluorescence assays on the surgically resected specimens, Peng et al. have identified the presence of cuboidal and columnar epithelial hyperplasia

Abbreviations: CC10, Club Cell 10 kDa Protein; SPC, Surfactant protein C; TTF-1, thyroid transcription factor-1; CD151, cluster of differentiation 151.

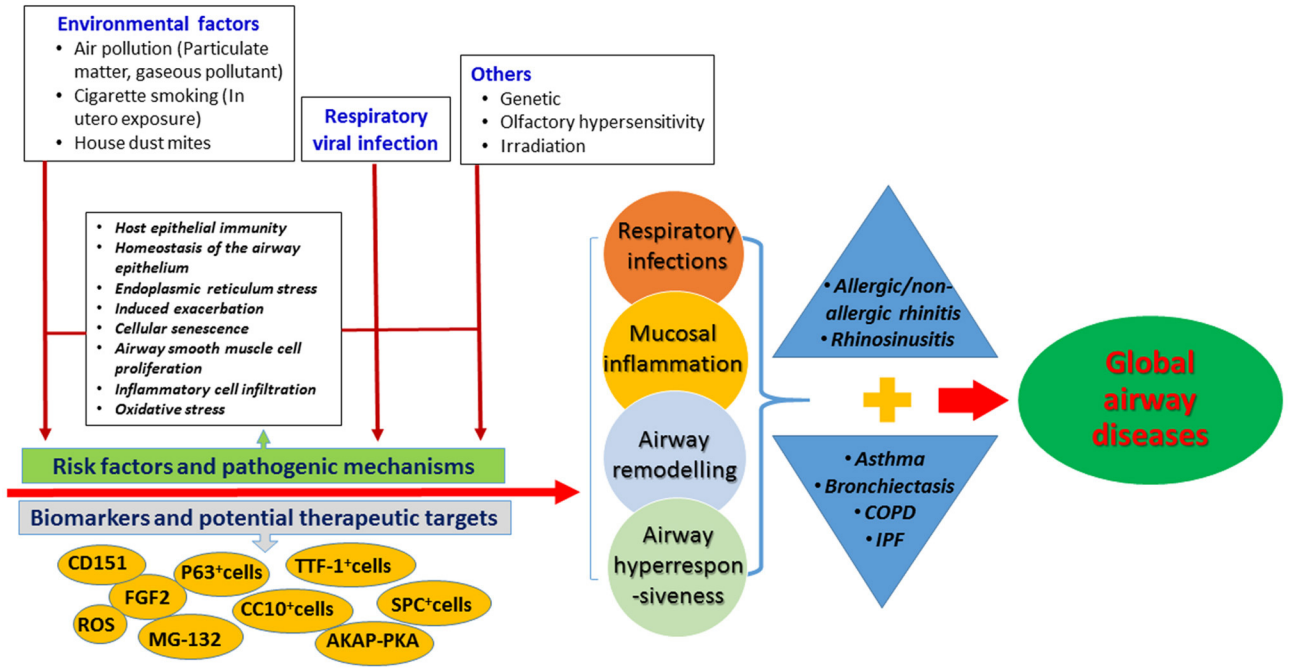

FIGURE 1 | Summary of the risk factors, molecular mechanisms and biomarkers for the global airway diseases in this specific topic. AKAP-PKA, A-kinase anchoring proteins and phosphokinase A; ASM, airway smooth muscle cells; CC10, Club Cell 10 kDa Protein; SPC, Surfactant protein C; TTF-1, thyroid transcription factor-1; CD151, cluster of differentiation 151; FGF2, fibrocyte growth factor-2; ROS, reactive oxygen species.

with disarrangement of thyroid transcription factor-1 (TTF-1)$^{+}$ cells in patients with bronchiectasis. Importantly, most progenitor cell markers (Clara Cell 10, surfactant protein C and p63) co-localized with TTF-1 in the dilated bronchiole sub-epithelium. Expression of surfactant protein C co-localized with TTF-1 in regions with cuboidal epithelial hyperplasia, whereas TTF-1 co-localized with P63 and surfactant protein C in regions with columnar epithelial hyperplasia. These pilot studies link the abnormality of airway progenitor cells to the pathogenesis of bronchiectasis, thus unraveling the therapeutic targets for bronchiectasis.

Figure 1 summarizes the emerging understandings of the causes of the diseases, molecular mechanisms, and possible targets for therapy. We are now in a better position to appreciate the molecular mechanisms responsible for the tissue and cellular injury. A systemic approach (which takes into account the epithelial barrier function, airway remodeling, immune dysregulation, oxidative stress, and dysbiosis) is needed for an integrated clinical assessment and management of the global airway diseases.

AUTHOR CONTRIBUTIONS

WG, TT, and D-YW: wrote the paper. All authors: critical review and approval.

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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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REVIEW
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Endoplasmic Reticulum Stress and Mitochondrial Function in Airway Smooth Muscle

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Inflammatory airway diseases such as asthma affect more than 300 million people worldwide. Inflammation triggers pathophysiology via such as tumor necrosis factor α (TNFα) and interleukins (e.g., IL-13). Hypercontraction of airway smooth muscle (ASM) and ASM cell proliferation are major contributors to the exaggerated airway narrowing that occurs during agonist stimulation. An emergent theme in this context is the role of inflammation-induced endoplasmic reticulum (ER) stress and altered mitochondrial function including an increase in the formation of reactive oxygen species (ROS). This may establish a vicious cycle as excess ROS generation leads to further ER stress. Yet, it is unclear whether inflammation-induced ROS is the major mechanism leading to ER stress or the consequence of ER stress. In various diseases, inflammation leads to an increase in mitochondrial fission (fragmentation), associated with reduced levels of mitochondrial fusion proteins, such as mitofusin 2 (Mfn2). Mitochondrial fragmentation may be a homeostatic response since it is generally coupled with mitochondrial biogenesis and mitochondrial volume density thereby reducing demand on individual mitochondrion. ER stress is triggered by the accumulation of unfolded proteins, which induces a homeostatic response to alter protein balance via effects on protein synthesis and degradation. In addition, the ER stress response promotes protein folding via increased expression of molecular chaperone proteins. Reduced Mfn2 and altered mitochondrial dynamics may not only be downstream to ER stress but also upstream such that a reduction in Mfn2 triggers further ER stress. In this review, we summarize the current understanding of the link between inflammation-induced ER stress and mitochondrial function and the role played in the pathophysiology of inflammatory airway diseases.

Keywords: mitofusin, IRE1, XBP1, asthma, inflammation

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INTRODUCTION

Inflammation triggers asthma pathophysiology via pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 13 (IL-13). Two hallmarks of asthma are human airway smooth muscle (hASM) hypercontractility and cell proliferation (James, 2005; Joubert and Hamid, 2005; Black et al., 2012; Prakash, 2013, 2016; Wright et al., 2013a,b; Delmotte and Sieck, 2015). It is likely that with asthma, hASM exists in both hyper-contractile and proliferative states, thus contributing to a thicker, more contractile airway. An emergent theme in this context is the role of inflammation-induced endoplasmic reticulum (ER) stress and mitochondria. Previously,

we showed that cytokine exposure increases agonist-induced hASM force and ATP consumption due to an increase in contractile protein expression (Dogan et al., 2017). Initially, the increase in hASM ATP demand is met by increased mitochondrial O_2 consumption and ATP production, but at the expense of reactive oxygen species (ROS) formation and oxidative stress. There is increasing evidence that mitochondria and the ER, although structurally separate organelles, are functionally interdependent units, which must establish links for normal cellular function, including $[Ca^{2+}]_{cyt}$ regulation, energy production and cell proliferation (Hajnoczky et al., 2000; Franzini-Armstrong, 2007; Romagnoli et al., 2007; Liesa et al., 2009; Kornmann and Walter, 2010; Antico Arciuch et al., 2012; Glancy and Balaban, 2012; Dorn and Maack, 2013; Kornmann, 2013; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). These links are established through specialized ER-mitochondria encounter structures (ERMES) comprising both ER and mitochondrial transmembrane proteins that provide a tethering force between the two organelles to ensure proximity and communication (Franzini-Armstrong, 2007; Kornmann and Walter, 2010; Dorn and Maack, 2013; Kornmann, 2013; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). Mitofusin-2 (Mfn2) is an ERMES component that serves to tether mitochondria to the ER. Mfn2 also serves to fuse mitochondria, which together with other fusion proteins [e.g., Mfn1, optic atrophy 1 (Opa1)] elongate mitochondria making them more filamentous, whereas fission proteins such as dynamin related protein 1 (Drp1) and fission 1 protein (Fis1) act to fragment mitochondria. Together these fusion/fission proteins act to dynamically remodel mitochondria under a variety of conditions (Smirnova et al., 2001; James et al., 2003; Lee et al., 2004; Song et al., 2009; Sheridan and Martin, 2010; Palmer et al., 2011; Ranieri et al., 2013). The tethering of mitochondria to the ER allows mitochondrial proximity to ER Ca^{2+} release sites representing a microdomain of higher $[Ca^{2+}]_{cyt}$ ("hotspot" > 2 μM) that is essential for mitochondrial Ca^{2+} influx [by activating the mitochondrial Ca^{2+} uniporter (MCU)] (Raffaello et al., 2012). In the absence of mitochondrial Ca^{2+} buffering, the transient $[Ca^{2+}]_{cyt}$ response to agonist stimulation is elevated, thereby enhancing hASM force generation. This review will discuss the link between ER stress, Mfn2 expression, mitochondrial tethering to the ER, mitochondrial Ca^{2+} influx, and mitochondrial function in the context of airway inflammation and potential consequences on ASM hyper-contractile and proliferative states.

INFLAMMATION AND ER STRESS IN HUMAN ASM

One consequence of inflammation is the unfolding of proteins that accumulate in the lumen of the ER, exposing binding sites for the chaperone protein, binding immunoglobulin protein (BiP). With an accumulation of unfolded proteins, BiP dissociates from three proteins localized at the ER membrane resulting in their activation. The resulting physiological response referred as ER stress or unfolded protein response attempts to restore normal ER function by increasing chaperones proteins expression, halting protein translation and activating protein degradation (Yoshida et al., 2001; Bravo et al., 2012; Garg et al., 2012; Verfaillie et al., 2012; Hasnain et al., 2013; Sano and Reed, 2013; Vannuvel et al., 2013; Delmotte and Sieck, 2015; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Morris et al., 2018). These three ER stress protein markers involved in this signaling cascade are: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1α, also called serine/threonine-protein kinase/endoribonuclease IRE1α) (**Figure 1**) (Hai et al., 1989; Nikawa and Yamashita, 1992; Cox et al., 1993; Harding et al., 1999; Li et al., 2000). Phosphorylation of IRE1α (pIRE1α) catalyzes the alternative splicing of XBP1 mRNA (XBP1s) and expression of the active XBP1s transcription factor. Generally, the pIRE1α/XBP1s pathway is associated with upregulation of chaperone protein expression that serves to promote protein refolding and restore ER homeostasis. The RNAse activity of IRE1α is also involved in the regulation of mRNAs through a mechanism called regulated IRE1-dependent decay of mRNA (RIDD) (Hollien and Weissman, 2006). Interestingly, IRE1α could also cleave several pre-miRNAs which could potentially regulate a number of mRNA targets (Upton et al., 2012). As a result, RIDD and therefore, ER stress could affect directly and indirectly a large number of mRNA targets. ATF6 translocates to the Golgi apparatus and is cleaved first by site 1 protease (S1P) and second by site 2 protease (S2P) leading to an active ATF6 transcription factor. As for the pIRE1α/XBP1s pathway, the ATF6 pathway is usually associated with upregulation of chaperone protein expression but also with autophagy, lipid synthesis and endoplasmic-reticulum-associated protein degradation (ERAD) (Yoshida et al., 2001; Bravo et al., 2012; Garg et al., 2012;

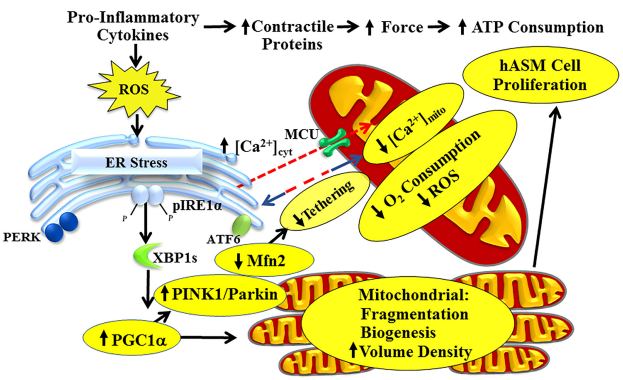

FIGURE 1 | Pro-inflammatory cytokines activate the pIRE1α/XBP1s ER stress pathway in hASM, leading to increased PGC1α and reduced Mfn2 expression. Reduced Mfn2 disrupts tethering of mitochondria to the ER, thereby decreasing mitochondrial Ca^{2+} influx and reducing O_2 consumption of individual mitochondrion. Increased PGC1α induces mitochondrial biogenesis and increases mitochondrial volume density to meet increased ATP demand. Cytokines also increase hASM force and ATP consumption by increasing contractile protein expression, thereby increasing energetic demand of individual hASM cells. This is mitigated by inducing hASM cell proliferation.

Verfaillie et al., 2012; Hasnain et al., 2013; Sano and Reed, 2013; Vannuvel et al., 2013; Delmotte and Sieck, 2015; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Morris et al., 2018). The role of ATF6 in the upregulation of XBP1 and the transcription factor C/EBP homologous protein (CHOP, ER stress-induced apoptosis) is also well documented and reviewed in Hu et al. (2018). Finally, PERK phosphorylates the translation-initiator factor eukaryotic initiation factor 2 (eIF2α), resulting in the translation of activating transcription factor 4 (ATF4). ATF4 is involved in the upregulation of CHOP, ERAD and mitophagy pathways (Adolph et al., 2012; Hasnain et al., 2012, 2013; Dromparis et al., 2013; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Hu et al., 2018; Morris et al., 2018). In cell types other than hASM, inflammation has been shown to induce ER stress (Adolph et al., 2012; Garg et al., 2012; Hasnain et al., 2012, 2013; Baban et al., 2013; Martino et al., 2013). These studies also demonstrated that the ER stress response is highly variable depending on cell type, species and experimental condition, which subsequently leads to various downstream physiological effects. Inflammation-induced ER stress is most likely a consequence of increased ROS generation (Adolph et al., 2012; Garg et al., 2012; Hasnain et al., 2012, 2013; Baban et al., 2013; Martino et al., 2013), although it is unclear whether ROS is the only mechanism involved. In a recent study, we showed that, TNFα selectively activates the pIRE1α/XBP1s in non-asthmatic hASM cells (Yap et al., 2019). Whether cytokines other than TNFα also selectively activate the pIRE1α/XBP1s pathway is not known. Interestingly, TNFα increases superoxide formation in hASM and Tempol, a superoxide scavenger, reduces the effect of TNFα on the activation of pIRE1α/XBP1s pathway (Yap et al., 2019). To date no other study has explored whether inflammation induces ER stress in hASM and whether an ER stress response in hASM cells from asthmatic patients exists and/or is affected by inflammation. A few studies suggest that the ER stress response is exaggerated in airway epithelial cells or immune cells in the context of asthma (Kim and Lee, 2015; Jeong et al., 2017; Pathinayake et al., 2018). In a mouse model of asthma, chemical chaperones have been used to reduce the ER stress response and attenuate airway hyperresponsiveness (Makhija et al., 2014; Miller et al., 2014; Kim and Lee, 2015; Siddesha et al., 2016).

Mfn2 AND ER STRESS RESPONSE

In cells other than hASM, the relationship between the ER stress response and mitochondria has gain considerable interest. These previous studies have suggested that Mfn2 and altered mitochondrial dynamics are upstream to ER stress such that a reduction in Mfn2 triggers ER stress (Munoz and Zorzano, 2011; Ngoh et al., 2012; Schneeberger et al., 2013; Bhandari et al., 2015). In a recent study, we suggested that a reduction in Mfn2 in hASM cells is downstream to ER stress (Yap et al., 2019), creating the possibility of a vicious cycle with reduced Mfn2 expression and altered mitochondrial function at the center. Currently, the link between ER stress and downstream regulation of Mfn2 expression has been largely unexplored. A limited number of studies have examined specific downstream targets of activation of the pIRE1α/XBP1s pathway (Calfon et al., 2002; Fonseca et al., 2005; Lipson et al., 2006, 2008; Zeng et al., 2009), but none of these studies have examined effects on Mfn2 expression. As mentioned above, TNFα selectively activates the pIRE1α/XBP1s pathway and reduces Mfn2 expression (Yap et al., 2019), but how IRE1α phosphorylation and XBP1 mRNA splicing affects Mfn2 expression has not been examined in any cell type. Potential targets of interest include peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), mitophagy-related proteins PTEN-induced kinase 1 (PINK1) and Parkin, and miRNAs (**Figure 1**). Several studies found that XBP1s increases expression of PGC1α (Arensdorf et al., 2013; Cheang et al., 2017). Interestingly, PGC1α activates the PINK1/Parkin mitophagy pathway, which is involved in ubiquitination of Mfn2 (Chen and Dorn, 2013; Basso et al., 2018; McLelland et al., 2018). Similarly, a growing list of miRNA has been implicated in the downregulation of Mfn2 but it is not clear if they are expressed in hASM and whether XBP1 is involved in their regulation (Kuhn et al., 2010; Dileepan et al., 2016; Purohit et al., 2019). Previous studies have also shown that XBP1 induces several miRNA but their potential effect on Mfn2 has not been explored and again it's not known if these miRNA are expressed in hASM (Kuhn et al., 2010; Dileepan et al., 2016; Purohit et al., 2019). The PERK and ATF6 pathway have also been suggested to affect Mfn2 expression, either through PGC1α, mitophagy or ERAD pathways (Morris et al., 2018). It is not known if cytokines other than TNFα activate the PERK and ATF6 pathway in hASM and whether they are activated or amplified in asthmatic hASM. Conversely, the effect of Mfn2 knockdown on IRE1α phosphorylation and XBP1 mRNA splicing has only been examined by four studies – two in neurons, and one each in embryonic fibroblasts and Drosophila (Ngoh et al., 2012; Munoz et al., 2013; Schneeberger et al., 2013; Bhandari et al., 2015).

Mfn2 AND DYNAMIC REMODELING OF MITOCHONDRIA

In hASM, mitochondria are tubular or filamentous but this mitochondria morphology is highly dynamic with mitochondria constantly fusing (fusion) or breaking (fission) from one another. Mitochondria morphology is therefore, the result of this balance between fusion vs. fission (Chen and Chan, 2005; Chan, 2006, 2012; Liesa et al., 2009; Youle and van der Bliek, 2012). This dynamic remodeling is thought to be essential for mitochondrial DNA stability, respiratory function, and to adapt cellular stress resulting from ROS formation (Chan, 2012). Mitochondrial fusion involves the GTPases Mfn1 and/or Mfn2 responsible for the fusion of the outer membrane, and optic atrophy protein 1 (OPA1) for the fusion of the mitochondrial inner membrane (**Figure 2**). Mfn1 is located only at the mitochondrial outer membrane whereas Mfn2 is localized both at the mitochondrial membrane and in the cytosol. The dimerization of Mfn2 (Mfn2/Mfn2) and/or Mfn1 (Mfn1/Mfn2) tethers outer membranes of neighboring mitochondria (Song et al., 2009;

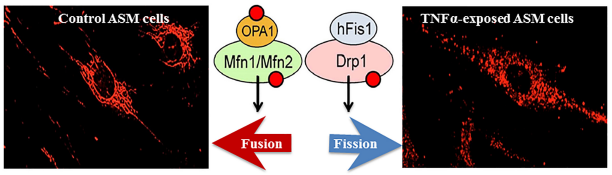

FIGURE 2 | In hASM, TNFα increases mitochondrial fragmentation (fission, visualized using MitoTracker Red); an effect mediated through a reduced expression of fusion proteins (Mfn2, Mfn1, and Opa1) and an increased expression of fission proteins (Drp1 and hFis1).

Palmer et al., 2011; Ranieri et al., 2013). Mitochondrial fission depends on the recruitment of cytosolic Drp1 by Fis1 to specific positions around mitochondria and known as constriction sites ultimately leading to fragmentation/fission of the mitochondria (Smirnova et al., 2001; James et al., 2003; Lee et al., 2004; Sheridan and Martin, 2010). The extent of fusion or fission of mitochondria can be quantified using morphological parameters such as form factor (perimeter2/4π × area) and/or aspect ratio (ratio of long and short axis) (Koopman et al., 2005a,b, 2006). We previously reported that Mfn2 expression was reduced in asthmatic hASM, and that this was correlated with an increase in mitochondria fragmentation (Aravamudan et al., 2014). A similar increase in mitochondrial fragmentation in hASM cells was observed after siRNA knockdown of Mfn2 (Aravamudan et al., 2017). In a recent study, we also found that TNFα reduces Mfn2 expression in hASM cells (Yap et al., 2019). As mentioned before, the relation between ER stress and Mfn2 expression and mitochondria fragmentation has been suggested but has not been clearly established.

ROLE OF Mfn2 IN TETHERING MITOCHONDRIA TO ER

There is converging evidence in other cell types that Mfn2 plays an essential role in tethering mitochondria to the ER (Hajnoczky et al., 2002; Patergnani et al., 2011; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). Mfn2 located at the ER membrane can dimerize with Mfn2 (Mfn2/Mfn2) and/or Mfn1 (Mfn1/Mfn2) located at the mitochondrial membrane to tether mitochondria to the ER. In hASM cells, a transient $[Ca^{2+}]_{cyt}$ response induced by 1 μM ACh stimulation is accompanied by a transient $[Ca^{2+}]_{mito}$ response (Delmotte et al., 2012; Delmotte and Sieck, 2015). The transient $[Ca^{2+}]_{mito}$ response is blunted by inhibiting the MCU using Ru360. Mitochondrial Ca^{2+} influx via the MCU (Baughman et al., 2011; De Stefani et al., 2011) is only activated by microdomains of higher $[Ca^{2+}]_{cyt}$ ("hotspots" > 2 μM) (Gunter et al., 2000; Gunter and Gunter, 2001; Rizzuto and Pozzan, 2006; Gunter and Sheu, 2009; Rizzuto et al., 2009; Giacomello et al., 2010), which exceed the normal global transient $[Ca^{2+}]_{cyt}$ response to agonist stimulation in hASM (~500–600 nM) (Pabelick et al., 1999; Sieck et al., 2008; Sathish et al., 2009, 2011; Delmotte et al., 2012). Higher levels of $[Ca^{2+}]_{cyt}$ do occur after 24-h TNFα exposure in response to muscarinic stimulation (Delmotte et al., 2012; Delmotte and Sieck, 2015; Dogan et al., 2017; Sieck et al., 2019), but are still well below levels required to activate the MCU (Gunter et al., 2000; Gunter and Gunter, 2001; Rizzuto and Pozzan, 2006; Gunter and Sheu, 2009; Rizzuto et al., 2009; Giacomello et al., 2010). However, much higher levels of $[Ca^{2+}]_{cyt}$ ("hotspots") are observed in regions in close proximity to the ER Ca^{2+} efflux channels (IP_3 and RyR). Thus, during agonist stimulation, mitochondria must be tethered to the ER in order to establish close proximity of mitochondria to $[Ca^{2+}]_{cyt}$ "hotspots" for mitochondrial Ca^{2+} influx. We previously showed that TNFα disrupts mitochondrial proximity to the ER in hASM cells (Delmotte et al., 2017), but this study only suggests the potential involvement of reduced Mfn2 expression in hASM. Further studies will be necessary to provide direct evidence for the involvement of Mfn2. In hASM cells, it has not been established that Mfn2 is essential for tethering mitochondria to the ER, and thus, for establishing proximity of mitochondria to the ER and microdomains of higher $[Ca^{2+}]_{cyt}$ ("hotspots" > 2 μM). Such interactions are cell and context specific, so establishing the role of Mfn2 in hASM is critical. The potential effect of ER stress on microdomains of higher $[Ca^{2+}]_{cyt}$, and mitochondrial Ca^{2+} influx is likewise not clearly established.

EXCITATION-ENERGY COUPLING VIA MITOCHONDRIAL Ca^{2+} INFLUX

Based on biochemical studies, it is well known that mitochondrial production of ATP (oxidative phosphorylation) depends on dehydrogenase enzyme activities of the tricarboxylic acid (TCA) cycle (or citric acid cycle). Some of these dehydrogenase enzymes [i.e., pyruvate dehydrogenase (PDH), NAD-isocitrate dehydrogenase (ICDH), and oxoglutarate dehydrogenase (OGDH)] are Ca^{2+} dependent (Rizzuto et al., 2000; Parekh, 2003; Franzini-Armstrong, 2007; Maack and O'Rourke, 2007; Romagnoli et al., 2007). Additionally, an increase in $[Ca^{2+}]_{cyt}$ stimulates mitochondrial shuttle systems such as the glycerol phosphate shuttle and the aspartate/glutamate transporters resulting in an increase in NADH levels in the mitochondria (Palmieri et al., 2001; Denton, 2009). Thus, mitochondrial Ca^{2+} influx during transient elevation of $[Ca^{2+}]_{cyt}$ stimulates dehydrogenase enzyme activities within the TCA cycle and increases, O_2 consumption, electron transport chain (ETC) flux and ATP production – excitation-energy coupling (**Figure 3**). Conversely, it is well known that increased ATP consumption leads to transport of ADP into mitochondria via the adenosine nucleotide transporter (ANT), which stimulates ATP synthase (complex V) activity to match ATP production with ATP consumption (**Figure 3**). Together, these two energy sensing pathways form a normal homeostatic mechanism for excitation-energy coupling in a variety of cell types including hASM. Pathophysiology and mitochondrial dysfunction involve disruptions in these mitochondrial energy-sensing/signaling pathways. As mentioned, most of these studies involved biochemical studies and in some cases isolated mitochondria. While they are critical in our understanding of mitochondrial

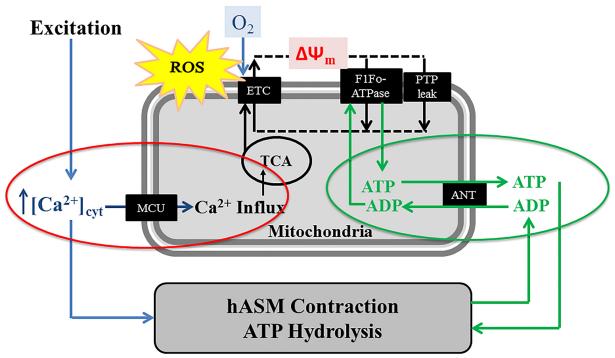

FIGURE 3 | In hASM cells, the agonist-induced transient increase of $[Ca^{2+}]_{cyt}$ (excitation) is accompanied by activation of the mitochondrial Ca^{2+} uniporter (MCU) and a transient increase in mitochondrial Ca^{2+} influx, thereby increasing activities of mitochondrial dehydrogenases of the TCA cycle and the electron transport chain (ETC). Mitochondrial O_2 consumption and ATP synthesis are also linked ATP hydrolysis (and therefore, hASM force) via changes in the ATP/ADP ratio and the adenine nucleotide transporter (ANT). A portion of the O_2 consumed in the ETC results in ROS formation.

function, there's a considerable need for new tools allowing to studies these mechanisms within single mitochondrion in live cells and tissues. A few imaging and/or molecular tools to measure mitochondrial membrane potential $\Delta\Psi_m$, succinate dehydrogenase (SDH) activity (Sieck et al., 1986, 1989, 1995, 1996), ATP consumption (Jones et al., 1999a,b; Dogan et al., 2017), ATP/ADP (Berg et al., 2009), and NAD/NADH ratio (Hung et al., 2011; Cohen et al., 2018) have been developed for use in live cells or tissue but have never been used in hASM.

ROLE OF ER STRESS IN MITOCHONDRIAL BIOGENESIS AND INCREASED MITOCHONDRIAL VOLUME DENSITY

A few studies reported that mitochondrial biogenesis in asthmatic hASM is increased but the mechanisms mediating this mitochondrial biogenesis were not explored (Trian et al., 2007; Girodet et al., 2011). These studies also did not examine mitochondria morphology and mitochondrial fragmentation/fission. An increase in mitochondrial volume density is an alternative mechanism to increase ATP production to meet increased ATP demand in hASM after exposure to pro-inflammatory cytokines. In this case, O_2 consumption in individual mitochondrion can be reduced to minimize formation of ROS. Recent evidence also suggests that reduced Mfn2 and mitochondrial fragmentation is required for mitochondrial biogenesis (Antico Arciuch et al., 2012; Peng et al., 2017), supporting our hypothesis that increased PGC1α and reduced Mfn2 are a coordinated homeostatic response to cytokine-induced activation of the pIRE1α/XBP1s ER stress pathway.

CYTOKINE EXPOSURE INCREASES ROS GENERATION IN hASM

A number of studies have reported that ROS generation increases in asthmatic patients (Katsumata et al., 1990; Comhair and Erzurum, 2010; Zuo et al., 2013) which has the potential to triggers ER stress in hASM. We recently showed exposure of non-asthmatic hASM to TNFα progressively increases superoxide anion formation (Yap et al., 2019). We also found that incubating hASM cells with Tempol (superoxide anion scavenger) mitigated the effects of TNFα in inducing ER stress as well as the reduction in Mfn2 (Yap et al., 2019). It is possible that an increase in ROS generation is triggered, in part by increased ATP consumption and mitochondrial O_2 consumption. Acute activation of the pIRE1α/XBP1s ER stress pathway leads to a transient reduction in Mfn2 thereby decreasing: (1) mitochondrial tethering to the ER (**Figure 1**); (2) mitochondrial Ca^{2+} influx (**Figures 1**, **3**); (3) TCA cycle dehydrogenase enzyme activity (**Figures 1**, **3**); (4) O_2 consumption (**Figures 1**, **3**); and as a result, (5) ROS formation (**Figures 1**, **3**). Without this homeostatic break on mitochondrial O_2 consumption, the increase in hASM force and ATP consumption induced by pro-inflammatory cytokines will increase ROS formation and further exacerbating ER stress.

CYTOKINE EXPOSURE INCREASES hASM FORCE, ATP CONSUMPTION AND TENSION COST

In previous studies, we showed that 24-h exposure of hASM cells to TNFα increases both $[Ca^{2+}]_{cyt}$ and force responses to 1 μM muscarinic (ACh) stimulation (White et al., 2006; Sathish et al., 2009, 2011; Delmotte et al., 2012; Jia et al., 2013; Delmotte and Sieck, 2015; Dogan et al., 2017; Sieck et al., 2019). However, hASM sensitivity to muscarinic stimulation is also increased after TNFα, which largely accounts for the enhanced $[Ca^{2+}]_{cyt}$ response, but not the force response (Sieck et al., 2019). In recent studies, we found that the increase in ASM force induced by 24-h TNFα exposure is due to an increase in contractile protein expression (Dogan et al., 2017; Sieck et al., 2019). Importantly, the increase in hASM force generation induced by TNFα exposure is associated with an increase in ATP consumption and tension cost (Dogan et al., 2017). This study used an NADH-linked fluorescence technique in permeabilized hASM in which the level of Ca^{2+} activation and force generation can be controlled. In previous studies, we showed that in ASM force generation is directly related to ATP hydrolysis rate (Jones et al., 1999a,b; Dogan et al., 2017). During isometric activation of hASM, ATP hydrolysis rate is initially faster and then declines with time to a sustained level even though isometric force is maintained (the "latch" state). Thus, there is a time-dependent decline in both ATP hydrolysis rate and tension cost that is likely due to cytoskeletal remodeling (Jones et al., 1999b). When actin polymerization in hASM is inhibited, force decreases while ATP hydrolysis rate increases; thereby increasing tension cost (Jones et al., 1999a,b). Normally,

tension cost of hASM is dramatically lower than skeletal muscle, but work efficiency is remarkably high (Sieck et al., 1998). Thus, the energetics of hASM are perfectly suited to sustain force at low energy cost. In hASM cells, an increase in ATP consumption is met by stimulation of ATP synthase activity (complex V) and an increase in O_2 consumption and ATP production (**Figures 1**, **3**). However, stimulation of mitochondrial O_2 consumption results in increased ROS formation that can trigger protein unfolding and an ER stress response. Thus, we propose that the pIRE1α/XBP1s ER stress pathway represents a homeostatic response directed toward reducing O_2 consumption and ROS formation in an individual mitochondrion, while increasing mitochondrial biogenesis and mitochondrial volume density to meet the increase in ATP demand. This leads to the question of how energy demand and supply are matched with continued exposure to pro-inflammatory cytokines. Sustained contractility at reduced tension cost is a hallmark of smooth muscle function, and any perturbation should be met with a homeostatic response. One possibility is that hASM cell proliferation (hyperplasia response) provides a mechanism to maintain contractility but at reduced ATP cost per cell.

ROLE OF Mfn2 IN hASM CELL PROLIFERATION

Recent evidence suggests that Mfn2 affects several pro-proliferative pathways and that dynamic mitochondrial remodeling (balance between fusion and fragmentation/fission) governs cell proliferation (Liesa et al., 2009; Antico Arciuch et al., 2012). During cell division, the number of mitochondria or therefore mitochondrial biogenesis needs to increase so each subsequent cell has a similar number of mitochondria (Antico Arciuch et al., 2012). As a result, mitochondria fuse then fragment to generate more mitochondria. Two studies in vascular smooth muscle show that Mfn2 is critical in cell division (Liesa et al., 2009; Antico Arciuch et al., 2012). Notably, the authors show that Mfn2 interacts with several pro-proliferative kinases such as extracellular signal-regulated kinase (ERK1/2) and participates in their inactivation (Liesa et al., 2009; Antico Arciuch et al., 2012). As a result, overexpression of Mfn2 in vascular smooth muscle inhibits cell proliferation (Liesa et al., 2009; Antico Arciuch et al., 2012). Importantly, ERK1/2 activation is believed to play a critical role in hASM proliferation induced by inflammatory cytokines (Lee et al., 2001; Kip et al., 2005; Yu et al., 2013; Dragon et al., 2014; Movassagh et al., 2014). While the relation between ER stress and Mfn2 is not clearly established, studies suggest that ER stress induces cell proliferation in many cell types (Vandewynckel et al., 2013; Chen et al., 2018). Whether ER stress induces hASM cell proliferation is unknown.

THERAPEUTIC APPROACHES TARGETING ER STRESS

An increase in ROS generation is likely responsible for inflammation-induced ER stress. Based on increased ROS generation associated with asthma, the benefits of antioxidant therapeutic have been explored (Heffner and Repine, 1989; Bast et al., 1991; Buhl et al., 1996; Jain and Chandel, 2013). However, one of the challenges with antioxidant treatment is specificity both in terms of ROS targeting and localization (extracellular, cytosol or mitochondrial). It is also now recognized that ROS regulate many cellular signaling cascades and have the potential to be more harmful than beneficial. An alternative therapeutic strategy of reducing ER stress in ASM is the use of chemical chaperone. Well tolerated even at high dose, chemical chaperones are effective in reducing ER stress *in vivo*. Bunezile, the US brand name for sodium phenylbutyrate or 4-phenylbutyrate (4-PBA), is currently used for patients with urea cycle disorders. Chemical chaperones such 4-PBA or tauroursodeoxycholic acid (TUDCA) have gained considerable interest as a potential therapy for a number of other diseases including but not limited to cystic fibrosis [national clinical trial (NCT)00590538 for 4-PBA and NCT00004441 for TUDCA], amyotrophic lateral sclerosis (NCT00107770 for 4-PBA and NCT03800524 for TUDCA) and some types of cancer (NCT00006019 for 4-PBA). A recent phase 1 clinical trial for TUDCA in patients with asthma has been initiated (NCT03878654). Studies in mice showed that 4-PBA attenuated airway inflammation and also reduced airway hyperreactivity in mice model of asthma further indicating a promising therapeutic role for chemical chaperones in the pathogenesis of asthma (Hoffman et al., 2013; Kim et al., 2013; Makhija et al., 2014). The effect of 4-PBA or TUDCA on ASM were not examined and it's not clear how the chemical chaperone achieved its effect, further illustrating the need to better understand how inflammation induces ER stress in hASM.

CONCLUSION AND PERSPECTIVES

Inflammation, airway hyper-contractility and proliferative remodeling are key aspects of airways diseases such as asthma. The role of inflammation-induced ER stress with downstream impact on Mfn2 and mitochondrial function is of particular interest. The ER stress pathways have been implicated in a growing number of downstream effects ranging from cell death to cell survival. Mfn2 is involved in mitochondrial tethering to the ER, mitochondrial Ca^{2+} influx, O_2 consumption, and ROS formation. Surprisingly, ER stress and Mfn2 have been largely overlooked in hASM. Mitigation of inflammation-induced ER stress in hASM may represent a novel target for therapeutic intervention.

AUTHOR CONTRIBUTIONS

PD and GS wrote the manuscript.

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REVIEW
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Why Do Intrauterine Exposure to Air Pollution and Cigarette Smoke Increase the Risk of Asthma?

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The prevalence of childhood asthma is increasing worldwide and increased *in utero* exposure to environmental toxicants may play a major role. As current asthma treatments are not curative, understanding the mechanisms underlying the etiology of asthma will allow better preventative strategies to be developed. This review focuses on the current understanding of how *in utero* exposure to environmental factors increases the risk of developing asthma in children. Epidemiological studies show that maternal smoking and particulate matter exposure during pregnancy are prominent risk factors for the development of childhood asthma. We discuss the changes in the developing fetus due to reduced oxygen and nutrient delivery affected by intrauterine environmental change. This leads to fetal underdevelopment and abnormal lung structure. Concurrently an altered immune response and aberrant epithelial and mesenchymal cellular function occur possibly due to epigenetic reprograming. The sequelae of these early life events are airway remodeling, airway hyperresponsiveness, and inflammation, the hallmark features of asthma. In summary, exposure to inhaled oxidants such as cigarette smoking or particulate matter increases the risk of childhood asthma and involves multiple mechanisms including impaired fetal lung development (structural changes), endocrine disorders, abnormal immune responses, and epigenetic modifications. These make it challenging to reduce the risk of asthma, but knowledge of the mechanisms can still help to develop personalized medicines.

Keywords: asthma, fetus, placental, smoking, particulate matter

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INTRODUCTION

Asthma is a disease that generally affects 5–20% of children globally (Global Asthma Network, 2018; Enilari and Sinha, 2019). It is a complex condition in which symptoms are mainly caused by bronchoconstriction (Thomson and Hasegawa, 2017). Airway constriction occurs rapidly in response to a variety of inhaled substances, for example, allergens such as pollen and house dust mite, and environmental sources such as dust and smoke, which usually can be fully or partially reversed by bronchodilators. Pathologically it is defined by airway remodeling, typified by increased smooth muscle and epithelial layer thickness, and increased numbers of inflammatory cells. However, the type of inflammation varies. For example, sputum based phenotyping of inflammation categorizes people into eosinophilic, neutrophilic, or paucigranulocytic asthma.

The other factors that can add to the complexity of asthma including the age of onset, etiological cause (if known), co-existence of other respiratory diseases, comorbidities, the degree of reversibility, and the ability for the symptoms being effectively controlled by pharmaceutical interventions.

The susceptibility to asthma is complex, which involves both genetic susceptibility, environmental insults (both pre and post birth), and is further complicated by asthma symptoms initiating and sometimes ceasing at different ages, as well as differences in asthma prevalence between the male and female sexes (Carey et al., 2007b).

It is known that boys are more susceptible than girls before puberty, but less than girls after puberty. Many theories exists to explain this phenomena including: dysnapsis due to different sized lungs in boys and girls, increased allergy (more IgE production in boys), different innate and adaptive immune responses in boys and girls, and the influence of sex hormones (Shames et al., 1998; Papadopoulos et al., 2004; Mohammad et al., 2016). The incidence of asthma is also related to the use of life saving medical interventions in premature and newborn children such as oxygen supplementation or mechanical ventilation due to physical permanent damage to the newborn's lungs (Davidson and Berkelhamer, 2017).

However, it has increasingly been recognized that certain factors during the intrauterine period affects childhood asthma susceptibility. In particular, maternal smoking (MSE) and particulate matter (PM) exposure (Burnett et al., 2014; Thacher et al., 2014), are the best described/researched *in utero* challenges which affect asthma susceptibility. This review will discuss the current understanding of multiple mechanisms underlying these two factors, which may help to develop personalized medicines.

EPIDEMIOLOGY OF ASTHMA

The prevalence of allergic disorders has been rising since the early 1980s. The average global rate of allergic disorders is 22%, ranging from 15 to 35% of the population in different countries (Warner et al., 2006). According to the WHO, the number of children with asthma is around 14% globally (Asthma fact sheet World Health Organisation, 2017). Severe asthma is common in children. A recent study reported that the prevalence of severe asthma was 4.9% in 6–7 years old children, however, the incidence was increased to 6.9% in 13–14 years olds. These phenomena demonstrated that age is an important factor for the onset of asthma (Lai et al., 2009).

Environmental toxicant exposure during pregnancy is a significant factor that has been shown to increase the incidence of asthma (Crinnion, 2012). In particular, MSE is the largest modifiable risk factor for the development of asthma. Although the harmful effect of smoking is well-known in the general public, smoking mothers find it difficult to quit due to nicotine addiction, even during pregnancy when nicotine metabolism is faster than non-pregnant status (Taghavi et al., 2018). A systematic review and meta-analysis in the Lancet showed that the top 3 countries with the highest smoking rate during pregnancy are Ireland (38.4%), Uruguay (29.7%), and Bulgaria (29.4%) (Lange et al., 2018). Even in Australia where anti-smoking legislation is one of the most aggressive in the world, the smoking rate in pregnant women is 11.7% (Laws et al., 2010).

Epidemiological studies have demonstrated a dose-dependent increase in asthma risk in offspring due to MSE (**Table 1**). Currently, several cohort studies have confirmed the association between MSE and asthma risk in the offspring (Strachan and Cook, 1998; Gilliland et al., 2001; Doherty et al., 2009; Burke et al., 2012). For example, a birth cohort study has found that women smoking during pregnancy could increase asthma incidence in the offspring with an adjusted hazard ratio of 1.79 (95% CI 1.20–2.67) (Grabenhenrich et al., 2014). The same outcome has been found in another cohort study where MSE during pregnancy caused higher asthma risk in the child in the first year of life with an odds ratio (OR) of 1.83 (Gold et al., 1999). Similarly, a systematic review of 14 studies revealed a wheezing [OR 1.41 (95% CI 1.19–1.67)] and asthma risk [OR 1.85 (95% CI 1.35–2.53)] in 2 years old and younger children, followed by a higher asthmatic risk in 5–18 years old children [OR 1.23 (95% CI 1.12–1.36)] caused by smoking during pregnancy (Burke et al., 2012). One study found a strong asthma risk in 14 year old girls whose mothers smoked during pregnancy, however this was not found in boys (Alati et al., 2006); whereas a different study found that boys at the age of 11 are more susceptible to the maternal and postnatal secondhand smoke (Hu et al., 2017). These differences might be related to the changes in asthma prevelance in boys and girls around puberty.

Around 91% of the world's population are living in the areas where the levels of air pollutants exceed the WHO limits (Balakrishnan et al., 2019). Epidemiological studies demonstrated a strong association between pulmonary disease and particular matter (PM) exposure (Burnett et al., 2014). Compared to cigarette smoking which can be avoided through quitting, the dangers of airborne pollution are hard to avoid in heavily polluted countries, such as China and India. In China, 74,000 premature deaths were attributed to $PM_{2.5}$ exposure in the year 2013 (Ji et al., 2019). It was estimated that 22% of these deaths could have been avoided if indoor $PM_{2.5}$ level met National Class I standards (Ji et al., 2019).

There are many different types of airborne pollution, but simplistically these can be divided into gasses and particulate matter (PM). PM is considered as particularly dangerous as respirable particles can remain airborne over large distances.

As shown in **Table 2**, prenatal PM exposure is also associated with childhood asthma. A cohort study found that prenatal PM_{10} exposure could cause pulmonary function changes with higher minute ventilation in newborns (Latzin et al., 2009). Another birth cohort study including pre-school and school-age children demonstrated that prenatal PM_{10} exposure increased the risk of developing asthma in both age groups, especially for those pregnant mothers who lived near the highways (Sbihi et al., 2016). The correlation between maternal PM exposure and asthma risk in different genders was also investigated. High levels of $PM_{2.5}$ exposure during mid-gestation increased the development of asthma by the age of 6 years in boys, but not in girls (Hsu et al., 2015). The above evidence indicates that maternal PM

TABLE 1 | Maternal smoking during pregnancy and the risk of asthma in children.

Smoking exposure	Age	Relative risk Odds ratio (95% CI)		References
		Male	Female	
Smoker at some stage	14 years	1.15 (1.01–1.72)	1.25 (0.85–1.22)	Alati et al., 2006
>20 cigarettes (early and late)	14 years	0.57 (0.20–1.60)	1.09 (0.47–2.51)	Alati et al., 2006
Total of 1–9 cigarettes/day	4–16 years	1:19 (0.98–1.43)		Thacher et al., 2018
<10 Cigarettes per day	7 years	1.20 (1.04–1.38)		Jaakkola and Gissler, 2004
Total of ≥10 cigarettes/day	<5 years	1.68 (1.10–2.58)		Martinez et al., 1992
>10 Cigarettes per day	7 years	1.31 (1.09–1.58)		Jaakkola and Gissler, 2004
Total of ≥10 cigarettes/day	4–16 years	1:66 (1.29–2.15)		Thacher et al., 2018
Smoking during pregnancy	First 3 years	1.88 (1.14–3.12)		Murray et al., 2004
Smoking during pregnancy	4–6 years	1.65 (1.18–2.31)		Neuman et al., 2012
Smoking during pregnancy	2–7 years	1.7 (1.2–2.2)		Harju et al., 2016
Smoking during pregnancy	5–9 years	0.97 (0.51–1.84)		Sherman et al., 1990
Smoking during pregnancy	14 years	1.49 (0.91–2.45)		Hollams et al., 2014
Smoking during pregnancy	7–16 years	0.99 (0.78–1.25)		Strachan et al., 1996

TABLE 2 | Maternal PM exposure and the development of asthma in offspring.

Pollutant	Age	Concentration increase	Relative risk	References
$PM_{2.5}$	6 years	1.7 $\mu g/m^3$ (per IQR)	1.15 (1.03–1.26)	Lee A. et al., 2018
$PM_{2.5}$	3–4 years	1 $\mu g/m^3$ (exposure interval)	0.95 (0.91–1.00)	Clark et al., 2009
$PM_{2.5}$	0–5 years	1.45 $\mu g/m^3$ (per IQR)	0.99 (0.97–1.01)	Sbihi et al., 2016
$PM_{2.5}$	6–10 years	1.46 $\mu g/m^3$ (per IQR)	1.01 (0.97–1.06)	Sbihi et al., 2016
$PM_{2.5}$	0–6 years	3.7 $\mu g/m^3$ (per IQR)	1.01 (0.99–1.04)	Lavigne et al., 2018
PM_{10}	3–6 years	12 $\mu g/m^3$ (per IQR)	0.89 (0.68–1.16)	Deng et al., 2016
PM_{10}	3–4 years	1 $\mu g/m^3$ (exposure interval)	1.09 (1.05–1.13)	Clark et al., 2009
PM_{10}	0–5 years	1.3 $\mu g/m^3$ (per IQR)	1.12 (1.05–1.19)	Sbihi et al., 2016
PM_{10}	6–10 years	1.36 $\mu g/m^3$ (per IQR)	1.09 (0.96–1.24)	Sbihi et al., 2016

IQR, interquartile range.

exposure during pregnancy has similar effects to MSE in terms of increasing the risks of developing asthma in childhood.

The difference of asthma prevalence between boys and girls and the change in prevalence which occurs around puberty naturally gives credence to the involvement of sex hormones. Animal models of estrogen receptor knockouts suggests that estrogen promotes the development of the asthma (Carey et al., 2007a); while male mice lacking testosterone showed more severe asthma symptom (Yu et al., 2002). These studies help to explain why boys are more susceptible to asthma before puberty, and girls more susceptible after puberty. However, the etiology of asthma is complex and is multifactorial.

THE ROLE OF OXIDATIVE STRESS IN THE DEVELOPMENT OF ASTHMA IN CHILDREN

Various chemicals can be found in both cigarette smoke and PM. It is unlikely that a single chemical is responsible for all the adverse effects of *in utero* exposure to cigarette smoke or PM on lung health in the offspring. Cigarette smoke and PM are two major environmental sources of inhaled free radicals and strong oxidants. The balance between excessive oxidant activity and the antioxidant capacity can tip in favor of excess oxidants causing oxidative stress. However, it is important to note that the production of oxidants is necessary to maintain healthy cell function, and important in regulating processes such as inflammatory responses. Oxidative stress induces adverse effects in tissues. The developing fetus is highly vulnerable to oxidative stress injury, as the immune system remains immature during the prenatal period (Lee A. G. et al., 2018). Free radicals and chemicals inhaled during MSE and maternal PM exposure can pass the blood-placental barrier to directly increase the level of oxidative stress in the offspring. Therefore, we propose the first common and prominent mechanism underlying these two factors to induce pathological changes in the offspring is oxidative stress.

Our previous studies in mice have repeatedly shown that MSE can reduce the level of endogenous antioxidant Manganese Superoxide Dismutase in the brain, kidney, and lungs of adult offspring accompanied by increased Reactive Oxygen Species (ROS) levels in those organs; interestingly, antioxidant supplementation during pregnancy could completely or partially reverse the adverse effects on those organs induced by MSE (Chan et al., 2017; Sukjamnong et al., 2017, 2018). The endogenous antioxidant enzyme system is established in the second and third trimester of pregnancy and continues to develop in early

childhood (Fanucchi, 2004). Interestingly, lung development also matures in the early postnatal period, suggesting that the antioxidant system may protect early life lung development from the adverse impacts of environmental oxidant pollutants (Pinkerton and Harding, 2014). After all, the function of the respiratory system is vital for survival immediately after birth. Vitamin C is an antioxidant which contributes to cellular antioxidant defense (Preston, 1991; Tous et al., 2019). A study in pigs found that vitamin C deficiency during pregnancy could cause brain damage in the offspring (Schjoldager et al., 2015). Giving smoking women vitamin C during pregnancy was shown to improve lung function (better airflow and less wheezing) in children during the first year of life (McEvoy et al., 2014). This again provided evidence that oxidative stress and insufficient capacity of antioxidants play a key role in organ dysfunction in the offspring due to MSE. PM consists of metals and endotoxins (polycyclic aromatic hydrocarbons) which also can generate ROS (Billah, 2015) and produce oxidative damage (Valavanidis et al., 2006). Therefore, the pathways associated with oxidative stress are regarded as playing an important role in inducing adverse respiratory outcomes after the exposure to environmental pollutants (Breland et al., 2002; Romieu et al., 2008).

In utero, any adverse effects that occur during fetal development can have long-lasting negative influences on organ development and later function after birth (Aycicek et al., 2005; Noakes et al., 2007). In fact, local tissue oxidative stress and injury due to the imbalance between free radicals and antioxidant capacity is a key factor in asthma pathogenesis. As such we propose that oxidative stress is the pathological insult that drives changes in the intrauterine environment and disturbs normal fetal development which subsequently increases the risks of developing asthma. It is also worth noting that maternal smoking is a strong risk factor for miscarriage, a process also linked to oxidative stress (Stone et al., 2014).

INTRAUTERINE GROWTH RESTRICTION – THE BARKER HYPOTHESIS

In 1990, the epidemiologist David Barker presented his hypothesis which linked chronic and degenerative diseases, such as heart disease, to the poor intrauterine environment caused intrauterine growth retardation (IUGR), low birth weight, and premature birth. This theory inspired scientists and has been expanded to the other organ systems including the respiratory system (Zacharasiewicz, 2016). Numerous studies have confirmed that environmental toxicant exposure during pregnancy, such as cigarette smoke, can cause IUGR and subsequently abnormal lung development in the offspring (Zacharasiewicz, 2016). Nicotine is the most widely studied component in cigarette smoke due to its addictive effects. Early studies showed that cotinine, the stable metabolite of nicotine, can be found in fetal circulation and body fluids (Sabra et al., 2017). This indicates that chemicals in cigarette smoke can cross the blood-placental barrier and reach the fetus. A more recent study by Geelhoed et al. (2011) showed that MSE can decrease blood flow in the ascending aorta because of higher arterial resistance in the uterus, which can reduce the oxygen and nutrient delivery to the growing fetus resulting in IUGR. Inadequate nutrient availability in the developing fetus, especially during the periods of rapid lung growth, has been shown to induce lung developmental defects (Chen et al., 2004; McMullen et al., 2005) and respiratory morbidity in the offspring (Harding et al., 2004; Maritz et al., 2005). Animal studies have demonstrated a decrease in both alveolarization and vessel density in the lung of sheep with IUGR (Rozance et al., 2011).

HOW DO MSE AND MATERNAL PM EXPOSURE IMPACT ON FETAL LUNG DEVELOPMENT?

In brief, MSE can induce such effects in two ways: the direct influence on the developing fetus, and indirect effects on the fetoplacental unit. Recently, studies have demonstrated that a small fraction of the circulating nicotine in the mothers can cross the trophoblastic membrane and reach the unborn child, and as such cotinine can accumulate in the fetal circulation and fluids in measurable concentrations (Jauniaux and Burton, 1992; Jauniaux et al., 1999). Furthermore, a similar concentration of cotinine in both fetal lung tissue and blood was found, suggesting cotinine may bind to the receptors in the lung to directly affect fetal lung development (McEvoy and Spindel, 2017). Maternal air pollution exposure can also cause fetal growth restriction (Bonzini et al., 2010). Polycyclic aromatic hydrocarbons on the surface of PM can easily cross the blood-placental barrier and circulate in the fetal blood because of its small size (Jauniaux et al., 1999). Therefore, lung development in the fetus can be directly affected by the PM inhaled by the mothers.

The fetoplacental unit has a significant influence on fetal development. The damage to fetoplacental unit caused by maternal smoking can be seen during early pregnancy. For example, MSE significantly increases villous membrane thicknesses and trophoblastic layer in the placenta during the first trimester (Jauniaux and Burton, 1992). There are also signs of reduced capillary volume in placental vasculature in pregnant smokers (Burton et al., 1989). The consequence of reduced capillary volume is nutrient delivery decrement. Intrauterine nutrient deficiency has been suggested as the major factor contributing to fetal growth restriction and low birth weight due to MSE (Figueras et al., 2008). Low birth weight can increase the asthma risk in later life, evidenced by a meta-analysis including 1.1 million people (Xu et al., 2014). In rat models, maternal PM exposure was found to change placental morphology, and decrease placental weight, size and surface area (de Fátima Soto et al., 2017). Similar findings have also been confirmed in humans, where PM_{10} exposure can decrease placental weight with higher anti-angiogenic factors in cord blood (van den Hooven et al., 2012). As a result, increased vascular resistance can be predicted, which will reduce uteroplacental perfusion and lead to various maternal and fetal complications, such as low birth weight and miscarriage (Kaufmann et al., 2003; Ness and Sibai, 2006; Schlembach et al., 2007).

The abovementioned evidence indicates that MSE and maternal PM exposure during pregnancy can impair fetal lung development through a direct effect on the fetus and indirect influence on placental morphology and function. However, the molecular mechanisms underlying the increased risk of asthma due to MSE and maternal PM exposure are not well understood. In monkeys, MSE upregulated nicotinic acetylcholine receptors in the fetal lung, associated with lung function decline after birth (Sekhon et al., 1999, 2001). Several *in vitro* and *in vivo* animal models have also shown that both MSE and PM exposure during pregnancy affects the development of the neonatal immune system, lung structure, and lung function in the offspring, making them more susceptible to the development of asthma (Collins et al., 1985; Mauad et al., 2008). These will be discussed in greater detail later.

THE DEVELOPMENT OF ASTHMA IN CHILDREN

The Role of Altered Lung Structure

Just as discussed above, MSE and maternal PM exposure during pregnancy can result in oxidative stress, and cause nutrition deficiency resulting in IUGR, which eventually alters lung development and structure. Fetal lung development starts from embryo Weeks 3–5 when the laryngotracheal groove forms on the floor of the foregut and matures during the early postnatal year. Therefore, inhaled environmental toxicants by pregnant mothers may change lung morphology and function as early as gestational Weeks 5–17 when epithelial and smooth muscle cell differentiation takes place. Epidemiological evidence well supports this theory, where significant lung function impairment was found in the newborns of mothers who smoked during pregnancy or inhaled high levels of PM (Carlsen et al., 1997; Latzin et al., 2009). Such lung function disorders can last until later childhood (Jedrychowski et al., 1997, 2010). It needs to be noted that lung function deficiency in early life has been correlated with increased asthma incidence later on (Borrego et al., 2009).

Lung dysfunction after birth can be attributed to lung structural changes during fetal development. Animal studies have shown that both MSE and maternal PM exposure could decrease lung volume, alveoli number and mean linear intercept in the offspring as well as reduced alveolar–bronchiolar attachment points (Collins et al., 1985; Elliot et al., 2001; Mauad et al., 2008). Nicotine as the "addictive substance" in tobacco smoke has often been used in animal models to investigate the potential mechanisms underlying the adverse effects of maternal tobacco smoking. For example, increased airway collagen deposition and altered vascular structure were found in a monkey model after prenatal nicotine exposure (Sekhon et al., 1999, 2004). However, it is uncertain if these results can be translated to humans as nicotine replacement therapy during pregnancy has not been found to be associated with the same adverse outcomes as

TABLE 3 | Clinical evidence of the adverse impacts of MSE and maternal PM exposure.

Pollutant	Sample collecting time (gestation)	Adverse impact	References	
Maternal smoking	9–14 weeks	High villous membrane and trophoblastic layer thicknesses	Jauniaux and Burton, 1992	Placenta
Maternal smoking	–	Smaller villous capillaries and high basement membrane thickness	Van der Velde et al., 1983	
Maternal smoking	–	High villous membrane thickness	Burton et al., 1989	
Maternal smoking	28 ± 1 weeks	Decreased uterine artery volume	Castro et al., 1993	
Maternal smoking	1st trimester	More NK cells and macrophages, less regulatory T cells	Prins et al., 2012	Immune cells regulation
Maternal smoking	34th week	Lower Treg cell numbers	Herberth et al., 2014	
Maternal smoking	After delivery	Attenuated innate immune responses	Noakes et al., 2006	
Maternal smoking	During gestation	DNA methylation in cord blood cells	Joubert et al., 2016	Epigenetics
Maternal smoking	6–28 weeks infants	Lower antioxidant level and high oxidative stress level	Aycicek et al., 2005	Oxidative stress
Maternal smoking	3 months infants	Higher markers of oxidative stress	Noakes et al., 2007	
PM_{10}	1st and 2nd-trimester	Lower Pro- and anti-angiogenic factors and PlGF	van den Hooven et al., 2012	Placenta
$PM_{2.5}$	Early/late gestation	Higher CD3 + and CD4 + lymphocytes and lower CD19 + and NK cell number during early gestation, which were opposite in the late gestation	Herr et al., 2010	Immune cells regulation
$PM_{2.5}$	After delivery	Higher GSTP1 methylation	Lee A. G. et al., 2018	Epigenetics
$PM_{2.5}$	During gestation	Higher 3-NTp levels (oxidative stress)	Saenen et al., 2016	Oxidative stress

GSTP1, Glutathione S-Transferase Pi 1; 3-NTp, 3-nitrotyrosine; MSE, maternal smoke exposure; NK cells, natural killing cells; PlGF, placental growth factor; PM, particulate matter; Treg cells, T regular cells.

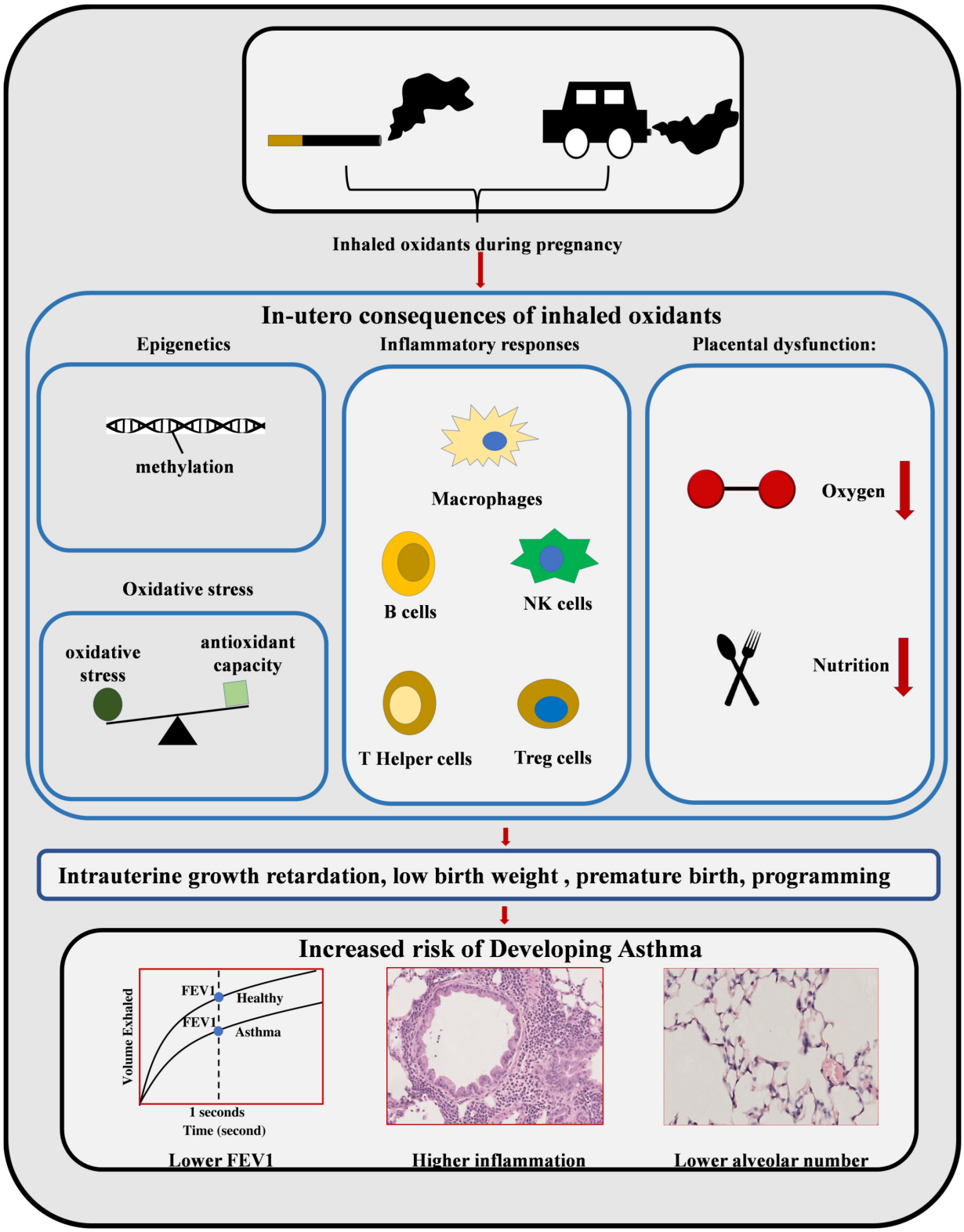

FIGURE 1 | Maternal smoke exposure and maternal PM exposure can increase the rate of childhood asthma. MSE and maternal PM exposure can induce various adverse impacts on the fetus during different intrauterine developmental stages, such as DNA methylation, oxidative stress, inflammatory responses, and placental dysfunction. The resulting intrauterine growth retardation, low birth weight, and premature birth can increase the risk of childhood asthma with a lower alveolar number and reduced lung function, as well as increased lung inflammation.

maternal cigarette smoking (Dhalwani et al., 2015) or nicotine administration in animal models (Sekhon et al., 1999, 2004). This suggests that the whole constituent of tobacco smoke is needed to study the mechanism in animals.

The Role of Endocrine Disorders

Endocrine disruption during pregnancy is a potential cause of adverse pregnancy outcomes. Endocrine glands form an important part of the fetoplacental unit that can secrete a significant amount of hormones including the estrogen to support pregnancy. Estrogen plays a key role in regulating neuroendocrine homeostasis in the developing fetus and promotes Th2 immune cell development in the fetus (Xu et al., 2003; Wood, 2014). A human study demonstrated that abnormal estrogen level in pregnant mothers affects fetal development (Migliaccio et al., 1996). A reduction in estrogen and estrone (a weak estrogen) levels in the cord blood has been found if the mother smoked during pregnancy (Varvarigou et al., 2009). This is because smoking can produce an anti-estrogenic effect and induce androgenisation in pregnant mothers to disturb hormonal homeostasis (Håkonsen et al., 2014). Such changes may influence the risk of asthma in offspring (Rangaraj and Doull, 2003).

The evidence to prove the relationship between maternal PM exposure and its impact on endocrine homeostasis are scarce. It has been shown that the endocrine-disrupting chemicals (EDCs) on the surface of PM can disrupt sex hormone synthesis (Lauretta et al., 2019). Polycyclic aromatic hydrocarbons in both tobacco smoke and PM, can also affect steroidogenesis through inhibiting steroidogenic enzymes (Rocha Monteiro et al., 2000). However, there is no direct evidence suggesting the correlation between hormone change induced by maternal PM exposure and fetal lung development, neither is known about the risk of asthma in the offspring (Street et al., 2018).

However, the information collected from cord blood at birth can't accurately reflect the changes in fetal lung development during particular sensitive windows of embryo development induced by MSE and Maternal PM exposure. Amniocentesis is an alternative method to measure hormone levels at different time points and explore endocrine disruption, but access is limited. Animal modeling may shed a light on the correlation between

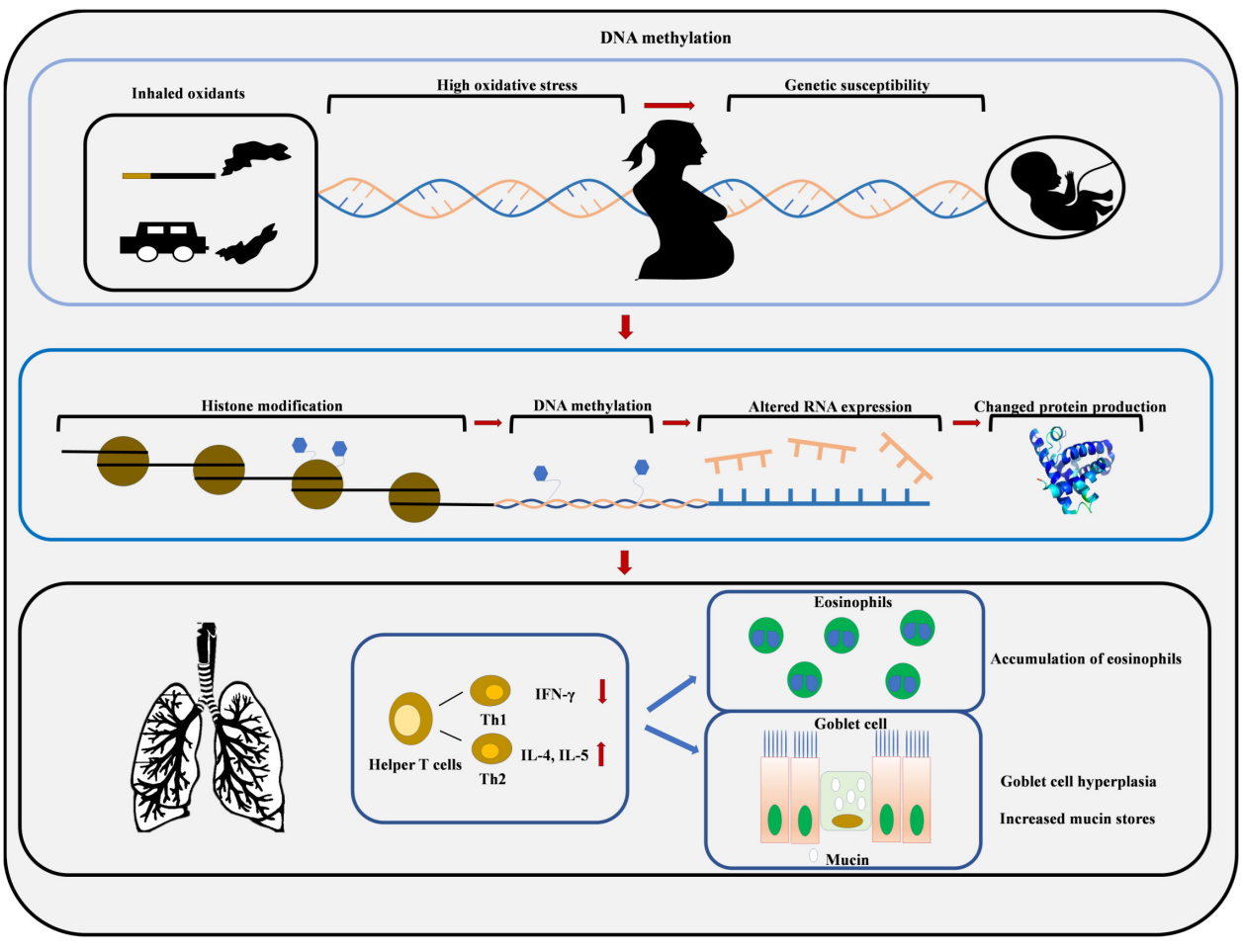

FIGURE 2 | Maternal smoke exposure and maternal PM exposure increase oxidative stress in the womb which increases the risk of developing asthma due to the epigenetic modification of fetal DNA. Environmental toxicants can induce histone modifications and DNA methylation, which results in Th2 cytokine overproduction, eosinophils accumulation, goblet cell hyperplasia, and mucin hypersecretion.

placental hormone changes and fetal lung development, as well as postnatal lung function and susceptibility to asthma. Future research can focus on this aspect to better understand the niche factors contributing to lung development and the risk of asthma.

The Role of Epigenetic Programing

Programing is a term used to describe an altered phenotype due to changes in the *in utero* environment. Epigenetic programing describes stable inheritable phenotypic changes without the alteration in the DNA sequence. Such a process controls mRNA expression and protein production through changing the transcriptome, including DNA methylation and histone modifications. Mounting evidence has closely linked asthma to epigenetic programing due to intrauterine environmental changes. For example, asthma is also an inheritable disease (Eder et al., 2006). The parent-of-origin effect which is usually due to epigenetic mechanism, also shows a prominent influence on the development of asthma, e.g., asthmatic mothers are more likely to have offspring with asthma than the asthmatic fathers (Moffatt and Cookson, 1998). As mitochondrial DNA is 100% inherited from the mothers, epigenetic modification of this genome may largely contribute to this phenomenon. In addition, the fetal period is a vulnerable stage and thus very sensitive to environmental toxicant exposure, when maternal protection is vital. During embryogenesis, cells divide rapidly and therefore the genome is in a relatively unstable status. During this period, oxidative stress induced by environmental toxicant exposure may easily interrupt genomic duplication process (Foley et al., 2009), leading to abnormal epigenetic modifications or even mutation, rendering the fetus susceptible to future chronic diseases after birth, such as asthma.

In a cohort study on MSE, CpGs methylation has been found on genes responding to the pollutants in tobacco smoke in the newborns of smokers who smoked during pregnancy (Joubert et al., 2014). In addition, CpG methylation was also found in the genes involved in fetal development in cord blood by MSE, suggesting a mechanism by which MSE results in intrauterine underdevelopment (Joubert et al., 2014). Previous studies have shown that maternal PM exposure could alter DNA methylation in the offspring. Prenatal PM_{10} exposure induced superoxide dismutase 2 (SOD2) protomer methylation in cord blood cells (Zhou et al., 2019), which is related to phthalate and diisocyanate-induced asthma (Yucesoy et al., 2012; Wang and Karmaus, 2017). As the epigenetic changes are inheritable, they will change gene expression to affect normal embryo development and persist throughout life, resulting in the susceptibility to chronic diseases in later life (Montgomery and Ekbom, 2002). It may also result in the transfer of certain respiratory diseases to subsequent generations, such as asthma, establishing a family history. For a detailed review on epigenetic changes due to *in utero* oxidative challenges, please see Zakarya et al. (2019).

The Role of the Immune Response

The mother's immune system plays a central role in the protection of fetal development. The fetus and newborns need maternal antibodies (Ig) to protect them from infectious diseases (Niewiesk, 2014). Previous studies have shown that parental smoking and PM exposure increased Ig E levels in the cord blood

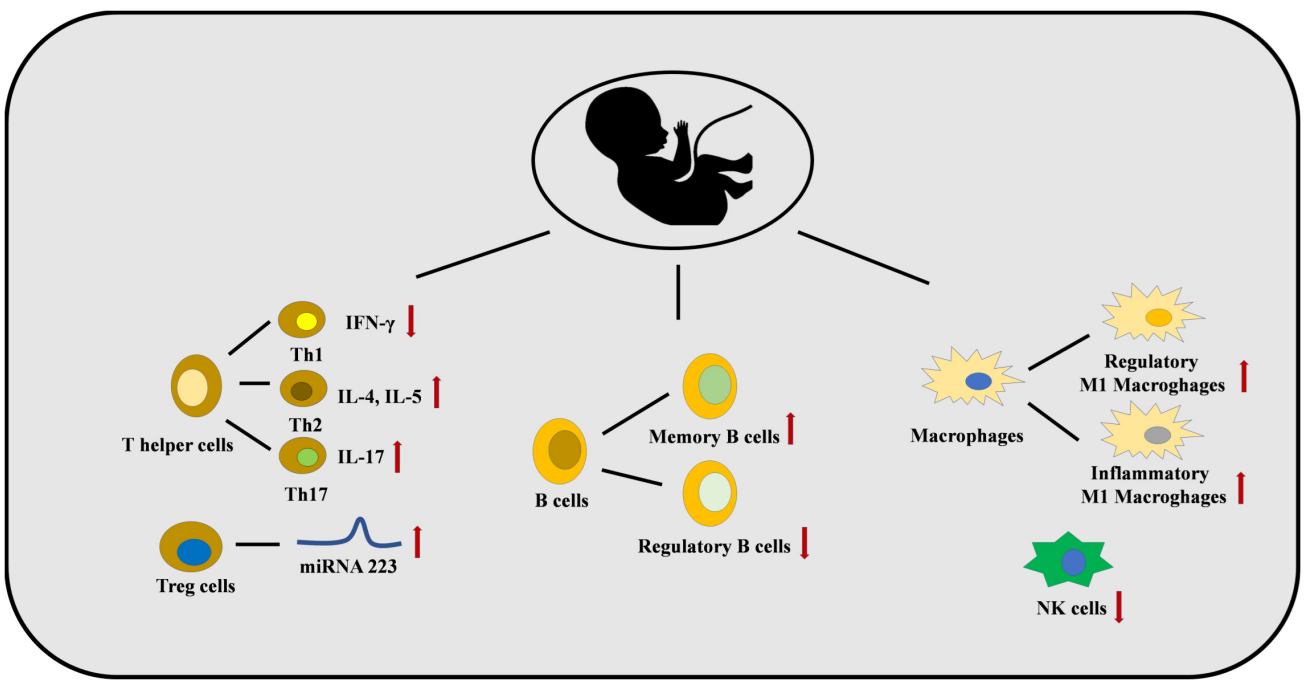

FIGURE 3 | Maternal smoke exposure and maternal PM exposure can dysregulate the immune system in the fetus. The numbers of Th2 and Th17 cells are increased with a lower number of Th1 cells. This is caused by several epigenetic mechanisms, for example, miRNA 223 is increased in Treg cells. B cell and macrophages differentiation are also affected, and a lower number of NK cells are found.

(Valavanidis et al., 2006; Liu et al., 2007). MSE and maternal PM exposure can also alter immune responses through activating inflammatory macrophages and memory B cells in the offspring (Prins et al., 2012; Yoshida et al., 2012). These changes in immune responses suggest that MSE and maternal PM exposure can alter the innate and adaptive immune response in the offspring. In addition, MSE and maternal PM exposure have also been shown to delay the maturation of immune system (Ege et al., 2006; Noakes et al., 2006), which may also make such offspring more susceptible to allergic disorders.

Toll-like receptors (TLRs) play an important role in the neonatal immune response (Yoon, 2010). MSE can inhibit neonatal immune system maturation through impairing TLR mediated responses (such as TLR2 and TLR9) (Noakes et al., 2006). We also have similar observations in the brains of mice who are offspring which had MSE. At postnatal day 1, mRNA expression of TLR4 was decreased in the offspring from MSE compared to those from Sham-exposed mothers, suggesting suppressed immune response or delayed maturation of immune response (Chan et al., 2016). However, TLR4 mRNA expression was increased in 13 weeks old offspring which had MSE along with increased inflammatory cytokines expression (Chan et al., 2016), suggesting that MSE has a sustainable influence on the immune system leading to heightened inflammatory cytokines production. Maternal PM exposure could induce similar adverse effects. High levels of TLR2 and TLR4 expression were found in the human offspring and animals from mothers exposed to increased levels of PM during pregnancy (Ege et al., 2006).

Asthma is typified by T cell dysregulation, including Th1, Th2, and Th17 cells (Kaiko et al., 2008). In most asthmatic patients, accumulating evidence shows the suppression of Th1 cytokines (for example IFNγ) with higher Th2 cytokine expression (IL-4, IL-5, and IL-13) (Mazzarella et al., 2000). Furthermore, clinical data showed that allergic responses are more prevalent among the children who have developed attenuated Th1 responses during infancy (Shirakawa et al., 1997). Similar changes were found in animal studies. In pregnant C57BL/6 mice, intranasal exposure to diesel exhaust particles has been shown to increase the Th2 cell percentage in the bronchoalveolar lavage fluid with higher levels of pro-inflammatory cytokines (IL-4 and IL-5) in the offspring with asthma (Manners et al., 2014). MSE was also shown to increase Th2 cytokines (IL-4 and IL-5) and other pro-inflammatory cytokines (such as IL6) with suppressed Th1 cytokines (IFN-γ) due to reduced NK cell activities (Singh et al., 2011; Prins et al., 2012).

However, the immune response is complicated, and difficult to investigate from a broader spectrum. A study has found that $PM_{2.5}$ exposure differentially impacts the immune system at different stages of gestation. High level of CD3 + and CD4 + lymphocytes and low percentage of CD19 + lymphocytes and NK cells can be found in the cord blood during the early gestation; however, the opposite changes with low level of CD3 + and CD4 + lymphocytes and high percentage of CD19 + lymphocytes and NK cells were found if PM exposure occurs during late gestation (Herr et al., 2010). These studies suggest that immune response has been programed by *in utero* exposure to air pollution, however, future studies are needed to fully understand the extent of the changes in this system.

CONCLUSION AND PERSPECTIVES

In conclusion, cigarette smoking and PM exposure during pregnancy is detrimental to fetal development and increase the risk of childhood asthma (**Table 3**). As summarized in **Figures 1–3**, oxidants inhaled by the mother result in increased oxidative stress in the intrauterine environment. This results in persistent changes to both the structure of the lung and the epigenome, altering immune and endocrine systems. Collectively these changes increase the risk of childhood asthma. Although smoking cessation is preferred, the success rate remains low during pregnancy. Given the similarity between MSE and maternal PM exposure, antioxidant supplementation during pregnancy may be a plausible prophylactic strategy, which is yet to be confirmed by large clinical trials.

AUTHOR CONTRIBUTIONS

BW, HC, and BO designed and wrote the manuscript. YC and GW contributed to the grammar checking.

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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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REVIEW
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CD151 in Respiratory Diseases

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The tetraspanin, Cluster of Differentiation 151 (CD151), is ubiquitously expressed in adult tissue, especially in the lungs where it has been implicated in lung cancer, asthma, influenza, and idiopathic pulmonary fibrosis (IPF). CD151 interacts with laminin-binding integrins and growth factor receptors, and is reported in cancer-promoting processes such as tumor initiation, metastasis, and angiogenesis. In asthma, CD151 was shown to promote airways hyperresponsiveness through calcium signaling whereas in influenza, CD151 was shown to be a novel host factor for nuclear viral export signaling. Furthermore, CD151 was shown to be associated with increased disease severity and poorer survival outcome in asthma and lung cancer, respectively. In this review, we provide an update on the current understanding of CD151 with regards to its contribution to lung pathophysiology. We also summarize factors that have been shown to regulate CD151 expression and identify key areas that need to be taken into consideration for its utility as a screening or prognostic tool in disease management and/or as a therapeutic target for the treatment of lung diseases.

Keywords: CD151, lung cancer, asthma, influenza, idiopathic pulmonary fibrosis, biomarker, targeted therapy, tetraspanin

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INTRODUCTION

Respiratory diseases account for significant illness and premature mortality around the world. The global impact of respiratory diseases is difficult to quantify due to discrepancies or insufficient data across regions, however, the socioeconomic burden of these conditions cannot be ignored. More than 9.5 million deaths globally were attributed to respiratory diseases – the most common of which were asthma, lower respiratory infections, chronic obstructive pulmonary disease, and lung cancer – and the total cost of respiratory diseases in the European Union alone was estimated to total over €380 billion annually (Gibson et al., 2013; Ferkol and Schraufnagel, 2014). Whilst risk factors such as air pollution, tobacco smoke exposure, occupational agents, and genetics (Gibson et al., 2013) have been identified, respiratory diseases are not curable in some cases and current treatment options are suboptimal for the majority of patients with chronic respiratory diseases. Hence, the development of novel therapies is required to alter the progression of disease severity and/or prevent disease onset.

In this review, we provide an update on the current understanding of CD151 with regards to its contribution to lung physiology and pathophysiology (**Figure 1**). We also summarize factors that have been shown to regulate CD151 expression and identify key areas that need to be taken into consideration for its utility in disease management as a screening or prognostic tool and as targeted or adjuvant therapy.

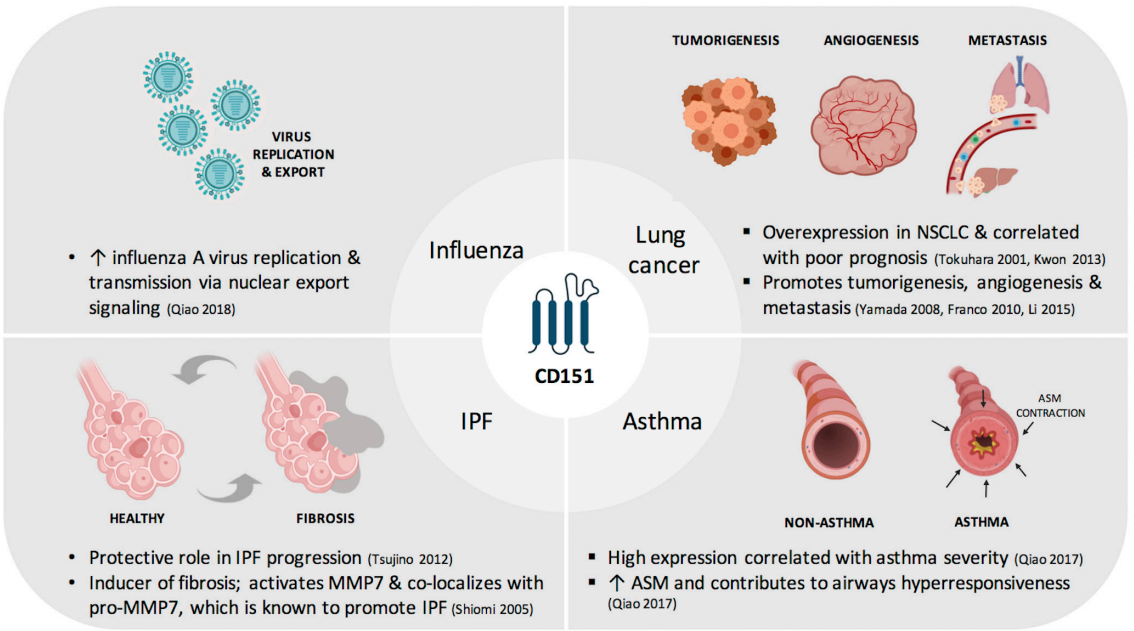

FIGURE 1 | Contribution of CD151to respiratory diseases. ASM, airway smooth muscle; IPF, idiopathic pulmonary fibrosis; MMP7, matrix metalloproteinase-7; NSCLC, non-small cell lung cancer.

CD151

Cluster of Differentiation 151 was first identified as a platelet surface glycoprotein, gp27, where it was found to induce platelet aggregation and mediator release (Ashman et al., 1991). Following successful cloning of the cDNA encoding gp27, it was shown to belong to the transmembrane 4 superfamily, tetraspans, and renamed Platelet-Endothelial cell Tetra-span Antigen-3 (PETA-3) (Fitter et al., 1995). The following year, another team independently identified the same protein but named it protein SFA-1 as it was induced by a T-cell leukemia cell line, SF-HT. The protein was subsequently reassigned as Cluster of Differentiation 151 (CD151) (Ashman, 2002). Whilst early reports identified CD151 as a surface protein, CD151 is also expressed intracellularly (Hwang et al., 2019), including cytoplasmic vesicles, endothelial cell junctions and perinuclear regions (Liu et al., 2007; Qiao et al., 2017).

Like other tetraspanin family members, CD151 forms lateral associations with multiple partner proteins within the tetraspanin-enriched microdomain (TEM). The most prominent partners of CD151 are laminin-binding integrins (Liu et al., 2007), whilst adhesion molecules, growth factor receptors, metalloproteinases, and intracellular signaling molecules have also been shown to be associated with CD151 or be localized within TEMs (Hemler, 2003; Stipp et al., 2003). In addition to membrane clustering and organization, they have been shown to modulate, stabilize or prevent the activities of their associate molecules to influence cell activation, proliferation, migration, adhesion, and signaling.

Cluster of Differentiation 151 is widely expressed in multiple cell types of normal human tissue (Sincock et al., 1997). Immunohistochemical staining showed that CD151 was found to be strongly positive in the lungs, specifically in airway smooth muscle (Qiao et al., 2017), epithelial layers, endothelial cells and blood vessels (Sincock et al., 1997). Given its abundance in lung tissue, the role of CD151 in normal and pathological processes in airways is especially relevant.

CD151 in Development and Maintenance of Lung Structure

The role of CD151 in normal lung physiology is not fully explored but inferences about its critical role can be made from clinical cases of CD151 gene mutation and from studies in mice where CD151 is deleted. With regards to clinical cases of CD151 gene mutation, there have been six reported case studies of patients with CD151 gene mutations resulting in truncated or altered forms of the protein. Nephropathy was reported in four of the cases as well as epidermolysis bullosa and sensorineural deafness in two cases (Karamatic Crew et al., 2004; Vahidnezhad et al., 2018). The remaining two cases, with a single base substitution, exhibited no significant clinical outcomes (Karamatic Crew et al., 2008). Differences in lung function or morphology were not reported in these patients – whether genetic mutations of CD151 had no effect on lung physiology and therefore it was not reported, or whether the investigators did not look at other organ phenotypes is not known. In CD151-null mice, several studies have recapitulated the significant role of CD151 in kidney, skin and inner ear function (Wright et al., 2004; Sachs et al., 2006). Interestingly, Tsujino et al. (2012) reported that CD151 is essential for the normal function of alveolar epithelial cells (APCs) as the deletion of CD151 in APCs resulted in

the loss of epithelial integrity (loss of epithelial adhesion to the basement membrane). In addition, there were increases in collagen deposition, collagen-1 expression and hydroxyproline content in the lungs of the CD151-null mice and lung compliance was also reduced in these mice as compared to wildtype control mice. The authors concluded that CD151 functions to protect against pulmonary fibrosis by maintaining epithelial integrity.

Besides these reports, findings from other groups may be informative in understanding the physiological role of CD151. Treatment with anti-CD151 antibody resulted in the loss of epithelial integrity in skin epithelial cells (Shigeta et al., 2003) whilst CD151 knockdown was shown to disrupt integrin-mediated adhesion to the basement membrane (Yamada et al., 2008). Both these processes are crucial for the development and maintenance of lung structure. Given the strong interaction between CD151 and its laminin-binding partners, defects in bronchi morphogenesis and lung deficiencies observed with the deletion of integrin α3β1 (Kreidberg et al., 1996) and laminin-α5 (Nguyen et al., 2002), respectively, cannot be overlooked and further studies have to be conducted to conclusively determine whether the physiological conditions observed are a direct consequence of CD151 dysregulation or a secondary effect of CD151 due to disruption of its laminin-binding partners.

Tumor Promoter in Lung Cancer

Lung cancer has a high degree of molecular heterogeneity thus giving rise to diverse pathological features. Cases are classified according to respective histological characteristics leading to the distinction between small and non-small cell lung cancer (NSCLC) (Herbst et al., 2008). The onslaught of molecular targets has driven a shift in therapeutic management from conventional cytotoxic drugs to targeted molecular targeted therapy (Hirsch et al., 2017; Herbst et al., 2018). The pathogenesis of lung cancer and its management has been covered extensively by others in the preceding reviews. CD151 was the first tetraspanin to be identified as a tumor promoter (Testa et al., 1999). The authors found that the monoclonal antibody raised against CD151 was capable of inhibiting both spontaneous and experimental metastasis in the chick embryo chorioallantoic membrane model. Since then, CD151 overexpression has been implicated as a negative prognostic indicator in numerous solid malignancies (Ang et al., 2004; Chien et al., 2008; Ke et al., 2009; Suzuki et al., 2011; Kwon et al., 2012; Lee et al., 2012; Kang et al., 2013; Romanska et al., 2013; Fisher et al., 2016; Medrano et al., 2017).

In NSCLC, several studies have shown that CD151 overexpression, measured by gene expression and immunohistochemical analysis, is associated with poorer prognosis in terms of survival rate (Tokuhara et al., 2001) or overall and disease-free survival (Kwon et al., 2013). Exosome protein profiling comparing a cohort of lung cancer patients with non-cancer patients revealed that CD151 was one of the markers found to be upregulated in lung cancer (Sandfeld-Paulsen et al., 2016). This study showed that whilst CD151 expression was detected on structural or immune cells, these cells also secreted CD151-containing exosomes in circulation to act as components for crosstalk between cancer initiating cells and their environment (Yue et al., 2015). This highlights their key role in cancer and as a mediator of cell-cell communication.

Use of gene deletion technology and/or use of monoclonal antibodies directed at CD151 have been employed to explore the functional significance of CD151. In lung cancer cells, CD151 knockdown resulted in impairment of several cancer-promoting processes including cell survival, migration, invasion, and matrix adhesion. These changes were mainly due to CD151 interaction with partner or associated proteins such as integrin α3β1 (Yamada et al., 2008), matrix metalloproteinase-9, vascular endothelial growth factor (Li et al., 2015) and hepatocyte growth factor (Franco et al., 2010), and subsequently downstream signaling of these proteins. In spontaneous lung metastasis models, CD151-null mice exhibited a marked delay in tumor initiation and a decrease in number of metastastic lesions (Deng et al., 2012; Copeland et al., 2013). Similarly, when tumor cells were injected at a separate primary site, CD151-null mice also showed reduced lung metastasis and tumor cell residence (Takeda et al., 2007, 2011; Sadej et al., 2010).

Hyperresponsiveness Mediator in Asthma

Asthma is a chronic airway disease characterized by three main features, airways hyperresponsiveness, airway wall remodeling and airway inflammation (Barnes, 1996; Kudo et al., 2013). Current asthma treatment is mainly focused on long-term clinical control of asthma and rapid-acting symptom relief. However, these drugs are suboptimal in controlling disease, for example in the case of the widely prescribed inhaled corticosteroids which are limited in its clinical efficacy due to varying responsiveness in the population and increased risk of adverse effects when administered at higher doses (Bateman et al., 2008; Reddel et al., 2015). Therefore, a better understanding of underlying mechanisms of disease and therapeutic targeting of these mechanisms may contribute greatly to improved asthma management.

Expression of CD51 is clinically relevant to asthma as immunohistochemical analysis of human bronchial biopsy specimens for CD151 expression in airway smooth muscle bundles were highest in mild or moderate asthma subjects compared with non-asthmatic subjects (Qiao et al., 2017). In the same study, CD151 was implicated to play a role in airways hyperresponsiveness – loss- and gain-of-function studies showed that CD151 enhances G protein-coupled receptor (GPCR)-induced calcium and protein kinase C signaling, which are key signaling pathways implicated in airway smooth muscle cell contraction. In support, CD151-null mice did not exhibit increased airway reactivity to contractile agonists and were shown to have increased lung compliance compared to the wild-type group (Qiao et al., 2017). This study is significant as it provides a better understanding of the mechanism for airways hyperresponsiveness which may help in the design of more efficacious and emerging therapies for asthma. What remains to be determined is whether high CD151 expression is a result of asthma or whether elevated expression of CD151 results in asthma. Furthermore, the impact of CD151 on the remaining

key features of asthma, airway wall remodeling, and airway inflammation, is yet to be established.

Host Factor in Influenza Viral Export

An estimated 5% of adults develop influenza symptoms annually with severe cases leading to conditions such as bronchitis and pneumonia. These seasonal epidemics are largely due to influenza viruses A and B. Influenza viruses are segmented, negative-sense RNA viruses within a lipid envelope containing the surface glycoproteins hemagglutinin and neuraminidase – involved in viral entry and progeny release, respectively (Nicholson et al., 2003; Shim et al., 2017). Tetraspanins are reported to play critical roles in multiple virus life cycle processes such as virus entry, endocytosis, intracellular trafficking, assembly and budding (van Spriel and Figdor, 2010). CD151 was previously implicated in several viral infections including papillomavirus, cytomegalovirus and human immunodeficiency virus (Thali, 2011; Scheffer et al., 2014; Hochdorfer et al., 2016). CD151 plays a critical role in influenza A virus signaling (IAV) (Qiao et al., 2018). The knockdown of CD151 expression in patient-derived nasal epithelial cells resulted in a significant reduction in IAV progeny viral load. In addition, CD151-null mice were more resistant and tolerant to IAV infection with higher survival rates as compared to the wild-type infected mice. With regards to respiratory infection, the anti-viral responses were attributed to CD151 mediating the export of progeny viruses through its binding to newly synthesized viral proteins (NP, M1, and NEP) and host nuclear export protein (CRM1). The novel action of CD151 on viral nuclear export signaling associated with H3N2 and H1N1 infection (Qiao et al., 2018) highlights the potential of CD151 to be developed as a novel therapy to treat influenza and other viral infections. Targeting CD151 is especially beneficial given that it targets a host mechanism thus making it less susceptible to constant changes in viral strains – due to antigenic shift and antigenic drift – that render current antiviral drugs or vaccines ineffective after each season.

Protective or Progressive Role in Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a form of interstitial pneumonia that features the chronic and progressive scarring of the lung. Initially regarded as an inflammatory disease, recent reports have highlighted the importance of AECs activation in causing repetitive injury in IPF (King et al., 2011; Richeldi et al., 2017). This triggers the recruitment of immune cells and fibroblasts in the lung microenvironment and initiates the remodeling of the extracellular matrix and lung regeneration (Martinez et al., 2017; Mora et al., 2017).

Interestingly, CD151-null mice spontaneously developed pulmonary fibrosis features observed at 30 weeks and exhibited accelerated bleomycin-induced lung injury in the pulmonary fibrosis model, suggesting a protective role of CD151 in IPF. This was attributed to the critical function of CD151 in maintaining AECs integrity, and that CD151 deletion attenuates AECs adhesion to the basement membrane, upregulates transforming growth factor-β1 (TGF-β1) signaling in AECs and promotes epithelial-mesenchymal transition changes. In support of this, CD151-negative AECs were focally observed in lung biopsies from IPF patients. However, it is unclear if the IPF phenotype observed in CD151-null mice is strain-specific as differences in renal function in tumor formation (Li et al., 2013) has been observed between FVB/N background [generated using 129SvEv ES cells (Takeda et al., 2007; Tsujino et al., 2012)] versus C57BL/6 mice [generated from 129/Ola and C57BL/6 ES cells (Wright et al., 2004)]. Importantly, whether similar phenotypes are observed in patients with CD151 mutations remains to be explored.

In contrast to the report above describing the protective role of CD151 in IPF, Fujishima et al. (2010) proposed that interactions between CD151 and MMP-7 (for which expression is elevated in IPF patients) could contribute to worsening of IPF condition. They show that CD151 facilitates MMP-7 activation by acting as a docking molecule (Shiomi et al., 2005) and was colocalized with pro-MMP-7 in sections of lung tissue from IPF patients. However, CD151 levels in these IPF patient samples were not directly measured in this study (Fujishima et al., 2010). In support, CD151 has been implicated in other fibrotic processes, such as liver fibrosis and skin wound healing (Mazzocca et al., 2002; Geary et al., 2008). Therefore, more studies are warranted to clearly define the role of CD151 in fibrosis in general and IPF more specifically.

REGULATION OF CD151 EXPRESSION

CD151 Expression Modulators

Given the importance of CD151 in respiratory diseases, it is interesting to note that little is known about regulators of CD151 expression changes. To date, there are only two studies that report that the expression of CD151 mRNA and protein abundance are modulated by therapies – anti-epileptic drugs and anti-cancer drugs, respectively (Hua et al., 2001; Hwang et al., 2019). The study conducted by Hua et al. (2001) showed that chronic administration of the anti-epileptic drugs, carbamazepine or valproate, significantly decreased CD151 transcript levels in rat frontal cortex following chronic (five-week) treatment. The functional significance of this reduction was not explored in this study and there have been no followup studies to date by this group or others. In contrast to that, a recent study by Hwang et al. (2019) showed that several anti-cancer drugs (gefitinib, lapatinib, cisplatin, oxaliplatin, camptothecin, and 5-fluorouracil) induced CD151 protein levels in A431 skin epidermoid carcinoma cells. They further showed that gefitinib upregulated CD151 protein levels in MDA-MB-231 breast and A549 lung carcinoma cell lines, respectively. In this study, the group showed that anti-cancer drug resistance may in part be attributed to CD151 upregulation, for which CD151 knockdown sensitized the cells to drug treatment. It remains to be seen whether these effects could be translated to *in vivo* conditions.

Besides drug-induced regulation, hypoxic conditions were shown to regulate CD151 expression and subsequently cell

adhesion and metastasis. Hypoxia is the condition of oxygen deficiency and is a major driver of cancer-promoting processes such as angiogenesis and migration. Hypoxia is mainly mediated through hypoxia-inducible factors (HIF) (Ke and Costa, 2006). In this study, the expression of CD151 was downregulated under hypoxic conditions via the HIF-1α-dependent pathway in colorectal cells. HIF-1α dependency was further confirmed when CD151 levels were inhibited by HIF-1α induction through treatment with desferrioxamine (hypoxia-mimetic agent) or overexpression with plasmid vectors (Chien et al., 2008). It remains to be observed whether the same hypoxic conditions can reduce CD151 in a lung cancer setting.

Mechanisms Underlying CD151 Regulation

Whilst CD151 expression modulation plays a critical role in determining disease progression, whether in promoting pathophysiology of lung cancer, asthma, and influenza or in potentially protecting against IPF, there is a distinct gap in the literature pertaining to the mechanisms underlying these changes. In the study reporting CD151 mRNA downregulation after treatment with anti-epileptic drugs, valproate and carbamazepine (Hua et al., 2001), there were no experiments conducted to explain gene expression changes or whether downstream protein expression was affected. Whereas in the anti-cancer drug-induced CD151 study (Hwang et al., 2019), the authors ruled out integrin dependence and proposed that CD151 upregulation may be due to diminished protein degradation. However, there are no reports to date to corroborate this proposal.

Interestingly, the mechanism underlying hypoxia-mediated downregulation of CD151 was determined to be at the transcriptional level (Chien et al., 2008). In addition to the decrease in CD151 protein expression under hypoxic conditions, CD151 mRNA levels were also reduced significantly in hypoxic compared to normoxic conditions. This reduction in mRNA levels was confirmed with desferrioxamine treatment. The group went on to identify a putative hypoxia-response element (HRE) in the human CD151 promoter region and intron II, suggesting a direct action on CD151 regulation by hypoxia exposure.

In addition to transcriptional regulation of CD151 expression, another area that may be explored is post-translational modifications. Several reports have previously identified six intracellular C- terminal cysteine sites on CD151 that are palmitoylated, that is, C11, C15, C79, C80, C242, and C243 (Berditchevski et al., 2002; Yang et al., 2002). Besides palmitoylation, CD151 may also undergo glycosylation at the asparagine residue, N159 (Baldwin et al., 2008). However, palmitoylation and glycosylation modifications have not exhibited differences in expression levels or staining pattern (Baldwin et al., 2008; Zevian et al., 2011). Also, the effect of drug treatment on these post-translational modifications has not been elucidated. Besides palmitoylation and glycosylation of CD151, the post-translational modification of ubiquitination should also be explored as it may be informative with regards to CD151 protein stability.

KEY CONSIDERATIONS FOR CD151 IN THE MANAGEMENT OF LUNG DISEASES

CD151 as a Prognostic and Diagnostic Tool

The prognostic value of CD151 was previously emphasized in low-grade prostate cancer, in which CD151 expression could predict the clinical outcome of patients more accurately than the traditional histological grading method (Ang et al., 2004). Given the strong association between CD151 and lung cancer (Tokuhara et al., 2001; Kwon et al., 2013), especially adenocarcinoma which is the most common NSCLC subtype, CD151 expression has proven to be especially informative on patient prognosis. Furthermore, the prognostic utility of CD151 expression may be extended to other diseases such as asthma, in which the expression of CD151 was associated with disease severity (Qiao et al., 2017).

Besides immunohistochemical analysis, an emerging technology in determining protein expression is exosome protein profiling. Tetraspanins, including CD151, are shown to be highly enriched in exosomes (Merino et al., 2014) which may be derived from plasma or urine samples (Jakobsen et al., 2015) making this method far less invasive than traditional biopsy techniques. The potential of CD151 expression detection in exosomes as a screening tool has been explored where a high level of accuracy (72%) in detecting cancer in adenocarcinoma patients was observed (Sandfeld-Paulsen et al., 2016). Effective screening methods are especially vital in NSCLC for which 60% of patients are only diagnosed at an advanced stage (Reck et al., 2013). Whilst this method requires further validation, it may be a promising avenue to complement or improve current diagnostic processes.

Targeted CD151 Therapy

Given the role of CD151 in a milieu of processes that contribute to lung disease pathophysiology, targeting CD151 in a clinical setting is justifiable. The most commonly reported method of targeting CD151 is with anti-CD151 monoclonal antibodies. Treatment with these antibodies has been shown to impair cellular processes at multiple cancer stages. Tumor growth potential, neoangiogenesis and metastasis following injection with a hepatocellular carcinoma cell line was also inhibited in mice that were treated with mAb 9B, which was shown to specifically disrupt the interaction between CD151 with integrin α6β1 (Ke et al., 2011). Two other studies recapitulated the anti-metastasis action in chick embryo models using the monoclonal antibodies, mAb 50-6 (Testa et al., 1999) and mAb 1A5 (Zijlstra et al., 2008), which were shown to reduce both spontaneous and experimental metastasis. Similarly, the mAb SFA1.2B4 was shown to impair pulmonary metastasis in mice injected with either colon cancer or fibrosarcoma cell lines (Kohno et al., 2002). Whether or not these monoclonal antibodies disrupt disease progression in respiratory diseases remain to be explored, although its anti-cancer properties for the treatment of lung cancer are promising.

Besides the use of monoclonal antibodies, CD151 gene deletion has been instrumental in improving markers of disease

outcomes in asthma, influenza, and tumor progression. As described above, CD151 knockdown, through siRNA technology, markedly reduced airway smooth muscle contraction whilst airway hyperresponsive processes decreased significantly in allergen-induced CD151-null mice. Following influenza A virus infection, CD151-null mice exhibited better survival and reduced viral titer, which was attributed to the role of CD151 in nuclear export of viral proteins confirmed using CD151 knockdown experiments. In the context of cancer, both experimental lung metastasis and tumor cell residence were reduced in CD151-null mice. Furthermore, pathologic angiogenesis was impaired in these CD151-null mice despite showing no vascular defects under normal developmental conditions. The impact of CD151 was also reported in breast cancer in which mammary tumor initiation, tumor growth, survival, and metastasis were impaired in CD151-null mice (Yang et al., 2008; Deng et al., 2012).

Despite the promising results with monoclonal antibodies and gene deletion, there are several obstacles that restrict the development of these therapeutic strategies. The ubiquitous expression of CD151 in normal human tissue (Sincock et al., 1997) and its role under physiological conditions (as described above) warrant further studies to determine whether diminishing CD151 expression for therapeutic benefit in the lungs may lead to unwanted, adverse effects to other healthy cells/organs. In this regard, advancements in targeted drug delivery would be extremely beneficial to avoid off-target effects. Developments in the field of nanotechnology and the advent of nanoparticles or nanocarriers in biomedical research provide another avenue for exploiting the importance of CD151 in lung diseases. Several reviews have summarized the clinical significance of nanomedicine in which binding molecules (such as peptides, aptamers, and antibodies) are utilized in cell-specific or tissue-localized delivery of drugs, compounds or genetic material (Poelstra et al., 2013; Rosenblum et al., 2018). For instance, using the nanoparticle-based system, Deshmukh et al. (2018) were able to deliver the small molecule EGFR-specific inhibitor, erlotinib, specifically to myofibroblasts by means of targeting a transmembrane receptor, platelet-derived growth factor receptor-beta, which is uniquely expressed on myofibroblasts in the liver. This specific delivery to hepatic myofibroblasts was more effective and well-tolerated compared to systemic administration of the drug alone (Deshmukh et al., 2018). No studies to date have reported the use of CD151-specific binding molecules as a targeting mechanism for drug delivery, but its potential could be explored given the importance of CD151 overexpression in various lung diseases and other human malignancies. Delivery of genetic material to inhibit the expression of CD151, such as in the case of short hairpin RNA or guide RNA for CRISPR/Cas9-based genome editing, is another approach that may be investigated. Also, advancements could be made to improve not only uptake but enhanced retention of drugs (Matsumura and Maeda, 1986; Klibanov et al., 1990; Blume et al., 1993; Blanco et al., 2015), as well as route of drug administration (local delivery via inhalation versus systemic delivery) (Yan et al., 2016; Cryer and Thorley, 2019; Mukherjee et al., 2019). In addition to that, the magnitude of CD151 reduction required in cases of overexpression will also need to be assessed, specifically whether CD151 expression levels can be ablated completely or must be brought back to a baseline expression to ensure physiological functions are not disrupted. Finally, drug-induced upregulation of CD151 must be taken into account given the importance of expression changes in disease progression and severity.

Cluster of Differentiation 151 may also play an important role as an adjuvant therapy alongside currently available therapeutics. Given the close association of CD151 with oncogenic drivers including growth factors, such as EGFR (Yang et al., 2008; Deng et al., 2012), HER2 (Yang et al., 2010; Deng et al., 2012; Romanska et al., 2015), and HGFR (c-Met) (Klosek et al., 2005; Franco et al., 2010; Ha et al., 2014), CD151 was shown to impair growth factor receptor-dependent processes such as tumor onset, cell growth, spreading and motility. CD151 ablation in these studies was sufficient to arrest or impair oncogenic responses. Furthermore, in the case of EGFR and HER2-specific inhibitors, drug sensitivity was restored when CD151 expression was abrogated (Hwang et al., 2019). Restoration of drug sensitivity with CD151 knockdown was shown to not be restricted to targeted therapy but also observed in chemotherapeutic drugs oxaliplatin, cisplatin, and 5-fluorouracil, via apoptotic induction.

CONCLUSION

Cluster of Differentiation 151 is involved in numerous physiological and pathophysiological processes. Therefore, it has been implicated in several diseases, including respiratory diseases such as lung cancer, asthma, influenza and IPF (**Figure 1**). The majority of studies link CD151 to disease onset, severity or progression. The mechanisms underlying CD151 upregulation or downregulation remain to be fully understood. Nonetheless, the clinical significance of CD151 expression shows that CD151 has great potential to be developed as a diagnostic biomarker which may be helpful for early detection or screening, or as a prognostic biomarker given its relevance in predicting lung cancer or asthma severity. Furthermore, CD151 monoclonal antibodies, gene deletion, and nanotechnology studies support the notion for its development as a novel targeted or adjuvant therapy in the treatment of respiratory diseases.

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REVIEW
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Potential Role of Cellular Senescence in Asthma

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Cellular senescence is a complicated process featured by irreversible cell cycle arrest and senescence-associated secreted phenotype (SASP), resulting in accumulation of senescent cells, and low-grade inflammation. Cellular senescence not only occurs during the natural aging of normal cells, but also can be accelerated by various pathological factors. Cumulative studies have shown the role of cellular senescence in the pathogenesis of chronic lung diseases including chronic obstructive pulmonary diseases (COPD) and idiopathic pulmonary fibrosis (IPF) by promoting airway inflammation and airway remodeling. Recently, great interest has been raised in the involvement of cellular senescence in asthma. Limited but valuable data has indicated accelerating cellular senescence in asthma. This review will compile current findings regarding the underlying relationship between cellular senescence and asthma, mainly through discussing the potential mechanisms of cellular senescence in asthma, the impact of senescent cells on the pathobiology of asthma, and the efficiency and feasibility of using anti-aging therapies in asthmatic patients.

Keywords: cellular senescence, asthma, telomere shortening, oxidative stress, senescence-associated secreted phenotype, autophagy, anti-senescence therapies

INTRODUCTION

Asthma is one of the most common non-communicable pulmonary diseases. Bronchodilators and inhaled/systemic corticosteroids are the most often used drugs for asthma (Fanta, 2009). According to disease severity and symptom control assessment, patients are managed with stepwise therapy until asthma symptoms are under control. Although these standardized therapies are highly effective in most asthmatics, approximately 10% patients are of steroid-refractory (Barnes, 2013). Even with the highest step of standardized treatment, in which targeted therapies like anti-IgE, anti-IL5, anti-IL5R, and anti-IL4R antibodies would be applied, uncontrolled asthma symptoms and exacerbation still frequently exist in some patients (Israel and Reddel, 2017; Chipps et al., 2018). These patients with difficult-to-treat asthma often have higher mortality and lower lung function (McGeachie et al., 2016; Coumou et al., 2018). Furthermore, with increasing morbidity, difficult-to-treat asthma might explain the stalled reduction in global asthma mortality (Ebmeier et al., 2017). Thus, more interventions and novel strategies are in great demand for asthma patients to achieve further decrease in mortality rate. Current studies are trying to explore new mechanisms involved in the pathogenesis of asthma and then identify potential therapeutic targets.

Asthma is mainly characterized by chronic airway inflammation, airway hyperresponsiveness and airway remodeling (Papi et al., 2018). It's a heterogenetic disease with various inflammatory phenotypes, including eosinophilic inflammation, neutrophilic inflammation, mixed inflammation, and non-inflammatory pattern (Israel and Reddel, 2017). Neutrophilic inflammation associates with the disease severity (Ray and Kolls, 2017). Structural changes like airway wall thickening and extracellular matrix deposition contribute to airway obstruction, leading to persistent airflow limitation, and reduced lung function. Thus, the abnormality of airway inflammation and alteration of airway structure constitute the basic pathophysiology of asthma.

Cellular senescence is a heterogenetic status in response to various stimuli. The main features of cellular senescence contain irreversible limitation of cell proliferation and the senescence-associated secretory phenotype (SASP), which is produced by primary senescent cells and induces senescence of surrounding cells in a paracrine manner (Nelson et al., 2012; Acosta et al., 2013). Senescent cells could be characterized by several properties, including reduced proliferative rate, increased senescence-associated β-Galactosidase (SA-β-Gal), upregulation of tumor suppressors and cell cycle inhibitors like p21, p16, p53, senescence-associated heterochromatic foci, enlarged or flat cell morphology, and secretion of multiple SASP components (Acosta et al., 2013; Muñoz-Espin and Serrano, 2014). Physiologically, cellular senescence is present during natural development and aging as a modulating mechanism, contributing to tumor suppression, wound healing (Demaria et al., 2014), and embryogenesis (Muñoz-Espín et al., 2013; Storer et al., 2013). In recent decades, increasing attentions have been addressed to its contributions to the pathogenesis of diseases and organ dysfunction. Persistent accumulation of senescent cells during aging induces low-grade inflammation through SASP (Acosta et al., 2013), impairs the immune system (Savale et al., 2009; Albrecht et al., 2014), and increases the vulnerability and susceptibility of organs to various pathological challenges (López-Otín et al., 2013). In respiratory system, cellular senescence has established role in the pathogenesis of aging-related diseases like chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) (Tsuji et al., 2006; Diaz de Leon et al., 2010; Kuwano et al., 2016; Álvarez et al., 2017; Yanagi et al., 2017; Rashid et al., 2018; Schuliga et al., 2018; Vij et al., 2018; Araya et al., 2019; Fang et al., 2019; Parikh et al., 2019b). However, little is known about the place of cellular senescence in the development of asthma.

Amassing data has showed that pulmonary cells of COPD and IPF exhibit a senescent phenotype, which is involved in promoting airway chronic inflammation, airway remodeling, and lung function decline (Tsuji et al., 2006; Yao et al., 2012; Álvarez et al., 2017; Yanagi et al., 2017; Rashid et al., 2018; Schuliga et al., 2018; Vij et al., 2018; Araya et al., 2019; Fang et al., 2019; Parikh et al., 2019b). Increased senescence-associated proteins p16 and p21 in alveolar cells are correlated with airflow limitation of patients with emphysema (Tsuji et al., 2006). Deficiency of p21 could attenuate airspace enlargement and lung function decline in cigarette smoke-exposed mice (Yao et al., 2012). Multiple SASP components including IL-6, IL-8, TGF, and MMPs are closely associated with persistent airway inflammation and abnormal extracellular matrix remodeling or pulmonary fibrosis in COPD and IPF (Barnes et al., 2015; Richeldi et al., 2017; Álvarez et al., 2017). As asthma resembles COPD and IPF in chronic inflammation, airway remodeling as well as lung function decline (McGeachie et al., 2016; Papi et al., 2018), would it be possible that cellular senescence also promotes the development of asthma?

Limited but undeniable data has showed that cellular senescence is associated with asthma. Bronchial fibroblasts from asthmatic patients had lower DNA synthesis with cell passage and *in vitro* lifespan than normal controls (Dubé et al., 1998). Myofibroblasts from asthmatics showed reduced proliferative activity in response to mitogens *in vitro*, but higher expression of SASP factors like GM-CSF and IL-8 when stimulated by IL-1α than those from non-asthmatics (Ward et al., 2008). Asthmatic bronchial fibroblasts demonstrated greater proportion of SA-β-Gal positive staining (Hadj Salem et al., 2015). Expression of p21, a cyclin-dependent kinase inhibitor, was elevated in bronchial epithelium of asthmatics, and had a tendency to be higher in severe asthma than mild asthma (Puddicombe et al., 2003). p53 is a tumor suppression protein regulating cell proliferation and also considered as a marker of cellular senescence. It has been showed that p53 was upregulated in bronchial smooth muscle cells from asthmatics (Trian et al., 2016). Based on these evidences, we highly speculate that cellular senescence might have a similar function in asthma as it does in COPD and IPF.

In this review, we will summarize the current knowledge and research focusing on the possible involvement of cellular senescence in asthma, particularly the potential mechanisms of cellular senescence, senescent cell types and their impact on the development of asthma, as well as the effect of current and latent anti-aging strategies on asthma.

POTENTIAL MECHANISMS OF CELLULAR SENESCENCE IN ASTHMA

Cellular senescence was initially discovered by Hayflick and Moorehead (1961) who described a state of cell proliferation arrest in cultured human cells after several divisions. Up to date, several stimuli causing cellular senescence have been reported, including telomere shortening due to replication exhaustion, DNA damage, mitochondrial dysfunction, oxidative stress, certain cytokines, and loss of tumor suppressor (Martínez-Zamudio et al., 2017). These factors and their downstream signal pathways constitute an intricate network leading to cell cycle arrest and SASP in target cells. Some of these stressors are found to be associated with asthma, such as telomere shortening, oxidative stress, inflammation, and autophagy.

Telomere Shortening

Telomere shortening is one of the common mediators of cell aging and correlates to several aging-related diseases (Gansner and Rosas, 2013). Telomere shortening is generally caused by exhaustive replication and brings about cell cycle

arrest (Hayflick and Moorhead, 1961). Telomeres locate at the end of chromosomes in mammalian cells and gradually shorten after each round of cell division because they remain unduplicated during DNA synthesis phase. When they reach the critical length, the ability of cell division will be restricted (Nikitina and Woodcock, 2004).

Scientists have observed decreased proliferation of bronchial fibroblasts and myofibroblasts in asthmatic subjects as compared to non-asthmatics, despite the apparent thickening of airway smooth muscle layer and high levels of inflammatory factors, and suggesting a premature status of cellular senescence in asthma (Dubé et al., 1998; Ward et al., 2008). To explore the underlying mechanism of cell replicative restriction in asthmatics, Hadj Salem et al. (2015) measured the telomere length in bronchial fibroblasts from asthmatic patients and healthy controls. They observed decrease of telomere length in asthmatic fibroblasts, correlating with the increase of the cellular senescence marker β-Galactosidase (Hadj Salem et al., 2015). Similarly, Lee et al. (2017) also found that relative telomere length in peripheral blood mononuclear cells (PBMCs) was shorter in asthmatic children and adolescences than that of non-asthmatics. Likewise, leukocytes from asthmatic patients seemed to have shorter telomere length compared to age-matched controls (Kyoh et al., 2013; Belsky et al., 2014). Shorter telomere length has been proposed as a biomarker of accelerating aging (López-Otín et al., 2013; Bernadotte et al., 2016). These evidences implicate the existence of cellular senescence of bronchial structure cells and immune cells of asthmatics.

Telomere shortening may not only explain the limited proliferation ability of cells in asthmatic patients, but also highly correlated to the clinical features and severity of asthma. For example, telomere shortening in bronchial fibroblasts was associated with airway hyperresponsiveness (Hadj Salem et al., 2015) and lower forced expiratory flow (Henckel et al., 2018). Furthermore, shorter telomere served as a biomarker of life-course-persistent asthma and was linked to eosinophilic inflammation (Belsky et al., 2014). Decrease of telomere length in bronchial fibroblasts was associated with increased severity of asthma (Kyoh et al., 2013). Telomere length may also reflect therapeutic effect for asthma. Asthmatic patients who received steroid treatment would have less telomere shortening than those did not (Lee et al., 2019). These results show that telomere shortening might be a critical biomarker correlating to the pathophysiology of asthma.

Although studies have described the closely association between asthma and telomere shortening, the controversy is that whether shorter telomere accelerates the development of asthma, or telomere shortening is resulted from asthma? According to a study, life-course-persistent asthma, along with higher eosinophilic inflammation, correlated with shorter telomere length than childhood-onset, adulthood-onset asthma, and no-asthma controls (Belsky et al., 2014). One possibility is that higher eosinophilic inflammation promotes telomere shortening. However, the authors found that there was no rapid change of telomere length between age 26 and age 38 in patients with life-course-persistent asthma, indicating that eosinophilic inflammation has little effect on the telomere shortening (Belsky et al., 2014). Thus, telomere shortening is more likely to be a cause of greater inflammation in asthma, rather than a result.

Various factors like environmental pollutants and lifestyle could influence the telomere length from young age (Mirabello et al., 2009; Cassidy et al., 2010). Studies have shown that the rate of telomere shortening accelerated in children exposed to air pollutants such as polycyclic aromatic hydrocarbons (PAHs), ozone (O_3) and fine particulate matter ($PM_{2.5}$) (Lee et al., 2017, 2019). Cigarette smoke exposure also has an adverse effect on the telomere length in children (Ip et al., 2017). Air pollutants and cigarette smoke are common extrinsic inducers to evoke oxidative stress and inflammation in the airway (Zhang X. et al., 2016; Chandrasekaran et al., 2017), leading to the vulnerability of telomere (Von Zglinicki, 2002; Venkatachalam et al., 2017). Prenatal stressor like higher tumor necrosis factor-α/interleukin 10 (TNF-a/IL-10) ratio could also lead to shorter telomere length in newborns (Lazarides et al., 2019). Nuclear factor kappa-B (NF-kB)-driven chronic inflammation could accelerate the rate of senescence in mice through enhancing the expression of cyclooxygenase-2 (COX-2) and reactive oxygen species (ROS), which promote DNA damage and telomere dysfunction (Jurk et al., 2014).

Although some of these stressors are involved in the development or exacerbation of asthma (Guarnieri and Balmes, 2014; Underner et al., 2015), whether telomere shortening induced by these childhood or prenatal risk factors would lead to asthma or not is still obscure. Suh et al. (2019) followed up 84 subjects and found that higher prenatal stress and shorter telomere length did not increase the risk of developing preschool asthma. Thus, more prospective and large-sample investigations are required to answer this question.

Oxidative Stress

Oxidative stress is long been considered as an inducer of premature senescence (Finkel and Holbrook, 2000). Various oxidants like peroxide hydrogen (H_2O_2), which is also an endogenous oxidant, are commonly used to trigger stress-induced premature senescence in experimental studies (Wang et al., 2013). Oxidative stress can be manifested into increased ROS production and decreased antioxidant capacity within the cells (Thannickal and Fanburg, 2000). ROS overproduction could result from mitochondrial oxidative metabolism (Chandel, 2010), respiratory burst, and exposure to environmental pollutants like O_3 and cigarette smoke (Chandrasekaran et al., 2017). Oxidative stress is aggravated during chronic inflammation, due to the release of ROS by multiple immune cells including activated neutrophils, macrophages, monocytes, and dendritic cells (Sánchez et al., 2015). Thus, allergens, environmental noxa, and inflammatory factors act on cells, resulting in altered function of mitochondria, elevated production of ROS, and then perpetuate inflammation as a positive feedback loop. The formation of this vicious cycle will finally lead to SASP by activating nucleotide-binding domain, leucine-rich repeat-containing family protein (NLRP)-3 inflammasome and releasing inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-18 (Davalli et al., 2016).

Evidences have revealed the role of oxidative stress in the pathogenesis of asthma (Bullone and Lavoie, 2017; Kleniewska and Pawliczak, 2017). The ROS in asthma could come from resident cells or immune cells. Mixed allergens could significantly induce ROS production in airway epithelial cells *in vitro* with the absence of immune cells (Chan et al., 2016, 2017; Chen J. et al., 2017). Pollutants like $PM_{2.5}$ could also promote ROS production in human lung alveolar epithelial A549 cells (Deng et al., 2013). ROS production is closely associated with neutrophilic and Th17 inflammation, which are involved in the development of asthma (Chesné et al., 2014; Ray and Kolls, 2017; Carr et al., 2018), and correlated to exacerbation and asthmatic patients with obesity (Suzuki et al., 2008; Kim et al., 2014; Ray and Kolls, 2017; To et al., 2018). Elevated ROS generation from neutrophils and macrophages in asthmatic subjects is correlated to increase of NLRP3 inflammation (Simpson et al., 2014), leading to airway hyperresponsiveness, and lung fibrosis (Kim et al., 2014; Sun et al., 2015).

The mechanism of cellular senescence induced by oxidative stress is involved with a complicated process. Chan et al. (2016, 2017) demonstrated that HDM challenge could enhance ROS generation and elevate the expression of DNA-damaging marker γH2AX. At the same time, DNA repair associated protein was also upregulated (Chan et al., 2016, 2017). The former response would lead to cell cycle arrest and cell death, while the latter could result in cell survival. Cellular senescence might be an intermediated state resulted from the conflict of oxidative stress-induced DNA damage and DNA repair, because senescent cells are still alive but with proliferation arrest (Hayflick and Moorhead, 1961). Probably these affecting cells are not killed because of insufficient DNA damage, and they stop cell diving due to inadequate DNA repair. From another perspective, exogenous and endogenous sources of ROS in asthma could simultaneously activate multiple signaling pathways, including NF-κB, p53, phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) and p38 mitogen-activated protein kinases (MAPK) (Finkel and Holbrook, 2000). p53 serves as a checkpoint protein and its downstream factor p21, a cell cycle dependent kinase inhibitor, could lead to cell cycle arrest (Surget et al., 2013). However, PI3K/Akt/mammalian target of rapamycin (mTOR) pathway could induce chronic inflammation, inhibit cell death, and promote cell proliferation (Bent et al., 2016). Their combinational effect finally brings about a senescent state in cells. This theory has been proved by a previous investigation, which demonstrated that both cell cycle blockage and growth stimulation were required for the development of cellular senescence (Demidenko and Blagosklonny, 2008).

Inflammation

Chronic inflammation serves as the principal hallmark of asthma. Previous studies had shown that aged people with asthma would have higher inflammation levels, which contributed to the therapy unresponsiveness (Busse et al., 2017; Dunn et al., 2018). Intimate association between senescence and inflammation has been depicted in various diseases, such as COPD, inflammatory bowel disease (IBD), cardiovascular disease, obesity and diabetes, autoimmune diseases, and cancer (Zhang J. et al., 2016). According to current understanding, the interrelationship between inflammation and cellular senescence is mainly mediated by the SASPs (Fougère et al., 2017).

Senescence-associated secreted phenotype was firstly defined by Coppé et al. (2008) in and now has been considered as a hallmark of cellular senescence. They found that these secretory phenotypes formed only after DNA damage in fibroblasts and epithelial cells (Coppé et al., 2008). SASPs include inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), growth regulators such as GRO and insulin-like growth factor binding protein-2 (IGFBP-2), cell survival modulators such as OPG and sTNF RI, and shed surface proteins such as uPAR and ICAM-1. Although the SASP in senescent fibroblasts and epithelial cells are not totally the same (Coppé et al., 2008), they execute similar functions in lung diseases, such as promoting cellular senescence, wound repair, and airway remodeling (Parikh et al., 2019b).

Senescence-associated secreted phenotypes reflect an active but abnormal metabolic state of senescent cells despite of quiescence in cell proliferation (Zhang J. et al., 2016). Since 1998, researchers had found that even though lung fibroblasts in asthmatics decreased in proliferation capability, they were still active in producing extracellular matrix proteins such as collagen (Dubé et al., 1998; Ward et al., 2008). SASP is primarily a DNA damage response (DDR) (Rodier et al., 2009). Its secretion is mediated by intracellular IL-1α/miR-146a/b/IL-6/C/EBP-β loop and p38/NF-κB and mTOR pathways (Tchkonia et al., 2013). Senescent cells have a bystander effect on the nearby healthy cells. Co-culture with senescent fibroblasts could increase the generation of DNA double-strand breaks (DSBs) foci in young fibroblasts, indicating that senescent cells could induce DDR in surrounding proliferating cells (Nelson et al., 2012). Soluble factors released into the culture medium from senescent cells hardly promote DNA damage in young cells, but they can transmit to the attached surrounding cells via cell-cell junctions. Blocking these gap junctions would attenuate the increase of DNA damage foci (Nelson et al., 2012). Some components of SASP could induce cellular senescence in a receptor-mediated manner. For example, Jin et al. (2016) found that MCP-1, one of the dominant components of SASP, enhanced senescence in mesenchymal stromal cells (MSCs) via activating the cognate receptor chemokine (c-c motif) receptor 2 (CCR2) and its downstream ROS-p38-MAPK-p53/p21 signaling cascade. Activated p53 would then elevate secretion of MCP-1 to form a positive feedback loop (Jin et al., 2016).

By now, it is still hard to exactly distinguish whether intrinsic inflammation in asthma induces cellular senescence or senescent cells result in airway chronic inflammation *via* SASP. On one hand, some stressors can lead to low-grade inflammation in the airway before asthma onset. Exposure to HDM extract could induce the upregulation of several components of SASP, such as MCP-1, IL-6 and IL-8 in human monocytes (Lee et al., 2008). Childhood obesity is a risk factor for asthma. Postnatal hyperalimentation could enhance the expression of inflammatory cytokines including IL-6, TNF-α, and IL-17A and then induced airway hyperresponsiveness in mice (Dinger et al., 2016). On the other hand, preexisting asthmatic inflammation has different

effect on cellular senescence. TSLP plays an important role in inducing Th2 inflammation and airway remodeling in asthma (Soumelis et al., 2002; Chen et al., 2013). Wu et al. (2013a) found that TSLP could trigger senescence in airway epithelial cells *in vitro*, indicated by the upregulation of p21, p16 and SA-β-Gal. However, Belsky et al. (2014) conducted a prospective study and demonstrated that higher eosinophilc inflammation did not accelerate telomere shortening rate in asthma. Thus, cellular senescence in asthma might be only induced with some specific inflammatory factors.

Autophagy

Autophagy is an intercellular self-degradation process responding to various stimuli including inflammation, pathogenic infection, environmental pollutants, and hypoxia to maintain cellular homeostasis. The role of autophagy in cellular senescence is quite debated, because both its activation and inhibition effects have been reported. Kang and Elledge (2016) suggested that there are two kinds of autophagy: selective autophagy and general autophagy. In selective autophagy, specific components rather than global bulk would be cleaned out through receptor-mediated phagocytosis into autophagosome or lysosome. Selective autophagy could suppress cellular senescence by degrading GATA4, which could initiate NF-κB pathway and induce SASP. On the contrary, general autophagy would promote senescence through TOR-autophagy spatial coupling compartment (TASCC) to facilitate the production of SASP-associated factors (Kang and Elledge, 2016).

Autophagy has been implicated in asthma pathogenesis, but whether it serves as a protective or promoting role is also controversial. Genetic variants of autophagy gene 5 (ATG5) have been found to correlate with asthma exacerbation (Martin et al., 2012) and prebronchodilator FEV_1 in asthmatic patients (Poon et al., 2012). In patients with severe asthma, the level of autophagy in peripheral blood cells and eosinophils is higher than that in non-severe asthma or healthy controls (Ban et al., 2016). McAlinden et al. (2019) found that autophagy is activated in HDM-induced asthma mice with increased Beclin 1 and ATG5 in airway epithelium and airway smooth muscle, and autophagy inhibitor chloroquine could significantly reduce airway inflammation, hyperresponsiveness, and structure remodeling. Conversely, another study showed that autophagy stimulator Simvastatin could alleviate Th2 inflammation and extracellular matrix deposition in asthmatic mouse model (Gu et al., 2017). Suzuki et al. (2016) also discovered that ATG5-mediated autophagy could attenuate airway hyperresponsiveness and neutrophil inflammation, while ATG5 depletion would lead to development of glucocorticoid resistance, and severe IL17A-dependent neutrophil inflammation.

The relationship between cellular senescence and autophagy in asthma is also vague. In respiratory system, it was proposed that insufficient autophagy or mitophagy would induce cellular senescence in COPD and IPF (Fujii et al., 2012; Ito et al., 2015; Kuwano et al., 2016; Tsubouchi et al., 2018; Vij et al., 2018; Araya et al., 2019). For example, insufficient autophagy of mitochondria would increase ROS production and therefore lead to oxidative stress (Gomes and Scorrano, 2013). P62 could combine with polyubiquitinated substrates and Atg8/LC3 to form an important component of autophagosome, and regulate the delivery of ubiquitinated proteins for selective autophagic degradation. Thus, cumulative p62 and ubiquitinated proteins are thought to be the indicators of insufficient autophagy (Komatsu et al., 2007). Transient and insufficient activation of autophagy induced by cigarette smoke extract (CSE) lead to the accumulation of p62 and ubiquitinated proteins, resulting in increased cellular senescence and SASP in human bronchial epithelial cells (HBECs) (Fujii et al., 2012; Ito et al., 2015). Sufficient autophagy activated by Torin1 could avoid amassing of p62 and ubiquitinated proteins, and therefore prevent developing into cellular senescence (Fujii et al., 2012). In asthma, p62 plays a pivotal role in mediating Th2 inflammation in allergic airway diseases (Martin et al., 2006). Although p62 was decreased in airway epithelium and smooth muscle layer of HDM-induced asthma mice (McAlinden et al., 2019), it was upregulated in $CD11c^+$ cells to promote higher neutrophilic airway inflammation and hyperreactivity (Suzuki et al., 2016). Suzuki et al. (2016) found that impaired autophagy in $CD11c^+$ cells, but not in epithelial cells, contribute to severe airway inflammation and steroid resistance. Therefore, the effect of autophagy on asthma depends on not only the kind of autophagy, but also the type of target cells.

According to the previous studies, insufficient autophagy and non-selective autophagy might contribute to cellular senescence (Fujii et al., 2012; Kang and Elledge, 2016). However, most of current investigations have neglected the type of autophagy when studying the correlation of autophagy and asthma. Autophagy is a complicated biological process so that it's difficult to figure out which type it is. In order to identify the function of autophagy on asthma, probably an easier way is to detect whether the affected cells are senescent or not. The link of autophagy, cellular senescence and asthma should be identified. Thus, it is necessary for future studies to pay attention to the type of autophagy, the type of target cells and the outcome of the affected cells.

SENESCENT CELLS AND THEIR INFLUENCES ON THE DEVELOPMENT OF ASTHMA

Epithelial Cell Senescence

Airway epithelium physiologically functions as the first line of defense in innate immunity, preventing intrusion of extraneous particles such as pathogens, allergens, and environmental pollutants from inhaled air into lung. In asthma, epithelial cells were damaged and functioned abnormally by promoting pathologically tissue repair and inducing chronic airway inflammation through the release of cytokines like TSLP, IL-25 and IL-33 (Lambrecht and Hammad, 2012; Gon and Hashimoto, 2018; Papi et al., 2018). Epithelial senescence plays a pivotal role in the initiation of chronic airway diseases. In COPD and IPF, senescence of airway epithelial cells is mainly mediated by mitochondrial dysfunction and DNA damage (Mora et al., 2017; Zhang et al., 2017; Fang et al., 2019). In aged people, the barrier function of airway epithelium was impaired, making them more

vulnerable to infections, which could initiate the exacerbation of chronic diseases such as asthma and COPD (Boulet et al., 2017; Yanagi et al., 2017). Telomere shortening and cellular senescence in type II alveolar epithelial cells (AECs), rather than mesenchymal cells such as myofibroblasts, resulted in airway remodeling and lung fibrosis (Naikawadi et al., 2016). Besides, increased inflammatory cell infiltration in the bronchoalveolar lavage fluid (BALF) was accompanied with higher senescent type II AECs in telomere repeat binding factor 1 (TRF1) -depleted mice (Naikawadi et al., 2016). Thus, senescence in airway epithelial cells plays a key role in initiating airway remodeling and inflammation.

Although there is only a few direct evidence showing senescent epithelial cells in the lung tissues of asthmatic patients (Puddicombe et al., 2003), one study has proved that TSLP could induce cellular senescence in airway epithelial cells *in vitro* (Wu et al., 2013a). Low dose exposure of air pollutants PM_{10} could lead to airway inflammation through inducing oxidative stress and mitochondrial dysfunction (Chan et al., 2019), which has been implicated to result in epithelial cell senescence (Tezze et al., 2017). Epithelium senescence might promote asthma development through damaging the epithelial integrity and barrier function. ITGB4 is a critical structural adhesion molecule maintaining the integrity of airway epithelium. One study found that ITGB4 expression was downregulated in OVA-challenged mice accompanied with reduced wound repair ability and anti-oxidant capacity (Liu et al., 2010a). ITGB4 was also found to be decreased in asthmatic patients (Liu et al., 2010b). Deficiency of ITGB4 could result in cellular senescence in airway epithelial cells through p53 signaling pathway (Yuan et al., 2019). Besides, ITGB4 deficiency could also result in severe airway inflammation and airway hyperresponsiveness in asthma (Liu et al., 2010a). Thus, epithelial cell senescence induced by the downregulation of ITGB4 or increased TSLP leading to airway epithelium dysfunction, might be an important mechanism of asthma pathogenesis. However, more evidences are needed to further certify that epithelial cell senescence initiates the development of asthma.

Mesenchymal Cell Senescence

Mesenchymal cells in the airway include lung fibroblasts, myofibroblasts and airway smooth muscle cells (ASMCs). Fibroblasts might be the most commonly used model for studying cellular senescence. Previous studies have detected premature senescence of bronchial fibroblasts and myofibroblasts in asthmatic lungs (Dubé et al., 1998; Ward et al., 2008; Hadj Salem et al., 2015). SASP-related cytokines, chemokines, matrix-remodeling proteases expressed by senescent lung fibroblasts could result in low-level inflammation and fibrosis (Schafer et al., 2017; Álvarez et al., 2017). Clearance of these senescent fibroblasts by senolytic drugs would render the resolution of fibrosis (Schafer et al., 2017). The effect of fibroblast senescence on the pathobiology of asthma is not clear yet, but we could still find some clues. Some investigators have demonstrated that the activation of transcription factor signal transducer and activator of transcription 3 (STAT3) might contribute to lung fibroblast senescence in patients with IPF (Waters et al., 2018, 2019). They found that nuclear localization of STAT3 was elevated in senescent fibroblasts while inhibition of STAT3 activity would attenuate the accumulation of SA β-Gal and mitochondrial dysfunction. STAT3 plays a vital role in lung inflammation and airway remodeling in asthma, and it has been well proved as the downstream signal of TSLP (Wu et al., 2013b; Gavino et al., 2016). Therefore, the activation of STAT3 in asthma may induce chronic inflammation and airway remodeling via promoting lung fibroblast senescence. Further evidences are still needed to confirm the role of fibroblast senescence in airway inflammation and remodeling of asthma.

Airway smooth muscle cells is another integral cell type constituting the airway structure. One featured symptom of asthmatic patients is airway hyperresponsiveness, which is mainly induced by the contraction of ASMCs in response to specific stimuli. Persistent chronic inflammation and secreted growth factors could lead to increased airway smooth muscle mass and then promote irreversible airway obstruction. Some asthma-related factors have shown to induce senescence in smooth muscle cells. *In vitro* study showed that hypoxia induced cellular senescence in fetal ASMCs, leading to the upregulation of proinflammatory and profibrotic mediators, as well as increased contractility, which conduces to inflammation, tissue remodeling and airway obstruction (Parikh et al., 2019a). IgE and its receptor play an important role in the pathogenesis of allergic diseases like asthma. Recent study found that IgE induced senescence of smooth muscle cells via upregulating lincRNA-p21 and p21 in OVA-asthma model (Guo et al., 2019).

However, it is unclear whether ASMCs from asthmatics are senescent or not. Some studies suggested that the proliferation rate of ASMCs from asthmatic subjects was enhanced (Johnson et al., 2001; Trian et al., 2016). Although Trian T and coworkers found that senescent marker p53 was increased in asthmatic ASMCs, it seems that p53 had lost its anti-proliferative function in asthma (Trian et al., 2016). On the other hand, some research failed to detect the increase of nuclei numbers or proliferative markers like Ki67 in airway muscle bundles of asthma (Benayoun et al., 2003; Moir et al., 2003). By using bronchial biopsies from 14 subjects with mild to moderate asthma and 15 control subjects, Woodruff et al. (2004) demonstrated that there was hyperplasia but not hypertrophy in smooth muscle. Conversely, with a larger sample (about 50 subjects per group), James et al. (2012) found that ASMCs hypertrophy was present in both fatal and non-fatal asthma while hyperplasia only occurred in fatal asthma. In acute asthmatic murine model, Ki67 was upregulated in ASMCs. However, in chronic model (with significant airway remodeling), ASMCs exhibited hypertrophic cell shape instead of increased proliferation rate (Plant et al., 2012). Thus, persistent course of asthma or severe asthma might result in ASMC hypertrophy instead of hyperplasia. Furthermore, increased oxidative stress burden in asthma is also more likely to induce hypertrophy of ASMCs. Genome-wide microarray analysis identified increased expression of NADPH oxidase (NOX) subtype 4 (Nox4) in primary airway smooth muscle of asthma (Sutcliffe et al., 2012). TGF-β1 could also promote human ASMC hypertrophy through inducing Nox4 expression (Sturrock et al., 2007). Interestingly, Nox4 overexpression could not only induce hypertrophy of

vascular smooth muscle cells (VSMCs) but also lead to stronger SA-β-Gal staining (McCrann et al., 2009). Besides, Zhou et al. (2004) found that increased expression of p21 could result in hypertrophy and cell cycle arrest in human ASMCs. With respect to these reports, it's highly possible that hypertrophic ASMC is a senescent phenotype as it has enlarged cell morphology and proliferation suspension, but future studies still need to use more senescence-associated markers to identify whether hypertrophic ASMCs are senescent or not.

Immune Cell Senescence

The third cell type that might suffer from cellular senescence in asthmatics is immune cell. As we've mentioned above, PBMCs and leukocytes from asthmatic patients have experienced telomere shortening, which is one of the characteristics of cellular senescence (Kyoh et al., 2013; Belsky et al., 2014; Lee et al., 2017). In clinical studies, immune cells isolated from peripheral blood might be the most commonly used subjects for studying cellular senescence in diseases as they are easy-obtained. Accelerated aging of leukocytes from asthmatics patients was associated with longer course of disease (Belsky et al., 2014). Brandenberger and Mühlfeld (2017) concluded that immune senescence in aged people impaired both innate and adaptive immunity, making organisms more susceptible to infection, and contributing to the development of chronic lung diseases. Affected immune cells in the elderly may include macrophages, neutrophils, natural killer (NK) cells, dendritic cells, B cells and T cells, leading to higher levels of IL-6, IL-8, and TNF-α. The inflammatory response was more severe while the ability of pathogen clearance declined. Furthermore, senescence of T cells would also alter the T-cell mediated immunity and its regulatory immune function, facilitating the development of autoimmune diseases (Lynch et al., 2017).

Most of the studies related to immune senescence were reported in aged people, while there are still a few studies that reveal the effect of immune senescence on the disease development in young people. Balint et al. (2013) presented a premature immune senescence in multiple sclerosis (MS) children who had lower numbers of naive T cells as well as reduced recent thymic emigrants of Treg cells compared to their healthy counterparts, indicating the impairment of T cells hemostasis. This finding may provide further support to the relationship of immune senescence and pathogenesis of asthma, with which some patients exhibit Th17/Treg bias (Carr et al., 2018; Papi et al., 2018). Th17/Treg bias is more common in aged people with asthma. The Th17/Treg ratio rises up with aging and contributes to a proinflammatory status (Schmitt et al., 2013). Th17 cells differentiated from naïve T cells when stimulated by IL-6 and TGF-β, which are common components of SASP. This might be the reason of why Th17 cells increase along with aging. Interestingly, IL-17 could also enhance the secretion of SASP cytokines in bronchial fibroblasts, such as GM-CSF, TNF-α, IL-1β, and IL-6 (Molet et al., 2001). Though Treg cells also increase with aging, its function to suppress Th17 cell expansion is deficient (Jagger et al., 2014). Th17/Treg bias in asthma, probably affected by immune cell senescence, contributes to neutrophil inflammation, and difficult-to-treat phenotype (Papi et al., 2018). Recent study found that it is impaired autophagy of immune cells, but not epithelial cell, contributed to severe Th17-mediated neutrophil inflammation, and steroid resistance (Suzuki et al., 2016). Impaired autophagy is thought to trigger cellular senescence (Fujii et al., 2012), thus this study indicates that immune cell senescence might have greater contribution to chronic airway inflammation in asthma.

THERAPEUTIC ROLE OF ANTI-AGING STRATEGIES IN ASTHMA

Though the role of cellular senescence in the asthma development is still under investigation, some of the anti-aging strategies have been proven to improve the airway inflammation or airway remodeling in asthma. According to our previous discussion, senescence-associated triggers like telomere shortening, oxidative stress, inflammation, and autophagy are greatly associated with the development of asthma. Thus, the therapy purpose is to inhibit these potential mediators. As current therapies for asthma have reached an impasse, anti-senescence strategies might provide a new perspective for asthma treatment.

To avoid natural aging, multiple strategies have been put forward, including healthy lifestyle, caloric restriction and weight loss in obese, as well as some pharmacological interventions (de Cabo et al., 2014). Some drug candidates have been introduced, such as azithromycin, metformin, resveratrol, rapamycin, and roxithromycin. Although their underlying mechanisms are undetermined, it's proposed that they could block aging progress *via* direct or indirect activation of autophagy in target cells (de Cabo et al., 2014). Most of the anti-aging drugs are repurposing from existing drugs, with the advantage of being thoroughly screened for safety and clear mechanisms of action (Snell et al., 2016). Here we will describe the current anti-senescence therapies that might be effective for improving asthmatic symptoms and pathobiology.

Caloric Restriction and Weight Loss

Previous reports showed that intermittent fasting or calorie restriction might improve immune function and ameliorate inflammation in some conditions (Buono and Longo, 2019; Collins et al., 2019). These two studies demonstrated that cycled fasting would help inhibit inflammation response to various stimuli and elevate the anti-infection effect of immune system. For example, normal mice needed to spend 1 week to totally clear off the invasive pathogens, while fasting mice just consumed 2 days. Furthermore, inflammation level was reduced in adults after fasting for 19 h. This is of importance because inflammation is a double-edged sword. Persistent or excessive inflammation would facilitate the development of various chronic diseases or cancer, as well as induce cellular senescence and organ aging. Thus, caloric restriction and weight loss have been proposed to be one of the efficient interventions to delay aging process (de Cabo et al., 2014).

Although the mechanism involved is not fully understood, caloric restriction may prevent cellular senescence through eliminating inflammation- and oxidative-induced damage and activates selective autophagy to remove the present damage components (Fontana et al., 2018). Besides, calorie restriction can also ameliorate the circulating insulin growth factor 1 (IGF-1) level and mTOR activation, which could lead to premature senescence in cells (Fontana et al., 2018). Asthma coexistence with obesity tends to become more severe and difficult-to-treat (Kim et al., 2014; To et al., 2018). To et al. (2018) demonstrated that obesity-derived oxidative stress was to blame for the asthma outcomes. Obesity is more associated with Th17 and neutrophilic inflammatory phenotypes, leading to NLRP3 inflammasome activation (Kim et al., 2014), which would induce cellular senescence and SASP (Davalli et al., 2016). Johnson et al. (2007) found that after alternate days of calorie restriction, serum levels of oxidative substances and inflammation were reduced and the levels of antioxidant uric acid were increased in obese people with asthma. A systematic analysis also concluded that caloric restriction and weight loss were beneficial for disease control, lung function and life quality in asthmatic patients (Forte et al., 2018). However, whether caloric restriction exerts similar effect in asthmatic patients with normal weight is unknown.

Senolytic Drugs

Majority of senolytic agents are selected from FDA-approved drugs and repurposed through *in vitro* or *in vivo* senescent models. Although senolytic drugs are still in their infancy in clinical trials, some drugs with potential anti-aging effect have been proved to be medicative in asthma. Azithromycin (AZM), a 15-membered macrolide originated from erythromycin, is not only with bactericidal effect, but also deemed to be anti-inflammatory and capable of regulating inflammatory response (Kanoh and Rubin, 2010). Clinical benefits in asthma contain improvements of peak expiratory flow, symptoms and life quality (Reiter et al., 2013; Gibson et al., 2017). AZM has been shown previously to strengthen the airway epithelial barrier and therefore decrease the invasion of inhaled allergens and pathogens (Slater et al., 2016). Recently, Ozsvari et al. (2018) identified AZM as a novel senolytic drug to clean about 97% of senescent human lung fibroblasts *in vitro*, indicating that AZM might help remove senescent fibroblasts and reduce SASP-related factors in asthmatic lungs, and then attenuate airway inflammation and airway remodeling. According to this study, the senolytic activity of AZM might be through inducing selective autophagy to preferentially target senescent cells and accelerate their death. Metformin, a widely used hypoglycemic drug, has been demonstrated to improve the clinical outcomes of patients with coincidence of asthma and diabetes when compared with placebo controls (Li and Li, 2016; Wu et al., 2019). However, such results did not happen in the patients using insulin (Chen C. Z. et al., 2017), indicating that metformin may achieve this effect through other mechanism instead of just lowering blood glucose level. Metformin is also known as senolytic drug candidate, suggested to activate AMP-activated protein kinase (AMPK), the upstream regulator of autophagy, to protect cells against apoptosis and senescence (Chen et al., 2016; Garg et al., 2017). Activation of AMPK by metformin could attenuate CSE-induced inflammation in airway epithelial cells and elastase-induced airspace enlargement. This effect is probably through metformin's senolytic activity as it could reduce the expression of senescence-related genes such as p21 and p16, as well as SASP components like IL-6, IL-8, and MCP-1 in CSE-treated epithelial cells and elastase-stimulated mice (Cheng et al., 2017). SASP not only contributes to chronic inflammation, but also is involved in airway remodeling via expression of profibrotic factors and extracellular matrix (Parikh et al., 2019b). Park et al. (2012) found that metformin could reduce eosinophilic inflammation and peribronchial fibrosis, smooth muscle layer thickening, and mucin secretion though activating AMPK and decreasing oxidative stress in murine model of chronic asthma. However, further investigations need to ascertain if metformin is through inhibiting SASP and eliminating senescent cells in asthma to attenuate airway inflammation, ECM deposition and airway wall thickening. Other senolytic drugs including resveratrol, rapamycin and roxithromycin also have been demonstrated to protect asthmatic patients from persistent airway inflammation and airway remodeling, such as attenuating airway fibrosis and reducing bronchial smooth muscle mass (Shimizu et al., 1994; Black et al., 2001; Chen et al., 2015; Hua et al., 2015; Wu et al., 2015). These data give us a new insight into therapeutic role of senolytic drugs in treatment of asthma. Besides, their medicative effect on asthma also provides additional evidences to suggest the role of cellular senescence in the pathogenesis of asthma.

Stem Cells Transplantation

Remarkable experimental and clinical trials have demonstrated the therapeutic effect of mesenchymal stem cells (MSCs) in the diseases of various organs or systems with the capabilities of regeneration and immunomodulation (Uccelli et al., 2008). In recent years, with consideration of their merits of cell replacement and improving airway microenvironment, increasing clinical studies of intravenous injection of MSCs to patients with aging-related diseases including aging frailty, cardiovascular diseases, IPF and COPD have been conducted (Weiss et al., 2013; Golpanian et al., 2016; Bartolucci et al., 2017; Glassberg et al., 2017; Tompkins et al., 2017). MSCs transplantation was shown to improve the physical performance, immune function, FEV_1 and quality of life in patients with aging frailty, which was featured by exhaustion of stem cells or precursor cells, and chronic inflammation (Golpanian et al., 2016; Tompkins et al., 2017). In patients with aging-related respiratory diseases such as COPD and IPF, MSCs infusion would alleviate inflammation and lung fibrosis (Royce et al., 2014). In murine asthma models, intravenous injection of MSCs would suppress inflammatory cells infiltration and cytokines secretion, and ameliorate histopathological changes (Bonfield et al., 2010; Firinci et al., 2011). MSC exosomes could promote proliferation of Treg cells to restore Th17/Treg homeostasis in aged people and some difficult-to-treat asthmatics through its immunosuppression effect (Du et al., 2018). MSCs infusion could also downregulate the expression of SASP-associated

cytokines (TNF-α, IL-1β, MCP-1, and IL-6) and proteases (MMP9 and MMP12) in lung with cigarette smoke exposure (Guan et al., 2013), indicating that MSCs play an important role in ameliorating SASP. However, whether stem cell therapy could selectively clean out the senescent cells is not yet demonstrated. Thus, Further studies need to detect the change of senescence-associated markers in asthmatic patients after MSCs administration.

SPECIFIC ROLE OF CELLULAR SENESCENCE IN ASTHMA DIFFERENT FROM COPD AND IPF

The contribution of cellular senescence in the pathogenesis of COPD and IPF is quite well established. Similar to COPD and IPF, asthma is also initiated from airway epithelium injury, sharing the analogical pathobiology features including chronic airway

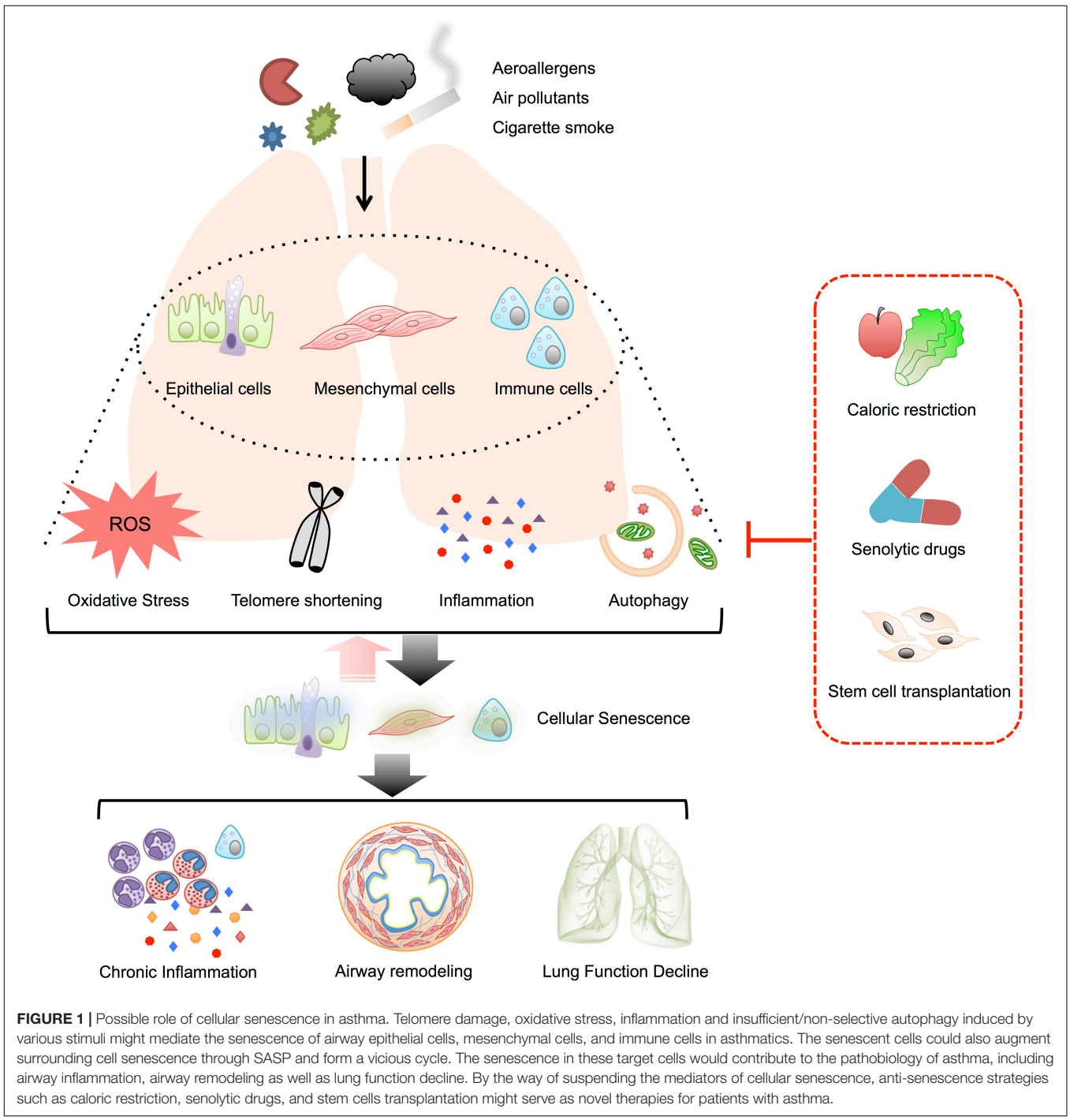

FIGURE 1 | Possible role of cellular senescence in asthma. Telomere damage, oxidative stress, inflammation and insufficient/non-selective autophagy induced by various stimuli might mediate the senescence of airway epithelial cells, mesenchymal cells, and immune cells in asthmatics. The senescent cells could also augment surrounding cell senescence through SASP and form a vicious cycle. The senescence in these target cells would contribute to the pathobiology of asthma, including airway inflammation, airway remodeling as well as lung function decline. By the way of suspending the mediators of cellular senescence, anti-senescence strategies such as caloric restriction, senolytic drugs, and stem cells transplantation might serve as novel therapies for patients with asthma.

inflammation and airway remodeling. Thus, based on the facts that cellular senescence could promote chronic inflammation and airway fibrosis in COPD and IPF, and cellular senescence could be detected in asthmatic subjects (Dubé et al., 1998; Puddicombe et al., 2003; Ward et al., 2008; Hadj Salem et al., 2015), we highly propose that cellular senescence is a potential mechanism for asthma development.

However, some characteristics of asthma are different from COPD and IPF, suggesting that cellular senescence might be triggered or effect differently in this disease. First of all, asthma can be child-onset, while COPD and IPF are often diagnosed at adulthood and old age. This indicates that there is premature senescence triggered at young age in asthma. In this regard, studies have demonstrated that hypoxia in infants, air pollutants exposure, and allergen challenge in childhood/adolescence could induce underlying senescence process (Deng et al., 2013; Chan et al., 2016, 2017; Lee et al., 2017, 2019; Parikh et al., 2019a). Such stressors might induce cellular senescence in airway epithelial cells, mesenchymal cells or immune cells, leading to their dysfunction and facilitating the initiation of asthma. Second, asthma is a heterogenetic disease with various inflammation phenotypes such as eosinophilic, neutrophilic, mixed inflammation, and non-inflammatory patterns. IgE could induce senescence of smooth muscle cells in asthmatic model (Guo et al., 2019). TSLP could lead to bronchial epithelial cell senescence (Wu et al., 2013a). Neutrophil and Th17 inflammation is more likely correlated to steroid-resistant asthma (Israel and Reddel, 2017). IL-17 could enhance the secretion of SASP cytokines in bronchial fibroblasts, such as GM-CSF, TNF-α, IL-1β, and IL-6 (Molet et al., 2001). Vice versa, SASP associated factors (IL-1β, IL-6, IL-8, and GM-CSF) is closely associated with increase of both neutrophil and eosinophil inflammation (Busse et al., 2017). Third, although epithelial senescence-induced barrier dysfunction is also important in the pathogenesis of COPD and IPF, the mediator of epithelial cell senescence might be different in asthma. In IPF, we could find out a lot of gene mutations correlating to the premature aging of epithelial cells (Richeldi et al., 2017), while those gene mutations haven't been reported in asthma. Instead, according to current studies, ITGB4 might be a pivotal gene to link epithelial cell senescence and the development of asthma (Liu et al., 2010a,b; Yuan et al., 2019), while it has not been demonstrated in COPD and IPF.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we summarized the current evidences illustrating the possible correlation of cellular senescence and the pathophysiology of asthma. We assume that asthma-related risk factors like invasive allergens, environmental pollutants or cigarette smoke could induce telomere shortening, oxidative stress, inflammation and insufficient/unselective autophagy, leading to the cellular senescence in epithelial cells, mesenchymal cells, and immune cells. Aging of these cells will then break the epithelial barrier, induce airway remodeling and sustain airway inflammation through SASP, which could augment cellular senescence in surrounding proliferating cells. Such a feedback loop promotes the pathogenesis of asthma. Thus, breaking this vicious cycle by anti-senescence strategies may help restrain the development of asthma (**Figure 1**).

Current understanding on the involvement of cellular senescence in asthma is hampered for several reasons. First, most of studies have neglected the detection of cellular senescence markers when investigating the role of telomere shortening, oxidative stress, inflammation and autophagy in the pathogenesis of asthma. Second, prospective data is limited so that it is difficult to figure out whether cellular senescence is a cause or a result of asthma. Third, few investigations have focused on the mechanisms of anti-senescence therapies for asthma. Thus, future studies need to put additional emphasis on ascertaining the place of cellular senescence in asthma by using and the mechanisms of anti-senescence therapies for patients with asthma. Sufficient methods for detecting premature senescence are necessary for future studies, including SA-β-Gal staining, proliferation assay, cell morphology, formation of senescence-associated heterochromatin foci, and secretion of SASP components.

AUTHOR CONTRIBUTIONS

Z-NW and R-NS contributed to the writing and revising of the manuscript. B-YY designed the figure and helped to draft the manuscript. K-XY and L-FY contributed to the searching of the related articles and reviews, and helped to draft the manuscript. YY provided knowledge on the molecular biology and critically revised the manuscript. Z-GC conceived the original idea and fixed the final outline. All authors read and approved the manuscript for publication.

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Irradiation Induces Epithelial Cell Unjamming

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The healthy and mature epithelial layer is ordinarily quiescent, non-migratory, solid-like, and jammed. However, in a variety of circumstances the layer transitions to a phase that is dynamic, migratory, fluid-like and unjammed. This has been demonstrated in the developing embryo, the developing avian airway, the epithelial layer reconstituted *in vitro* from asthmatic donors, wounding, and exposure to mechanical stress. Here we examine the extent to which ionizing radiation might similarly provoke epithelial layer unjamming. We exposed primary human bronchial epithelial (HBE) cells maintained in air-liquid interface (ALI) to sub-therapeutic doses (1 Gy) of ionizing radiation (IR). We first assessed: (1) DNA damage by measuring p-H2AX, (2) the integrity of the epithelial layer by measuring transepithelial electrical resistance (TEER), and (3) the extent of epithelial cell differentiation by detecting markers of differentiated airway epithelial cells. As expected, IR exposure induced DNA damage but, surprisingly, disrupted neither normal differentiation nor the integrity of the epithelial cell layer. We then measured cell shape and cellular migration to determine the extent of the unjamming transition (UJT). IR caused cell shape elongation and increased cellular motility, both of which are hallmarks of the UJT as previously confirmed. To understand the mechanism of IR-induced UJT, we inhibited TGF-β receptor activity, and found that migratory responses were attenuated. Together, these observations show that IR can provoke epithelial layer unjamming in a TGF-β receptor-dependent manner.

Keywords: epithelium, irradiation, migration, unjamming, metastasis, cancer

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INTRODUCTION

The healthy and mature epithelial layer is ordinarily quiescent, non-migratory, solid-like, and jammed. In this jammed layer, epithelial cells maintain cobblestone-like shapes and rarely rearrange with their neighbors (Bi et al., 2014; Park et al., 2015; Atia et al., 2018). However, in a variety of circumstances the layer transitions to a phase that is dynamic, migratory, fluid-like and unjammed. In the unjammed layer, epithelial cells become elongated in shape and rearrange cooperatively with their neighbors (Sadati et al., 2013; Fredberg, 2014; Park et al., 2015, 2016; Pegoraro et al., 2016; Atia et al., 2018). This phase transition from the jammed to the unjammed phase, called the unjamming transition (UJT), was discovered in a living system using well-differentiated human bronchial epithelial (HBE) cells exposed to mechanical compression that mimics the mechanical effect of bronchospasm (Park et al., 2015). Since this discovery of these dynamic and structural hallmarks of epithelial UJT (Park et al., 2015; Atia et al., 2018), work from our team and others

now suggests that the UJT seems to be a normal and perhaps essential part of epithelial biology but, nonetheless, can become hijacked in disease. For example, in the healthy embryo *in vivo* the UJT is triggered during ventral furrow formation during gastrulation in *Drosophila* (Atia et al., 2018), during elongation of the vertebrate body axis in the embryonic zebrafish (Mongera et al., 2018), and during airway epithelial branching in the embryonic avian lung (Spurlin et al., 2019). The UJT is therefore observed across vastly diverse biological contexts, in normal development and disease, both *in vitro* and *in vivo*, but its mechanisms and stimuli provoking the UJT are unknown.

Radiation therapy is often used to kill or shrink tumors (Molina et al., 2008; Chaffer and Weinberg, 2011). However, a growing body of evidence indicates that radiation therapy sometimes results in increased cancer metastasis among surviving cells (Moncharmont et al., 2014; Vilalta et al., 2016). The mechanism underlying this paradoxical radiation-induced metastasis remains unidentified but may involve a fluidization of the healthy, non-cancerous stroma which normally restrains the tumor. Here, we hypothesize that this paradoxical result is due to the unwanted effect of radiation on epithelial unjamming and resulting cellular migration. To test this hypothesis, we used primary HBE cells as a well-established model of the jammed layer, which can undergo the UJT. To test if radiation can provoke the UJT, we exposed the jammed HBE cell-layer to ionizing radiation (IR).

RESULTS

Ionizing Radiation Induces DNA Damage

Air-liquid interface (ALI) culture recapitulates the environment in which bronchial epithelial cells exist within the airway *in vivo*. Over 14 days in ALI, progenitor basal cells differentiate into a diverse population of epithelial cells as found *in vivo* (Fulcher et al., 2005). To assess DNA damage, we exposed cultures of primary HBE cells in ALI conditions to 1 Gy on ALI day 14. To determine the level of DNA damage, we performed immunofluorescent staining to detect p-H2AX, a marker for DNA-double strand breaks (DSBs) (Kuo and Yang, 2008). As previously reported in a different type of cells (Mariotti et al., 2013), we observed a maximal increase in p-H2AX at 1 h post-irradiation (data not shown). This maximal p-H2AX was reduced back to baseline by 6 h post-irradiation (data not shown). Compared to time-matched control cells, irradiated cells showed robust increases in the level of p-H2AX, indicating that exposure to IR indeed leads to DNA damage (**Figure 1B**). We observed positive p-H2AX in both apical and basolateral HBE cells as demonstrated by orthogonal side-view imaging (**Figure 1C**). We also observed increased p-H2AX protein by western blot (**Figure 1D**). Collectively, these data indicate that exposure of HBE cells to IR induces DNA damage.

Ionizing Radiation Does Not Disrupt Normal Epithelial-Cell Functions

To determine if DSBs induced by IR led to disruption of normal epithelial functions, we assessed barrier integrity, cellular

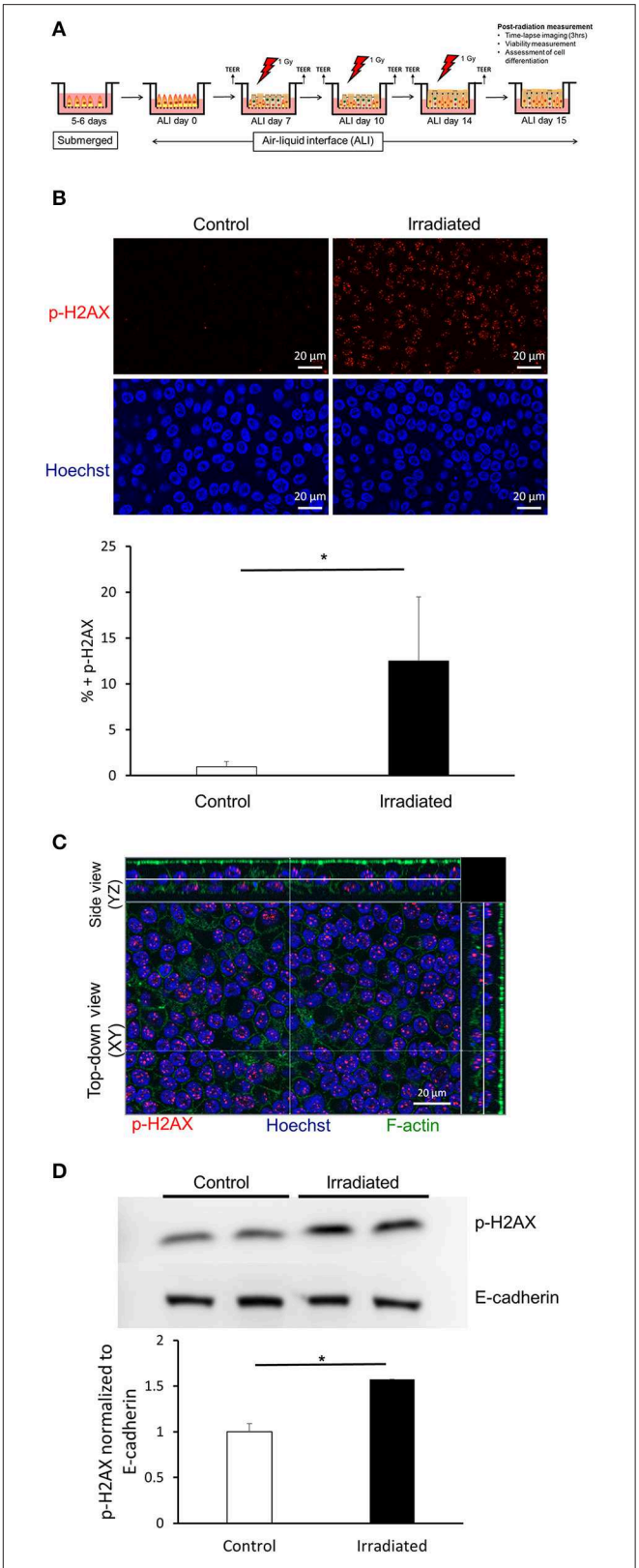

FIGURE 1 | Ionizing radiation induces DNA damage. **(A)** Timeline of the experimental protocol performed to investigate epithelial cell unjamming induced by ionizing radiation. In primary HBE cells maintained in air-liquid

(Continued)

FIGURE 1 | interface culture exposed to ionizing radiation (IR), we determined DNA damage, barrier integrity, cellular viability, epithelial differentiation, as well as cellular shape and migration. **(B)** Representative images of p-H2AX (top, red) and nuclei stained with Hoechst (bottom, blue) from six independent experiments. IR exposure induced p-H2AX indicating increased DNA damage. Images were captured with a 63X objective (scale bars = 20 μm). The quantification of the mean area of positive p-H2AX staining is presented in the graph. Error bars represent the standard deviation from four FOVs ($n = 4$) from one representative experiment. **(C)** Representative orthogonal images of p-H2AX (red), F-actin (green), and nuclei stained with Hoechst (blue). Images were captured with a 63X objective (scale bar = 20 μm). **(D)** Western blotting confirms that IR induced p-H2AX. Quantified p-H2AX normalized to E-cadherin is presented in the graph. Error bars represent the standard deviation from two samples ($n = 2$).

viability, and epithelial differentiation. On ALI days 7, 10, and 14, barrier integrity of the HBE-cell layer was measured by transepithelial electrical resistance (TEER) at 1 h before and after each IR exposure (1 Gy) (**Figure 2A**). As expected for the normally maturing airway epithelial layer, TEER increased over ALI days, as previously reported (Park et al., 2015). In control HBE cells there was no difference in TEER before and after sham treatment. To our surprise, however, in irradiated cells there was also no difference in TEER before and after radiation exposure. Across four independent HBE-cell donors, TEER values post-irradiation on ALI day 14 showed no reduction compared with control (**Figure 2B**), thus indicating that repeated exposure to IR (1 Gy) did not compromise barrier function. At 24 h after the final exposure to radiation, we also examined cell viability by dead-cell staining with ethidium homodimer (EthD-1) that is only permeable to dead cells (Somodi and Guthoff, 1995). As a positive control for cell death, we treated cells with 70% methanol and detected robust reductions in cellular viability as expected. However, we observed no significant difference in cellular viability between the control and the irradiated cells, (**Figure 2C**) further indicating that repeated exposure to radiation (1 Gy) did not affect cellular viability.

During ALI culture conditions, basal HBE cells differentiate into goblet and ciliated cells (Stewart et al., 2012; **Figure 2D**). In control and irradiated cells, we observed a similar degree of differential populations of HBE cells by immunofluorescence staining for epithelial cell-type markers: MUC5AC for goblet cells; FOXJ1 and β4-tubulin for ciliated cells; p63α for basal cells (**Figure 2D**). Higher magnification, orthogonal side-view images of each cell marker shows that irradiated cells indeed differentiate, as exhibited by their expected localization in the baso-lateral plane (**Supplementary Figure 1**).

Quantification of the differential cell markers is technically challenging in the pseudostratified epithelium. Therefore, we analyzed mRNA expression of differentiated epithelial cell-type markers by RT-qPCR analysis (**Figure 2E**). We also measured mRNA expression of *TEKT1*, a gene encoding tektin-1 protein expressed in cilia (Yoshisue et al., 2004; Park and Tschumperlin, 2009). In control and irradiated cells, there was no difference in the levels of mRNA expression of *MUC5AC*, *FOXJ1, TEKT1*, and *TP63*. Collectively, these data demonstrate that repeated exposure of the primary HBE cells to radiation (1 Gy) induces DNA damage but does not affect epithelial layer integrity, cellular viability, or cellular differentiation.

Ionizing Radiation Induces Epithelial Unjamming in a TGF-β Receptor-Dependent Manner

To determine UJT in HBE cells, we measured cellular shape and motility. Because structural signatures reflect dynamic process of the UJT, we first used cell shape analysis to assess UJT (Park et al., 2015; Atia et al., 2018). We fixed cells at 24 h after the final exposure to IR and stained for F-actin to mark cell boundaries (**Figure 3A**). Control cells maintained a cobblestone-like shape whereas irradiated cells became elongated (**Figure 3A**). To determine systematic differences of cell shapes between control and irradiated cells, we measured cellular aspect ratio (AR) (Mashburn et al., 2012). Mean AR showed a significant difference between control and irradiated cells (**Figure 3B**), suggesting that irradiation induced cell shape elongation. In addition, we took time-lapse images of the pseudostratified epithelial layers starting at 5 h post-irradiation and analyzed cell motility and dynamics. Through optical flow analysis, we computed root-mean-square (RMS) displacements over 3 h. Compared to the time-matched controls, exposure to IR significantly increased the average RMS displacement of the HBE cells (Control: 0.79 ± 0.09 μm vs. IR: 6.21 ± 1.95 μm) (**Supplementary Video 1**).

To test whether inhibition of TGF-β receptor activity prevents the IR-induced UJT, HBE cells were pre-treated with the TGF-β receptor inhibitor, SB431542 (SB, 10 μM) (or vehicle) for 1 h prior to each IR exposure. Pre-treatment with the TGF-β receptor inhibitor partially attenuated the IR-increased average displacement of the cells (veh + IR: 6.21 ± 1.95 μm vs. SB + IR: 2.60 ± 0.75 μm; **Figures 4A,B**). Taken together, these data indicate that exposure of airway epithelial cells to IR provokes a UJT, accompanied by cell shape change. This IR-induced UJT is partially mediated via TGF-β receptor signaling. Because the activation of TGF-β receptor pathway is known to induce epithelial-to-mesenchymal transition (EMT) (Xu et al., 2009; Nieto et al., 2016), we examined if irradiated cells were undergoing EMT. While the cells treated with TGF-β1, as a positive control for the EMT, showed substantially increased expression of EMT marker genes, including *FN-EDA, VIM,* and *ZEB1*, irradiated cells were unchanged in their mRNA expressions (**Supplementary Figure 2**).

MATERIALS AND METHODS

Culture of Primary Human Bronchial Epithelial Cells

Primary HBE cells were obtained from the CF Center Tissue Procurement and Cell Culture Core, the University of North

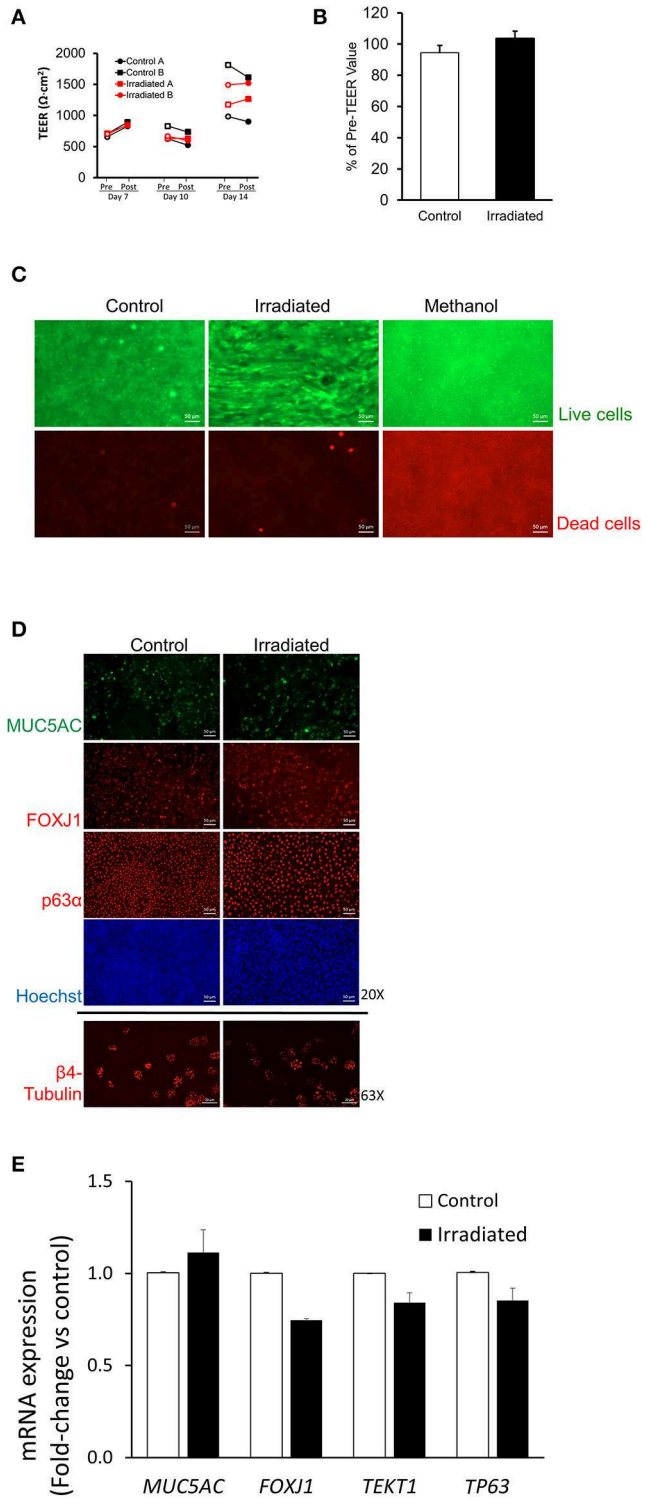

FIGURE 2 | Ionizing radiation does not disrupt normal epithelial cell functions. **(A)** On ALI days 7, 10, and 14, TEER measured 1 h before (pre-exposure, open symbols) and 1 h after (post-exposure, closed symbols) indicates that IR exposure did not reduce TEER. Black lines represent TEER measured from two independent wells (Control A and Control B) of control and red lines represent TEER measured from two independent wells (Irradiated A and Irradiated B) of irradiated cells from one donor. **(B)** On ALI day 14, the percent change in TEER comparing 1 h pre-, and 1 h post-IR exposure showed no difference between control (white bar) and irradiated (black bar) cells, indicating that IR did not reduce TEER. Error bars represent the standard error of the mean from four independent donors ($n = 4$). **(C)** Methanol treatment used as a positive control for cell death resulted in prominent cell death as indicated by EthD-1 staining (red), whereas neither control nor IR exposure induced cell death (scale bars = 50 μm). Representative images **(D)** of MUC5AC, FOXJ1, p63α (20X), and β4-tubulin (63X). RT-qPCR **(E)** showing mRNA expression of *MUC5AC, FOXJ1, TEKT1,* and *TP63* indicate no differences in cellular differentiation between control and irradiated cells. Scale bar = 50 μm (top 8 panels), 20 μm (bottom 2 panels) in **(D)** and error bars represent the standard deviation from two representative donors ($n = 2$) in **(E)**.

Carolina at Chapel Hill (courtesy of Dr. Scott Randell). As previously described (Park et al., 2015), these cells were isolated from lungs unsuitable for transplant. The lungs were obtained under protocol #03-1396 approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Informed consent was obtained from authorized representatives of all organ donors. As previously described (Park et al., 2015; Mitchel et al., 2016), HBE cells were seeded on transwells coated with 0.05 mg/ml rat tail collagen 1 and maintained in ALI culture until the cells were well-differentiated as determined by immunofluorescent staining for goblet cells marked for MUC5AC, ciliated cells marked for FOXJ1 and b4-tubulin, and basal cells marked for p63α (**Figure 2D**).

Exposure of Primary Airway Epithelial Cells to Ionizing Radiation

For the exposure of HBE cells to ionizing radiation, we used a RadSource RS 2000 Biological Research Irradiator (RadSource, Brentwood, TN). HBE cells were exposed to radiation over the course of ALI cultures, on days 7, 10, and 14. A dose of 1 Gy was achieved by setting the instrument to 160 kV, 25 mA for 87 s. During the radiation exposure, time-matched control (non-irradiated) cells were placed outside of the RadSource machine (**Figure 1A** demonstrates a timeline of the experimental procedures).

In experiments where TGF-β receptor was inhibited, SB431542 (10 μM) was added to the basolateral culture medium 1 h prior to each IR exposure. For vehicle of SB431542, DMSO (1%) was used.

Immunofluorescent Staining

HBE cells on the transwell were fixed in 4% paraformaldehyde (PFA) for 30 min and subsequently washed with PBS. Cells were then permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBST) for 10 min. Permeabilized cells were blocked in 10% non-specific goat serum and 1% bovine serum albumin (BSA) in PBST for 1 h. Primary antibody against p-H2AX (20E3, Cell Signaling Technology, Danvers, MA) was diluted at 1:200. Secondary antibody conjugated to Alexa Fluor 594 (ThermoFisher Scientific) was diluted at 1:500. Stained cells were mounted on coverslips and imaged with a fluorescent microscope (Axio Observer, Zeiss, Germany). To observe the differentiation of the ALI cultures, we stained fixed cells with primary antibodies

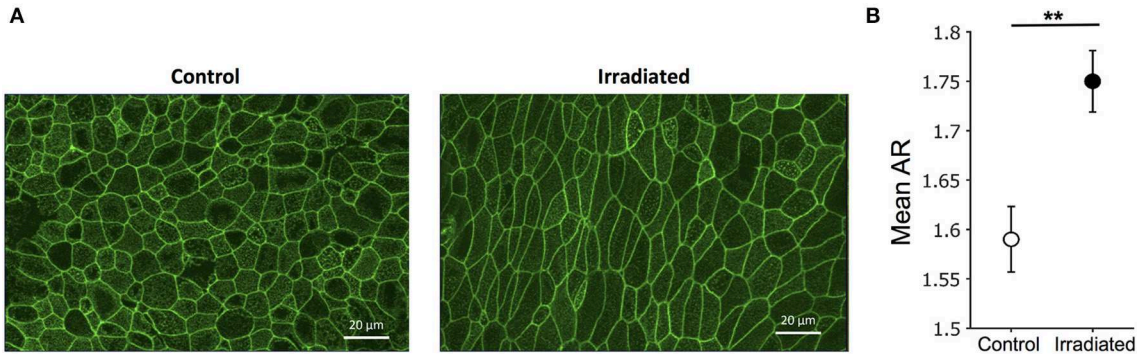

FIGURE 3 | Ionizing radiation induces cell shape changes. **(A)** Representative images of F-actin staining indicate cobblestone-like shape in control (left) and elongated shape in irradiated cells (right) (scale bars = 20 μm). **(B)** Segmented images of F-actin staining (as shown in **A**) were used to calculate aspect ratio (AR = length of long axis/length of short axis). Mean aspect ratio, mean (AR), was calculated in four fields of view. Error bars represent the standard error of the mean from five independent experiments ($n = 5$).

for MUC5AC (45M1, ThermoFisher Scientific), FOXJ1 (2A5, ThermoFisher Scientific), β4-tubulin (ONS, Millipore Sigma), and p63α (D2K8X, Cell Signaling Technology). All primary antibodies were diluted at 1:500, followed by appropriate secondary antibody (AlexaFluor mouse/rabbit). Stained cells were counter stained for F-actin using phalloidin conjugated to AlexaFluor 488 (ThermoFisher Scientific) diluted at 1:40 and with Hoechst 33342 (ThermoFisher Scientific) diluted at 1:5000.

Western Blotting

Protein lysates were separated in a 10% Mini-PROTEAN TGX pre-cast gel (Biorad). Proteins were transferred to a PVDF membrane which was subsequently blocked in 5% skim milk. The PVDF membrane was incubated with antibody against p-H2AX (20E3, Cell Signaling Technology) or E-cadherin (24E10, Cell Signaling Technology) and subsequently with secondary antibodies.

RT-qPCR

RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One μg of total RNA was used to synthesize cDNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). RT-qPCR was performed using 25 ng of cDNA, primers (**Table 1**) and 2X SYBR Green PCR Mastermix.

Cell Shape Analysis

To determine if cell shape changes were affected by IR exposure, cells were stained for F-actin as described above. Then, maximum intensity projections of F-actin images were created in ImageJ software (Schindelin et al., 2012) and cells were segmented using SeedWaterSegmenter (Anaconda2). As previously reported (Atia et al., 2018), cellular aspect ratio (AR = long axis/short axis) was calculated and the mean AR was computed.

Transepithelial Electrical Resistance

To determine if the integrity of airway epithelial cell layer is disrupted after radiation exposure, we used a widely accepted assay, TEER measurement, using an epithelial volt/ohm meter (EVOM2) (World Precision Instruments, Sarasota, FL), following the manufacturer's instruction, as we previously used (Park et al., 2015). In each well, TEER was measured 1 h before and 1 h after control treatment or irradiation. In some experiments, TEER was measured subsequently on ALI days 7, 10, and 14 (**Figure 2A**) following each exposure, whereas in other

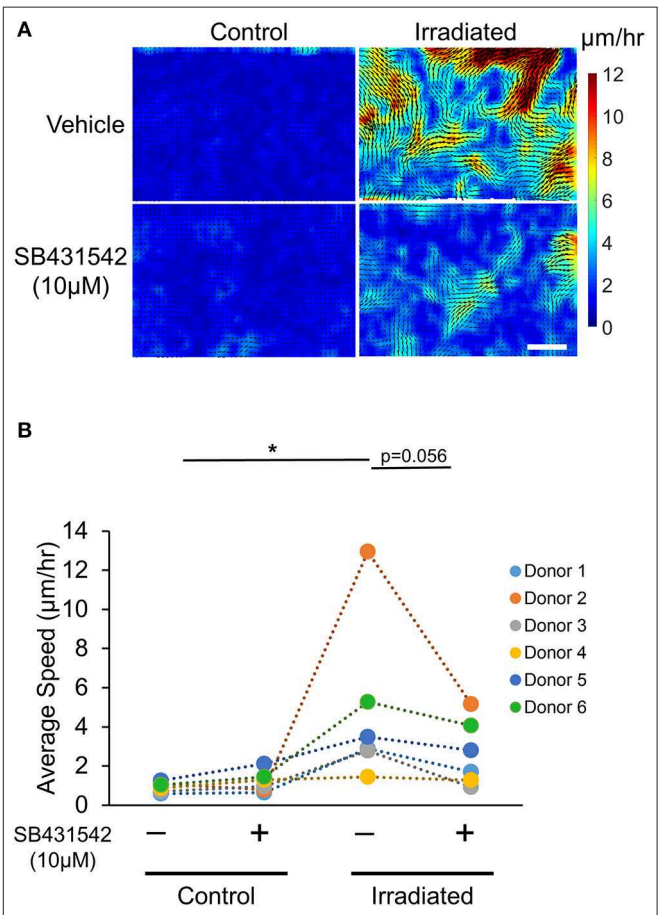

FIGURE 4 | Irradiation-induced collective cell migration partially depends on TGF-β receptor. Representative displacement maps overlaid with velocity heat maps **(A)** and the quantified cellular motility **(B)** indicate that IR induced cellular motility, which was attenuated by SB431542 (a TGF-β receptor inhibitor) (Scale bar = 100 μm). Six donors ($n = 6$) were used as indicated.

TABLE 1 | Primer sequences used in RT-qPCR.

Genes	Primer sequences	References
GAPDH	FW: 5′-TGGGCTACACTGAGCACCAG-3′ RV: 5′-GGGTGTCGCTGTTGAAGTCA-3′	Primer express 3 (Chu et al., 2006)
MUC5AC	FW: 5′-GGAACTGTGGGGACAGCTCTT-3′ RV: 5′-GTCACATTCCTCAGCGAGGTG-3′	Primer express 3
FOXJ1	FW: 5′-ATCCGCCACAACCTGTCTCT-3′ RV: 5′-CTTGCCTGGTTCGTCCTTCTC-3′	Primer express 3
TEKT1	FW: 5′-GCCCTTGCACATCACTGAGA-3′ RV: 5′-TCAATGCCAATGCGCTTCT-3′	Primer express 3
TP63	FW: 5′-GGACCAGCAGATTCAGAACGG-3′ RV: 5′-AGGACACGTCGAAACTGTGC-3′	Primer express 3
FN-EDA	FW: 5′-GAGCTATTCCCTGCACCTGATG-3′ RV: 5′-CGTGCAAGGCAACCACACT-3′	Doerner and Zuraw, 2009
VIM	FW: 5′-TGTCCAAATCGATGTGGATGTTTC-3′ RV: 5′-TTCTACCATTCTTCTGCCTCCTG-3′	Primer express 3
ZEB1	FW: 5′-GATGATGAATGCGAGTCAGATGC-3′ RV: 5′-ACAGCAGTGTCTTGTTGTTGT-3′	Tian et al., 2015

experiments (**Figure 2B**), TEER was measured only on ALI day 14 following the last exposure.

Live/Dead Stain

To assess the viability of the cells post-radiation, cells were stained with calcein-AM and EthD-1 (Viability/Cytotoxicity kit, ThermoFisher Scientific, Waltham, MA) was performed according to manufacturer's protocol. As a positive control for cell death, other cells from independent culture wells were incubated with 70% methanol for 30 min. Briefly, at 24 h after the final IR, cells grown on transwells were incubated in PBS containing 2 μM calcein AM and 4 μM EthD-1 for 30 min. Then, stained cells were placed on glass coverslips and imaged at a single z-plane with a fluorescent microscope (Axio Observer, Zeiss, Germany).

Time-Lapse Imaging

To monitor the motility of the cells after the final IR exposure, time-lapse images of the cells were taken at 3 min-intervals for 18 h. The imaging chamber was supplied with 37°C, 5% CO_2 humidified air. A 10X objective, mounted on an Axio Observer (Zeiss, Germany) was used to collect phase contrast images. Each condition was run in duplicate and images from six fields of view per well were randomly collected. The average speed of cellular migration was assessed by Farneback optical flow analysis using Matlab (MathWorks, Natick, MA).

Statistics

Statistical analysis was performed in GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA). Data are presented as mean + SD or + SEM as indicated, except **Figure 3**, which is represented by Mean ± SEM. In experiments with two groups, a two-tailed, paired Student's *t*-test was utilized. In experiments with more than two groups, a one-way ANOVA with Tukey's *post-hoc* test was utilized. *P*-values < 0.05 were considered to be significant.

DISCUSSION

The goal of this study is to provide the insight into the paradoxical effect of therapeutic radiation on cell motility (Moncharmont et al., 2014; Vilalta et al., 2016; Blyth et al., 2018). To address this question, we utilized *in vitro* culture of primary human airway epithelial cells as a model of the healthy, mature epithelium. In this epithelial cell layer system, irradiation caused DNA damage but did not disrupt normal differentiation or layer integrity. Irradiation did, however, induce cellular migration and cell shape elongation, both hallmarks of the UJT (Bi et al., 2015; Park et al., 2015; Atia et al., 2018). Our data therefore indicate that IR induced the UJT of well-differentiated, healthy HBE cells in a TGF-β receptor-dependent manner. This UJT of the non-cancerous healthy cells might create a fluidized environment, in which tumor cells might efficiently disseminate.

Under homeostatic conditions, the epithelium is typically non-migratory. However, under a variety of circumstances, the non-migratory layer becomes migratory. The transition occurs under both physiological circumstances, including wound healing, embryonic development, and pathophysiological circumstances, including tissue remodeling, cancer invasion, and metastasis. Transition of the non-migratory confluent epithelium toward the migratory state has traditionally been attributed to EMT or partial-EMT (pEMT) (Hay, 1982, 1995; Boyer et al., 1989; Savagner, 2015; Nieto et al., 2016; Brabletz et al., 2018), but in some cases can be attributed to the UJT (Sadati et al., 2013; Fredberg, 2014; Park et al., 2015, 2016; Pegoraro et al., 2016; Atia et al., 2018; Mitchel et al., 2019). The UJT together with associated fluidization of the confluent living tissue was first discovered in our laboratory (Trepat et al., 2009; Sadati et al., 2013; Park et al., 2015; Atia et al., 2018). This discovery was rooted firmly in airway biology. Since that time, the concept of cell jamming has diffused widely into the study of other collective cellular systems, and growing evidence from us and others now suggests that the capacity to jam and unjam may be an innate property of many epithelia both in normal development (Atia et al., 2018; Mongera et al., 2018; Spurlin et al., 2019) and in disease (Sadati et al., 2013; Haeger et al., 2014; Park et al., 2015; Bi et al., 2016; Gamboa Castro et al., 2016; Oswald et al., 2017; Atia et al., 2018; Mongera et al., 2018; Palamidessi et al., 2018, 2019; Fujii et al., 2019; Spurlin et al., 2019), including asthma (Angelini et al., 2011; Nnetu et al., 2012; Garcia et al., 2015; Park et al., 2015, 2016; Malinverno et al., 2017; Atia et al., 2018), and cancer (Haeger et al., 2014; Oswald et al., 2017; Palamidessi et al., 2019).

In cancer clinics, radiation is the most commonly used to kill or shrink tumors but sometimes results in the undesirable side effect of promoting migration and metastasis in surviving cancer cells. Further, tumor cells cultured in pre-irradiated stroma become highly metastatic, known as the tumor bed-effect (Monnier et al., 2008). However, these mechanism(s) remain unclear. In A549 cells (adenocarcinoma alveolar type II cells), IR (at 6 or 12 Gy) augments migration of the cells into a scratched-wound (Jung et al., 2007). This augmented migration is accompanied by cell-shape elongation (Jung et al., 2007). During cancer therapy, not only cancer cells, but also surrounding healthy epithelial cells are subjected to radiation. IR doses delivered to tumors often exceed a cumulative dose of 60 Gy, delivered over multiple treatment days (Kong et al., 2014). Delivery of high doses with minimal off-target damage is achieved by three-dimension-conformal treatment, which mitigates risk of exposure to nearby healthy tissue. Advances in radiotherapy delivery techniques have allowed for a sharp focus on the target tissue, limiting the damage to the surrounding stroma (Intensity Modulated Radiation Therapy Collaborative Working Group, 2001; Verellen et al., 2007). The resulting dose delivered to surrounding tissue is thus significantly lower than that which the tumor receives. Here, we utilized a 1 Gy dose, which is much lower than the therapeutic dose used in the clinic. Given three times over ALI culture of healthy primary HBE cells, the cumulative dose was 3 Gy. By the use of radiation at this dose, we did not observe cell-death but observed a striking migratory response, which could in turn affect physical behavior of neighboring cancer cells. Often, the radiated healthy epithelial cells are considered to promote cancer cell survival (Blyth et al., 2018). It has also been shown that low doses (0.8 Gy) of radiation may contribute to metastasis by increasing angiogenesis at the tumor site (Sofia Vala et al., 2010). However, the effect of radiation on cellular migration in healthy, mature, confluent airway epithelium had been unknown. Using primary HBE cells maintained in ALI, we examined the effects of irradiation on the homeostatic function of epithelial cells, as well as cellular migration.

Exposure of the cells to IR causes DNA damage by cleaving the phosphodiester bond in the backbone of the DNA (Lomax et al., 2013), leading to a DSB. Upon DSB, ATM Kinase phosphorylates H2AX followed by the cascade of DNA repair (Burma et al., 2001). Thus, we first determined if IR causes DSBs in HBE cells cultured at ALI. As we expected, exposure of HBE cells to IR (1 Gy) indeed caused DNA damage that is marked by p-H2AX (**Figure 1C**). Among the cellular events induced by IR, this observation can be particularly linked to asthma. In patients with asthma, the airway epithelium possesses an increased level of p-H2AX, suggesting that DNA damage is greater, or that DNA repair mechanisms are impaired in asthmatic airways (Chan et al., 2016). Furthermore, in a house dust mite (HDM) mouse model of allergic asthma, p-H2AX is increased in the airway epithelium (Chan et al., 2016). Surprisingly, a recent study suggests that IL-13 could be a therapeutic target for radiation-induced pulmonary fibrosis (Chung et al., 2016). IL-13 is a type 2 cytokine that is strongly associated with asthma (Rael and Lockey, 2011) and induces asthmatic airway remodeling, including increased subepithelial fibroblast proliferation (Kraft et al., 2001). Thus, there may be some common pathways that are shared between radiation-induced lung injury and asthma.

Despite substantial DNA damage marked by p-H2AX, radiation exposure at this sub-therapeutic dose (1 Gy) affected neither epithelial layer integrity nor normal epithelial differentiation, both of which are critical for maintaining proper epithelial functions (**Figure 2**). It is possible that radiation resulted in less p63α positive basal cells (**Figure 2D**), which may indicate the transition of basal into more differentiated cell types after exposure to DNA damage. However, we did not detect significant differences in *TP63* mRNA expression. In irradiated cells, we observed a small reduction in both marker genes for ciliated cells, *FOXJ1 and TEKT1* (Ryan et al., 2018), but it was not statistically significant (**Figure 2E**). Furthermore, we did not see any meaningful differences in any cell type marker proteins marked by immunofluorescent staining (**Figure 2D**), indicating that irradiated cells maintain their ability to differentiate similarly to non-radiated cells.

Without disruption of the epithelial barrier in the confluent epithelial layer, IR substantially induced cellular migration. To identify the mode of this increased cellular migration, we analyzed the cell shape and the speed of cellular motility to determine if this migration is attributable to the UJT. Although IR did not break cell-cell junctions as indicated by the lack of reduction in TEER (**Figures 2A,B**), IR caused significant changes in cell shape from cobblestone-like to elongated (**Figure 3**). IR also increased cellular motility (**Figure 4**), which was attenuated by pretreatment with a TGF-β receptor inhibitor. The TGF-β signaling pathway plays an important role in irradiation-induced fibrotic remodeling (Monson et al., 1998) and the inhibition of TGF-β signaling reduces irradiation-induced fibrosis in humans (Xavier et al., 2004). Furthermore, the increase in TGF-β expression in the mouse lung post-IR depends on IL-13, a type 2 cytokine (Chung et al., 2016), which is also tightly associated with asthma (Vignola et al., 1996; Minshall et al., 1997; Doran et al., 2017). TGF-β is a pleotropic factor that promotes a variety of asthmatic airway remodeling processes, including myofibroblast differentiation (Michalik et al., 2009), development of airway hyperresponsiveness (Leung et al., 2006), and airway smooth muscle proliferation (Makinde et al., 2007). Furthermore, TGF-β is a strong stimulator for epithelial cell migration through the EMT (Jakowlew, 2006; Al-Alawi et al., 2014; Nieto et al., 2016; Brabletz et al., 2018). In irradiated cells, our data indicate no signs of EMT (**Supplementary Figure 2**) but strong signs of UJT.

CONCLUSION

In the present study, we investigated the unjamming transition, an emergent phenomenon in epithelial cell migration, induced by radiation exposure. Despite substantial DNA damage detected in the radiated cells, the integrity of the epithelial-cell layer, cellular viability, and degree of epithelial differentiation were not disrupted by radiation. Radiation induced the UJT that is characterized by increased collective cellular migration and

elongated cell shape changes. Furthermore, blocking of TGF-β receptor attenuated the degree of radiation-induced cell migration, indicating a role for TGF-β receptor signaling in HBE-cell unjamming.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MO'S, JM, ZN, and J-AP designed the experiments. MO'S and JM performed experiments. MO'S, JM, AD, and SK analyzed the results. MO'S, JM, AD, SK, HL, DB, ZN, and J-AP interpreted the data and revised the manuscript. MO'S, JM, and J-AP drafted 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/fcell.2020.00021/full#supplementary-material

Supplementary Video 1 | In control (left) and irradiated (right) cells, time-lapse images were taken between 5 and 8 h after exposure at an acquisition rate of every 3 min. In the converted movies, images are shown at 20 frames per second (one second of video is equal to 1 h of experimental time).

Supplementary Figure 1 | Representative orthogonal (top-down view: X-Y and side view: Y-Z) and single z-plane (center) images from irradiated cells as shown in **Figure 2D**: MUC5AC (green), FOXJ1 (red), β4-tubulin (red), p63α (red), and Hoechst (blue). White line through orthogonal section indicates z-plane displayed (scale bar = 20 μm).

Supplementary Figure 2 | In primary HBE cells, radiation did not induce EMT. To determine EMT, we measured mRNA expressions of EMT-related proteins, including fibronectin-EDA, vimentin and Zeb1 by RT-qPCR. In the cells exposed to TGFβ (10 ng/ml) as a positive control for the EMT, we detected a significantly increased expression of three genes, whereas in the cells exposed to radiation, we detected no meaningful increase in three genes, suggesting no sign of EMT.

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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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Aberrant Epithelial Cell Proliferation in Peripheral Airways in Bronchiectasis

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Dilation of bronchi and bronchioles caused by destruction and excessive epithelial remodeling is a characteristic feature of bronchiectasis. It is not known how epithelial progenitor cells contribute to these pathologic conditions in peripheral airways (bronchioles) in bronchiectasis. We aimed to explore the expression levels of signature airway progenitor cells in the dilated bronchioles in patients with bronchiectasis. We obtained the surgically resected peripheral lung tissues from 43 patients with bronchiectasis and 33 control subjects. Immunostaining was performed to determine the expression patterns of thyroid transcription factor-1 (TTF-1, for labeling progenitor cells in distal airways), P63 (basal cells), club cell 10 kDa protein (CC10, club cells), and surfactant protein C (SPC, alveolar type II epithelial cells) in epithelium or sub-epithelium. Here, we reported significantly lower percentage of TTF-1^+ cells and $CC10^+$ cells, and higher percentage of $P63^+$ cells within the epithelium of dilated bronchioles compared with control bronchioles. In airway sub-epithelium of the dilated bronchioles, epithelial hyperplasia with disarrangement of TTF-1^+ cells yielded cuboidal (100%) and columnar (93.0%) type among bronchiectasis patients. Most progenitor cell markers co-localized with TTF-1. The median (the 1st, 3rd quartile) percentage of $P63^+$TTF-1^+, $CC10^+$TTF-1^+, and SPC^+TTF-1^+ cells was 16.0% (8.9, 24.0%), 14.5% (7.1, 20.8%), and 52% (40.3, 64.4%), respectively. For cuboidal epithelial hyperplasia, 91.0% (86.5, 94.0%) of areas co-stained with SPC and TTF-1. Columnar epithelial hyperplasia was characterized by TTF-1 co-staining with $P63^+$TTF-1^+ and $CC10^+$TTF-1^+ cells. Taken together, aberrant proliferation of airway progenitor cells in both epithelium and sub-epithelium are implicated in bronchiectasis.

Keywords: bronchiectasis, progenitor cells, epithelium, bronchiole, hyperplasia

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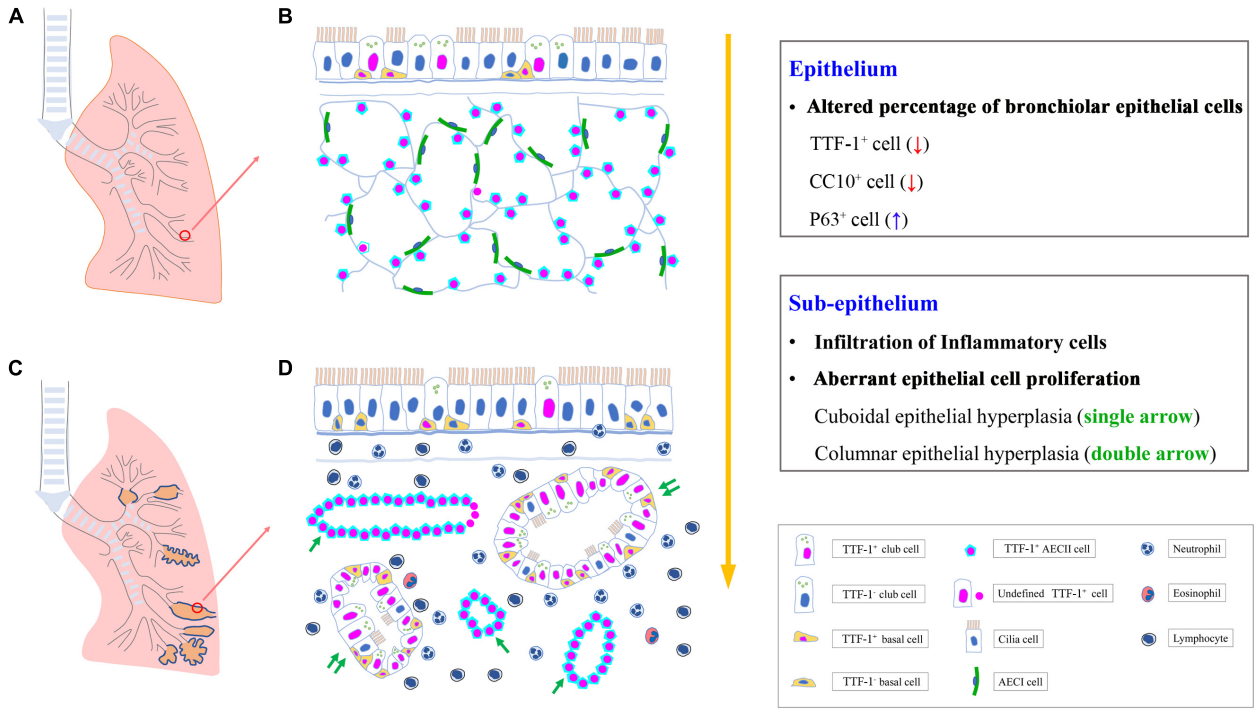


GRAPHICAL ABSTRACT | Aberrant proliferation of airway progenitor cells in both epithelium **(A,B)** and sub-epithelium **(C,D)** in peripheral airways in bronchiectasis. AECI, type I alveolar epithelial cell; AECII, type II alveolar epithelial cell; CC10, club cell 10 kDa protein; TTF-1, thyroid transcription factor-1.

INTRODUCTION

Bronchiectasis is characterized by recurrent infections and heightened inflammatory responses that are responsible for progressive lung injury and irreversible dilatation of bronchi and bronchioles (Boyton and Altmann, 2016). In many cases, the pathologic dilatation of bronchioles may co-exist with bronchiectasis of the large to medium-sized airways. Despite the vicious cycle model, the pathophysiology of bronchiectasis remains poorly understood. Impaired mucociliary clearance and aberrant repair (including hyperplasia) of airway epithelium has recently been implicated in bronchiectasis (Chen et al., 2018). However, few effective therapies are available to bronchiectasis management.

Advances in pathophysiology have provided fundamental insights into the role of airway epithelium in the milieu of recurrent infections and inflammation. Endogenous airway progenitor cells are crucial to lung homeostasis and regeneration because of their self-renewal capacity through differentiation into normal airway epithelial cells (ECs) (Nikolić et al., 2018; Liu et al., 2019). Abnormalities of progenitor cells have been associated with airway inflammatory diseases. For instance, basal cells (BCs) counts were lower and self-renewal capacity was impaired in chronic obstructive pulmonary disease (COPD) (Ghosh et al., 2018). Identification of the critical drivers (particularly the identity and sub-populations of airway progenitor cells) would help unravel the mechanisms of pathogenesis from a novel perspective and explore targets for therapeutic interventions for bronchiectasis (Nikolić et al., 2018).

The primordial lung bud was first demarcated with the expression of thyroid transcription factor-1 (TTF-1, also known as Nkx2 homeobox 1) (Whitsett et al., 2015). TTF-1 is a "master gene" in maintaining lung morphogenesis and cytodifferentiation of certain EC lineages (Akram et al., 2016). TTF-1 has been regarded as the earliest known marker of the lung epithelial cell lineage, and is expressed in all epithelial lineages of the lower respiratory tract during development (Kotton and Morrisey, 2014). It has also been reported that the respiratory lineage initiates from the differentiation of TTF-1-positive progenitor cells that ultimately form the gas-exchange surface (Sui et al., 2019). In human lungs, TTF-1 is required for expression of several epithelial markers in the distal developing airways, including BCs and club cells of the distal airways, and alveolar type II EC (AECII) (Hösgör et al., 2002; Hawkins et al., 2017; Wang G. et al., 2019). When subject to injury, these ECs must be replaced rapidly to maintain normal lung structure and function. BCs play a key role in the maintenance of normal airway epithelial architecture through self-renewal (differentiation into ciliated and club cells), whereas club cells (formerly known as Clara cells) and AECII are largely responsible for bronchiolar and alveolar repair,

Abbreviations: AECII, type II alveolar epithelial cell; BC, basal cell; CC10, club cell 10 kDa protein; DAPI, 4′ 6-diamidino-2-phenylindole; FEV1, first second percentage; FVC, forced vital capacity; HPF, high power field; HRCT, high-resolution computed tomography; HRP, horseradish peroxidase; IF, immunofluorescent; IHC, immunohistochemistry; SPC, surfactant protein C; TTF-1, thyroid transcription factor-1.

respectively (Walters et al., 2013; Hannan et al., 2015; Liu et al., 2019). However, there has not been a systematic investigation directly linking the abnormality of airway progenitor cells to bronchiectasis.

We hypothesized that abnormality of airway progenitor cells that led to failure of airway injury repair would be key to bronchiectasis pathogenesis. Here, we sought to investigate the spatial distribution and quantify airway progenitor cells with histologic assessment of specific markers, including TTF-1^+ cells for multiple airway progenitors, $P63^+$ for BCs, Club Cell 10 kDa Protein $(CC10)^+$ for club cells and surfactant protein C $(SPC)^+$ for AECII in the bronchiolar or alveolar epithelium of distal airways in bronchiectasis (Rawlins et al., 2009; Mou et al., 2012; Crystal, 2014; Jacob et al., 2017). We have identified two major patterns of epithelial hyperplasia (cuboidal or columnar) which can be extensively labeled by TTF-1^+ in sub-epithelium of the dilated bronchioles. Our results provide a basis for promoting EC repair through "normalizing" airway progenitor cells, which might help reverse the trend of bronchiectasis progression.

MATERIALS AND METHODS

Patient Recruitment

Study protocol approval was obtained from the institutional review boards of The First Affiliated Hospital of Guangzhou Medical University. All study participants provided informed consent. The diagnosis of control subjects with benign tumor and patients with bronchiectasis was based on chest high-resolution computed tomography (HRCT) (**Figures 1A,B**) and the documentation of respiratory symptoms (including chronic cough and sputum production) from medical charts. The modified Reiff score was recorded to evaluate the radiologic severity of bronchial dilatation (tubular: 1 point, varicose: 2 points, cystic: 3 points), with the maximal score of 18 for six lobes (with the lingula lobe being a separate lobe) (Chalmers et al., 2014). The site of bronchiectasis was categorized as central, peripheral, or mixed (central + peripheral) airways.

The duration of respiratory symptoms (particularly cough) and the most recent spirometry findings [performed according to international standards (Miller et al., 2005)] were extracted from medical charts. Cystic fibrosis was not routinely screened because of the extremely low prevalence in China. Smokers were defined as current cigarette smokers if they had smoked for more than 10 pack years. No study participant had a physician's primary diagnosis of asthma.

We evaluated peripheral lung tissues with the dilated bronchioles from 43 bronchiectasis patients and peripheral normal lung tissues from 33 controls subjects [including 20 peripheral lung tissues from healthy donors, 13 adjacent normal tissue (>1 cm away from the affected tissue) from subjects with benign tumor (six with pulmonary hamartoma, five with atypical adenomatous hyperplasia and two with intrapulmonary lymph nodes)] who underwent lung transplantation, lobectomy or pneumonectomy between June 2017 and June 2019. Because of the policy on anonymization of data from healthy donors, the clinical characteristics of control subjects could only be retrieved from 13 patients with benign tumor (**Table 1**).

Tissue Preparation, Immunohistochemistry, and Immunofluorescent Staining

Three-micrometer thick tissue sections were dewaxed in xylene, rehydrated in graded alcohols, and rinsed in distilled water. Sections were then subject to heat-induced antigen retrieval in Tris–EDTA buffer (pH 9.0) at 95°C for 15 min and cooled at room temperature.

For immunohistochemistry (IHC), staining of TTF-1 (1:300, anti-rabbit, ab76013, Abcam, United States), CD4 (1:200, anti-rabbit, ab133616, Abcam, United States), CD8 (1:50, anti-mouse, ab17147, Abcam, United States) and neutrophil elastase (1:600, anti-mouse, clone NP57, Dako A/S, Glostrup, Denmark) was performed with a modified horseradish peroxidase (HRP) technique with Dako Cytomation EnVision 1 System-HRP (Dako A/S). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Sections were stained with primary antibody at 4°C for overnight incubation. Slides were incubated with Dako EnVision 1 System-HRP (Dako A/S) at room temperature for 30 min, followed by the addition of HRP substrate (diaminobenzidine), and counterstained with hematoxylin. Images were obtained with digital pathological section scanner (PRECICE500B, Beijing, China).

For immunofluorescent (IF) staining, sections were incubated with primary polyclonal antibodies of P63 (1:50, anti-mouse, ab735, Abcam, United States), CC10 (1:500, anti-mouse, sc-365992, Santa Cruz Biotechnology, United States) and SPC (1:100, anti-mouse, sc-518029, Santa Cruz Biotechnology, United States), respectively. We performed triple co-staining with TTF-1 (1:300, anti-rabbit, ab76013, Abcam, United States), α-tubulin (1:100, anti-chicken, ab89984, Abcam, United States) and one of the above-mentioned markers for overnight incubation at 4°C, followed by incubation with Alexa Fluor 488-, Alexa Fluor 555-, and Alexa Fluor 647-conjugated secondary antibodies (1:500, Life Technologies, Carlsbad, CA, United States) at 37°C for 1 h. The nuclei were visualized by staining with 4′-6-diamidino-2-phenylindole (Life Technologies, Carlsbad, CA, United States). Images were acquired with fluorescence microscopy (Leica DM6, Wetzlar, Germany).

For negative controls, primary antibodies were substituted with species- and subtype-matched antibodies of the same concentration.

Morphometric Analysis

In control subjects, the peripheral airway bronchioles were defined as having the diameter of <2 mm (small airways), and consist of: (1) proximal bronchioles: 1–2 mm in diameter; (2) distal bronchioles: <1 mm in diameter; (3) terminal bronchioles: small non-alveolarized bronchioles in the vicinity of respiratory bronchioles; (4) respiratory bronchioles: alveolarized bronchioles (Okuda et al., 2019). Because of the difficulty to differentiate proximal and distal from terminal bronchioles solely based on the airway diameters (readily subject to artifacts associated with post-fixation processing), we specifically focused on the inclusion criteria for normal bronchioles as follows: (1) non-alveolarized bronchioles 0.3–2 mm in diameter; (2) a narrow collapsible lumen, lined with simple

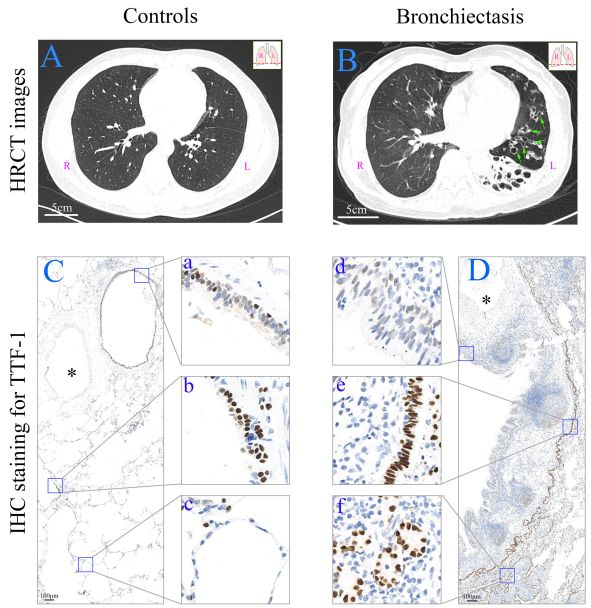


FIGURE 1 | HRCT scan images and expression of TTF-1 protein determined by means of immunohistochemistry in the peripheral lung tissue. **(A)** HRCT scan image from a control subject (age: 45; sex: male). Scale bar = 5 cm. **(B)** HRCT scan image from a patient with bronchiectasis [age: 65; sex: female; HRCT score: 8; dilated bronchioles in the peripheral left lower lobe (green arrows)]. Scale bar = 5 cm. **(C,D)** In a control subject (from a donor) **(C)**, TTF-1$^+$ cells are present in bronchiole (a), respiratory bronchiole (b) and alveoli (c), respectively. In a patient with bronchiectasis (age: 14; sex: male; HRCT score: 3) **(D)**, TTF-1$^+$ cells are decreased in the epithelium of the dilated bronchioles (d), and both columnar (e) and cuboidal (f) epithelial hyperplasia of TTF-1$^+$ cells are present. The similar accompanying vessel are shown in *. Scale bar = 100 μm. HRCT: high-resolution computed tomography; IHC: immunohistochemistry; L: left side; R: right side; TTF-1: thyroid transcription factor-1.

columnar ciliated epithelium without cartilage plates and sub-epithelial glands.

In patients with bronchiectasis, the dilated non-alveolarized bronchioles were defined as: (1) the non-alveolarized bronchioles >2 mm in diameter, and presented with inflammatory cell infiltration surrounding the sub-epithelium; (2) with pseudostratified ciliated columnar or simple columnar ciliated epithelium without cartilage plates and sub-epithelial glands; (3) with a diameter larger than the accompanying thick-walled vessel.

Marker Expression Profiling of Bronchiolar Epithelium

The total number of ECs was assessed by manually counting all cellular nuclei located at epithelial areas. We specifically enumerated all ECs, TTF-1$^+$ cells, P63$^+$ BCs and CC10$^+$ club cells. The percentage of TTF-1$^+$, P63$^+$ and CC10$^+$ cells was calculated as the number of positively stained cells divided by 200 ECs multiplied by 100%. Five to ten areas of epithelium staining from tissue sections were taken randomly from each sample in a blinded manner at high power fields (HPFs).

TABLE 1 | Baseline characteristics of study participants.

	Control subjects	Patients with bronchiectasis
Subjects, *n*	33	43
Healthy donors, *n* (%)	20 (60.6)	–
Patients with benign tumor, *n* (%)†	13 (39.4)	–
Sex (M/F), *n*	6/7	14/29
Age, years	48.9 ± 13.5	47.4 ± 14.6
Smokers, *n* (%)	0 (0)	3 (7.0)
BMI (kg/m^2)‡	22.3 ± 2.7	21.3 ± 2.8
FEV_1 % predicted‡	105.6 ± 12.9	81.8 ± 23.9
FEV_1/FVC (%)‡	83.4 ± 5.1	78.3 ± 10.6
Duration of disease, years	–	6.3 ± 8.0
Modified Reiff score of HRCTs	–	6.3 ± 4.3
The percentage of inflammatory cells§		
Eosinophils	–	8.3 ± 5.9
Neutrophils	–	9.8 ± 5.0
CD4$^+$ T cell	–	33.1 ± 9.7
CD8$^+$ T cell	–	15.8 ± 7.5
Inflammatory cell infiltration, *n* (%)¶		
Eosinophilic infiltration		20 (46.5)
Neutrophilic infiltration		19 (44.2)

†*The characteristics of control subjects were recorded from patients with benign tumor (n = 13).* ‡*These information from four patients with bronchiolectasis were miss.* §*Differential cell counts of the inflammatory cells were expressed as the percentage number of positive staining cells/200 leukocytes × 100%.* ¶ *Eosinophilic or neutrophilic infiltration is categorized by each inflammatory cell count being greater than 10% of the total leukocyte count. The data of age, BMI, FEV_1% predicted, FEV_1/FVC (%), duration of disease, modified Reiff score of HRCTs and the percentage of inflammatory cell were presented as mean ± standard deviation. BMI, body mass index; F, female; FEV_1 = forced expiratory volume in 1 s; FVC, forced vital capacity; HRCT, high-resolution computed tomography; M, male.*

Marker Expression Profiling of Sub-Epithelium

The total number of abnormal hyperplastic progenitor cells in sub-epithelium was assessed by counting all TTF-1$^+$ cells with cuboidal or columnar epithelial hyperplasia. Ten areas with abnormal TTF-1$^+$ epithelial hyperplasia (either cuboidal or columnar) were randomly counted for each sample (n = 43) in 5∼10 HPFs in a blinded manner (**Supplementary Figure S2**). We calculated the percentage of cuboidal and columnar epithelial hyperplasia (total N = 430). Most areas of sub-epithelial hyperplasia in the dilated bronchioles can be extensively labeled with TTF-1$^+$, most of which co-stained with P63, CC10 or SPC. The fluorescence intensity was not analyzed because it was highly influenced by the quality of the material. Therefore, the percentage of P63$^+$, CC10$^+$ and SPC$^+$ cells were expressed as the percentage of positively stained cells divided by 200 sub-epithelial TTF-1$^+$ cells multiplied by 100%, respectively.

Inflammatory Cell Analysis

Five individual fields with infiltration of inflammatory cells were selected for total and differential cell counts (**Supplementary Figure S3**). Total cells counts were derived from counting 200 leukocytes (under 400× magnification). Differential cell counts of the inflammatory cells were expressed as the percentage

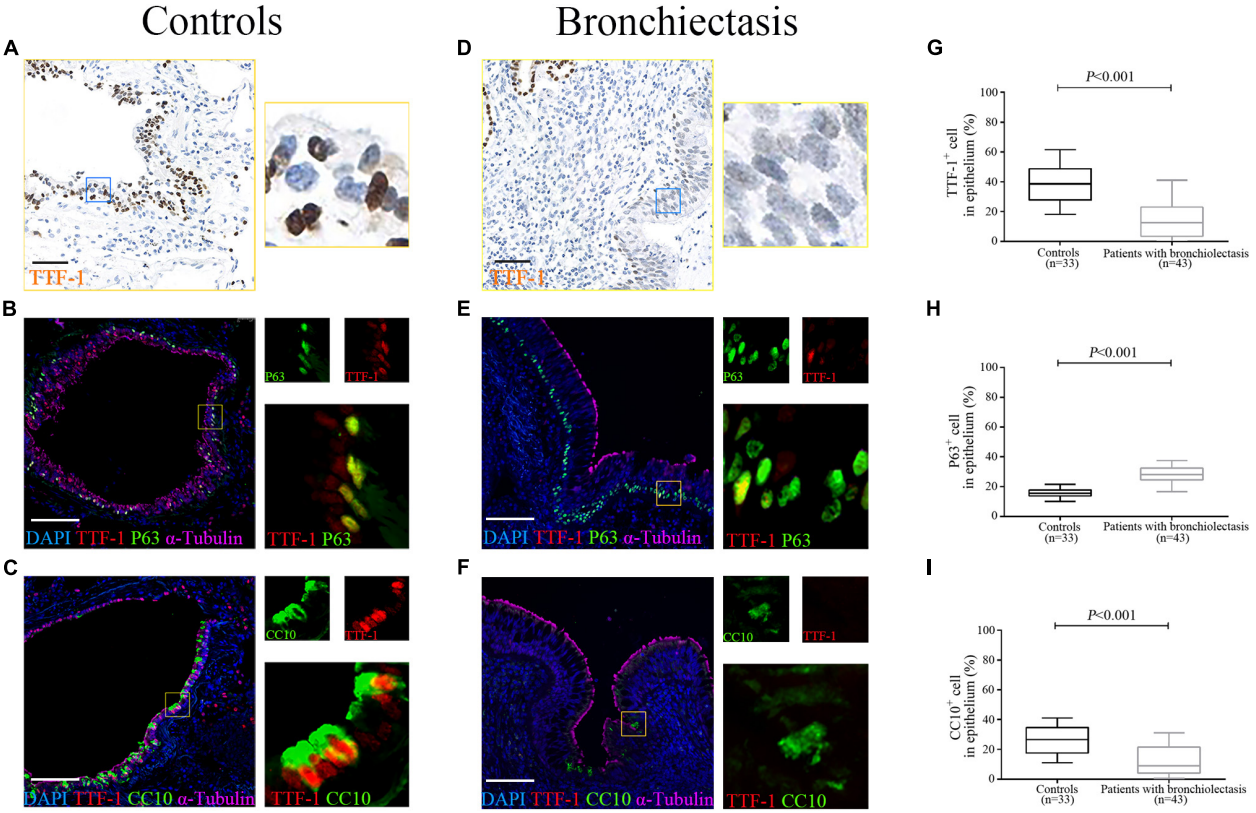


FIGURE 2 | Aberrant progenitor marker expression within the dilated bronchiolar epithelium. The IF staining reveal the expression of progenitor cell markers within bronchiolar epithelium in both control subjects and bronchiectasis patients. TTF-1 (red) co-localizes with P63 (green), whereas TTF-1 (red) co-stains with CC10 (green), in a subset of ECs **(A–F)**. A significantly lower percentage of TTF-1^{+}ECs and CC10^{+}ECs and higher percentage of P63^{+}ECs could be identified in bronchiectasis patients compared with control subjects **(G–I)**. Scale bar = 100 μm. CC10: club cell 10kDa protein; EC: epithelial cell; TTF-1: thyroid transcription factor-1.

number of positive staining cells/200 leukocytes × 100%. For each inflammatory cell count (e.g., CD4^{+} T cells, CD8^{+} T cells, eosinophils and neutrophils), the actual percentage number was recoded. Eosinophilic or neutrophilic infiltration was defined by eosinophils or neutrophils count being greater than 10% of the total leukocyte count, respectively (Chen et al., 2018).

Statistical Analysis

Statistical analyses were conducted with SPSS 21.0 software (IBM, Chicago, IL, United States) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, United States). The normal distribution was tested, and the Mann-Whitney two-sided non-parametric test was used as appropriate to compare the continuous variables between two groups. Correlation analysis was performed with Spearman's model. $P < 0.05$ was deemed statistically significant for all analyses.

RESULTS

Subject Characteristics

The clinical characteristics of control subjects and bronchiectasis patients are shown in **Table 1**. Bronchiectasis patients yielded significantly lower percent predicted of forced expiratory volume in 1 s (FEV_1) (mean: 81.8% vs. 105.6%, $P < 0.01$) than control subjects. 93.0% of bronchiectasis patients were never-smokers. 60.5% ($n = 26$) patients had bilateral bronchiectasis. In 17 patients with unilateral disease, 52.9% ($n = 9$) had right lung involvement. 93.0% ($n = 40$) of patients had both central and peripheral bronchiectasis, and the remainders had peripheral bronchiectasis only. The mean modified Reiff score was 6.3 among 43 bronchiectasis patients, of whom 48.8% ($n = 21$) had cystic bronchiectasis. For 43 bronchiectasis patients, the mean ± standard deviation percentage of eosinophils, neutrophils, CD4^{+} T cells and CD8^{+} T cells was 8.3 ± 5.9, 9.8 ± 5.0, 33.1 ± 9.7, and 15.8 ± 7.5, respectively. Furthermore, Eosinophilic and neutrophilic infiltrations are found in 46.5% (20/43) and 44.2% (19/43) of bronchiectasis patients, respectively.

Aberrant TTF-1 Expression in the Dilated Bronchioles

The IHC staining shows normal peripheral airway structure in controls subjects (**Figure 1C**). Consistent with previous findings (Hösgör et al., 2002), negative TTF-1 staining

was found in the epithelial areas of trachea (A), proximal bronchi (B) and distal bronchi (C) from healthy adult donors (**Supplementary Figure S1**). TTF-1 was extensively expressed in AECII and a subset of normal bronchiolar ECs (**Figures 1C-a–c**). By contrast, we noted significantly dilated bronchioles in bronchiectasis patients (**Figure 1D**). The percentage of TTF-1$^+$

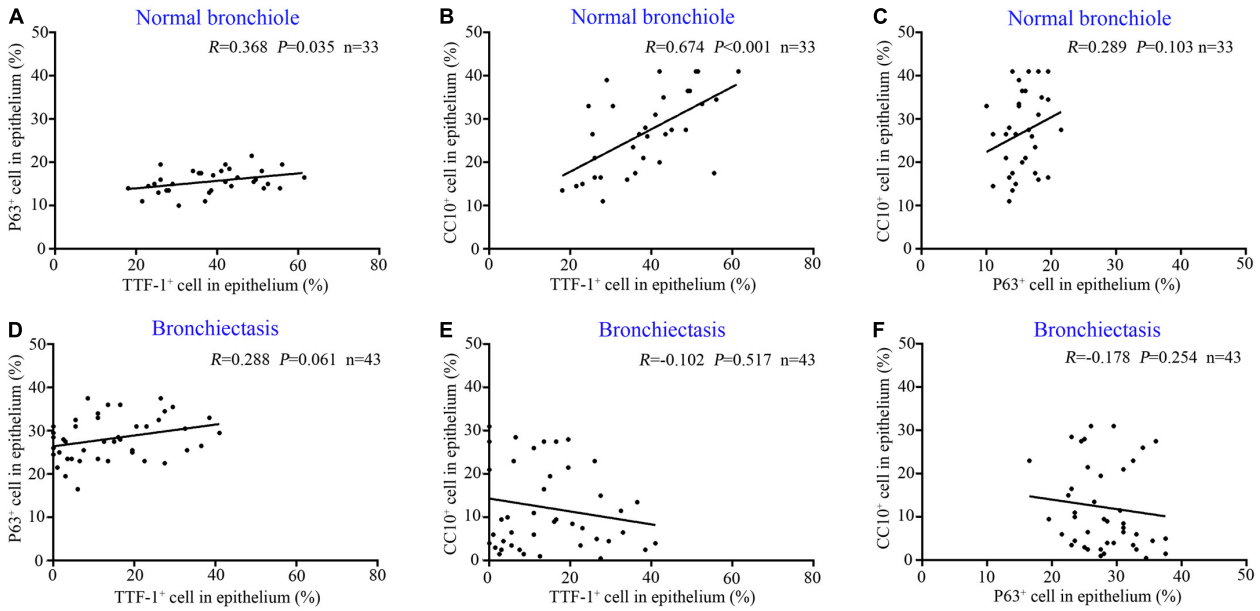


FIGURE 3 | Correlation among the expression of TTF-1$^+$ECs, P63$^+$ECs or CC10$^+$ECs in control subjects and patients with bronchiectasis. In the epithelium of normal bronchioles, there is a positive correlation between TTF-1$^+$ECs and P63$^+$ECs **(A)** and between TTF-1$^+$ECs and CC10$^+$ECs **(B)**, but not in P63$^+$ECs and CC10$^+$ECs **(C)**. In the epithelium of dilated bronchioles, no significant correlation is identified **(D–F)**. CC10: club cell 10 kDa protein; EC: epithelial cell; TTF-1: thyroid transcription factor-1.

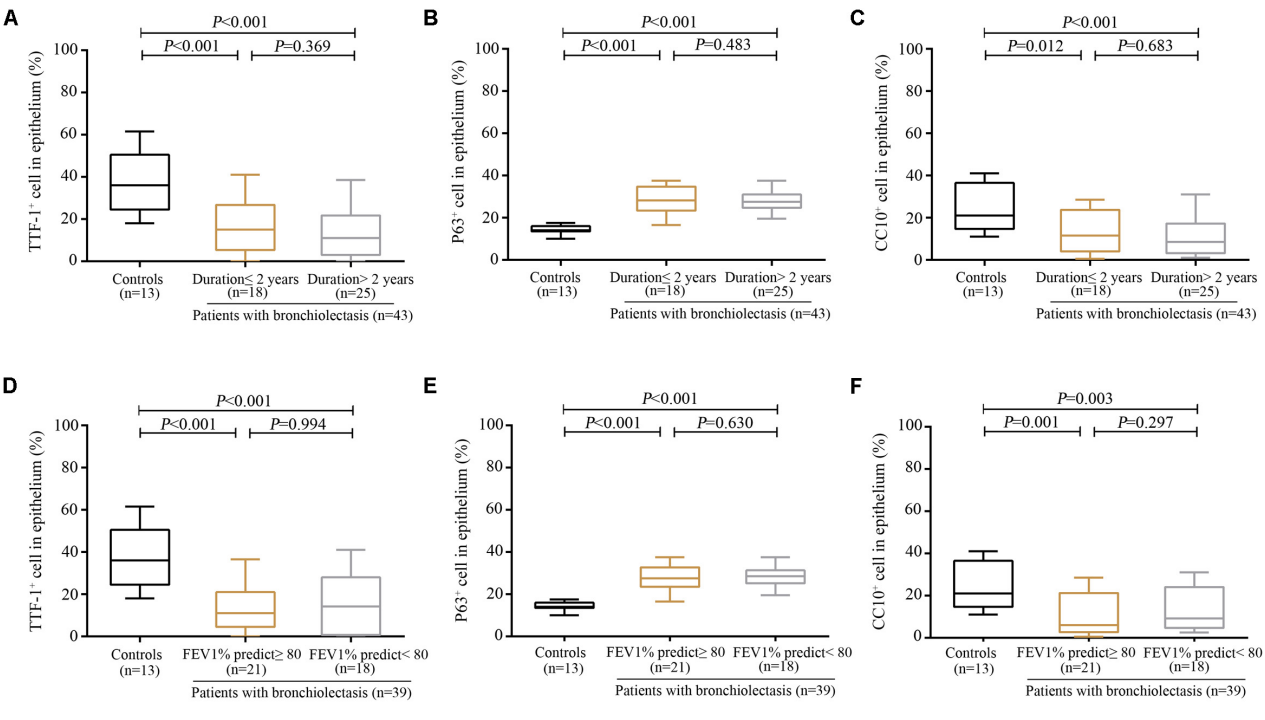


FIGURE 4 | Continued

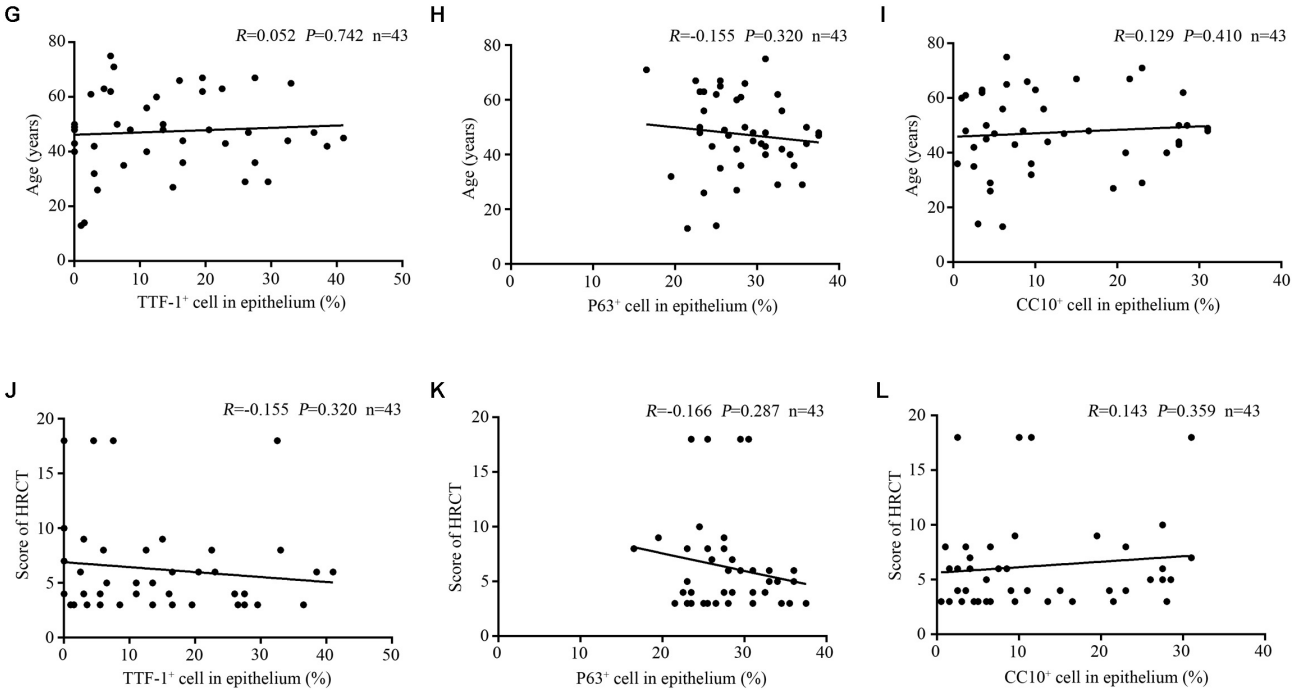


FIGURE 4 | Association between the expression of TTF-1$^+$ECs, P63$^+$ECs or CC10$^+$ECs and the clinical characteristics of patients with bronchiectasis. **(A–C)** A significantly decreased percentage of TTF-1$^+$ECs and CC10$^+$ECs and a higher percentage of P63$^+$ECs in the dilated bronchioles are identified in bronchiectasis when stratifying patients based on the duration of symptoms (cut-off: 2 years). **(D–F)** A significantly decreased percentage of TTF-1$^+$ECs and CC10$^+$ECs and a higher percentage of P63$^+$ECs in the dilated bronchioles are identified in bronchiectasis when stratifying patients based on the magnitude of lung function impairment (cut-off: 80% for FEV_1 predicted %). No significant correlation between the percentage of TTF-1$^+$ECs, P63$^+$ECs, and CC10$^+$ECs and the age **(G–I)** as well as the modified Reiff score **(J–L)**. CC10: club cell 10kDa protein; EC: epithelial cell; FEV1: first second percentage; HRCT: high-resolution computed tomography; TTF-1: thyroid transcription factor-1.

cells within the dilated bronchiolar epithelium was markedly lower compared with that within the control bronchioles (**Figure 1D-d**).

Two patterns of aberrant TTF-1 expression dominated the sub-epithelium of dilated bronchioles: (1) columnar epithelial hyperplasia (including wall-like, canalicular, or pseudoglandular distribution) (**Figure 1D-e**); (2) cuboidal epithelial hyperplasia (including wall-like or canalicular distribution) (**Figure 1D-f**).

Aberrant Progenitor Marker Expression Within the Dilated Bronchiolar Epithelium

The IF staining revealed the expression of progenitor cell markers within bronchiolar epithelium in both control subjects and bronchiectasis patients. TTF-1 partially co-localized with P63, whereas TTF-1 partially co-stained with CC10, in a subset of ECs (**Figures 2A–F**). We noted a significantly lower percentage of TTF-1$^+$ECs and CC10$^+$ECs and higher percentage of P63$^+$ECs in bronchiectasis patients compared with control subjects (all $P < 0.05$, **Figures 2G–I**).

In control subjects, the percentage of TTF-1$^+$ECs correlated positively with that of both P63$^+$ECs (**Figure 3A**) and CC10$^+$ECs in the bronchiolar epithelium (**Figure 3B**), which partially co-localized with TTF-1. However, the percentage of P63$^+$ECs did not correlate with that of CC10$^+$ECs within the epithelium of control bronchioles ($P > 0.05$). Furthermore, there was no significant correlation between the percentage of TTF-1$^+$ECs and P63$^+$ECs or CC10$^+$ECs in the dilated bronchiolar epithelium in bronchiectasis (both $P > 0.05$, **Figures 3C–F**).

Progenitor Marker Expression Within the Bronchiole Epithelium Was Not Different When Stratified by the Key Clinical Characteristics and the Infiltration of Inflammatory Cells of Bronchiectasis

Next, we stratified bronchiectasis patients according to the key clinical metrics. None of the 13 control subjects (with benign tumors, data from 20 healthy donors were excluded because of the policy on anonymization) presented with chronic cough or abnormal lung function. Compared with control subjects, significantly decreased percentage of TTF-1$^+$ECs and CC10$^+$ECs and a higher percentage of P63$^+$ECs were identified in the dilated bronchioles in bronchiectasis (all $P < 0.05$). However, the cell count did not differ statistically when stratified by the duration of symptoms (cut-off: 2 years) and lung function impairment (cut-off: 80% for FEV_1 predicted %) among bronchiectasis patients (**Figures 4A–F**). However, the percentage of TTF-1$^+$ECs,

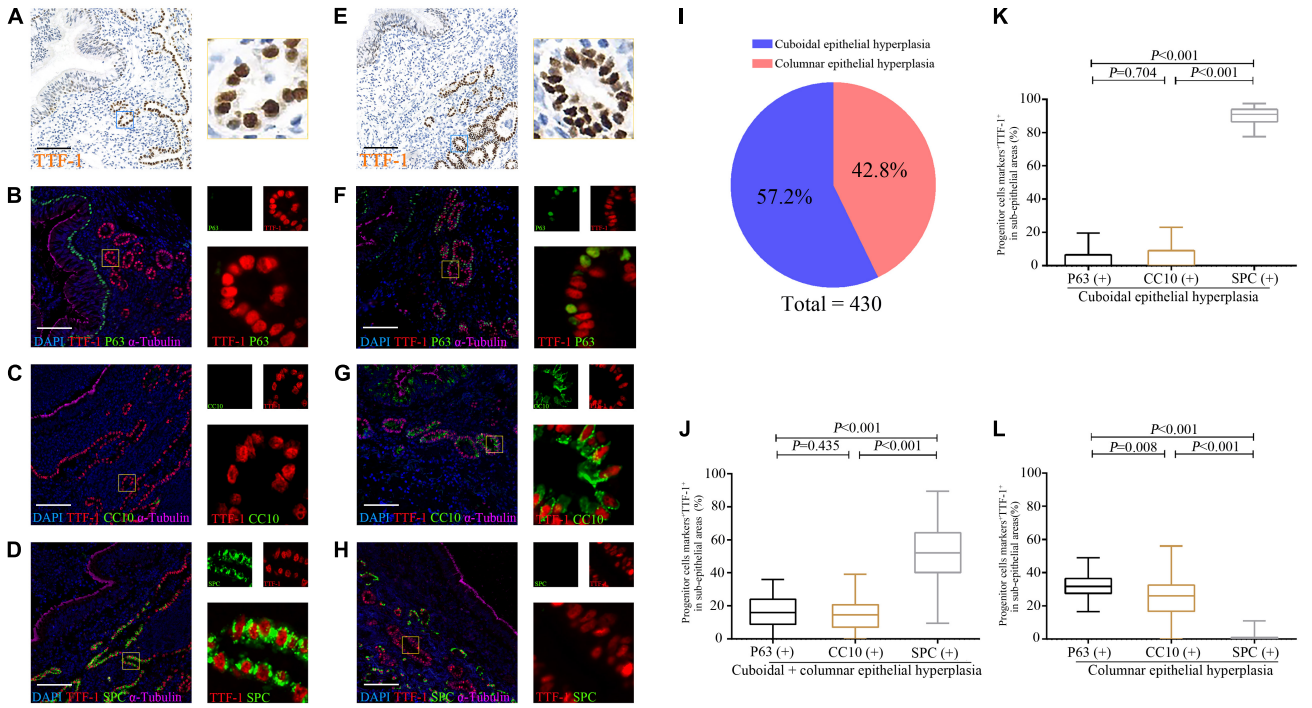


FIGURE 5 | Expression of TTF-1, P63, CC10 and SPC protein in the sub-epithelium areas of the dilated bronchioles in bronchiectasis. Two major abnormal patterns are observed in the sub-epithelium areas of control and dilated bronchioles from patients with bronchiectasis with TTF-1 staining: cuboidal epithelial hyperplasia **(A–D)** and columnar epithelial hyperplasia **(E–H)**. We confirm that most progenitor markers (P63, CC10 or SPC) (green) co-localize with TTF-1 (red) **(B–D,F–H)**. We count 430 areas (10 areas per patient) with epithelial hyperplasia. 57.2% (n = 246) of areas present with cuboidal epithelial hyperplasia, whereas 42.8% (n = 184) of areas yield columnar epithelial hyperplasia **(I)**. The percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$ and $SPC^+TTF\text{-}1^+$ are assessed in both cuboidal and columnar epithelial hyperplasia **(J)**, only for cuboidal epithelial hyperplasia **(K)** and only for columnar epithelial hyperplasia **(L)**, respectively. α-tubulin (pink) is stained as a ciliary marker for epithelium which is helpful to distinguish the sub-epithelium areas. Scale bar = 100 μm. CC10: club cell 10 kDa protein; SPC: surfactant protein C; TTF-1: thyroid transcription factor-1.

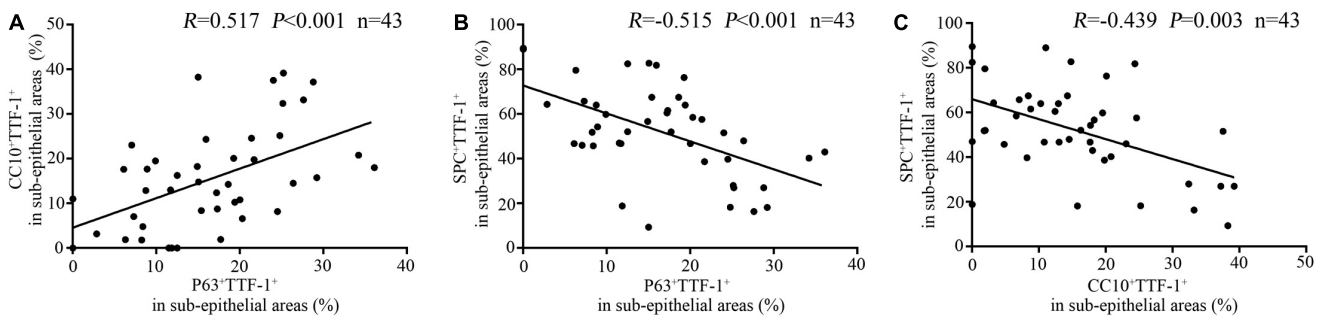


FIGURE 6 | Correlation among the percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$, and $SPC^+TTF\text{-}1^+$ cells in the sub-epithelium of the dilated bronchioles in bronchiectasis. The percentage of $P63^+TTF\text{-}1^+$ cells correlates positively with that of $CC10^+TTF\text{-}1^+$ cells **(A)**, and negatively with that of $SPC^+TTF\text{-}1^+$ cells in the dilated bronchiolar sub-epithelium **(B)**. The percentage of $CC10^+TTF\text{-}1^+$ cells also correlates negatively with that of $SPC^+TTF\text{-}1^+$ cells **(C)**. CC10: club cell 10 kDa protein; SPC: surfactant protein C; TTF-1: thyroid transcription factor-1.

$P63^+$ECs and $CC10^+$ECs did not correlate with the age or the modified Reiff score (**Figures 4G–L**).

Overall, there was no significant association between each inflammatory cell count (e.g., $CD4^+$ T cells, $CD8^+$ T cells, eosinophils, and neutrophils) and the marker expression profiles of progenitor cells ($TTF\text{-}1^+$ECs, $P63^+$ECs, and $CC10^+$ECs) of dilated bronchiole (except for the significant association between $P63^+$ ECs cell count and $CD8^+$ cell count) (**Supplementary Figure S4**). Furthermore, the percentage of epithelial progenitor cells (TTF-1, P63, and CC10) in the dilated bronchioles was not statistically different between eosinophilic and non-eosinophilic inflammation, nor between neutrophilic and non-neutrophilic inflammation (cut-off: 10% of all leukocytes) (**Supplementary Figure S5**).

Aberrant Progenitor Marker Expression in the Dilated Bronchiolar Sub-Epithelium

Next, we explored the association between progenitor marker expression and cuboidal/columnar epithelial hyperplasia in the dilated bronchiolar sub-epithelium (**Figures 1D-e,f**). TTF-1 was stained to indicate epithelial hyperplasia in the dilated bronchiolar sub-epithelium. We also applied IF triple co-staining with α-tubulin (indicating the dilated bronchiolar epithelium), TTF-1 (indicating multiple airway progenitors) and one of the epithelial cell markers (P63 for BCs, CC10 for club cells and SPC for AECII) in the bronchiolar or alveolar epithelium of the distal airways).

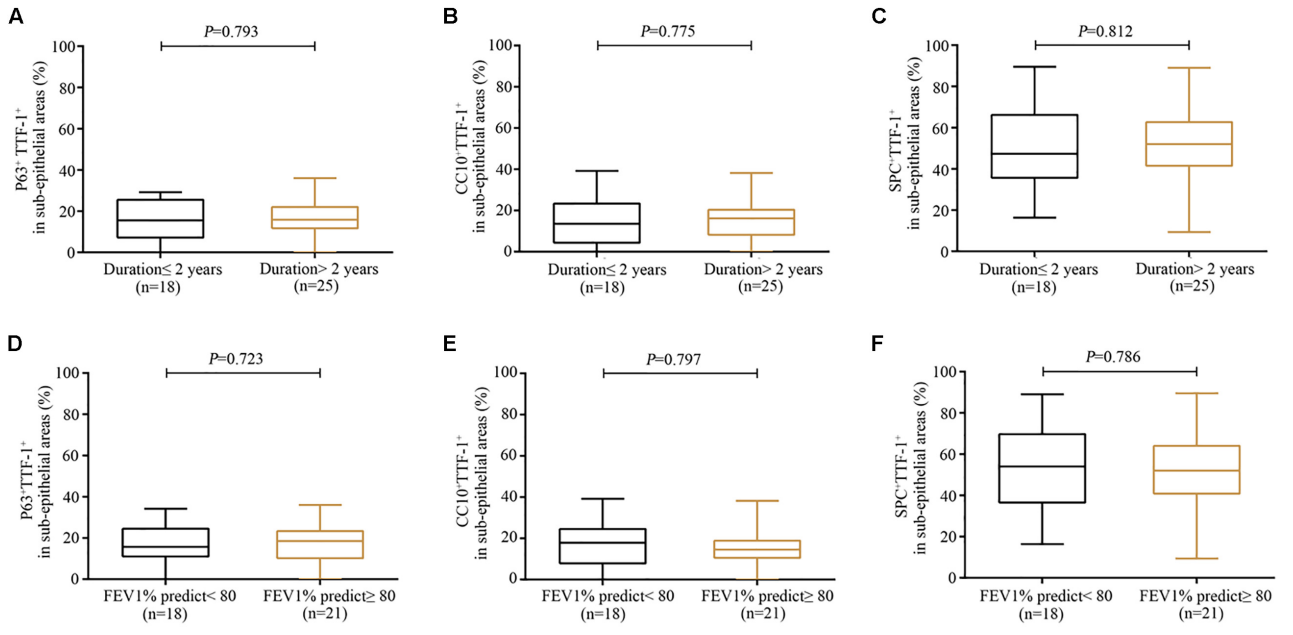
We confirmed that most epithelial markers in the distal airways (P63, CC10, and SPC) co-localized with TTF-1 in the dilated bronchiolar sub-epithelium (**Figures 5A–H**). We also noted remarkable inflammatory cell (i.e., lymphocytes, neutrophils) infiltration that precluded an accurate cell enumeration. In this regard, we enumerated TTF-1^+ cells that yielded abnormal patterns of epithelial hyperplasia. We noted two main types of epithelial hyperplasia with disarrangement of TTF-1^+ cells: cuboidal (100%) and columnar (93.0%) among the 43 bronchiectasis patients. We next counted 430 areas (10 areas per patient) with epithelial hyperplasia. 57.2% (n = 246) of areas presented with cuboidal epithelial hyperplasia, whereas 42.8% (n = 184) of areas yielded columnar epithelial hyperplasia (**Figure 5I**).

To evaluate sub-epithelial hyperplasia, we randomly counted 200 abnormal TTF-1^+ cells in 5∼10 HPFs in the dilated bronchiolar sub-epithelium per patient. The median (the 1st, 3rd quartile) percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$, and $SPC^+TTF\text{-}1^+$ cells was 16.0% (8.9, 24.0%), 14.5% (7.1, 20.8%), and 52% (40.3, 64.4%), respectively (**Figure 5J**).

We then evaluated cuboidal and columnar epithelial hyperplasia, respectively. For cuboidal epithelial hyperplasia with TTF-1^+ cells, 91.0% (86.5, 94.0%) of areas co-stained with SPC, which accounted for a significantly greater percentage of cell count compared with the cells co-stained with P63 or CC10 (**Figure 5K**). For columnar epithelial hyperplasia with TTF-1^+ cells, the percentage of cells co-stained with progenitor markers followed the following order: $P63^+ > CC10^+ > SPC^+$ (**Figure 5L**).

Furthermore, the percentage of $P63^+TTF\text{-}1^+$ cells correlated positively with that of $CC10^+TTF\text{-}1^+$ cells (**Figure 6A**), and negatively with that of $SPC^+TTF\text{-}1^+$ cells in the dilated bronchiolar sub-epithelium (**Figure 6B**). Moreover, the percentage of $CC10^+TTF\text{-}1^+$ cells also correlated negatively with $SPC^+TTF\text{-}1^+$ cells (**Figure 6C**). We also found no significant correlation between the percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$ and $SPC^+TTF\text{-}1$ + cells in the sub-epithelium and the duration of symptoms, nor was a significant correlation found with FEV_1 % predicted, age and the modified Reiff score in bronchiectasis patients (**Figure 7**).

Finally, we noted there was no remarkable difference between each inflammatory cell count (e.g., $CD4^+$ T cells, $CD8^+$ T cells, eosinophils, and neutrophils) and the percentage of different types of sub-epithelial progenitor cells ($P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$ and $SPC^+TTF\text{-}1^+$) (**Supplementary Figure S6**). The percentage of different types of sub-epithelial progenitor cells was also not statistically different between eosinophilic and non-eosinophilic inflammation, nor between neutrophilic and non-neutrophilic inflammation (**Supplementary Figure S7**).



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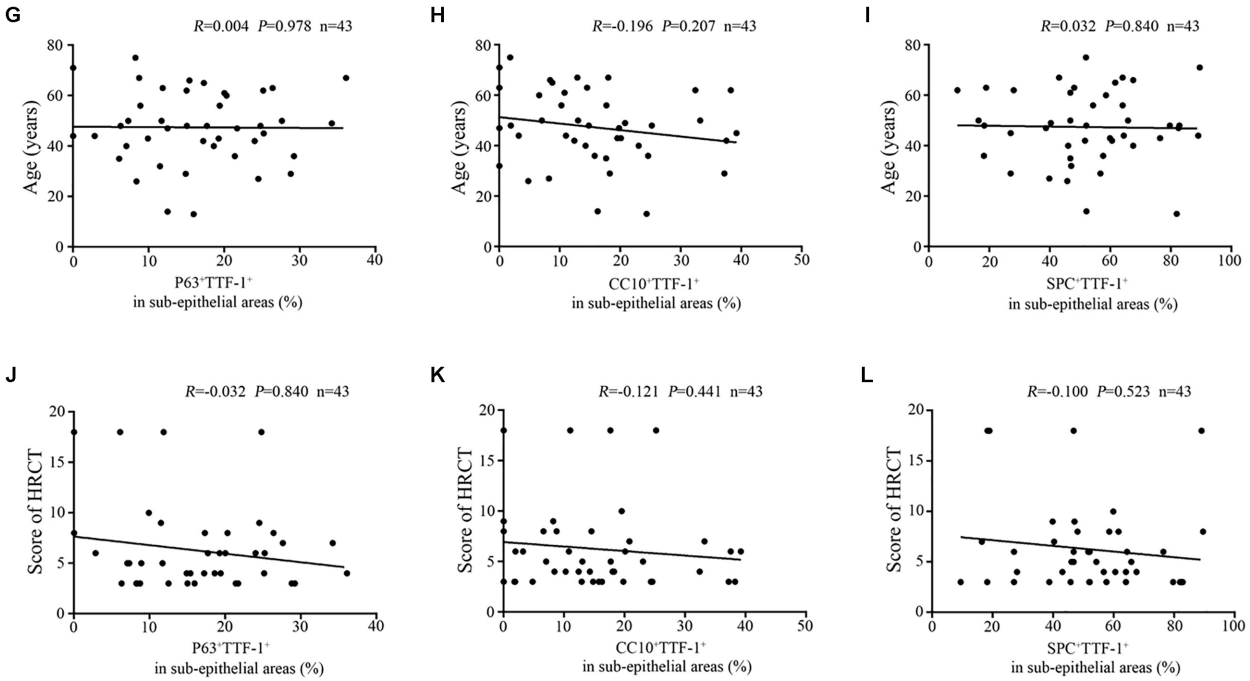

FIGURE 7 | Association between the expression of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$, and $SPC^+TTF\text{-}1^+$ in the sub-epithelium of dilated bronchioles and the clinical characteristics of bronchiectasis. **(A–C)** A similar percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$ and $SPC^+TTF\text{-}1^+$ in the sub-epithelium of the dilated bronchioles based on the duration of symptoms (cut-off: 2 years). **(D–F)** A similar percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$, and $SPC^+TTF\text{-}1^+$ in the sub-epithelium of dilated bronchioles based on lung function impairment (cut-off: 80% for FEV_1 predicted %). No significant correlation between the percentage of $P63^+TTF\text{-}1^+$, $CC10^+TTF\text{-}1^+$, and $SPC^+TTF\text{-}1^+$ and the age **(G–I)** as well as the modified Reiff score **(J–L)**. CC10: club cell 10 kDa protein; EC: epithelial cell; FEV1: first second percentage; HRCT: high-resolution computed tomography; SPC: surfactant protein C; TTF-1: thyroid transcription factor-1.

DISCUSSION

Airway ECs constitute the first barrier against pathogens and xenobiotics, and play a critical role in maintaining homeostasis (Whitsett and Alenghat, 2015). Structural and functional abnormalities of airway epithelium may dampen host defenses, immune or inflammatory responses, and repair processes, leading to progressive epithelial injury and recurrent infections in bronchiectasis (Maeda et al., 2011; Guan et al., 2018a; Nikolic, 2018).

To our knowledge, this is the first study that has systematically investigated the expression profiles of progenitor cells in peripheral airways of bronchiectasis. We have revealed abnormal cell proliferation in both epithelium and sub-epithelium of the dilated bronchioles (previously regarded as clinically silent zones) in bronchiectasis. Despite extensive validation, the vicious cycle model falls short of elucidating the pathologic changes associated with the progressive airway injury that cannot readily be reversed by antibiotic or mucolytic therapies (Polverino et al., 2017). Our findings have pointed to the critical roles of defective progenitor cell renewal, suggesting candidate targets for paradigm shift in future disease management (**Graphical abstract**).

In control subjects, progenitor cells in distal airway epithelium consist of BC, club cells (both in bronchioles) and AECII (in alveoli) which partially yielded TTF-1 expression. Different from the distal airway, we did not identify any TTF-1-positive cells in the normal epithelial areas of proximal airways (trachea and bronchi) from healthy donors (n = 4) (**Supplementary Figure S1**). Notably, we have identified the aberrant expression of progenitor cell markers (TTF-1, P63, and CC10) in bronchiolar epithelium in bronchiectasis. The two dominant patterns, including cuboidal epithelial hyperplasia (TTF-1 mostly co-localized with SPC, **Figure 5K**) and columnar epithelial hyperplasia (TTF-1 mainly co-localized with P63 or CC10, **Figure 5L**) in the sub-epithelium of the dilated bronchioles, indicated the dysregulated proliferation of the injured epithelium. Previous reports have documented TTF-1 expression in ECs of human fetal lungs at 11–12 weeks' gestation. ECs of the peripheral developing airways during pseudoglandular period (weeks 12–16) and canalicular period (weeks 16–28) strongly expressed TTF-1, presenting with the morphology highly similar with the cuboidal and columnar epithelial hyperplasia as seen in our study (Kimura and Deutsch, 2009; Kaarteenaho et al., 2010). Collectively, pathologic dilatation of bronchioles may be linked to defective lung repair or regeneration.

Recent studies have documented the aberrant structure or function of ECs (e.g., ciliated cell, BC and goblet cells) in chronic airway inflammatory diseases (Guan et al., 2018b). For instance, up-regulation of P63 contributes to epithelial remodeling in nasal polyps (Zhao et al., 2017). Zuo et al. (2015) reported that $p63^+$

keratin 5 (Krt5)$^+$ distal airway stem cells give rise to multiple epithelial cell lineages, including bronchiolar secretory cells as well as alveolar type I and type II pneumocytes, to regenerate the distal lung in response to influenza-induced lung damage. In peripheral airways, AECII failed to repair the damaged epithelium as a result of defective proliferation, migration, and/or differentiation, which reportedly led to interstitial scarring in pulmonary fibrosis (Sisson et al., 2010). The greater type I alveolar differentiation potential in the distal airway stem cells in COPD (Wang Y. et al., 2019), coupled with the metaplastic transition to a muco-secretory phenotype in the terminal airways of patients with asthma and COPD (Jeffery, 2001), have provided further evidence of aberrant proliferation of the peripheral airways in chronic airway inflammatory diseases.

Main strengths of our study included the comprehensive profiling of progenitor cell markers that helped unravel dysregulated epithelial proliferation in the peripheral airways of bronchiectasis which have been rarely investigated. Our findings might form a novel basis for exploring airway proliferation as a therapeutic target of bronchiectasis. However, our study is limited by the inclusion of the heterogeneous etiologies of bronchiectasis, and the limited sample size from a single center. External validation of our findings is therefore warranted. The IHC staining also fell short of providing quantitative assessment of the expression levels or the activity of progenitor cell markers. We did not assess airway pathogens because of the archival tissues. Incorporation of airway infection status and inflammatory phenotypes with paired sputum or bronchoalveolar lavage samples might provide greater insights into the interaction between the host-defense and airway infection and inflammation.

In summary, we have unraveled the aberrant expression of progenitor cells in bronchiole epithelium and sub-epithelium in bronchiectasis. The dysregulated epithelial cell proliferation might represent a novel research direction and provide potential targets for future therapeutic interventions of bronchiectasis.

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 institutional review boards of The First Affiliated Hospital of Guangzhou Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NZ, D-YW, and WG: conceived the experiments. YP, YH, and XH: collection of samples. YP, AX, and SC: performed the experiments. YP, AX, and SC: data analysis. YP and WG: wrote the manuscript. NZ and D-YW: critical review and approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00088/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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Allergen Immunotherapy–Induced Immunoglobulin G4 Reduces Basophil Activation in House Dust Mite–Allergic Asthma Patients

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It is unclear if allergen immunotherapy (AIT) can reduce allergy effector cell activation. We evaluated the basophil response during *Dermatophagoides pteronyssinus* (Der p) subcutaneous immunotherapy (SCIT) and its relationship to allergen-specific immunoglobulin G4 (sIgG4) in allergic rhinitis and/or asthma patients. The study included 55 subjects, of which 35 cases received Der p SCIT and 20 controls received standard medications. Symptom and medication scores (SMSs), sIgG4 levels, specific immunoglobulin E (sIgE) levels, allergen-induced basophil activation tests (BATs) in whole blood, and BAT inhibition assays in serum were determined at weeks 0, 4, 12, 16, 52, and 104 of SCIT. Levels of Der p sIgG4 in SCIT patients significantly increased after 12 weeks of treatment compared to week 0. Serum obtained from SCIT patients significantly inhibited basophil activation after 12 weeks of treatment. Removal of immunoglobulin G4 (IgG4) antibodies at week 104 reduced the ability of serum to block basophil activation. An increase of Der p sIgG4 rather than reduction of Der p sIgE correlated with the reduction of basophil activation during SCIT. The sIgG4 antibodies may compete with sIgE binding to allergens to form an immunoglobulin E (IgE)–allergen complex. SCIT reduced the sensitivity of allergen-triggered basophil activation in Der p allergic rhinitis and/or asthma patients through induction of sIgG4.

Keywords: asthma, rhinitis, allergen immunotherapy, basophil activation test, serum specific immunoglobulin G4 (IgG4)

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INTRODUCTION

Allergen-specific immunotherapy (AIT) is an effective treatment for many allergens including house dust mite allergens (Wang et al., 2006; Durham et al., 2012; Nelson, 2014), which are a major cause of allergic rhinitis and allergic asthma in China (Li et al., 2009). The therapeutic mechanisms of AIT involve modulation of cellular reactions and related antibody responses as well as inhibition of anaphylactic cell release of their mediators (Till et al., 2004).

Cellular changes include generation of allergen-specific regulatory subsets of T and B cells and inhibition of allergen-specific T helper type 2 (Th2) cells (Wachholz et al., 2002). Antibody

Abbreviations: AHR, airway hyperresponsiveness; AIT, allergen immunotherapy; BAT, basophil activation test; Der f, *Dermatophagoides farina*; Der p, *Dermatophagoides pteronyssinus*; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; SCIT, subcutaneous allergen immunotherapy; sIgE, specific immunoglobulin E; sIgG4, specific immunoglobulin G4; SMS, combined symptom medication score.

responses are involved in the induction of allergen-specific immunoglobulin G (IgG) antibodies, in particular, IgG4 antibodies. IgG4 antibodies may have blocking activities, as they compete with specific immunoglobulin E (sIgE) for binding to the allergen. This inhibits allergen–immunoglobulin E (IgE) complex formation on sIgE receptor–expressing cells such as mast cells and basophils. The responses of basophils to allergen stimulation are dose dependent. *Reactivity* is defined as the maximal response plateau to allergen stimulation located on the dose-response curve. Cellular *sensitivity* represents the threshold of allergen sensitivity under submaximal allergen concentration stimulation (MacGlashan, 1993). The basophil activation test (BAT) is a useful tool for allergy diagnosis (Gonzalez-Munoz et al., 2008; Santos et al., 2014; Imoto et al., 2015); studies have provided evidence for its usefulness in monitoring the induction of immune tolerance by immunotherapy (Bidad et al., 2014; Kepil Ozdemir et al., 2014). Potapinska et al. demonstrated that BAT has high sensitivity and specificity values in the diagnosis of atopic diseases (Potapinska et al., 2009; Ozdemir et al., 2011). In a peanut immunotherapy study, Santos et al. found that depletion of IgG4 reduced the inhibitory effect of peanut-induced basophil activation (Santos et al., 2015). Our previous study demonstrated that IgG4 responses are prominent during *Dermatophagoides pteronyssinus* (Der p) subcutaneous immunotherapy (SCIT) (Lai et al., 2013). In this study, we investigated the relationship between basophil response and IgG4 antibodies to demonstrate that Der p SCIT might reduce basophil reactivity and/or sensitivity through induction of IgG4 in dust mite–sensitive subjects.

MATERIALS AND METHODS

Study Design and Population

The study included a total of 55 subjects, 21 children (age ≤ 14 years) and 34 adults (age 15–57 years), with mild-to-moderate asthma and/or rhinitis. Of these patients, 35 cases received Der p SCIT, and 20 controls received regular medications, serving as the medication group (**Table 1**). All patients came from the allergy and clinical immunology department of the Guangzhou Institute of Respiratory Diseases, fulfilled the ARIA guideline for allergic rhinitis and/or GINA guideline for mild-to-moderate asthma (Bousquet et al., 2007, 2008), and had a positive skin prick test (SPT) and sIgE to Der p ≥ 0.7 kU/L (ImmunoCap, Pharmacia, Sweden). All patients had a forced vital capacity in the first second (FEV_1) greater than 70% of the predicted value before enrolling into the study. Patients visited the hospital for treatments and clinical evaluations. Serum samples were collected before initiation of SCIT and at weeks 4, 12, 16, 52, and 104 during the treatment. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University and registered at http://www.chictr.org.cn (ChiCTR-OOC-15006207). Written informed consent was obtained from all adult patients or the parents of children. We estimated an appropriate sample size based on our data focusing on SCIT patients and containing paired samples and independent samples. We used a one-tailed test for independent samples with the alpha level set at 0.05 and the effect size equal to 0.8, with a statistical power of 0.90. The allocation ratio of the SCIT group to the medication group was 1.5. The estimation indicated that at least 35 individuals in the SCIT group and 23 individuals in the medication group would be required.

TABLE 1 | Baseline information for patients in SCIT and medication groups.

Items	SCIT group	Medication group	*P* Value
	(*n* = 35)	(*n* = 20)	
Gender m/f (cases)	25/10	12/8	>0.05
Age distribution (y)	20.3 ± 2.2	22.3 ± 2.5	>0.05
Children (≤14)	15 (9.1 ± 0.5)	6 (8 ± 0.6)	>0.05
Adults (>14)	20 (28.8 ± 2.6)	14 (30 ± 1.9)	>0.05
Case distribution cases (%)			
Asthma	5 (14.3)	2 (10.0)	>0.05
Rhinitis	4 (11.4)	3 (15.0)	>0.05
Asthma combined	26 (74.3)	15 (75.0)	>0.05
Rhinitis			
Symptom and medication score	3.67 ± 0.42	3.82 ± 0.45	>0.05
FEV_1 (%predicted)	91.16 ± 2.25	91.64 ± 1.81	>0.05
FVC (%predicted)	98.05 ± 1.50	93.85 ± 2.17	>0.05
Total IgE (kU/1)	477 ± 65	562 ± 47	>0.05
Specific IgE to Der p (kU/1)	85 ± 11	87 ± 11	>0.05

Data are presented as mean ± SE. FEV_1, forced expiratory volume at 1s; FVC, forced vital capacity; IgE, immunoglobulin E.

Detection of Serum IgE and IgG4

The levels of total IgE and sIgE against Der p were measured by a Pharmacia CAP fluorescence enzyme immunoassay system (ThermoFisher, Sweden). The sIgE results are reported as kU/L, with a lower limit of 0.35 kU/L and an upper detection limit of 100 kU/L. Serum Der p allergen-specific IgG4 (sIgG4) levels were measured using a four-layer sandwich enzyme-linked immunosorbent assay (ELISA) system as previously reported (Lai et al., 2013).

SCIT Protocol

The patients were treated with subcutaneous injections of standardized aluminum-formulated Der p Alutard-SQ vaccine (ALK-Abello A/S, Horsholm, Denmark). The treatment protocol followed the recommended up-dosing schedule of 16 weeks before reaching a maintenance dose of 100,000 Alutard-SQ, for a duration of 2 years of SCIT.

Clinical Evaluations

The patients were asked to complete a symptom and medication diary routinely during the whole course of treatment. Patients were asked to rate symptoms of asthma (daytime: 0–5; nighttime: 0–4) and rhinitis (day- or nighttime: 0–2) according to the severity and frequency of the symptoms in disturbing daily activities and sleep (Wang et al., 2006). The daily medication score was calculated by assigning a score of 1 for each 160 μg of budesonide or the equivalent dose of inhaled corticosteroid,

or each 130 μg of budesonide or the equivalent dose of nasal corticosteroid, as well as for each puff of salbutamol/terbutaline or the equivalent dose of another inhaled β2-agonist and for each 10 mg of oral loratidine or the equivalent dose of another antihistamine tablet. A symptom and medication score (SMS) was defined as the sum of symptom scores and medication scores (Canonica et al., 2007).

Evaluation of Basophil Activation via Measurement of CD63 Expression

Basophil activation tests were performed as reported in Santos et al. (2014) at weeks 0, 4, 12, 16, 52, and 104. First, we performed dose-finding experiments in 10 HDM-allergic patients and 5 non-atopic controls. Heparinized whole blood (100 μl) was stimulated for 30 min at 37°C with Der p extract (ALK-Abello A/S, Horsholm, Denmark) diluted in phosphate-buffered saline (PBS, Sigma Diagnostics, St. Louis) at serial sixfold dilutions (150, 15, 1.5, 1.5×10^{-1}, 1.5×10^{-2}, 1.5×10^{-3} μg/ml). The two allergen concentrations that evoked maximal (i.e., 15 μg/ml) and submaximal (i.e., 0.15 μg/ml) cell stimulation were chosen for the study to investigate the time course of BAT during the SCIT. Before erythrocyte lysis, cells were stained with CD123-PE-Cy5 (BD), CD203c-PE, HLA-DR-ECD, and CD63-FITC (Beckman Coulter). Basophils gated as $SSC^{low}/CD203c^{+}/CD123^{+}/HLA\text{-}DR^{-}$ were detected by flow cytometry (Beckman Coulter Epics XL-MCL, United States) and analyzed using FCS Express software (version 4).

Basophil Activation Inhibition Assays

Heparinized whole venous blood was obtained from three HDM atopic adult volunteers. The sIgE values for Der p were 90, 104, and 159 kU/L, respectively. The percentage of CD63 was pre-determined by the BAT as previously described (the basic basophil activation is 22, 25, and 26% at 0.15 μg/ml Der p, and 62, 63, and 65% at 15 μg/ml Der p, respectively). Serum (10 μl) from SCIT patients or medication subjects was incubated with 30 μl of Der p allergen (the final concentration of Der p was 0.15 or 15 μg/ml) at 37°C for 1 h. We then added 100 μl of HDM atopic donor blood and incubated the sample at 37°C for 30 min. The following steps were performed as BAT (i.e., stained with antibodies and detected by flow cytometry).

IgG4 Antibody Depletion and Retest of BAT Inhibition Assays

IgG1 anti-IgG4 antibody (Fitzgerald) was coupled to cyanogen bromide (CNBr)–activated Sepharose (GE Healthcare, Hertfordshire, United Kingdom) as described by Kavran and Leahy (2014). Mock-coupled Sepharose beads were prepared and incubated with coupling buffer lacking antibody to generate a negative control. The following depletion steps were performed as described by Santos et al. (2015). Briefly, the remaining reactive CNBr sites were blocked with 1 mol/L ethanolamine and then washed in alternating pH using 0.1 mol/L acetic acid/sodium acetate at pH 4.0 and 0.1 mol/L Tris–HCl at pH 8.0 for three cycles. Fifteen serum samples from patients after 104 weeks of SCIT, with the largest ratio of basophil CD63 expression at week 0 and week 104 according to the BAT inhibition assay results, were diluted 1:5 in PBS-AT (0.3% BSA, 0.1% Tween 20, and 0.05% NaN3 in PBS). Diluted serum samples were incubated with anti-IgG4- or mock-coupled Sepharose beads overnight and collected by means of centrifugation. The specificity of the depletion of Der p specific antibody was confirmed by a four-layer sandwich ELISA as previously demonstrated (Lai et al., 2013). After IgG4 antibody depletion, samples were assayed for BAT inhibition assays as previously described. The percentage of inhibition calculation formula was as follows:

$$\begin{aligned}&\%\ \text{inhibition}\\&= (\%\ \text{CD63 cells sensitized with serum from an HDM atopic volunteer and 0.15 μg/ml Der p} - \%\ \text{CD63cells sensitized with test serum and 0.15 μg/ml Der p})\\&/(\%\ \text{CD63 cells sensitized with serum from an HDM atopic volunteer and 0.15 μg/ml Der p}).\end{aligned}$$

Statistical Analysis

An independent-samples *t*-test was used to analyze group differences in SMS, Der p sIgG4, BAT, and BAT inhibition assays. A paired-samples *t*-test was used to analyze within-group differences. Data are presented as mean ± SE. Linear regression was employed to analyze the relationship between Der p sIgG4, Der p sIgE, and BAT inhibition assays. Differences were considered significant at $P < 0.05$.

RESULTS

Patient Characteristics

The demographic data, SMS, and antibody levels of all subjects are shown in **Table 1**. There were no differences between SCIT and medication group in gender, SMSs, and serum IgE levels.

Changes of Clinical Outcomes

The combined symptom medication score (SMS) of asthma and rhinitis decreased significantly after 12 weeks of treatments compared to baselines in both groups (**Supplementary Figure S1A**), with more significant declines seen in the SCIT subjects compared to medication-treated subjects at weeks 52 and 104 (**Supplementary Figure S1A**). FEV_1% did not change significantly during treatment in either group (**Supplementary Figure S1B**).

Allergen Concentration Curves for BAT

Basophil activation in mite allergy patients ($n = 10$) and non-atopic controls ($n = 5$) was assayed with six mite extract concentrations. The analysis of allergen concentration curves for the BAT showed no significant change in CD63 in non-atopic controls ($P > 0.05$). In contrast, BAT results were dose dependent in the mite-allergic patients (**Supplementary Figure S1C**). The two allergen concentrations that evoked maximal (i.e., 15 μg/ml)

and submaximal (i.e., 0.15 μg/ml) cell stimulation were chosen to further investigate the time course of basophil activation.

Time Course of Basophil Activation During SCIT

Activated basophils gated as $SSC^{low}/CD203c^{+}/CD123^{+}/HLA\text{-}DR^{-}$ (**Figure 1A**). Basophil activation responses to the 0.15 μg/ml allergen concentrations decreased significantly in the SCIT group from week 16 to 104 (16 weeks = 21.5%, $P = 0.023$; 52 weeks = 19.7%, $P = 0.009$; 104 weeks = 20.0%, $P = 0.001$) compared to baseline (0 week = 25.6%, **Figure 1B**). However, basophil activation was not significantly changed after stimulation with 15 μg/ml of allergen extracts at the six time points in course of SCIT ($P > 0.05$, **Figure 1B**). In the medication group, no significant changes were observed at the six time points in the basophil CD63 response to 15 and 0.15 μg/ml of allergen extract compared to baseline ($P > 0.05$, **Supplementary Figure S2A**).

Time Course of the BAT Inhibition Assay

Serum obtained from SCIT patients significantly increased the capacity to inhibit basophil activation upon challenge with 0.15 μg/ml Der p allergen, starting from week 12 (0 week = 61.6 ± 2.3%, 4 weeks = 60.7 ± 2.0%, 12 weeks = 56.2 ± 1.9%, 16 weeks = 36.4 ± 2.0%, 52 weeks = 24.0 ± 1.4%, 104 weeks = 16.0 ± 1.1%; **Figure 1C**). This effect was not found when basophils were challenged with 15 μg/ml Der p allergen (**Figure 1D**). Serum from medication subjects did not alter basophil responses to either 0.15 or 15 μg/ml Der p allergen (**Supplementary Figures S2C,D**).

Changes of Serum Der p sIgG4 and Der p sIgE Antibodies

Der p sIgG4 levels significantly increased in SCIT patients starting from week 12, with 10- to 71-fold increases from week 16 to week 104 (**Figure 2A**). No significant changes were observed

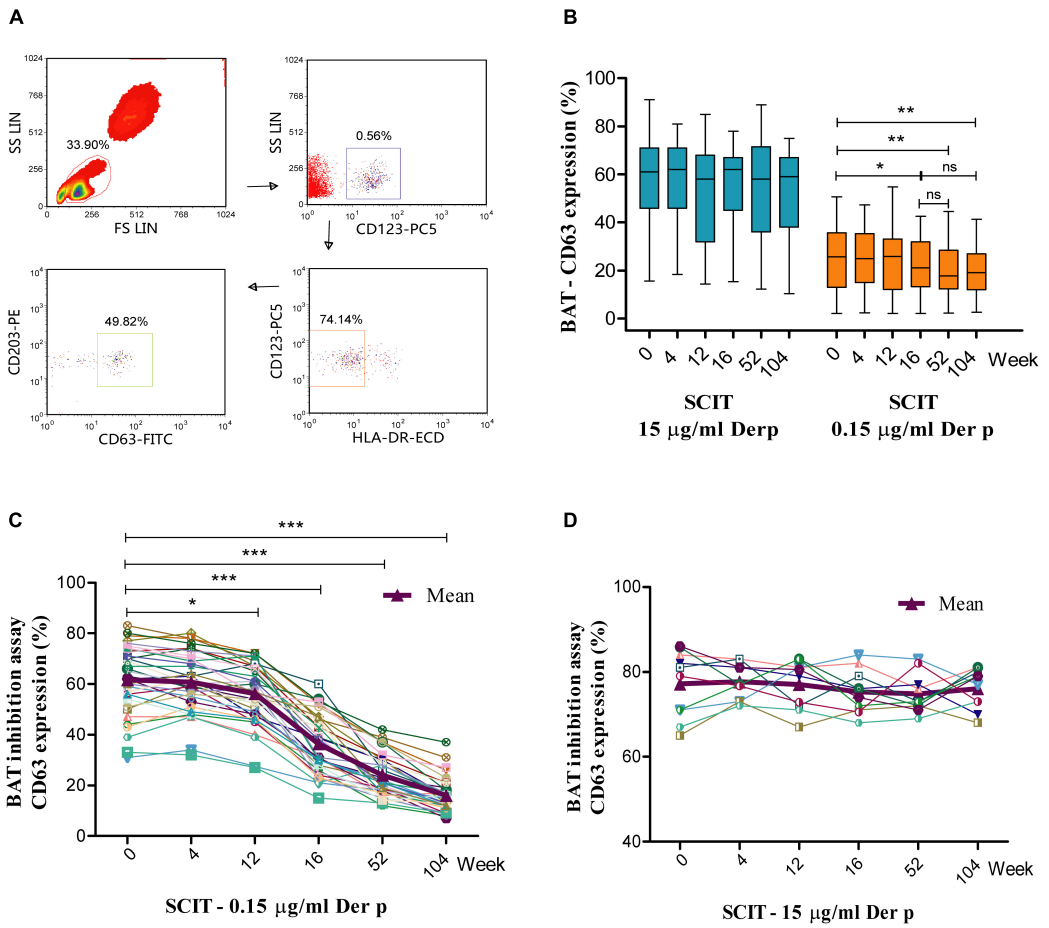

FIGURE 1 | Time course of basophil activation test (BAT) and BAT inhibition assay during subcutaneous immunotherapy (SCIT). Lymphocytes are identified by their scatter properties, and the activated basophils gated as $SSC^{low}/CD203c^{+}/CD123^{+}/HLA\text{-}DR^{-}$ **(A)**. The basophil CD63 responses to 0.15 and 15 μg/ml of Der p extract during the time course of BAT **(B)**. The central box represents the values from the lower to the upper quartile (25th and 75th percentiles); the line within the box indicates the median, and the whiskers show the 5th and 95th percentiles. The BAT inhibition assay was performed with serum from SCIT patients incubated with 0.15 μg/ml (**C**; $n = 35$) or 15 μg/ml (**D**; $n = 10$) Der p allergen before performance of the BAT. Der p = *Dermatophagoides pteronyssinus*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with week 0; ns = non-significant.

in the medication group (**Supplementary Figure S2B**). The difference between the two groups was significant starting from week 16 (data not shown). Significantly lower levels of Der p sIgE were observed at week 104 in SCIT subjects (Der p sIgE: 0 week = 85 ± 11 kU/L, 4 weeks = 87 ± 12 kU/L, 12 weeks = 94 ± 12 kU/L, 16 weeks = 95 ± 14 kU/L, 52 weeks = 84 ± 9 kU/L, 104 weeks = 73 ± 9 kU/L) when compared to baseline ($P < 0.05$) (**Figure 2B**). No significant differences were seen in the medication group ($P > 0.05$) (**Figure 2B**).

Removal of IgG4 Antibodies Reduced the Ability of Serum to Block Basophil Activation

The ability to block basophil activation was reduced after IgG4 depletion from the serum of SCIT patients at week 104 (median inhibition, mock depleted = 57.1%, IgG4 depleted = 27.9%, $P < 0.001$, $n = 15$) (**Supplementary Figure S1D**).

Correlations Between sIgG4, sIgE, and BAT Inhibition Assay

Der p sIgG4 had a significant linear association with the basophil activation inhibition assay in the SCIT group at all time points during the 2 years of treatment ($R^2 = 0.58$, $P < 0.001$; **Figure 2C**). There was no correlation between Der p sIgE levels and the BAT inhibition assay in the SCIT group at all time points during the 2 years of treatment ($R^2 = 0.01$, $P > 0.05$; **Figure 2D**).

DISCUSSION

In this study of SCIT with Der p extract, we found that SCIT could significantly improve asthma symptoms and reduce medication requirements starting after 12 weeks of treatment. We demonstrated that allergen-induced basophil activation decreased after 16 weeks of AIT at submaximal allergen concentrations compared to week 0. SCIT-induced sIgG4

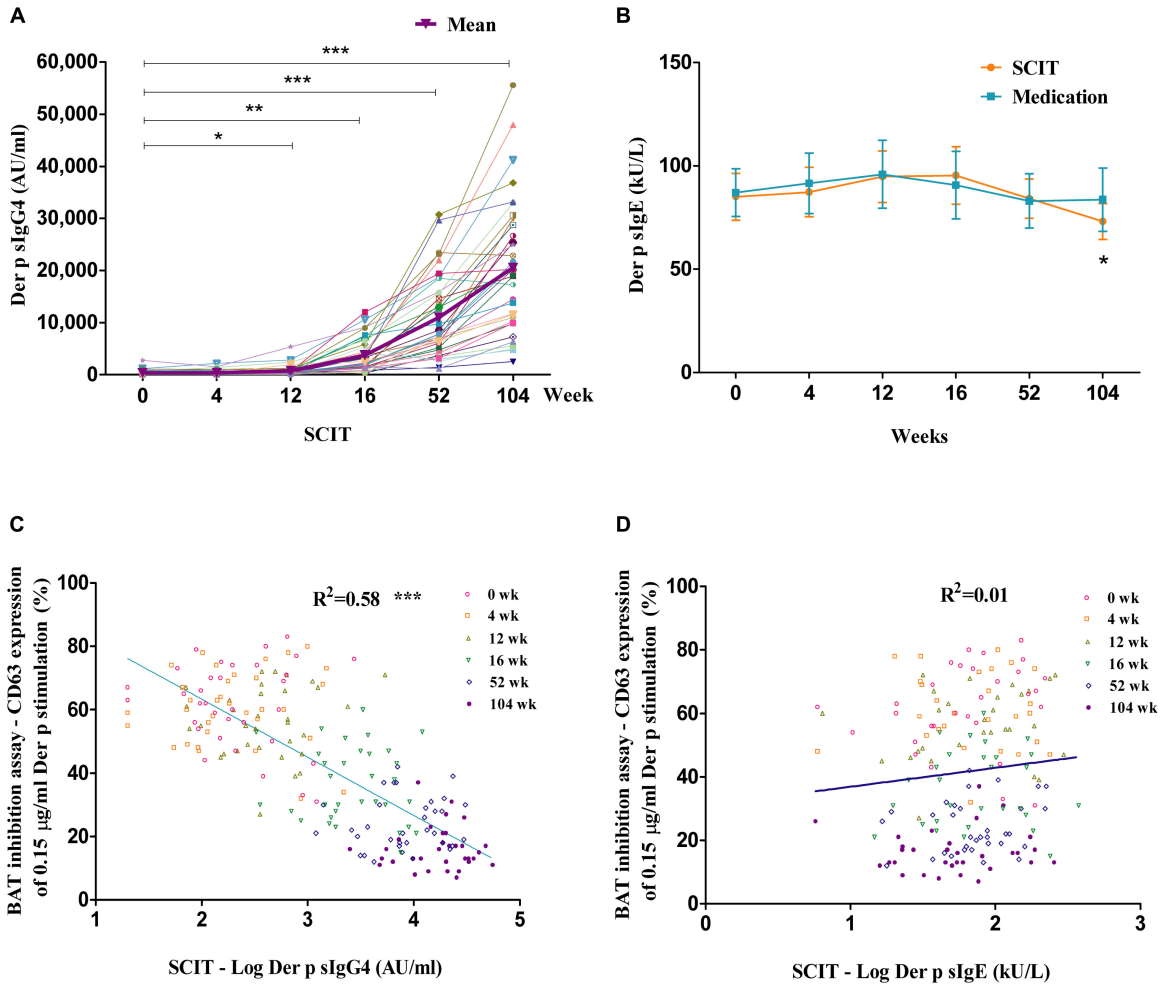

FIGURE 2 | Time course of allergen-specific IgG4 (sIgG4) and specific IgE (sIgE), and the relationship with BAT inhibition assay. Time course of Der p sIgG4 **(A)** and Der p sIgE **(B)** in SCIT group. Linear regression between Der p sIgG4 and BAT inhibition assay in the SCIT group at all time points during the 2 years of treatment **(C)** and the relationship between BAT inhibition assay and Der p sIgE **(D)**. The *x*-axis is a log scale. IgG = immunoglobulin G; IgE = immunoglobulin E. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with week 0.

antibodies substantially increased, and serum obtained from SCIT patients significantly inhibited basophil activation after 12 weeks of treatment compared to week 0. Removal of IgG4 antibodies reduced the ability of blocking basophil activation, and Der p sIgG4 and basophil activation inhibition assays had a significant relationship according to linear regression analysis.

Although it was impracticable to perform a double-blind, placebo-controlled study in this 2-year clinical observation, the current investigation is consistent with a previous study (Wang et al., 2006; Lai et al., 2013) and other studies (Keles et al., 2011; Devillier et al., 2016), which confirmed that HDM AIT is an effective treatment for allergic disease.

Induction of sIgG4 has long been regarded as a prominent immunological change induced by AIT (Sahin et al., 2016). In agreement with our previous findings and those of other studies (Wachholz et al., 2003; Shamji et al., 2012; Lai et al., 2013), we found that successful AIT could induce a substantial increase of sIgG4. The magnitude of increase in Der p sIgG4 concentrations is associated with the allergen concentration used for immunotherapy (Van Metre et al., 1980). Specific IgG4 has been proposed to block antibodies by competing with sIgE for allergens to form IgE–allergen complexes. This reduces the complex binding to sIgE receptor–expressing effector cells (Gehlhar et al., 1999; Francis et al., 2008) and prevents the allergen-dependent activation of T cells by interfering with IgE-facilitated antigen presentation (Van Neerven et al., 2006). IgG antibodies have also been shown to be associated with the inhibition of allergen-induced effector cell activation (Ball et al., 1999; Mothes et al., 2003) or with reduced allergen sensitivity (Reisinger et al., 2005). Although induction of allergen-specific serum IgG is regarded as a characteristic feature of the immunological response induced by AIT, treatment-induced changes in the levels of sIgE secretion remain a controversial issue. We found that the levels of Der p sIgE decreased significantly after 104 weeks of SCIT; this is supported by other studies (Arlian et al., 1993; Chen et al., 2017; Feng et al., 2018), However, other studies demonstrated that sIgE levels did not change after 1 year of AIT (Shamji et al., 2011; Sahin et al., 2016), and Blumberga et al. (2011) found that AIT-induced serum sIgE increased initially and then declined to baseline value after 1 year of treatment. In general, the decrease of sIgE during the late phase of AIT might be associated with the secretion of IL-10 and TGF-β by Treg cells as well as the switching of allergen-specific B cells toward IgG4 production instead of sIgE production (Frew, 2010).

We found that basophil activation triggered by submaximal allergen concentrations decreased after 16 weeks of AIT treatment. However, basophil activation was not significantly changed with maximal allergen concentrations in either the AIT or the medication group. This observation is consistent with other studies (Ebo et al., 2007; Lalek et al., 2010; Kepil Ozdemir et al., 2014), showing that basophil activation decrement was observed mainly in submaximal allergen stimulation in BAT experiments. However, in some studies, allergen-induced basophil CD203c expression did not change after 4 months of grass pollen sublingual immunotherapy (Horak et al., 2009). Erdmann et al. (2004) did not observe a change in basophil activation after 6 months of venom immunotherapy. Differences in the type of allergy, BAT markers used, time course of immunotherapy, and the allergen stimulation concentration may help explain the contradicting results in different studies.

This is the first study to assess the blocking function of serum IgG antibodies during HDM SCIT in allergic rhinitis and/or asthma patients by using the basophil activation inhibition assay. Studies have found that specific IgG4s have blocking activities by competing with sIgE for allergens to form allergen–IgE complexes. This inhibits complex binding to IgE receptor–expressing effector cells by means of the IgE-facilitated allergen binding assay (Wachholz et al., 2003; Shamji et al., 2012; Feng et al., 2018). These studies demonstrated that IgG antibodies could inhibit the allergen–IgE complex binding to the low-affinity IgE receptor (FcεRII, i.e., CD23) by use of a CD23-expressing Epstein–Barr virus-transformed B cell line and may thereby reduce allergen-specific T cell responses. We found that the serum obtained from SCIT patients significantly inhibited basophil activation and that basophils express high-affinity IgE receptor (FcεRI). Therefore, we demonstrated the effectiveness of IgG blocking activities in the high-affinity IgE receptor–expressing effector cell. We also found that the expression of CD63 in the basophil activation inhibition control (i.e., week 0) was higher than the basic basophil activation, and the Der p sIgE level significantly decreased at the late phase of SCIT. However, there was no correlation between the BAT inhibition assay and sIgE. The IgE-sensitized basophils may have already bound with IgE on the cell surface FcεRI, so that the FcεRI-bound IgE rather than serum free sIgE plays a more important role in the BAT inhibition assay.

Since the reduction of BAT appeared associated with the concentration of the triggering allergen, we considered the likely involvement of inhibiting antibodies. The inhibition experiments showed that serum from SCIT patients reduced basophil allergen threshold sensitivity but had no effect on basophil reactivity. We also depleted IgG4 from the serum of patients after completion of 2 years of SCIT and found that the IgG4-deficient serum demonstrated reduced inhibitory effects on basophil activation. Linear regression showed significant correlations between sIgG4 and the basophil activation inhibition assay. Thus, IgG4 antibodies appear to play an important role in reducing allergen sensitivity rather than reducing reactivity during AIT. IgG4 depletion could not remove the inhibition ability completely; the existence of other antibody isotypes such as IgA and IgG1 may also have inhibition ability (Santos et al., 2015). Further investigation on these antibody isotypes may help explain this phenomenon.

IgG4 is a unique antibody with a half-antibody exchange; it is also referred to as "Fab-arm exchange," which results in monovalent and non-crosslinking antibodies (Aalberse et al., 2009). IgG4 antibodies can bind to allergens and reduce the free allergen concentration but not induce effector cell activation. Our results are consistent with other studies. For example, Nopp et al. (2009) observed negative correlations between basophil activation and specific IgG antibodies (20-fold median increase of sIgG4 after 9 months of SCIT). Lalek et al. (2010) showed that birch-specific IgG antibodies are responsible for the reduction of basophil allergen threshold sensitivity

(fivefold median increase of sIgG4 after 2–4 months of SCIT). We reported 10-fold increases of sIgG4 after 4 months of SCIT. However, Horak et al. (2009) demonstrated that basophil responsiveness did not change after 4 months of grass sublingual immunotherapy and only found a twofold median increase in sIgG4 antibodies. These results may also explain why AIT had no or less effect on basophil reactivity (Lalek et al., 2010), as there were not sufficient antibodies to compete with IgE binding to the allergen.

Previous studies demonstrated that AIT could induce a substantial increase of sIgG4 antibodies and had blocking activity, but allergen-specific IgG does not always correlate with therapeutic effects (Bodtger et al., 2005). This could be explained by AIT not only increasing the level of serum IgG4 antibodies but also altering their specificity and/or affinity. Many studies have demonstrated that AIT not only increases the level of serum IgG4 antibodies but also alters their specificity (Michils et al., 1997, 1998). In venom immunotherapy, Michils et al. (1997) found that specificity change can occur at the beginning of treatment and long before the change of antibody titers could be detected. Kolbe et al. (1995) showed that administration of high concentrations of allergen in murine models alter not only antibody quantity but also affinity and specificity. However, Wachholz et al. (2003) showed that the binding affinities of allergen-specific IgG or IgE did not change after immunotherapy. Thus, the relationship between IgG4 and clinical efficacy requires further investigation.

CONCLUSION

We demonstrated the time course of both the cellular and humoral immune responses during HDM immunotherapy, including the induction of sIgG4 antibodies and reduction of basophil sensitivity to the allergen. We also demonstrated that an increase of Der p sIgG4 rather than a reduction of Der p sIgE correlates with the reduction of basophil activation. SCIT reduced the allergen basophil threshold sensitivity in Der p allergic rhinitis and/or asthma patients through induction of sIgG4.

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 the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JL designed the study, collected the data, and performed the statistical analysis. MF had responsibility for the experiments, performed the statistical analysis, and drafted the manuscript. XZ and RQ were involved in statistical analysis. QS, MX, and XS were involved in data collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00030/full#supplementary-material

FIGURE S1 | SMS and FEV1% over time. Allergen concentration curves for BAT, and IgG4 depletion BAT inhibition assay. Time course of mean SMS **(A)** and FEV_1% predicted **(B)** in the SCIT group and medication group. Der p induced basophil activation in blood samples from allergic patients with mite allergy (n = 10), and non-atopic controls (n = 5) were assayed with six mite extract concentrations. Results are expressed as percentages of $CD63^+$ basophils above basal values **(C)**. Fifteen serum samples from SCIT patients at week 104 underwent removal of IgG4 antibodies. IgG4-depleted and mock-depleted samples were incubated with 0.15 μg/mL Der p allergen before performance of the BAT. $^*P < 0.05$, $^{**}P < 0.01$ when compared with week 0; §$P < 0.05$ when compared with control. $^{***}P < 0.001$ refers to the comparison between IgG4- and mock-depleted paired samples. SMS = Combined symptom medication score; FEV_1 = Forced Expiratory Volume in one second.

FIGURE S2 | Time course of sIgG4, BAT, and BAT inhibition assay in Medication Group. Basophil CD63 responses to 0.15 and 15 μg/mL of Der p extract in Medication Group during the time course of BAT **(A)**. Time course of Der p sIgG4 in Medication Group **(B)**. The BAT inhibition assay was performed as serum from Medication Group patients incubated with 0.15 μg/mL (C; n = 35) or 15 μg/mL (D; n = 10) Der p allergen before performance of the BAT. Der p = *Dermatophagoides pteronyssinus*.

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Respiratory Viral Infections in Exacerbation of Chronic Airway Inflammatory Diseases: Novel Mechanisms and Insights From the Upper Airway Epithelium

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Respiratory virus infection is one of the major sources of exacerbation of chronic airway inflammatory diseases. These exacerbations are associated with high morbidity and even mortality worldwide. The current understanding on viral-induced exacerbations is that viral infection increases airway inflammation which aggravates disease symptoms. Recent advances in *in vitro* air-liquid interface 3D cultures, organoid cultures and the use of novel human and animal challenge models have evoked new understandings as to the mechanisms of viral exacerbations. In this review, we will focus on recent novel findings that elucidate how respiratory viral infections alter the epithelial barrier in the airways, the upper airway microbial environment, epigenetic modifications including miRNA modulation, and other changes in immune responses throughout the upper and lower airways. First, we reviewed the prevalence of different respiratory viral infections in causing exacerbations in chronic airway inflammatory diseases. Subsequently we also summarized how recent models have expanded our appreciation of the mechanisms of viral-induced exacerbations. Further we highlighted the importance of the virome within the airway microbiome environment and its impact on subsequent bacterial infection. This review consolidates the understanding of viral induced exacerbation in chronic airway inflammatory diseases and indicates pathways that may be targeted for more effective management of chronic inflammatory diseases.

Keywords: chronic airway inflammatory diseases, respiratory virus, acute exacerbation, upper airway, epithelium

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SEARCH STRATEGY

Search performed between July to November 2019, results is as of 15th November 2019

1. (virus OR viral) AND (chronic airway inflammat* OR airway inflamma* OR inflammat* OR asthma OR rhinosinusitis OR COPD OR Chronic Obstructive Pulmonary Disease) **61513 results**
2. (viral OR virus) AND (asthma OR rhinosinusitis OR COPD OR chronic obstructive pulmonary disease chronic OR chronic OR inflammation OR chronic inflammation) AND (airway OR lung OR nose OR nasal OR upper airway OR lower airway) **10622 results**
3. (virus OR viral) AND (chronic airway inflammat* OR airway inflamma* OR inflammat* OR asthma OR rhinosinusitis OR COPD OR Chronic Obstructive Pulmonary Disease) AND (epitheli*) **5029 results**
4. (viral OR virus) AND exacerbation AND (asthma OR rhinosinusitis OR COPD OR chronic obstructive pulmonary disease OR chronic OR inflammation OR airway) **1916 results**
5. (viral OR virus) AND exacerbation AND (asthma OR rhinosinusitis OR COPD OR chronic obstructive pulmonary disease chronic OR chronic OR inflammation OR chronic inflammation) AND (airway OR lung OR nose OR nasal OR upper airway OR lower airway) **641 results**
6. (viral OR virus) AND exacerbation AND (asthma OR rhinosinusitis OR COPD OR chronic obstructive pulmonary disease OR chronic OR inflammation OR airway) AND (epitheli*) **177 results**
7. (viral OR virus) AND exacerbation AND (asthma OR rhinosinusitis OR COPD OR chronic obstructive pulmonary disease chronic OR chronic OR inflammation OR chronic inflammation) AND (airway OR lung OR nose OR nasal OR upper airway OR lower airway) AND (epitheli*) **150 results**.

Additional literature was retrieved from citations within the articles of interest.

Article selection was performed with a focus on works from years 2009 to 2019.

INTRODUCTION

The prevalence of chronic airway inflammatory disease is increasing worldwide especially in developed nations (GBD 2015 Chronic Respiratory Disease Collaborators, 2017; Guan et al., 2018). This disease is characterized by airway inflammation leading to complications such as coughing, wheezing and shortness of breath. The disease can manifest in both the upper airway (such as chronic rhinosinusitis, CRS) and lower airway (such as asthma and chronic obstructive pulmonary disease, COPD) which greatly affect the patients' quality of life (Calus et al., 2012; Bao et al., 2015). Treatment and management vary greatly in efficacy due to the complexity and heterogeneity of the disease. This is further complicated by the effect of episodic exacerbations of the disease, defined as worsening of disease symptoms including wheeze, cough, breathlessness and chest tightness (Xepapadaki and Papadopoulos, 2010). Such exacerbations are due to the effect of enhanced acute airway inflammation impacting upon and worsening the symptoms of the existing disease (Hashimoto et al., 2008; Viniol and Vogelmeier, 2018). These acute exacerbations are the main cause of morbidity and sometimes mortality in patients, as well as resulting in major economic burdens worldwide. However, due to the complex interactions between the host and the exacerbation agents, the mechanisms of exacerbation may vary considerably in different individuals under various triggers.

Acute exacerbations are usually due to the presence of environmental factors such as allergens, pollutants, smoke, cold or dry air and pathogenic microbes in the airway (Gautier and Charpin, 2017; Viniol and Vogelmeier, 2018). These agents elicit an immune response leading to infiltration of activated immune cells that further release inflammatory mediators that cause acute symptoms such as increased mucus production, cough, wheeze and shortness of breath. Among these agents, viral infection is one of the major drivers of asthma exacerbations accounting for up to 80–90% and 45–80% of exacerbations in children and adults respectively (Grissell et al., 2005; Xepapadaki and Papadopoulos, 2010; Jartti and Gern, 2017; Adeli et al., 2019). Viral involvement in COPD exacerbation is also equally high, having been detected in 30–80% of acute COPD exacerbations (Kherad et al., 2010; Jafarinejad et al., 2017; Stolz et al., 2019). Whilst the prevalence of viral exacerbations in CRS is still unclear, its prevalence is likely to be high due to the similar inflammatory nature of these diseases (Rowan et al., 2015; Tan et al., 2017). One of the reasons for the involvement of respiratory viruses' in exacerbations is their ease of transmission and infection (Kutter et al., 2018). In addition, the high diversity of the respiratory viruses may also contribute to exacerbations of different nature and severity (Busse et al., 2010; Costa et al., 2014; Jartti and Gern, 2017). Hence, it is important to identify the exact mechanisms underpinning viral exacerbations in susceptible subjects in order to properly manage exacerbations via supplementary treatments that may alleviate the exacerbation symptoms or prevent severe exacerbations.

While the lower airway is the site of dysregulated inflammation in most chronic airway inflammatory diseases, the upper airway remains the first point of contact with sources of exacerbation. Therefore, their interaction with the exacerbation agents may directly contribute to the subsequent responses in the lower airway, in line with the "United Airway" hypothesis. To elucidate the host airway interaction with viruses leading to exacerbations, we thus focus our review on recent findings of viral interaction with the upper airway. We compiled how viral induced changes to the upper airway may contribute to chronic airway inflammatory disease exacerbations, to provide a unified elucidation of the potential exacerbation mechanisms initiated from predominantly upper airway infections.

SIGNIFICANCE OF VIRUS INFECTION IN EXACERBATION OF CHRONIC AIRWAY INFLAMMATORY DISEASES

Despite being a major cause of exacerbation, reports linking respiratory viruses to acute exacerbations only start to emerge in the late 1950s (Pattemore et al., 1992); with bacterial infections previously considered as the likely culprit for acute exacerbation (Stevens, 1953; Message and Johnston, 2002). However, with the advent of PCR technology, more viruses were recovered during acute exacerbations events and reports implicating their role emerged in the late 1980s (Message and Johnston, 2002). Rhinovirus (RV) and respiratory syncytial virus (RSV) are the predominant viruses linked to the development and exacerbation of chronic airway inflammatory diseases (Jartti and Gern, 2017). Other viruses such as parainfluenza virus (PIV), influenza virus (IFV) and adenovirus (AdV) have also been implicated in acute exacerbations but to a much lesser extent (Johnston et al., 2005; Oliver et al., 2014; Ko et al., 2019). More recently, other viruses including bocavirus (BoV), human metapneumovirus (HMPV), certain coronavirus (CoV) strains, a specific enterovirus (EV) strain EV-D68, human cytomegalovirus (hCMV) and herpes simplex virus (HSV) have been reported as contributing to acute exacerbations (Zheng et al., 2018). The common feature these viruses share is that they can infect both the upper and/or lower airway, further increasing the inflammatory conditions in the diseased airway (Mallia and Johnston, 2006; Britto et al., 2017).

Respiratory viruses primarily infect and replicate within airway epithelial cells (Costa et al., 2014). During the replication process, the cells release antiviral factors and cytokines that alter local airway inflammation and airway niche (Busse et al., 2010). In a healthy airway, the inflammation normally leads to type 1 inflammatory responses consisting of activation of an antiviral state and infiltration of antiviral effector cells. This eventually results in the resolution of the inflammatory response and clearance of the viral infection (Vareille et al., 2011; Braciale et al., 2012). However, in a chronically inflamed airway, the responses against the virus may be impaired or aberrant, causing sustained inflammation and erroneous infiltration, resulting in the exacerbation of their symptoms (Mallia and Johnston, 2006; Dougherty and Fahy, 2009; Busse et al., 2010; Britto et al., 2017; Linden et al., 2019). This is usually further compounded by the increased susceptibility of chronic airway inflammatory disease patients toward viral respiratory infections, thereby increasing the frequency of exacerbation as a whole (Dougherty and Fahy, 2009; Busse et al., 2010; Linden et al., 2019).

Furthermore, due to the different replication cycles and response against the myriad of respiratory viruses, each respiratory virus may also contribute to exacerbations via different mechanisms that may alter their severity. Hence, this review will focus on compiling and collating the current known mechanisms of viral-induced exacerbation of chronic airway inflammatory diseases; as well as linking the different viral infection pathogenesis to elucidate other potential ways the infection can exacerbate the disease. The review will serve to provide further understanding of viral induced exacerbation to identify potential pathways and pathogenesis mechanisms that may be targeted as supplementary care for management and prevention of exacerbation. Such an approach may be clinically significant due to the current scarcity of antiviral drugs for the management of viral-induced exacerbations. This will improve the quality of life of patients with chronic airway inflammatory diseases.

CURRENT UNDERSTANDING OF VIRAL INDUCED EXACERBATION OF CHRONIC AIRWAY INFLAMMATORY DISEASE

Once the link between viral infection and acute exacerbations of chronic airway inflammatory disease was established, there have been many reports on the mechanisms underlying the exacerbation induced by respiratory viral infection. Upon infecting the host, viruses evoke an inflammatory response as a means of counteracting the infection. Generally, infected airway epithelial cells release type I (IFNα/β) and type III (IFNλ) interferons, cytokines and chemokines such as IL-6, IL-8, IL-12, RANTES, macrophage inflammatory protein 1α (MIP-1α) and monocyte chemotactic protein 1 (MCP-1) (Wark and Gibson, 2006; Matsukura et al., 2013). These, in turn, enable infiltration of innate immune cells and of professional antigen presenting cells (APCs) that will then in turn release specific mediators to facilitate viral targeting and clearance, including type II interferon (IFNγ), IL-2, IL-4, IL-5, IL-9, and IL-12 (Wark and Gibson, 2006; Singh et al., 2010; Braciale et al., 2012). These factors heighten local inflammation and the infiltration of granulocytes, T-cells and B-cells (Wark and Gibson, 2006; Braciale et al., 2012). The increased inflammation, in turn, worsens the symptoms of airway diseases.

Additionally, in patients with asthma and patients with CRS with nasal polyp (CRSwNP), viral infections such as RV and RSV promote a Type 2-biased immune response (Becker, 2006; Jackson et al., 2014; Jurak et al., 2018). This amplifies the basal type 2 inflammation resulting in a greater release of IL-4, IL-5, IL-13, RANTES and eotaxin and a further increase in eosinophilia, a key pathological driver of asthma and CRSwNP (Wark and Gibson, 2006; Singh et al., 2010; Chung et al., 2015; Dunican and Fahy, 2015). Increased eosinophilia, in turn, worsens the classical symptoms of disease and may further lead to life-threatening conditions due to breathing difficulties. On the other hand, patients with COPD and patients with CRS without nasal polyp (CRSsNP) are more neutrophilic in nature due to the expression of neutrophil chemoattractants such as CXCL9, CXCL10, and CXCL11 (Cukic et al., 2012; Brightling and Greening, 2019). The pathology of these airway diseases is characterized by airway remodeling due to the presence of remodeling factors such as matrix metalloproteinases (MMPs) released from infiltrating neutrophils (Linden et al., 2019). Viral infections in such conditions will then cause increase neutrophilic activation; worsening the symptoms and airway remodeling in the airway thereby exacerbating COPD, CRSsNP

and even CRSwNP in certain cases (Wang et al., 2009; Tacon et al., 2010; Linden et al., 2019).

An epithelial-centric alarmin pathway around IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), and their interaction with group 2 innate lymphoid cells (ILC2) has also recently been identified (Nagarkar et al., 2012; Hong et al., 2018; Allinne et al., 2019). IL-25, IL-33 and TSLP are type 2 inflammatory cytokines expressed by the epithelial cells upon injury to the epithelial barrier (Gabryelska et al., 2019; Roan et al., 2019). ILC2s are a group of lymphoid cells lacking both B and T cell receptors but play a crucial role in secreting type 2 cytokines to perpetuate type 2 inflammation when activated (Scanlon and McKenzie, 2012; Li and Hendriks, 2013). In the event of viral infection, cell death and injury to the epithelial barrier will also induce the expression of IL-25, IL-33 and TSLP, with heighten expression in an inflamed airway (Allakhverdi et al., 2007; Goldsmith et al., 2012; Byers et al., 2013; Shaw et al., 2013; Beale et al., 2014; Jackson et al., 2014; Uller and Persson, 2018; Ravanetti et al., 2019). These 3 cytokines then work in concert to activate ILC2s to further secrete type 2 cytokines IL-4, IL-5, and IL-13 which further aggravate the type 2 inflammation in the airway causing acute exacerbation (Camelo et al., 2017). In the case of COPD, increased ILC2 activation, which retain the capability of differentiating to ILC1, may also further augment the neutrophilic response and further aggravate the exacerbation (Silver et al., 2016). Interestingly, these factors are not released to any great extent and do not activate an ILC2 response during viral infection in healthy individuals (Yan et al., 2016; Tan et al., 2018a); despite augmenting a type 2 exacerbation in chronically inflamed airways (Jurak et al., 2018). These classical mechanisms of viral induced acute exacerbations are summarized in **Figure 1**.

NOVEL MECHANISMS IN THE UPPER AIRWAY HYPOTHESIZED TO CONTRIBUTE TO VIRAL INDUCED ACUTE EXACERBATIONS

As integration of the virology, microbiology and immunology of viral infection becomes more interlinked, additional factors and

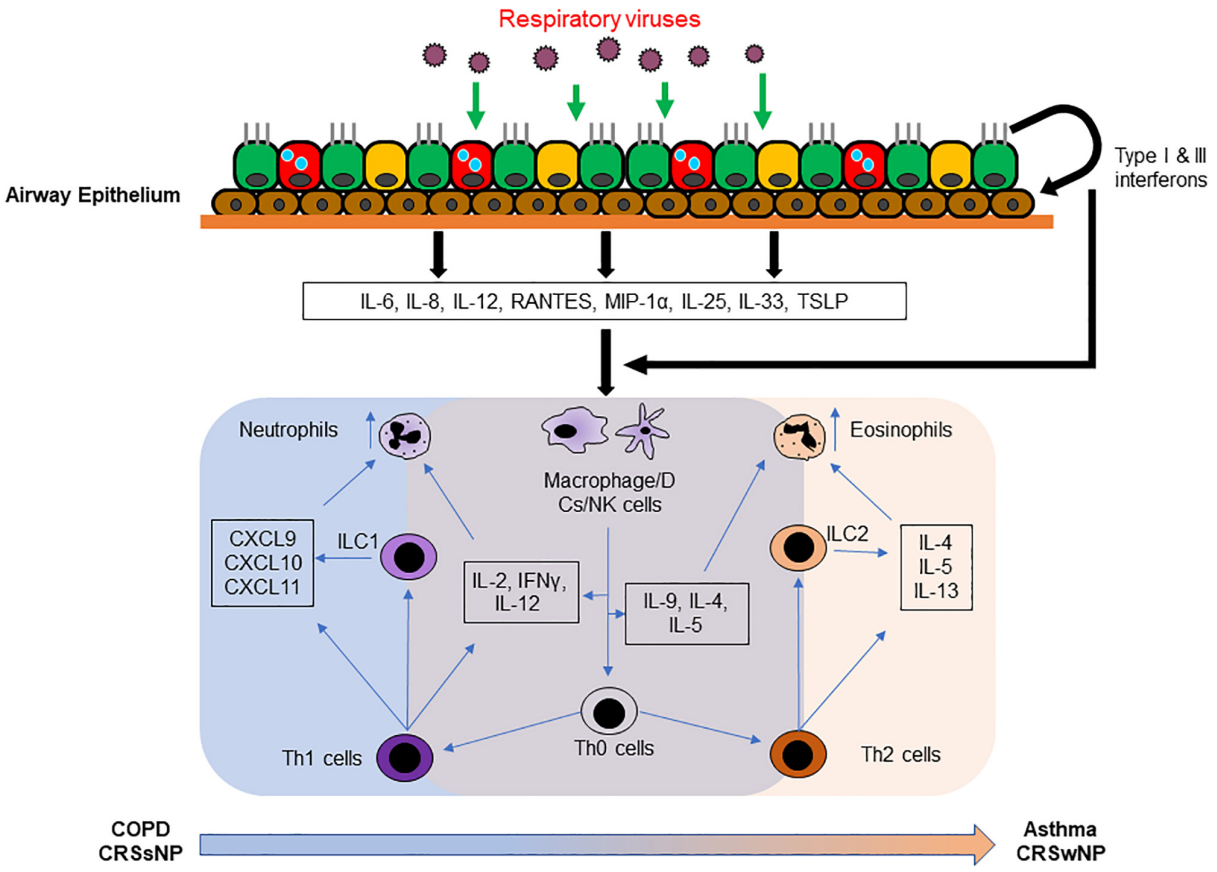

FIGURE 1 | Current understanding of viral induced exacerbation of chronic airway inflammatory diseases. Upon virus infection in the airway, antiviral state will be activated to clear the invading pathogen from the airway. Immune response and injury factors released from the infected epithelium normally would induce a rapid type 1 immunity that facilitates viral clearance. However, in the inflamed airway, the cytokines and chemokines released instead augmented the inflammation present in the chronically inflamed airway, strengthening the neutrophilic infiltration in COPD airway, and eosinophilic infiltration in the asthmatic airway. The effect is also further compounded by the participation of Th1 and ILC1 cells in the COPD airway; and Th2 and ILC2 cells in the asthmatic airway.

mechanisms have been implicated in acute exacerbations during and after viral infection (Murray et al., 2006). Murray et al. (2006) has underlined the synergistic effect of viral infection with other sensitizing agents in causing more severe acute exacerbations in the airway. This is especially true when not all exacerbation events occurred during the viral infection but may also occur well after viral clearance (Kim et al., 2008; Stolz et al., 2019) in particular the late onset of a bacterial infection (Singanayagam et al., 2018, 2019a). In addition, viruses do not need to directly infect the lower airway to cause an acute exacerbation, as the nasal epithelium remains the primary site of most infections. Moreover, not all viral infections of the airway will lead to acute exacerbations, suggesting a more complex interplay between the virus and upper airway epithelium which synergize with the local airway environment in line with the "united airway" hypothesis (Kurai et al., 2013). On the other hand, viral infections or their components persist in patients with chronic airway inflammatory disease (Kling et al., 2005; Wood et al., 2011; Ravi et al., 2019). Hence, their presence may further alter the local environment and contribute to current and future exacerbations. Future studies should be performed using metagenomics in addition to PCR analysis to determine the contribution of the microbiome and mycobiome to viral infections. In this review, we highlight recent data regarding viral interactions with the airway epithelium that could also contribute to, or further aggravate, acute exacerbations of chronic airway inflammatory diseases.

Increase Viral Susceptibility and Prolong Activation of Inflammation

Patients with chronic airway inflammatory diseases have impaired or reduced ability of viral clearance (Hammond et al., 2015; McKendry et al., 2016; Akbarshahi et al., 2018; Gill et al., 2018; Wang et al., 2018; Singanayagam et al., 2019b). Their impairment stems from a type 2-skewed inflammatory response which deprives the airway of important type 1 responsive CD8 cells that are responsible for the complete clearance of virus-infected cells (Becker, 2006; McKendry et al., 2016). This is especially evident in weak type 1 inflammation-inducing viruses such as RV and RSV (Kling et al., 2005; Wood et al., 2011; Ravi et al., 2019). Additionally, there are also evidence of reduced type I (IFNβ) and III (IFNλ) interferon production due to type 2-skewed inflammation, which contributes to imperfect clearance of the virus resulting in persistence of viral components, or the live virus in the airway epithelium (Contoli et al., 2006; Hwang et al., 2019; Wark, 2019). Due to the viral components remaining in the airway, antiviral genes such as type I interferons, inflammasome activating factors and cytokines remained activated resulting in prolong airway inflammation (Wood et al., 2011; Essaidi-Laziosi et al., 2018). These factors enhance granulocyte infiltration thus prolonging the exacerbation symptoms. Such persistent inflammation may also be found within DNA viruses such as AdV, hCMV and HSV, whose infections generally persist longer (Imperiale and Jiang, 2015), further contributing to chronic activation of inflammation when they infect the airway (Yang et al., 2008; Morimoto et al., 2009; Imperiale and Jiang, 2015; Lan et al., 2016; Tan et al., 2016; Kowalski et al., 2017). With that note, human papilloma virus (HPV), a DNA virus highly associated with head and neck cancers and respiratory papillomatosis, is also linked with the chronic inflammation that precedes the malignancies (de Visser et al., 2005; Gillison et al., 2012; Bonomi et al., 2014; Fernandes et al., 2015). Therefore, the role of HPV infection in causing chronic inflammation in the airway and their association to exacerbations of chronic airway inflammatory diseases, which is scarcely explored, should be investigated in the future. Furthermore, viral persistence which lead to continuous expression of antiviral genes may also lead to the development of steroid resistance, which is seen with RV, RSV, and PIV infection (Chi et al., 2011; Ford et al., 2013; Papi et al., 2013). The use of steroid to suppress the inflammation may also cause the virus to linger longer in the airway due to the lack of antiviral clearance (Kim et al., 2008; Hammond et al., 2015; Hewitt et al., 2016; McKendry et al., 2016; Singanayagam et al., 2019b). The concomitant development of steroid resistance together with recurring or prolong viral infection thus added considerable burden to the management of acute exacerbation, which should be the future focus of research to resolve the dual complications arising from viral infection.

Destruction of the Epithelial Barrier

On the other end of the spectrum, viruses that induce strong type 1 inflammation and cell death such as IFV (Yan et al., 2016; Guibas et al., 2018) and certain CoV (including the recently emerged COVID-19 virus) (Tao et al., 2013; Yue et al., 2018; Zhu et al., 2020), may not cause prolonged inflammation due to strong induction of antiviral clearance. These infections, however, cause massive damage and cell death to the epithelial barrier, so much so that areas of the epithelium may be completely absent post infection (Yan et al., 2016; Tan et al., 2019). Factors such as RANTES and CXCL10, which recruit immune cells to induce apoptosis, are strongly induced from IFV infected epithelium (Ampomah et al., 2018; Tan et al., 2019). Additionally, necroptotic factors such as RIP3 further compounds the cell deaths in IFV infected epithelium (Tan et al., 2019). The massive cell death induced may result in worsening of the acute exacerbation due to the release of their cellular content into the airway, further evoking an inflammatory response in the airway (Guibas et al., 2018). Moreover, the destruction of the epithelial barrier may cause further contact with other pathogens and allergens in the airway which may then prolong exacerbations or results in new exacerbations. Epithelial destruction may also promote further epithelial remodeling during its regeneration as viral infection induces the expression of remodeling genes such as MMPs and growth factors (Tan et al., 2017). Infections that cause massive destruction of the epithelium, such as IFV, usually result in severe acute exacerbations with non-classical symptoms of chronic airway inflammatory diseases. Fortunately, annual vaccines are available to prevent IFV infections (Vasileiou et al., 2017; Zheng et al., 2018); and it is recommended that patients with chronic airway inflammatory disease receive their annual influenza vaccination as the best means to prevent severe IFV induced exacerbation.

Augmentation of Infiltration by Increasing Barrier Leakiness

Another mechanism that viral infections may use to drive acute exacerbations is the induction of vasodilation or tight junction opening factors which may increase the rate of infiltration. Infection with a multitude of respiratory viruses causes disruption of tight junctions with the resulting increased rate of viral infiltration. This also increases the chances of allergens coming into contact with airway immune cells. For example, IFV infection was found to induce oncostatin M (OSM) which causes tight junction opening (Pothoven et al., 2015; Tian et al., 2018). Similarly, RV and RSV infections usually cause tight junction opening which may also increase the infiltration rate of eosinophils and thus worsening of the classical symptoms of chronic airway inflammatory diseases (Sajjan et al., 2008; Kast et al., 2017; Kim et al., 2018). In addition, the expression of vasodilating factors and fluid homeostatic factors such as angiopoietin-like 4 (ANGPTL4) and bactericidal/permeability-increasing fold-containing family member A1 (BPIFA1) are also associated with viral infections and pneumonia development, which may worsen inflammation in the lower airway (Li et al., 2015; Akram et al., 2018). These factors may serve as targets to prevent viral-induced exacerbations during the management of acute exacerbation of chronic airway inflammatory diseases.

Alteration of Airway Microbiome

Another recent area of interest is the relationship between asthma and COPD exacerbations and their association with the airway microbiome. The development of chronic airway inflammatory diseases is usually linked to specific bacterial species in the microbiome which may thrive in the inflamed airway environment (Diver et al., 2019). In the event of a viral infection such as RV infection, the effect induced by the virus may destabilize the equilibrium of the microbiome present (Molyneaux et al., 2013; Kloepfer et al., 2014; Kloepfer et al., 2017; Jubinville et al., 2018; van Rijn et al., 2019). In addition, viral infection may disrupt biofilm colonies in the upper airway (e.g., *Streptococcus pneumoniae*) microbiome to be release into the lower airway and worsening the inflammation (Marks et al., 2013; Chao et al., 2014). Moreover, a viral infection may also alter the nutrient profile in the airway through release of previously inaccessible nutrients that will alter bacterial growth (Siegel et al., 2014; Mallia et al., 2018). Furthermore, the destabilization is further compounded by impaired bacterial immune response, either from direct viral influences, or use of corticosteroids to suppress the exacerbation symptoms (Singanayagam et al., 2018, 2019a; Wang et al., 2018; Finney et al., 2019). All these may gradually lead to more far reaching effect when normal flora is replaced with opportunistic pathogens, altering the inflammatory profiles (Teo et al., 2018). These changes may in turn result in more severe and frequent acute exacerbations due to the interplay between virus and pathogenic bacteria in exacerbating chronic airway inflammatory diseases (Wark et al., 2013; Singanayagam et al., 2018). To counteract these effects, microbiome-based therapies are in their infancy but have shown efficacy in the treatments of irritable bowel syndrome by restoring the intestinal microbiome (Bakken et al., 2011). Further research can be done similarly for the airway microbiome to be able to restore the microbiome following disruption by a viral infection.

Disruption of Mucocilary Functions and Balance

Viral infections can cause the disruption of mucociliary function, an important component of the epithelial barrier. Ciliary proteins

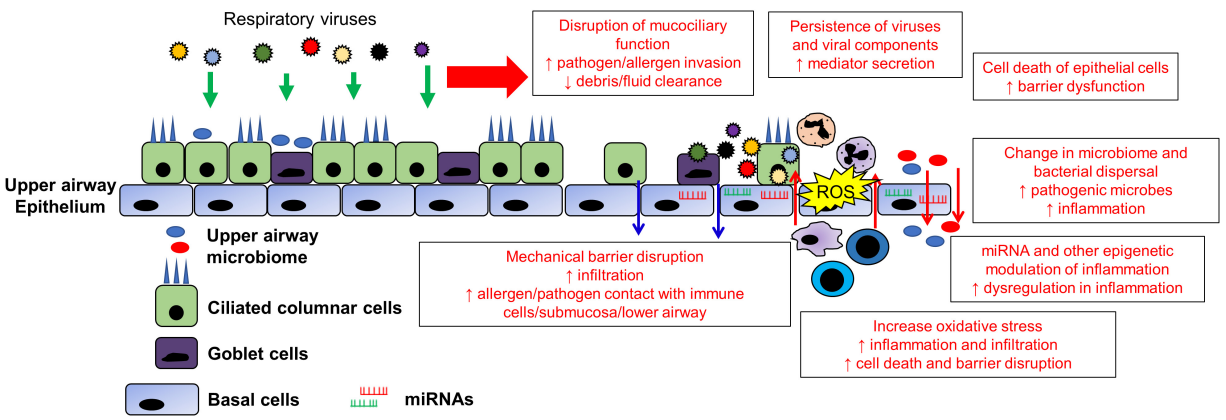

FIGURE 2 | Changes in the upper airway epithelium contributing to viral exacerbation in chronic airway inflammatory diseases. The upper airway epithelium is the primary contact/infection site of most respiratory viruses. Therefore, its infection by respiratory viruses may have far reaching consequences in augmenting and synergizing current and future acute exacerbations. The destruction of epithelial barrier, mucociliary function and cell death of the epithelial cells serves to increase contact between environmental triggers with the lower airway and resident immune cells. The opening of tight junction increasing the leakiness further augments the inflammation and exacerbations. In addition, viral infections are usually accompanied with oxidative stress which will further increase the local inflammation in the airway. The dysregulation of inflammation can be further compounded by modulation of miRNAs and epigenetic modification such as DNA methylation and histone modifications that promote dysregulation in inflammation. Finally, the change in the local airway environment and inflammation promotes growth of pathogenic bacteria that may replace the airway microbiome. Furthermore, the inflammatory environment may also disperse upper airway commensals into the lower airway, further causing inflammation and alteration of the lower airway environment, resulting in prolong exacerbation episodes following viral infection.

TABLE 1 | Summary of literature evidence of potential viral induced exacerbation mechanisms in chronic airway inflammatory diseases at the upper airway epithelium.

Types of exacerbation mechanism	Viral specific trait contributing to exacerbation mechanism (with literature evidence)
Increased viral susceptibility and prolonged activation of inflammation	Weak type 1 inflammation leading to skewed type 2 inflammation (RV, RSV) Persistence of virus and viral components (RV, RSV, AdV, hCMV, HSV) Development of steroid resistance (RV, RSV, PIV)
Destruction of the epithelial barrier	Diffused cell death in the epithelial layer (IFV, CoV)
Augmentation of infiltration by increasing barrier leakiness	Disruption of tight junctions (RV, RSV) Oncostatin M induction (IFV) ANGPTL4 induction (IFV) BPIFA1 changes (IFV)
Alteration of airway microbiome	Destabilization of the microbiome (RV) Disruption of biofilm colonies (IFV) Alteration of the airway nutrient profile (RV, IFV) Reduced bacterial immunity (RV, possibly IFV and RSV)
Disruption of mucociliary functions and balance	Infection targeting ciliated cells (RV, IFV, RSV) Alteration of ciliary gene expression (IFV) Destruction of cilia and disruption of ciliary function (RSV, CoV) Mucus overproduction (RV)
miRNA and other epigenetic modulation of inflammation	miRNA modulation (IFV, RV, RSV) DNA methylation and histone modifications (RV, RSV)
Oxidative stress	ROS production (RV, RSV, IFV, HSV)

As RV, RSV, and IFV were the most frequently studied viruses in chronic airway inflammatory diseases, most of the viruses listed are predominantly these viruses. However, the mechanisms stated here may also be applicable to other viruses but may not be listed as they were not implicated in the context of chronic airway inflammatory diseases exacerbation (see text for abbreviations).

that aid in the proper function of the motile cilia in the airways are aberrantly expressed in ciliated airway epithelial cells which are the major target for RV infection (Griggs et al., 2017). Such form of secondary cilia dyskinesia appears to be present with chronic inflammations in the airway, but the exact mechanisms are still unknown (Peng et al., 2018, 2019; Qiu et al., 2018). Nevertheless, it was found that in viral infection such as IFV, there can be a change in the metabolism of the cells as well as alteration in the ciliary gene expression, mostly in the form of down-regulation of the genes such as dynein axonemal heavy chain 5 (DNAH5) and multiciliate differentiation And DNA synthesis associated cell cycle protein (MCIDAS) (Tan et al., 2018b, 2019). The recently emerged Wuhan CoV was also found to reduce ciliary beating in infected airway epithelial cell model (Zhu et al., 2020). Furthermore, viral infections such as RSV was shown to directly destroy the cilia of the ciliated cells and almost all respiratory viruses infect the ciliated cells (Jumat et al., 2015; Yan et al., 2016; Tan et al., 2018a). In addition, mucus overproduction may also disrupt the equilibrium of the mucociliary function following viral infection, resulting in symptoms of acute exacerbation (Zhu et al., 2009). Hence, the disruption of the ciliary movement during viral infection may cause more foreign material and allergen to enter the airway, aggravating the symptoms of acute exacerbation and making it more difficult to manage. The mechanism of the occurrence of secondary cilia dyskinesia can also therefore be explored as a means to limit the effects of viral induced acute exacerbation.

miRNA and Other Epigenetic Modulation of Inflammation

MicroRNAs (miRNAs) are short non-coding RNAs involved in post-transcriptional modulation of biological processes, and implicated in a number of diseases (Tan et al., 2014). miRNAs are found to be induced by viral infections and may play a role in the modulation of antiviral responses and inflammation (Gutierrez et al., 2016; Deng et al., 2017; Feng et al., 2018). In the case of chronic airway inflammatory diseases, circulating miRNA changes were found to be linked to exacerbation of the diseases (Wardzynska et al., 2020). Therefore, it is likely that such miRNA changes originated from the infected epithelium and responding immune cells, which may serve to further dysregulate airway inflammation leading to exacerbations. Both IFV and RSV infections has been shown to increase miR-21 and augmented inflammation in experimental murine asthma models, which is reversed with a combination treatment of anti-miR-21 and corticosteroids (Kim et al., 2017). IFV infection is also shown to increase miR-125a and b, and miR-132 in COPD epithelium which inhibits A20 and MAVS; and p300 and IRF3, respectively, resulting in increased susceptibility to viral infections (Hsu et al., 2016, 2017). Conversely, miR-22 was shown to be suppressed in asthmatic epithelium in IFV infection which lead to aberrant epithelial response, contributing to exacerbations (Moheimani et al., 2018). Other than these direct evidence of miRNA changes in contributing to exacerbations, an increased number of miRNAs and other non-coding RNAs responsible for immune modulation are found to be altered following viral infections (Globinska et al., 2014; Feng et al., 2018; Hasegawa et al., 2018). Hence non-coding RNAs also presents as targets to modulate viral induced airway changes as a means of managing exacerbation of chronic airway inflammatory diseases. Other than miRNA modulation, other epigenetic modification such as DNA methylation may also play a role in exacerbation of chronic airway inflammatory diseases. Recent epigenetic studies have indicated the association of epigenetic modification and chronic airway inflammatory diseases, and that the nasal methylome was shown to be a sensitive marker for airway inflammatory changes (Cardenas et al., 2019; Gomez, 2019). At the same time, it was also shown that viral infections such as RV and RSV alters DNA methylation and histone modifications in the airway epithelium which may alter inflammatory responses, driving chronic airway inflammatory diseases and exacerbations (McErlean et al., 2014; Pech et al., 2018; Caixia et al., 2019). In addition, Spalluto et al. (2017) also showed that antiviral factors such as IFNγ epigenetically modifies the viral resistance of epithelial cells. Hence, this may indicate that infections such as RV and RSV that weakly induce antiviral responses may result in an altered inflammatory state contributing to further viral persistence and exacerbation of chronic airway inflammatory diseases (Spalluto et al., 2017).

Oxidative Stress

Finally, viral infection can result in enhanced production of reactive oxygen species (ROS), oxidative stress and mitochondrial dysfunction in the airway epithelium (Kim et al., 2018; Mishra et al., 2018; Wang et al., 2018). The airway epithelium of patients with chronic airway inflammatory diseases are usually under a state of constant oxidative stress which sustains the inflammation in the airway (Barnes, 2017; van der Vliet et al., 2018). Viral infections of the respiratory epithelium by viruses such as IFV, RV, RSV and HSV may trigger the further production of ROS as an antiviral mechanism (Liu et al., 2017; To et al., 2017; Aizawa et al., 2018; Wang et al., 2018). Moreover, infiltrating cells in response to the infection such as neutrophils will also trigger respiratory burst as a means of increasing the ROS in the infected region. The increased ROS and oxidative stress in the local environment may serve as a trigger to promote inflammation thereby aggravating the inflammation in the airway (Tiwari et al., 2002). A summary of potential exacerbation mechanisms and the associated viruses is shown in **Figure 2** and **Table 1**.

CLINICAL SIGNIFICANCE OF IDENTIFYING ADDITIONAL MECHANISMS OF ACUTE EXACERBATIONS

While the mechanisms underlying the development and acute exacerbation of chronic airway inflammatory disease is extensively studied for ways to manage and control the disease, a viral infection does more than just causing an acute exacerbation in these patients. A viral-induced acute exacerbation not only induced and worsens the symptoms of the disease, but also may alter the management of the disease or confer resistance toward treatments that worked before. Hence, appreciation of the mechanisms of viral-induced acute exacerbations is of clinical significance to devise strategies to correct viral induce changes that may worsen chronic airway inflammatory disease symptoms. Further studies in natural exacerbations and in viral-challenge models using RNA-sequencing (RNA-seq) or single cell RNA-seq on a range of time-points may provide important information regarding viral pathogenesis and changes induced within the airway of chronic airway inflammatory disease patients to identify novel targets and pathway for improved management of the disease. Subsequent analysis of functions may use epithelial cell models such as the air-liquid interface, *in vitro* airway epithelial model that has been adapted to studying viral infection and the changes it induced in the airway (Yan et al., 2016; Boda et al., 2018; Tan et al., 2018a). Animal-based diseased models have also been developed to identify systemic mechanisms of acute exacerbation (Shin, 2016; Gubernatorova et al., 2019; Tanner and Single, 2019). Furthermore, the humanized mouse model that possess human immune cells may also serves to unravel the immune profile of a viral infection in healthy and diseased condition (Ito et al., 2019; Li and Di Santo, 2019). For milder viruses, controlled *in vivo* human infections can be performed for the best mode of verification of the associations of the virus with the proposed mechanism of viral induced acute exacerbations (Ravi et al., 2019). With the advent of suitable diseased models, the verification of the mechanisms will then provide the necessary continuation of improving the management of viral induced acute exacerbations.

CONCLUSION AND FUTURE OUTLOOK

In conclusion, viral-induced acute exacerbation of chronic airway inflammatory disease is a significant health and economic burden that needs to be addressed urgently. In view of the scarcity of antiviral-based preventative measures available for only a few viruses and vaccines that are only available for IFV infections, more alternative measures should be explored to improve the management of the disease. Alternative measures targeting novel viral-induced acute exacerbation mechanisms, especially in the upper airway, can serve as supplementary treatments of the currently available management strategies to augment their efficacy. New models including primary human bronchial or nasal epithelial cell cultures, organoids or precision cut lung slices from patients with airways disease rather than healthy subjects can be utilized to define exacerbation mechanisms. These mechanisms can then be validated in small clinical trials in patients with asthma or COPD. Having multiple means of treatment may also reduce the problems that arise from resistance development toward a specific treatment.

AUTHOR CONTRIBUTIONS

KT, VC, and DW contributed to the initial conceptualization of the manuscript. KT, RL, JL, HO, and VT contributed to literature search. KT, HL, JL, HO, VT, HL, IA, VC, and DW contributed to literature selection. KT, RL, IA, and VC contributed to the writing of manuscript. KT, RL, HL, KC, IA, VC, and DW contributed to the review and finalization of the manuscript.

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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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Olfactory Sensitivity Is Related to Erectile Function in Adult Males

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Background: The olfactory system influences human social behavior, in particular the selection of a spouse. However, there is currently a lack of clinical research on the relationship between the olfactory system and erectile dysfunction (ED) in adult males.

Aim: We explored the association between olfactory sensitivity and erectile function and its possible mechanisms.

Results: A total of 574 patients, adult males aged between 19 and 42 years, diagnosed with ED in the Department of Infertility and Sexual Medicine of the Third Affiliated Hospital of Sun Yat-sen University from 2015 to 2018 were analyzed retrospectively. Among them, 115 patients (20.03%) had rhinologic diseases (RDs). In addition, in 201 adult male patients who underwent nasal surgery in the ENT department from 2012 to 2016, including 29 (14.43%) with ED, nasal congestion, nasal discharge, and hyposmia were the most common complaints based on the numerical rating scale (NRS). Furthermore, a prospective study was performed in a total of 102 sequential outpatients (male adults) with RD only (n = 46), ED only (n = 42) and both RD and ED (n = 14) in 2019, together with 40 healthy (male adults) volunteers as controls. The results showed that ED patients with RD had severe nasal discomfort and decreased erectile function ($P < 0.0001$). The olfactory sensitivity of patients with ED was lower than that of the controls, and patients with both ED and RD had the worst olfactory sensitivity ($P < 0.0001$). Spearman correlation analyses showed that sense of smell was positively correlated with the International Index of Erectile Function-5 score ($R = 0.507$, $P \leq 0.0001$) and the Erection Hardness Scale score ($R = 0.341$, $P < 0.0001$). Logistic regression analyses showed that having an olfactory disorder (OD), RD, age, and visual analog scale (VAS, over 5) score were risk factors for ED outcome, indicating that OD patients had a 16.479-fold increased risk for an ED outcome ($P < 0.05$).

Conclusion: A significant correlation was detected between olfactory sensitivity and erectile function in adult males. In particularly, impairment of olfactory sensitivity is more common in patients with both ED and RD than in patients suffering from a single disease.

Keywords: rhinologic diseases, erectile dysfunction, olfactory sensitivity, vomeronasal organ, Sniffin' Sticks test, IIEF-5

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INTRODUCTION

The olfactory system has a number of features associated with human ingestion, including the modulation of appetite, detection and identification of foods suitable for eating, and rejection of inedible foods (Stevenson, 2010). Olfaction plays a central role in mammalian sexual behavior as well (Pfeiffer and Johnston, 1994; Capparuccini et al., 2010). Olfactory regulation of social communication is vital to reproductive behavior, as it mediates emotional detection, inbreeding avoidance, and the selection of a spouse (Stevenson, 2010).

Previous studies have demonstrated that patients with rhinologic diseases (RDs), such as nasal polyps (NPs), rhinosinusitis (CRS), and allergic rhinitis (AR), have decreased erectile function, which improves significantly after treatment. Some clinical studies have shown that men with high olfactory sensitivity have high sexual desire and increased sexual experience. Therefore, olfaction appears to contribute to sexual arousal and sexual behavior, and people with impaired olfaction cannot benefit from olfaction increasing their sexual experience. In fact, the quality of life of patients with olfactory disorders (ODs) declines, affecting certain aspects of daily life, including social interaction and sexual contact (Croy et al., 2014). In a broad Internet-based survey of chemosensory impairment, more than 50% of participants complained that their chemosensory disorders had negative effects on their sexual activity (Merkonidis et al., 2015). One study showed that sexual desire decreased after olfactory loss in a large number of patients, moderated by the severity of olfactory symptoms and by depression. This effect is more pronounced in males than in females (Gudziol et al., 2009). ODs are often accompanied by a loss of mood and an impaired sexual life and social relationships, which makes patients more depressed and more dependent on their partners than controls (Schafer et al., 2019). In addition, better olfaction is related to better sexual experience. In healthy adults, the more sensitive the sense the smell, the greater the sexual pleasure and the more frequent the orgasm (Bendas et al., 2018). Another study showed a higher incidence of erectile dysfunction (ED) in patients suffering from NP (Gunhan et al., 2011). Surgery can improve the sexual functioning and sleep quality of patients with CRS and NPs, which indicates that CRS and NPs are independent risk factors for ED (Tai et al., 2016). Finally, patients with AR have a higher risk for ED in the future (Su et al., 2013).

These studies suggest a relationship between ODs and sexual behavior that may be regulated by social insecurity, depression, or other comorbidities. Patients with a combination of RDs have impaired erectile function, which improves when the RDs are treated or cured. To date, no study has investigated the direct effects of different manifestations of olfactory function on erectile function in males. In this study, we investigated the relationship between olfactory function and erectile performance in a male population. We speculated that higher olfactory sensitivity would be associated with better erectile function. We used the Erection Hardness Scale (EHS), the International Index of Erectile Function-5 (IIEF-5), a visual analog scale (VAS), and the Sniffin' Sticks test (Hummel et al., 1997) to fully assess nasal discomfort and olfactory sensitivity.

MATERIALS AND METHODS

Participants

Our study was carried out in accordance with the Declaration of Helsinki. Ethics approval was obtained from the institutional review boards of the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. All participants signed informed consent.

This study consisted of a retrospective cohort study with symptoms analysis and a prospective study with clinical measurements of olfactory sensitization and erectile functions in male adult patients. The demographic information of retrospective cohort study patients is shown in **Table 1**, for those of prospective study are described in **Table 2**.

In the first part of study, the patients with ED who were admitted to the Department of Infertility and Sexual Medicine of the Third Affiliated Hospital of Sun Yat-sen University from 2015 to 2018 were included. We collected their data of nocturnal penile tumescence and rigidity (NPTR),

TABLE 1 | The demographic information and symptoms analysis of the retrospective cohort study.

Characteristics	ED	RD
Original sample size	613	234
Qualified for inclusion	574	201
Age, mean (range)	30 (18–48)	31 (22–51)
Complication (*n*, %)	With RD(115, 20.03)	With ED (29, 14.43)
NRS for RD patients, mean (range)		
Nasal congestion	7 (4–9)	8 (5–10)
Nasal discharge	7 (3–10)	7 (6–10)
Hyposmia	5 (5–9)	6 (4–10)

NRS, numerical rating scale; RD, rhinologic disease; ED, erectile dysfunction.

TABLE 2 | Clinical characteristics, measures of olfactory sensitivity and erectile function of the four group patients in the prospective study.

Characteristics	Controls	RD only	ED only	RD with ED
Sample size	40	46	42	14
Mean age, (range)	26 (19–36)	27 (18–52)	30 (21–38)	30 (20–44)
IIEF-5	22.60 ± 1.23	21.28 ± 8.48	11.96 ± 5.61*	9.86 ± 6.67*#
EHS	3.70 ± 0.47	3.76 ± 0.43	2.62 ± 0.73*	2.25 ± 0.89*#
VAS scale	1.95 ± 1.50	5.61 ± 1.39	3.19 ± 1.87*	6.25 ± 1.88*#
Sniffin' Sticks test				
Discrimination test	11.30 ± 1.63	11.61 ± 1.42	11.05 ± 1.66	9.50 ± 3.68*#$
Identification test	11.95 ± 1.90	11.50 ± 2.47	10.55 ± 1.29*	9.21 ± 3.56*#$
TDI score	35.80 ± 3.11	32.91 ± 5.39	31.46 ± 2.64*	25.56 ± 9.59*#$

RD, rhinologic disease; ED, erectile dysfunction; IIEF-5, International Index of Erectile Function-5; EHS, Erection Hardness Scale; VAS, visual analog scale; data are given as mean ± SD or n (percentage). P-values are determined by ANOVA test unless mentioned otherwise. "" means the P-value is less than 0.05 and compared with the control group. "#" means compared with the ED only. "$" means compared with the RD only.*

records of outpatient visits, and prescription data for all patients with ED. Among the 613 ED patients, following patients were excluded due to organic ED (n = 19) based on NPTR, celibate patients (n = 6), mental disorder (n = 8) sever systemic disorders (n = 4) and patients older than 55 years (n = 2). The remaining 574 patients met the following inclusion criteria of physical ED based on NPTR, no other systemic disorders, aged between 18 and 55 years, ability to communicate, and detailed information at follow-up. The 115 patients who were diagnosed with RD in the outpatient clinic were selected and asked about their nasal symptoms using numerical rating scale (NRS), a 11-point rating scale (0–10) by phone, including nasal congestion, nasal discharge, sneezing, postnasal drip, loss of sense of smell, facial pain/stress, and headache.

In addition, we reviewed outpatient data from 234 adult male patients with RD who underwent nasal surgery in our hospital from January 2014 to December 2016. According to the inclusion criteria (without other severe systemic disorders, aged between 18 and 55 years, able to communicate, detailed information to follow-up, and no concurrent upper respiratory infection), 35 patients were diagnosed with ED, 6 patients had organic ED based on NPTR were excluded. Finally 201 patients proceeded for a telephone follow-up. Among them 29 patients with RD (non-organic ED) were asked about their nasal symptoms and severity which were graded by NRS.

In the prospective study (the second part), a total of 188 sequential adult male outpatients who visited the Departments of Infertility and Sexual Medicine and Otorhinolaryngology at the Third Affiliated Hospital of Sun Yat-sen University in 2019 were recruited. After screening with the same inclusion and exclusion criteria (in the first part), 102 patients were finally proceeded for all measurements including IIEF-5, EHS, the nasal symptoms VAS, and the Sniffin' Sticks test. Then, they were further divided into three groups: RD only, ED only, and RD with ED. Meanwhile, 40 healthy volunteers with age- and gender-matched graduate students or medical staff were recruited as a control group.

Nocturnal Penile Tumescence and Rigidity

As we reported previously (Zou et al., 2019), all patients underwent measurement of NPTR over two consecutive nights in the sleep unit of our clinic. Patients were prohibited from engaging in any activity that could interfere with sleep, including smoking; drinking tea, coffee, or alcohol; or taking hypnotics. A RigiScan Plus device (GOTOP Medical, Saint Paul, MN, United States) was strapped to the patient's thigh. Two self-calibrating loops were attached to the penis, with one loop at the tip and the other at the base. Data collected included the number of effective erectile events, total erection time, tumescence-activated units, rigidity-activated units, average event rigidity, and duration of erectile episodes with rigidity $\geq$60%. In accordance with EAU guidelines regarding male sexual dysfunction (Hatzimouratidis et al., 2010), an effective erectile event was defined as an erectile episode with penile tip rigidity $\geq$60% and a duration of no less than 10 min. Moreover, a patient was considered to have normal erectile function when at least one effective erectile event was recorded over two consecutive nights of measurements, indicating non-organic ED. If a patient had mechanical problems or a sleep disorder, or if monitoring time was <6 h, the patient was retested and excluded from the study.

Erectile Dysfunction

Erection Hardness Scale Score

Participants were asked to fill out the EHS for a subjective measurement of erection hardness. This questionnaire was originally used to assess the efficacy of sildenafil citrate for recovering erectile function (Goldstein et al., 1998). Then it was validated and standardized by Mulhall et al. (2007) as a reliable instrument for assessing erection hardness in clinical trials.

International Index of Erectile Function-5

Participants were asked to complete the IIEF, a brief, simple tool used to evaluate certain aspects of sexual function in adult males (Rosen et al., 1997). It contains five items on erection confidence and firmness, maintenance ability and frequency, and satisfaction. Each item is rated on a 5-point ordinal scale, where lower scores represent poorer erectile performance. The scores of all items are summed for an overall score ranging from 5 to 25, where a lower score represents more severe ED. The IIEF is used to diagnose and classify ED based on scores obtained on the erectile function domain: absence of ED (score: 22–25), mild (score: 17–21), mild to moderate (score: 12–16), moderate (score: 8–11), and severe (score: 5–7). These categories are based on our clinical understanding of the scores (Rosen et al., 1999).

Numerical Rating Scale for Nasal Disorders

In the retrospective cohort study, all participants underwent a phone call session to answer trial questions ("What is your pain level for your nasal symptoms?") using the 11-point NRS, where 0 is not painful and 10 is the most worse painful (Kazi et al., 2019).

Visual Analog Scale for Nasal Symptoms

In the prospective study, RD was categorized as mild, moderate, or severe based on the total VAS score, with anchor points of 0 (not troublesome) and 10 (worst possible trouble): mild = 0–3, moderate $\geq$3–7, and severe $\geq$7–10 (Fokkens et al., 2012). To evaluate severity, we asked the patient to indicate on the VAS his answer to the question "How troublesome are your nasal disease symptoms?"

Sniffin' Sticks Test

We used the Sniffin' Sticks test (Burghart, Wedel, Germany) to evaluate the olfactory sensitivity of the participants (Hummel et al., 1997). The sets were performed in a particular order using odorant (phenylethyl alcohol) assembled pens. The test is a multiple forced-choice test that is performed in a ventilated and quiet room. The open pen was positioned 2 cm in front of the nose for one to two breaths. Each pen was used once with an interval of at least 30 s to prevent olfactory desensitization.

Participants were blindfolded to reduce the possibility of visual detection. Three pens were positioned randomly for 3 s. Two pens contained an odorless solvent and the third contained a diluted odorant that was to be identified by the participant. We determined odor thresholds using the single staircase method (Bendas et al., 2018), starting at the lowest dilution and moving in the direction of increasing odor intensity. If participants correctly identified the odorant-containing pen two successive times, a reversal of the staircase was triggered; the opposite happened when the target pen was not identified properly. The threshold estimate was the mean of the last four staircase reversal points of the seven reversals. Higher values indicated higher olfactory sensitivity.

Statistical Analyses

Analyses of variance were performed to compare mean values for clinical characteristics (age), nasal and sexual outcome variables (VAS, IIEF-5, and EHS scores), and olfactory sensitivity (Sniffin' Sticks test: threshold test, discrimination test, identification test, and TDI score) among the four groups (ED, RD, ED with RD, and healthy controls; **Table 2**).

Spearman correlation analyses were used to assess potential relationships between the odor threshold (Sniffin' Sticks test) and measures of erectile function (IIEF-5 and EHS) and nasal symptoms (VAS score) in each group of patients (**Table 3**).

Logistic regression was used to specify which moderator variables predicted change in erectile function. All moderator variables (age, VAS score, and ED or RD) were included as predictors (**Table 4**).

$P < 0.05$ was considered significant. IBM SPSS® version 21 (IBM, Armonk, NY, United States) was used for all analyses.

RESULTS

ED With RD Complications

In the retrospective analyses of 574 patients, 115 patients (20.03%) had RDs, including 39 patients (33.91%) of AR, 23 patients (20%) of deviated septum, 18 patients (15.65%) of CRS, 9 patients (7.83%) of NPs, and 23 patients (22.61%) of chronic rhinitis. There were 341 effective responses during telephone follow-ups, and the main complaints were nasal congestion, nasal discharge, and hyposmia on the VAS score (>5), accounting for 82.42, 61.54, and 10.99% of the complaints, respectively.

Rhinologic Disease With ED Complications

A total of 201 patients undergoing nasal surgery, 29 patients (14.43%) were diagnosed with ED. All of them were contacted during the telephone follow-up. Nasal congestion, nasal discharge, and a decrease in olfactory sensitivity were the most common complaints, accounting for 85.19, 62.07, and 22.22% of the complaints, respectively.

TABLE 3 | Spearman correlational analysis between olfactory sensitivity and VAS score, IIEF-5, EHS.

Measurements of sexual function and nasal symptoms	Olfactory sensitivity via Sniffin' Sticks test			
	Threshold test	Discrimination test	Identification test	TDI score
IIEF-5				
Control	$R = -0.153$	$R = 0.133$	$R = 0.449$	$R = 0.184$
	$P = 0.347$	$P = 0.413$	$P = 0.004$	$P = 0.255$
RD	$R = 0.328$	$R = 0.117$	$R = 0.329$	$R = 0.425$
	$P = 0.026$	$P = 0.438$	$P = 0.025$	$P = 0.003$
ED	$R = 0.102$	$R = 0.111$	$R = 0.076$	$R = 0.120$
	$P = 0.355$	$P = 0.316$	$P = 0.491$	$P = 0.277$
RD + ED	$R = 0.224$	$R = 0.072$	$R = 0.176$	$R = 0.052$
	$P = 0.251$	$P = 0.718$	$P = 0.371$	$P = 0.794$
Combined	$R = 0.421$	$R = 0.195$	$R = 0.409$	$R = 0.507$
	$P = 0.000$	$P = 0.006$	$P = 0.000$	$P = 0.000$
EHS				
Control	$R = -0.182$	$R = -0.164$	$R = 0.423$	$R = 0.143$
	$P = 0.261$	$P = 0.313$	$P = 0.007$	$P = 0.378$
RD	$R = 0.293$	$R = 0.137$	$R = 0.198$	$R = 0.254$
	$P = 0.048$	$P = 0.363$	$P = 0.187$	$P = 0.089$
ED	$R = 0.079$	$R = 0.045$	$R = -0.087$	$R = -0.063$
	$P = 0.474$	$P = 0.681$	$P = 0.429$	$P = 0.571$
RD + ED	$R = 0.196$	$R = -0.070$	$R = 0.134$	$R = 0.055$
	$P = 0.317$	$P = 0.723$	$P = 0.497$	$P = 0.782$
Combined	$R = 0.293$	$R = 0.125$	$R = 0.320$	$R = 0.341$
	$P = 0.000$	$P = 0.098$	$P = 0.000$	$P = 0.000$
VAS socre				
Control	$R = -0.017$	$R = 0.430$	$R = -0.080$	$R = 0.197$
	$P = 0.916$	$P = 0.006$	$P = 0.625$	$P = 0.223$
RD	$R = 0.025$	$R = -0.084$	$R = -0.232$	$R = -0.159$
	$P = 0.867$	$P = 0.752$	$P = 0.121$	$P = 0.292$
ED	$R = 0.154$	$R = -0.006$	$R = -0.020$	$R = 0.097$
	$P = 0.161$	$P = 0.959$	$P = 0.854$	$P = 0.378$
RD + ED	$R = -0.007$	$R = 0.142$	$R = 0.143$	$R = 0.096$
	$P = 0.97$	$P = 0.471$	$P = 0.468$	$P = 0.626$
Combined	$R = -0.173$	$R = 0.022$	$R = -0.027$	$R = -0.096$
	$P = 0.021$	$P = 0.769$	$P = 0.716$	$P = 0.203$

RD, rhinologic disease; ED, erectile dysfunction; IIEF-5, International Index of Erectile Function-5; EHS, Erection Hardness Scale; VAS, visual analog scale.

The Relationship Between Erectile Function and Olfactory Sensitivity in Adult Males

One hundred and two patients and 40 healthy controls who were admitted in 2019 completed the IIEF, EHS, VAS, and the Sniffin' Sticks test to elucidate the relationship between erectile function and olfaction in males. ED patients with RD had increased nasal discomfort and worse erectile function than patients with ED ($P < 0.0001$; **Table 2**). The olfactory sensitivity of patients with ED (threshold test: 9.86 ± 1.77, discrimination test: 11.05 ± 1.66, identification test: 10.55 ± 1.29, and TDI score: 31.46 ± 2.64) was lower than that of the healthy controls (threshold test: 12.55 ± 1.96, discrimination

TABLE 4 | The logistic regression analysis for erectile dysfunction.

Risk factors	OR	95% CI	*P*-value
Age	1.099	(1.037, 1.164)	0.001
VAS	1.468	(1.176, 1.833)	0.001
With OD			
NO	–	–	–
Yes	17.479	(5.713, 53.477)	0.000
With RD			
NO	–	–	–
Yes	0.031	(0.009, 0.109)	0.000

OD, olfactory disorder.

test: 11.30 ± 1.63, identification test: 11.95 ± 1.90, and TDI score: 35.80 ± 3.11), and patients with both ED and RD had the worst olfactory sensitivity (threshold test: 9.86 ± 1.77, discrimination test: 9.50 ± 3.68, identification test: 9.21 ± 3.56, and TDI score: 25.56 ± 9.59; $P < 0.001$; **Table 2**). A positive correlation was detected between olfactory sensitivity and IIEF-5 score ($R = 0.507$, $P < 0.001$) and EHS score ($R = 0.341$, $P < 0.0001$; **Table 3**).

Factors Influencing Hyposmia

Logistic regression analyses were performed to identify risk factors for ED (**Table 4**). Patients with olfactory disease or RD, those who were older, and those with a higher VAS score were at higher risk for ED. Olfactory disease was associated with a 16.479-fold increase in ED outcomes ($P < 0.0001$). Older patients and people suffering from nasal discomfort had a 0.099 times and a 0.468 times increased risk for ED, individually ($P < 0.01$). We plotted the relationship between RD, ED, and olfactory disease and found that patients with RD with ED had a higher incidence of olfactory disease. This suggests that nasal congestion, runny nose, and erectile function caused by RD are positively correlated with olfactory disease. Combinations of ED and RD resulted in additive effects.

DISCUSSION

The predictive correlation between olfaction and sexual desire and sexual experience has been confirmed (Bendas et al., 2018; Schafer et al., 2019). Our retrospective analyses found that patients with ED were more likely than healthy controls to suffer from RD at the same time. A decline in olfactory sensitivity is often overlooked, because a minor decline in smell does not have a significant impact on daily life. Yet a recent study indicated that patients who complain of OD experience a significantly reduced quality of life regarding paid employment, household work, and social and family life (Bramerson et al., 2007). The present study found that nearly 15% of patients with RD have ED and that patients with RD and ED have the worst olfactory sensitivity. In summary, RDs are closely related to ED, which is consistent with previous results (Su et al., 2013). Olfactory sensitivity is associated with RD and ED. This is consistent with a study reporting that men who are born without a sense of smell exhibit a markedly reduced number of sexual relationships. The olfactory function test introduced in this study showed that olfactory sensitivity decreased in patients with RD and ED and that the olfactory threshold was significantly higher than in healthy controls (**Table 2**). Moreover, sense of smell decreased significantly, nasal discomfort increased, and overall erectile function was unsatisfactory in patients who suffered from both RD and ED. These results are consistent with previous findings of patients diagnosed with dysosmia losing sexual desire (Croy et al., 2013; Gudziol et al., 2009).

We detected a significant correlation between olfactory threshold and ED. Unfortunately, we did not observe a relationship between olfactory function and the severity of nasal discomfort. Studies have shown that the sense of smell is closely associated with the sexual process. Patients with decreased olfactory sensitivity are more likely to have ED. Therefore, smell could be considered an initiator of sexual activity, and penile erection is the effector. According to the literature, the normal operation of the effector results from a functional vomeronasal organ (VNO), because the human VNO provides important information on inhibiting chemical sensation, promoting heterosexual contact, and preventing inappropriate mating. Foltan and Sedy (2009) hypothesized that the adult VNO is indispensable in preventing the selection of an inappropriate spouse. Human pheromones extracted from armpit sweat and tears control ovulation, temper, hormone secretion, and attraction to different genetic partners (Wysocki and Preti, 2004). Periovulatory odors increase testosterone and cortisol levels in adult males, producing strong sexual desire (Cerda-Molina et al., 2013). Croy reported that the hypothalamus becomes activated in men with medium androstenedione concentrations (Croy et al., 2013). Thus, we hypothesize that smelling the chemosignal androstenedione via the VNO elicits activation of the hypothalamus. Our findings of reduced olfactory sensitivity in patients with ED support vomeronasal identification of pheromones and possible sexual behavior. In addition, our multi-element regression analyses determined that patients with an impaired sense of smell had a 16.479-fold risk for ED. We believe that the VNO has gradually degraded to non-organ form as a result of human evolution, yet it functions as olfactory cells that detect pheromones controlling human sexual activity.

Taken together, these results support the notion that olfactory function is associated with ED in males. VNO identification of pheromones to regulate sexual behavior will allow us to further explore the hypothesis that improving olfactory sensitivity can improve sexual experience. RDs and ED have obvious superimposing effects on ODs. Patients with RD do not typically report intimate ED problems, so routine medical care providers should inform about this common effect and ask for consultation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the Research Ethics Committee of the Institute of Basic Research in Clinical Medicine, The Third Affiliated Hospital of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

QY, HD, JF, and YZ conceived and designed the study. JF, HD, WK, WZ, GM, TH, SL, and YX acquired the data. JF and HD analyzed and interpreted the data, and drafted the manuscript. QY and YZ critically revised the manuscript for important intellectual content. All authors approved the final manuscript.

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REVIEW
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The Impact of $PM_{2.5}$ on the Host Defense of Respiratory System

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The harm of fine particulate matter ($PM_{2.5}$) to public health is the focus of attention around the world. The Global Burden of Disease (GBD) Study 2015 (GBD 2015 Risk Factors Collaborators, 2016) ranked $PM_{2.5}$ as the fifth leading risk factor for death, which caused 4.2 million deaths and 103.1 million disability-adjusted life-years (DALYs) loss, representing 7.6% of total global deaths and 4.2% of global DALYs. Epidemiological studies have confirmed that exposure to $PM_{2.5}$ increases the incidence and mortality of respiratory infections. The host defense dysfunction caused by $PM_{2.5}$ exposure may be the key to the susceptibility of respiratory system infection. Thus, this review aims to assess the impact of $PM_{2.5}$ on the host defense of respiratory system. Firstly, we elaborated the epidemiological evidence that exposure to $PM_{2.5}$ increases the risk of respiratory infections. Secondly, we summarized the experimental evidence that $PM_{2.5}$ exposure increases the susceptibility of different pathogens (including bacteria and viruses) in respiratory system. Furthermore, here we discussed the underlying host defense mechanisms by which $PM_{2.5}$ exposure increases the risk of respiratory infections as well as future perspectives.

Keywords: $PM_{2.5}$, respiratory system, infection, susceptibility, host defense

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INTRODUCTION

Exposure to air pollution, including gaseous pollution and particulate matter (PM) pollution, is a leading contributor to the Global Burden of Disease (GBD 2015 Risk Factors Collaborators, 2016). In recent years, more and more attention has been paid to the impact of PM pollution on public health. PM is a complex mixture of solids and liquids suspended in the air, which can be classified by its aerodynamic diameter as PM_{10} (<10 μm, inhalable particulate matter), $PM_{2.5}$ (<2.5 μm, fine particulate matter) and $PM_{0.1}$ (<0.1 μm, ultrafine particulate matter). PM originates from natural sources (such as dust, sea salt, and wildfires) and anthropogenic emissions (such as vehicles, household wood and coal burning as well as power plants and industry), and the latter accounts

Abbreviations: AMPs, antimicrobial peptides; AMs, alveolar macrophages; AQLI, Air Quality Life Index; ASL, airway surface liquid; CFUs, colony-forming units; COPD, chronic obstructive pulmonary disease; DALYs, disability-adjusted life-years; GBD, global burden of disease; ICAM-1, intercellular adhesion molecule-1; *K. pneumoniae*, *klebsiella pneumoniae*; *L. monocytogenes*, *Listeria monocytogenes*; LRTI; lower respiratory tract infection; *M. tuberculosis*, *Mycobacterium tuberculosis*; MRSA, Methicillin-resistant *Staphylococcus aureus*; NAC, N-acetylcysteine; NKs, natural killer cells; *P. aeruginosa*, *Pseudomonas aeruginosa*; PM, particulate matter; PM_{10}, inhalable particulate matter; $PM_{2.5}$, fine particulate matter; $PM_{0.1}$, ultrafine particulate matter; PMNs, polymorphonuclear granulocytes; ROS, reactive oxygen species; RSV, respiratory syncytial virus; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae*; SAG, salivary agglutinin; SPD, surfactant protein D; TJs, tight junctions; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; URTI, upper respiratory tract infection; VACES, versatile aerosol concentration enrichment system; WHO, World Health Organization.

for most of the PM pollution (Cho et al., 2018). The components of PM are extremely complex, including inorganic components (such as heavy and transition metals, elemental carbon, and sulfuric/nitric/ammonia salts), organic components (such as polycyclic aromatic hydrocarbons) and biological components (such as fungi, spores, and viruses) (Zhang et al., 2015; Cho et al., 2018). There are certain differences in the source and composition of different types of particulate matter, and the harm to public health varies as well (**Figure 1**). Among them, $PM_{2.5}$ was considered to be the most harmful one. $PM_{2.5}$ has a large surface area and can adsorb a variety of toxic and harmful substances (Hsu et al., 2016). Because of its small particle size, it can penetrate deep into the lungs and deposit in the terminal bronchioles and alveoli with breath, and even enter the circulatory system through the gas-blood barrier (Pinkerton et al., 2000; Schulze et al., 2017). Exposure to $PM_{2.5}$ can endanger multiple organs in the body, and even lead to systemic adverse effects (Chauhan and Johnston, 2003). Among them, the most common are the respiratory and cardiovascular systems (Xing et al., 2016). This review focuses on the respiratory system, the primary target organ for $PM_{2.5}$ exposure.

$PM_{2.5}$ may be the environmental risk factor that poses the greatest public health hazard. The GBD 2015 ranks $PM_{2.5}$ as the fifth leading risk factor for death, with exposure to $PM_{2.5}$ causing 4.2 million deaths (7.6% of global deaths) and loss of 10.31 million disability-adjusted life-years (DALYs) (4.2% of global DALYs) (Cohen et al., 2017). As claimed by the World Health Organization (WHO), 9 out of every 10 people in urban areas are exposed to high levels of $PM_{2.5}$ (annual average concentration >10 $\mu g/m^3$) from outdoor air pollution, and about 3 billion people using non-renewable fuels are exposed to serious indoor air pollution (World Health Organization [WHO], 2019). According to the Air Quality Life Index (AQLI), sustained exposure to an additional 10 $\mu g/m^3$ of $PM_{2.5}$ reduces life expectancy by 0.98 years[1]. In addition, an increase of 10 $\mu g/m^3$ per day of $PM_{2.5}$ concentration increases 0.29% of overall non-accidental mortality and 0.22% of respiratory disease mortalities (Chen et al., 2017).

A large number of epidemiological studies have shown that $PM_{2.5}$ exposure is closely related to a variety of respiratory diseases (Jo et al., 2017; Wang C. et al., 2019). It is noteworthy that exposure to $PM_{2.5}$ increases the susceptibility to respiratory infections. For instance, numerous clinical studies have found that $PM_{2.5}$ exposure is positively correlated with the number of outpatient visits, emergency visits, and hospitalizations for acute upper or lower respiratory infections (Li et al., 2017; Xia et al., 2017; Strosnider et al., 2019). Related animal models also support the notion that $PM_{2.5}$ exposure increases susceptibility to the lung infection (Yang et al., 2001; Sigaud et al., 2007; Zhao et al., 2014). However, the underlying mechanisms remain elusive. We speculate that $PM_{2.5}$ exposure may impair the host defense of the respiratory system, making the body more susceptible to infection. The purpose of this article is to review the existing epidemiological and experimental evidence to support the above hypothesis and to summarize the possible underlying mechanisms.

THE EPIDEMIOLOGICAL EVIDENCE

The respiratory system is the primary route for inhaled $PM_{2.5}$. Exposure to $PM_{2.5}$ can cause the development and progression of acute and chronic lung diseases, such as tracheal and pulmonary inflammation (Kampa and Castanas, 2008; Jedrychowski et al., 2013; Ge et al., 2018), asthma and its acute exacerbations (Habre et al., 2014; Zheng et al., 2015), chronic obstructive pulmonary disease (COPD) and its acute exacerbations (Faustini et al., 2013; Tsai et al., 2013). We are concerned with the fact that $PM_{2.5}$ exposure increases the susceptibility to respiratory infections.

Outpatient, emergency, and hospitalization-related data on respiratory infections confirmed that $PM_{2.5}$ exposure was positively associated with the increased respiratory infections. Li et al. (2017) studied the effects of air pollution on outpatients' acute respiratory outcomes and their study indicated that $PM_{2.5}$ exposure was positively correlated with outpatient visits for upper respiratory tract infection (URTI). A study in the U.S. state of Georgia also showed that pediatric emergency visits for URTI were associated with $PM_{2.5}$ concentrations (Strickland et al., 2016). Another study reported that $PM_{2.5}$ was significantly associated with emergency room visits for respiratory diseases, particularly for URTI and lower respiratory tract infection (LRTI) (Xu et al., 2016). Recently, Strosnider et al. (2019) confirmed that $PM_{2.5}$ exposure was positively correlated with emergency visits for multiple respiratory diseases, including respiratory infections. In addition, Xia et al. (2017) studied the association between different types of air pollution and respiratory hospitalization and found that short-term exposure of $PM_{2.5}$ was positively correlated with the number of hospitalizations for acute respiratory infections. Similarly, another four studies also supported a significant positive correlation between $PM_{2.5}$ and the number of hospitalizations for URTI and LRTI (Belleudi et al., 2010; Li et al., 2018; Liu et al., 2019b; Zhang D. et al., 2019).

The effect of $PM_{2.5}$ exposure on respiratory infections is not immediate, but there is a certain lag effect. For example, $PM_{2.5}$ exposure was positively associated with an increase in hospitalization for acute respiratory infections, but this correlation was delayed by 7–13 days (Xia et al., 2017). Liang et al. (2014) analyzed the time curve of $PM_{2.5}$ concentration and human influenza in Beijing urban area from 2008 to 2011, and proved that there was a significant correlation between $PM_{2.5}$ exposure and influenza, but this correlation showed a 1–2 months delay. In a study of 150,000 cases, Horne et al. (2018) investigated the relationship between $PM_{2.5}$ and the health status of patients with acute LRTI and discovered that the association of $PM_{2.5}$ exposure with respiratory syncytial virus (RSV) infection occurred in 2–4 weeks. The hysteresis effects varied in different studies. Therefore, when investigating the association between $PM_{2.5}$ exposure and respiratory infections, researchers may need to choose the appropriate follow-up time.

[1]https://aqli.epic.uchicago.edu/pollution-facts/?l=en

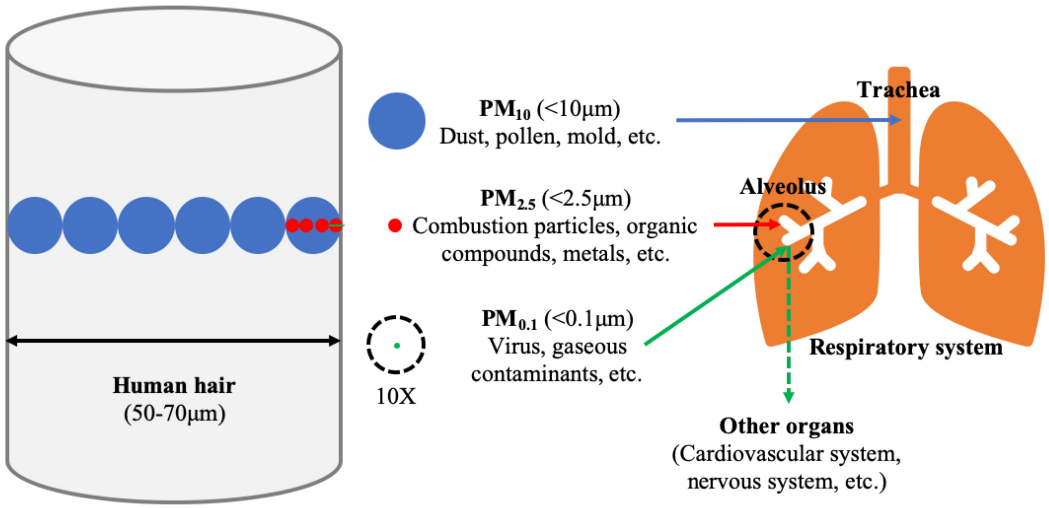

FIGURE 1 | The size, main composition and deposition site in the lung of the particulate matter (PM). The average diameter of human hair is 60 μm, which approximately equivalents to six inhalable particulate matter (PM_{10}, <10 μm in diameter, blue color), or twenty-four fine particulate matter ($PM_{2.5}$, <2.5 μm in diameter, red color) or six hundred ultrafine particulate matter ($PM_{0.1}$, <0.1 μm in diameter, green color). Particulate matter, with aerodynamic diameter 2.5–10 μm, is mainly deposited on the trachea. PM less than 2.5 μm in diameter poses the greatest problems, because it can get deep into the terminal bronchioles and alveoli, and some with <0.1 μm in diameter may even get into the bloodstream affecting other organs. 10X: The stuff in the dotted ring is magnified ten times.

Exposure to $PM_{2.5}$ increases the susceptibility to respiratory infections, especially in children and the elderly, as well as the vulnerable groups with hereditary or underlying diseases. A study on the relationship between $PM_{2.5}$ and the health status of patients with acute LRTI revealed that about 77% of the subjects were infants aged 0–2 years. RSV and influenza virus were the main pathogens (Horne et al., 2018). Another study on risks of respiratory infections associated with air pollution in China showed that children under the age of 14 were the predominantly susceptible population of acute respiratory infections caused by air pollution, accounting for 80% of hospitalized cases of respiratory system infections in the study (Xia et al., 2017). Similarly, Liu et al. (2019b) found that students aged 6–17 years were more vulnerable to $PM_{2.5}$ exposure. The maternal exposure to air pollution before birth may result in an impaired lung development and increase the risk of respiratory system infections (Pinkerton and Joad, 2006; Jedrychowski et al., 2013). In addition, by analyzing the association between $PM_{2.5}$ pollution and hospital emergency room visits for total and cause-specific respiratory diseases in urban areas in Beijing, Xu et al. (2016) found that people over 60 years of age demonstrated a higher risk of respiratory disease (including URTI and LRTI) after $PM_{2.5}$ exposure. Exposure to $PM_{2.5}$ is more likely to cause respiratory infections in people with congenital immune deficiencies due to hereditary diseases. For instance, exposure to $PM_{2.5}$ in patients with cystic fibrosis has been reported to be associated with the acquisition of *Pseudomonas aeruginosa* (*P. aeruginosa*). During the follow-up period, each additional $PM_{2.5}$ exposure of 10 $\mu g/m^3$ increased the risk of 24% *P. aeruginosa* acquisition (Psoter et al., 2015). Another study has found that each additional $PM_{2.5}$ exposure of 10 $\mu g/m^3$ increased the risk of methicillin-resistant *Staphylococcus aureus* (MRSA) by 68% (Psoter et al., 2017).

THE EXPERIMENTAL EVIDENCE

In vivo studies have shown that as a risk factor for respiratory infection, $PM_{2.5}$ exposure, can prime the lung for greater susceptibility to pathogens by impairing the respiratory host defense. Yang et al. (2001) found that PM exposure suppressed macrophage function and slowed the pulmonary clearance of *Listeria monocytogenes* (*L. monocytogenes*) in rats. Another research discovered that the colony-forming units (CFUs) of *P. aeruginosa* detected in the lung were significantly greater in the PM-exposed mice compared to the control mice (Liu et al., 2019a). Zhao et al. (2014) found that prior $PM_{2.5}$ exposure markedly increased the susceptibility of rats to subsequent *Staphylococcus aureus* (*S. aureus*) infection. Similarly, Duan et al. (2013) found that $PM_{2.5}$ exposure increased the susceptibility of rats to *Klebsiella pneumoniae* (*K. pneumoniae*) infection. Sigaud et al. (2007) and Migliaccio et al. (2013) established an exposure model in mice and subsequently infected with *Streptococcus pneumoniae* (*S. pneumoniae*). They found that PM exposure reduced bacterial clearance in the lungs of mice. In addition, Ma et al. (2017) discovered that exposure to $PM_{2.5}$ lowers influenza virus resistance. We summarized the *in vivo* experimental studies of $PM_{2.5}$ on respiratory host defense (**Table 1**).

In vitro experiments have also confirmed that $PM_{2.5}$ exposure increased the susceptibility of respiratory infection. For example, $PM_{2.5}$-pretreated A549 cells have a significantly increased risk of infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) (Rivas-Santiago et al., 2015), and PM can disrupt the airway epithelium through oxidative burst to promote *P. aeruginosa*

TABLE 1 | Summary of *in vivo* experimental studies of $PM_{2.5}$ on respiratory host defense.

Animal species	Pathogen	Exposure method	Effects on respiratory host defense	References
SD rats	Listeria	Intranasal instillation	Exposure to diesel exhaust particles (DEP) decreased the ability of macrophages to produce antimicrobial oxidants in response to Listeria, which may play a role in the increased susceptibility of rats to pulmonary infection	Yang et al., 2001
BALB/c mice	*S. pneumoniae*	Intranasal instillation	The combination of γ-interferon (IFN-γ) priming and concentrated ambient particles (CAPs) exposure led to an inflamed alveolar milieu where oxidant stress caused loss of antibacterial functions in alveolar macrophages (AMs) and recruited polymorphonuclear granulocytes (PMNs)	Sigaud et al., 2007
BALB/c mice	*S. pneumoniae*	Whole-body exposure (smoking)	Exposure to wood smoke-derived particulate matter decreased the ability of pulmonary macrophages to effectively mount a defense against infection, and appeared to be mediated via RelB activation	Migliaccio et al., 2013
SD rats	*K. pneumoniae*	Intranasal instillation	$PM_{2.5}$ exposure increased the susceptibility of the rats to *K. pneumoniae* infection and decreased bacterial clearance. Its mechanism may be related to the impairment of bronchial mucociliary system and interaction of cytokines.	Duan et al., 2013
Wistar rats	*S. aureus*	Intranasal instillation	Exposure to $PM_{2.5}$ increased susceptibility to respiratory *S. aureus* infection in rats via reducing pulmonary natural killer cells and suppressing the phagocytosis ability of AMs.	Zhao et al., 2014
Mice*	Influenza virus	Intranasal inhalation	Long-term exposure to $PM_{2.5}$ lowered influenza virus resistance via down-regulating pulmonary macrophage Kdm6a and mediated histones modification in IL-6 and IFN-β promoter regions	Ma et al., 2017
C57BL/6J mice	*P. aeruginosa*	Intracheal instillation	PM disrupted tight junctions (TJs) via oxidative stress to promote bacterial infection	Liu et al., 2019a

**The specific strain is not indicated in the literature.*

infection (Liu et al., 2019a). Similarly, Chen et al. (2018) found that PM suppressed airway antibacterial defense, causing an increased susceptibility to *P. aeruginosa*. In addition, adhesion is the key to microbial invasion of the respiratory tract. PM increased the binding of *S. pneumoniae* to both primary alveolar macrophages (AMs) and the murine macrophage cell line J774 A.1 but decreased internalization of bacteria (Zhou and Kobzik, 2007). Mushtaq et al. (2011) have discovered that urban PM increased the adhesion of *S. pneumoniae* to human tracheal epithelial cells. We also summarized the *in vitro* experimental studies of $PM_{2.5}$ on respiratory host defense (**Table 2**).

POSSIBLE MECHANISMS

$PM_{2.5}$ exposure impairs the host defense of respiratory system causing the body more susceptible to infection. We dissect the underlying mechanisms from the following three aspects: defective airway epithelial host defense functions, alterations in respiratory microecology, insufficiency and dysfunction of immune cells (**Figure 2**).

Defective Airway Epithelial Host Defense Functions

At the interface between the external environment and the host, the airway epithelium serves as the first line of host defense against pathogens. The airway epithelial host defense functions mainly include mucociliary clearance, the barrier functions of the epithelium, and the secretion of a number of proteins and peptides with antimicrobial activities (**Figure 3A**). Mucociliary clearance in the airway epithelium is a critical protective function and is essential for the clearance of respiratory pathogens. In general, most foreign bodies inhaled into the lungs can be removed in time by the mucociliary clearance system. However, it has been reported that $PM_{2.5}$ exposure decreased bacterial clearance by impairing the bronchial mucociliary system (Duan et al., 2013). Mucin hyperproduction or hypersecretion is a common reason for decreased mucociliary clearance. Val et al. (2012) found that the expression of MUC5AC, one of the predominant mucins produced by the airway epithelium, was upregulated via the epidermal growth factor receptor (EGFR) pathway after $PM_{2.5}$ exposure in mice (**Figure 3B**).

The adhesion of pathogens to host cells is a prerequisite for infection. A study has reported that exposure to urban PM increased the adhesion of *S. pneumoniae* to human airway epithelial cells, and the addition of N-acetylcysteine (NAC, an antioxidant) reversed this process, possibly be related to reactive oxygen species (ROS) produced by oxidative stress (Mushtaq et al., 2011). In addition, Liu et al. (2018) reported that ROS induced by $PM_{2.5}$ activated the AKT/STAT3/NF-κB pathway through IL-6 paracrine signaling, which then upregulated the expression of intercellular adhesion molecule-1 (ICAM-1, an important glycoprotein on the cell surface) in the lung to increase the adhesion of pathogens to the airway epithelium (**Figure 3B**). Woo et al. (2018) also found that $PM_{2.5}$ could enhance the adhesion of *P. aeruginosa* to epithelial cells, the mechanism of which depended on the increased bacterial surface hydrophobicity and damaged human cell plasma membrane by $PM_{2.5}$.

TABLE 2 | Summary of *in vitro* experimental studies of $PM_{2.5}$ on respiratory host defense.

Cell line	Pathogen	Culture method	Effects on respiratory host defense	References
Murine primary alveolar macrophages and the murine macrophage cell line (J774 A.1)	*S. pneumoniae*	Submerged	Soluble metal, especially iron, in the PM played an important role in the inhibition of macrophage phagocytosis killing of *S. pneumoniae*	Zhou and Kobzik, 2007
A549 cells and Human primary bronchial epithelial cells (HBEpC)	*S. pneumoniae*	Submerged	Urban PM increased adhesion of *S. pneumoniae* to human airway epithelial cells. PM-stimulated adhesion was mediated by oxidative stress and platelet-activating factor receptor (PAFR)	Mushtaq et al., 2011
A549 cells	*M. tuberculosis*	Submerged	Exposure of A549 cells to PM induced cellular senescence, a likely cause of the observed downregulation of HBD-2 and HBD-3 and the subsequent loss of *M. tuberculosis* growth control	Rivas-Santiago et al., 2015
BEAS-2B	*P. aeruginosa*	Submerged	PM impaired airway epithelial defense by impeding the induction of HBD-2 via an oxidative burst, potentially causing an increased susceptibility to infection	Chen et al., 2018
BEAS-2B	*P. aeruginosa*	Submerged	PM disrupted tight junctions (TJs) via oxidative stress to promote bacterial infection	Liu et al., 2019a

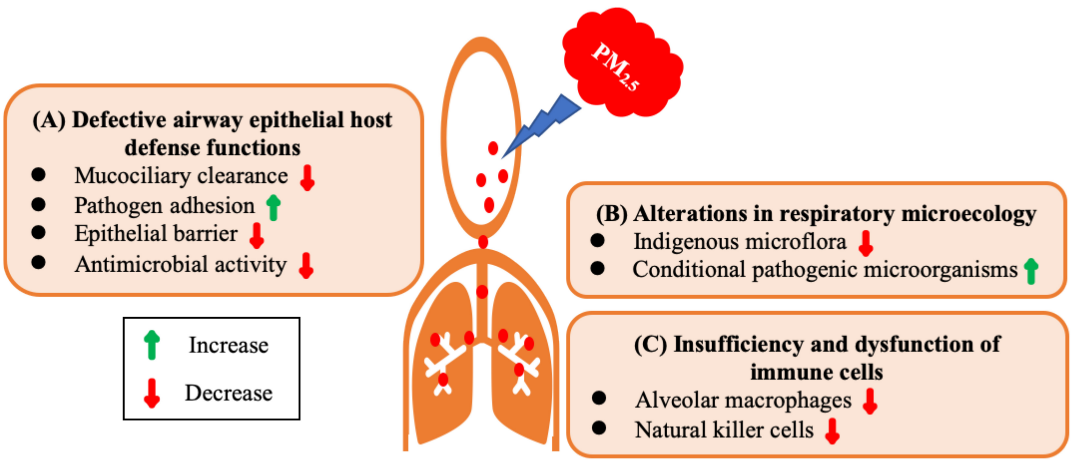

FIGURE 2 | Possible mechanisms underlying defective host defense of respiratory system by $PM_{2.5}$ exposure. **(A)** Defective airway epithelial host defense functions. $PM_{2.5}$ exposure increases adhesion of pathogens to epithelial cells, impairs epithelial barrier function, compromises mucociliary clearance, and reduces antimicrobial activity. **(B)** Alterations in respiratory microecology. $PM_{2.5}$ exposure decreases indigenous microflora and increases the content of conditional pathogenic microorganisms. **(C)** Insufficiency and dysfunction of immune cells. $PM_{2.5}$ exposure decreases the number of natural killer cells and impairs the phagocytic capacity of alveolar macrophages.

Tight junctions (TJs) are the significant protein complexes at cell-cell interfaces that connect adjacent cells with each other to form lung epithelial barrier against pathogens (Schlingmann et al., 2015). Lack of an intact TJs structure, the airway epithelial barrier cannot keep tight. It will allow pathogens to translocate across the barrier, making the lungs more susceptible to infection. A recent study reported that PM impaired TJs of airway epithelial barrier via oxidative stress to promote *P. aeruginosa* infection (Liu et al., 2019a). Claudin-1 is a major structural protein of TJs. Similarly, another study also discovered that exposure to PM downregulated claudin-1 expression in human airway cells via the ERK1/2 signaling pathway (Kim et al., 2017) (**Figure 3B**).

The airway epithelial cells are covered with a very thin fluid layer (airway surface liquid, ASL), which is an important component of the respiratory innate immunity. Antimicrobial peptides (AMPs) content is a significant and indispensable factor affecting the antibacterial effect of ASL. AMPs include salivary agglutinin (SAG), beta-defensins, lactoferrin, secretory IgA, and surfactant protein D (SPD) (Fabian et al., 2012; Kendall et al., 2013; Vargas Buonfiglio et al., 2018). Zhang S. et al. (2019) found that $PM_{2.5}$ exposure attenuated the antibacterial activity of airways by down-regulating the expression of SAG. In addition, several studies indicated that $PM_{2.5}$ exposure down-regulated airway β-defensin expression levels through oxidative stress (Rivas-Santiago et al., 2015; Vargas Buonfiglio et al., 2017; Chen et al., 2018). Collectively, these studies suggested that $PM_{2.5}$ could compromise the host defense function of airway epithelial cells by downregulating the expression of AMPs (**Figure 3B**).

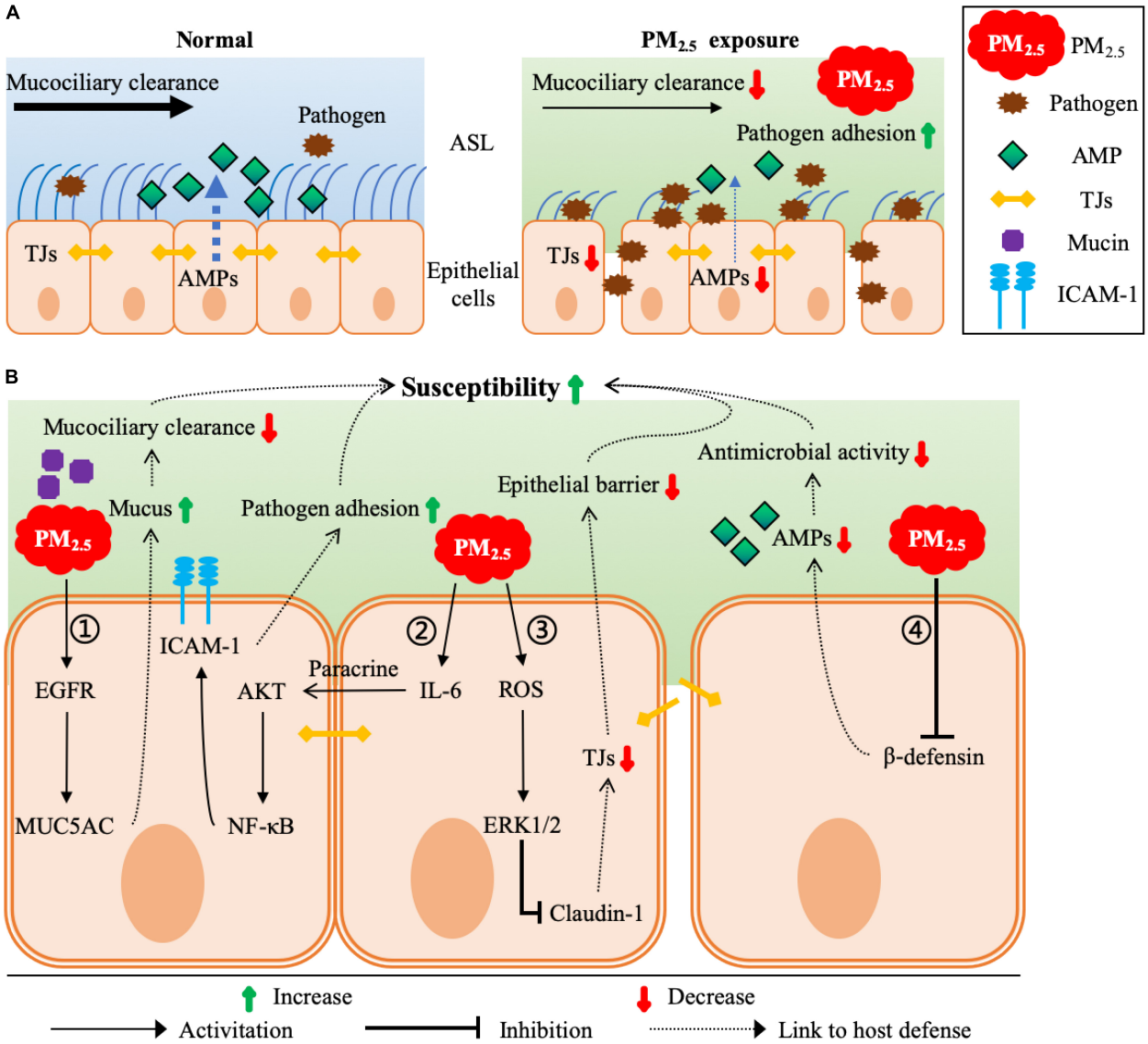

FIGURE 3 | Molecular mechanisms underlying $PM_{2.5}$-induced defective airway epithelial host defense functions. **(A)** Major host defense functions of normal and $PM_{2.5}$-exposured airway epithelia. Normal airway epithelia are protected from pathogens mainly by mucociliary clearance, the barrier functions of the epithelium, and the secretion of a number of AMPs with antimicrobial activities. However, $PM_{2.5}$ exposure disrupts these host defense functions, resulting in more pathogens in the airways. **(B)** Underlying molecular mechanisms: ① **$PM_{2.5}$ → EGFR ↑ → MUC5AC ↑ → Mucus ↑ → Mucociliary clearance ↓**: $PM_{2.5}$ up-regulates the expression of MUC5AC (one of the predominant mucins produced by the airway epithelium) by activating EGFR pathway, resulting in decreased mucociliary clearance. ② **$PM_{2.5}$ → IL-6 ↑ → AKT ↑ → NF-κB ↑ → ICAM-1 ↑ → Pathogen adhesion ↑**: $PM_{2.5}$ activates AKT/NF-κB pathway through IL-6 paracrine signaling, which then up-regulates the expression of ICAM-1 (an important glycoprotein on the cell surface) in the lung to increase the adhesion of pathogens to the airway epithelium. ③ **$PM_{2.5}$ → ROS ↑ → ERK-1/2 ↑ → Claudin-1 ↓ → TJs ↓ → Epithelial barrier ↓**: $PM_{2.5}$ down-regulates the expression of claudin-1 (a major structural protein of TJs) via generating ROS and activating ERK1/2 pathway, resulting in more pathogens to translocate across the disrupted epithelial barrier. ④ **$PM_{2.5}$ → β-defensin ↓ → AMPs ↓ → Antimicrobial activity ↓**: $PM_{2.5}$ inhibits the expression and secretion of β-defensin (one of the major AMPs in ASL), which allows more pathogens to survive and exacerbates respiratory infections.

Alterations in Respiratory Microecology

In healthy humans, the low respiratory tract is usually sterile, and without permanent bacterial colonization, while the upper respiratory tract (especially oropharynx) has a normal bacterial flora, which is also an important component of respiratory tract's natural immune defense, providing a biological barrier against foreign matter or pathogenic microorganisms through a space-occupying effect, nutritional competition, and secretion of bacteriostatic or bactericidal substances (Akata et al., 2016; Marsh et al., 2016; Wang L. et al., 2019). The oropharyngeal microecosystems of rats changed following PM exposure, including a decline of indigenous microflora and an increase of the content of conditional pathogenic microorganisms (Xiao et al., 2013). By analyzing 16S rRNA sequencing of respiratory

TABLE 3 | Strengths and weaknesses of intratracheal instillation and versatile aerosol concentration enrichment system (VACES) on $PM_{2.5}$ exposure to experimental rodents.

Method	Intratracheal instillation	Versatile aerosol concentration enrichment system (VACES)	
		Oral-inhalation exposure system	Whole-body exposure system
Equipment cost	Low	High	High
Operation difficulty	High	Low	Low
Animal activity level	Limited	Limited	Unlimited
Dosage	Instilled dosage (mg/kg of body weight or mg/animal)	Defined by the $PM_{2.5}$ concentration (mg/m^3)	
Deposition	Uneven distribution in the lung lobes	Even distribution in the lung lobes	
Application	Acute model, only affecting the lower respiratory tract	Acute or chronic model, affecting the whole respiratory tract	
Source of $PM_{2.5}$	$PM_{2.5}$ powder is usually obtained through high volume air sampler collection, ultrasonic elution and vacuum freeze drying. Different elution methods have an impact on the composition of $PM_{2.5}$ obtained	$PM_{2.5}$ is collected directly from the air and concentrated for the required exposure concentration. The composition of $PM_{2.5}$ will be affected by the spatial and temporal distribution of the collection sites	

tract lavage fluid, a recent study confirmed that there is a link between $PM_{2.5}$ exposure and alterations of the respiratory tract microecology (Yang et al., 2019).

Insufficiency and Dysfunction of Immune Cells

A variety of immune cells are resident in the respiratory tract, including AMs, polymorphonuclear granulocytes (PMNs), lymphocytes, etc. Their numbers and functions are essential to protect against pathogen invasion. Zhao et al. (2014) found that the reduction of phagocytic phagocytosis caused by $PM_{2.5}$ exposure was related to a decrease of NKs in a $PM_{2.5}$ exposure rat model and subsequently infected with *S. aureus*. Another research found that $PM_{2.5}$ exposure can trigger a Th2-type immune response and reduce the phagocytic capacity of AMs, which may be related to Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) (Zhao et al., 2012). In addition, Ma et al. (2017) found that long-term exposure to $PM_{2.5}$ lowered influenza virus resistance via down-regulating pulmonary macrophage Kdm6a and mediated histones modification in IL-6 and IFN-β promoter regions. Another two studies indicated that $PM_{2.5}$ can be used as an immunity inhibitor to reduce the phagocytic capacity of macrophages, thereby increasing the susceptibility to *S. pneumoniae* infection (Migliaccio et al., 2013; Zhao et al., 2014). PM exposure has also been indicated to decrease the ability of macrophages to produce antimicrobial oxidants in response to *L. monocytogenes*, which may play a role in the increased susceptibility of rats to respiratory infection (Yang et al., 2001).

CONCLUSION AND FUTURE PERSPECTIVES

In summary, there are sufficient epidemiological evidences from outpatient, emergency and hospitalization-related data that exposure to $PM_{2.5}$ increases susceptibility to respiratory infections. However, studies on the association between $PM_{2.5}$ exposure and age, gender, and specific pathogens remain controversial. In the future, a meta-analysis of existing research can be attempted to further confirm the susceptible population of $PM_{2.5}$ exposure to respiratory infections. We notice that there is a lag effect in the association between $PM_{2.5}$ exposure and respiratory infections. This may be due to the variations of the average incubation period of different pathogens. We also note that only a few epidemiological studies have reported the relationship between $PM_{2.5}$ exposure and the infection rates of specific pathogens associated with respiratory infections. Is there a specific pathogen preference for respiratory infections caused by $PM_{2.5}$ exposure? This still requires the unremitting efforts of the researchers.

In vivo and *in vitro* studies have shown that $PM_{2.5}$ exposure is beneficial to the adhesion, colonization and growth of microorganisms, but it is not conducive to the removal from the body. However, most of the current *in vivo* experiments establish a $PM_{2.5}$ exposure animal model by intratracheal instillation. This model usually affects the lower respiratory tract and is prone to uneven distribution in the lung lobes. Concentration and enrichment of $PM_{2.5}$ aerosol combined with the oral-inhalation or whole-body exposure system may be the best model for simulating human exposure. In recent years, an emerging technology has appeared, combing versatile aerosol concentration enrichment system (VACES) with oral-inhalation or whole-body exposure system. The advantages and disadvantages of these two models are shown in **Table 3**. Furthermore, current *in vitro* experiments are limited to epithelial cells- and alveolar macrophage-mediated innate immune responses, lacking attention to adaptive immune responses. Either *in vivo* or *in vitro*, the exposure doses currently used in the study are usually based on the corresponding toxicity experiments. How can such exposure levels represent real-world or clinical scenarios?

$PM_{2.5}$ exposure may impair the host defense system of respiratory system, making the organism susceptible to infection. In brief, the possible mechanisms include defective airway epithelial host defense functions, alterations of the respiratory tract microecology, and insufficient number or dysfunction of immune cells (**Figure 2**). However, there is still a lack of robust research on molecular mechanisms. Further efforts are desperately needed to elucidate the underlying mechanisms at the molecular level.

AUTHOR CONTRIBUTIONS

XT conceived and designed the manuscript. LY, CL, and XT wrote the manuscript and critically revised it. CL and XT generated the figures. XT provided guidance and edited the manuscript.

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REVIEW
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Airway Epithelial Dynamics in Allergy and Related Chronic Inflammatory Airway Diseases

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Allergic rhinitis, chronic rhinosinusitis, and asthma are highly prevalent, multifactorial chronic airway diseases. Several environmental and genetic factors affect airway epithelial dynamics leading to activation of inflammatory mechanisms in the airways. This review links environmental factors to host epithelial immunity in airway diseases. Understanding altered homeostasis of the airway epithelium might provide important targets for diagnostics and therapy of chronic airway diseases.

Keywords: asthma, chronic rhinosinusitis, epithelium, allergic rhinitis (AR), inflammation

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BULLET POINTS

Chronic airway diseases are mediated by several, in part unknown, host epithelial- and environment dependent mechanisms.

- The typical disease-leading host-environmental effects are on mucociliary clearance and other innate immunity functions of the epithelial barrier (such as epithelial junctions, pattern recognition, self-renewal, activation of adaptive immunity, metabolism).
- In AR, allergens are able to penetrate airway mucosa by their proteolytic, lipid-binding, and microbial-mimicking properties through the epithelial cells and/or between them.
- Airway viral infections have been implicated in development and exacerbations of AR and asthma, however, their role in CRS pathogenesis remains unclear.
- Airborne irritants, such as cigarette smoke, disrupt epithelial junction proteins and transepithelial resistance, and contribute to CRS alone or together with viral infection.
- Air pollutants might predispose to CRS via aberrant epithelial function.
- Microbiome dysbiosis likely contributes to CRS pathogenesis.
- *S. aureus* colonization indirectly/directly affects mucosal barrier function, leading to a Th2 type inflammation pattern.
- The role of fungi has been shown in at least two CRS phenotypes, fungal balls, and allergic fungal rhinosinusitis (AFRS).

INTRODUCTION

In allergic rhinitis (AR), allergens bind to specific immunoglobulin (Ig)E, leading to rhinorrhea, obstruction, itch, sneeze, and fatigue in sensitized subjects (Wise et al., 2018). AR is interlinked to co-morbidities including asthma, allergic conjunctivitis and atopic dermatitis. However, its role in chronic rhinosinusitis (CRS) is not clear. Chronic inflammation, mucus hypersecretion,

edema, variable obstruction, and fatigue characterize asthma. In both children and adults, asthma encompasses different, overlapping phenotypes (Wenzel, 2012; Kaur and Chupp, 2019). Allergic multi-morbidity and predominance in males characterize childhood-onset asthma, whereas adult-onset asthma is more common in females and includes a wide variety of allergic [T helper (Th) Type 2 (Th2)-high] and non-allergic (often Th1-high) phenotypes (Wenzel, 2012; Frohlich et al., 2017). Severe eosinophilic forms, e.g., non-steroidal anti-inflammatory drug (NSAID-) exacerbated respiratory disease (NERD), are more common in adults. So far, only few encouraging signals have been found in asthma prevention. The problem may be over-simplification of terminology. Asthma is not a single disease entity, but rather a complex, heterogeneous, and dynamic immunological disorder strongly influenced by gene – environment interactions.

AR and asthma affect over 300 million people worldwide, thus being major public health problems (Gupta et al., 2004; Nunes et al., 2017; GINA, 2018). The prevalence of AR is 15–50% (Pallasaho et al., 2006; Wiksten et al., 2018), its prevalence at teen-age is 13–38% (Pols et al., 2016; Blaiss et al., 2018; Sterner et al., 2019). The prevalence and socioeconomic impact are difficult to calculate since mild symptoms do not require medical treatment, and most patients outgrow their (especially food) allergies. The prevalence and incidence of, particularly childhood, asthma varies greatly in different parts of the world. After many decades of continuously increasing asthma rates in the Western world, we seem to have reached a plateau in asthma incidence since the beginning of 2000 in many developed countries. In some places even a decrease has been observed. Children migrating from low-income areas to higher socioeconomic areas have a lower prevalence of asthma, suggesting a critical time window for asthma onset in childhood. This suggests the possibility of asthma prevention, since there appear to be predisposing biological factors influenced by the environment. On the other hand, it is likely that within a population, there are genetic factors limiting the number of asthmatics. It should be kept in mind that up to 85% of asthma patients have AR, and on the other hand, 15–38% of AR patients have asthma (Mésidor et al., 2019). Of adults with asthma, 80% have rhinitis, and 50% have chronic rhinosinusitis (Jarvis et al., 2012).

Chronic rhinosinusitis (CRS) is a chronic symptomatic inflammation of the sinonasal tract, with a prevalence of 3–10% (Fokkens et al., 2012; Dietz de Loos et al., 2019; Hirsch et al., 2019). CRS presents with (CRSwNP) or without (CRSsNP) nasal polyps (NP), and is defined by typical subjective symptoms (facial pain, post-nasal drip, obstruction, discharge) lasting for at least 12 weeks, objectively confirmed by either positive endoscopic findings (oedema, mucus secretions, polyps) or positive radiologic findings (mucosal inflammation on sinus CT scans). NERD tends to lead to more severe forms of CRS, with NPs and asthma.

The pathomechanisms of asthma, CRS and AR are related to genetic predisposition and aberrant host-immune interactions during development. The environment strongly affects gene expression by epigenetic mechanisms. In addition to genetic predisposition, climate change, population growth, aging, and urbanization impact the increasing prevalence of chronic airway diseases (Kaur and Chupp, 2019).

Genetics and environmental factors can, during development, significantly modulate barrier homeostasis, influencing the predilection toward chronic airway inflammation. The respiratory epithelium is a part of the innate and adaptive immune system, with responsibility for several functions such as mucociliary clearance, pattern recognition, phagocytosis, antigen presentation, signaling, and self-renewal. Airway epithelial dysfunction is related to several airway diseases. The main focus of this review are the pathomechanisms of human airway epithelium in AR, CRS, and asthma. We also briefly discuss altered airway epithelium in bronchiectasis, primary ciliary dyskinesia (PCD), and cystic fibrosis (CF).

GENOME-SCALE EPITHELIAL FACTORS BEHIND AIRWAY DISEASES

Adult-onset asthma is mediated by activation of molecular pathways leading to persistent mucosal inflammation, variable airway obstruction, inflammation, and tissue remodeling. Genetic and epigenetic variation of the host play key roles (Willis-Owen et al., 2018), and airway dysbiosis may be an important trigger (Huang et al., 2015). Childhood-onset asthma appears to be triggered by allergic and infective immune responses, and barrier dysfunction, with a stronger genetic component and higher heritability (Pividori et al., 2019; Schoettler et al., 2019). Genome-wide association studies (GWAS)s have focused on childhood-onset allergic asthma, and the currently identified single nucleotide polymorphisms (SNP)s thus seem to have lower significance in adult-onset asthma (Pividori et al., 2019). Candidate genes for asthma include interleukin (IL)-4, IL-13 and IL-4R, ADRB2, MS4A2, tumor necrosis factor α (TNFα), cluster of differentiation (CD)14, human leukocyte antigen (HLA)-DRB1 and HLA-DQB1 (Toskala and Kennedy, 2015; Willis-Owen et al., 2018). Combined GWAS and transcriptome-wide association tests have identified several genetic loci that may be relevant in asthma, such as the chromosome 17q locus, HLA, IL-6, interferon regulatory factor 4 (IRF4), chemokine (C-C motif) ligand 20 (CCL20), mucin 5AC (MUC5AC), fatty acid desaturase 2 (FADS2), T-box transcription factor 21 (TBX21), runt-related transcription factor 1 (RUNX1), and cytokine and chemokine receptors and signaling molecules (Pividori et al., 2019; Vercelli and Bleecker, 2019). A study showed that asthma remission is associated with genes related to Th2-mediated inflammation, such as IL1RL1-, IL18R1-, and IL-13 (Vonk et al., 2018). A singular GWAS study of a Japanese adult-onset asthma population showed that SNPs of HLA-, thymic stromal lymphopoietin-WD repeat domain 36 (TSLP-WDR36)-, and ubiquitin specific peptidase 38-GRB2 associated binding protein 1 (USP38-GAB1) loci are associated with adult-onset asthma (Hirota et al., 2011).

A missense variant in arachidonate 15-lipoxygenase (ALOX15) is protective against the development of CRSwNP (Kristjansson et al., 2019). In nasal epithelial cells, IL-13 upregulates ALOX15 and promotes eoxtaxin 3 expression,

likely promoting tissue eosinophilia in CRSwNP patients (Li et al., 2019). This pathway may also have a role in epithelial remodeling and barrier dysfunction (Kristjansson et al., 2019). Our transcriptomics of healthy middle turbinate epithelium showed that 75% of protein encoding genes are expressed, suggesting that the epithelium is a very active organ (Hanif et al., 2019). The importance of barrier-environmental interactions was detected also in a single cell transcriptomics study of nasal epithelium, demonstrating that basal cell memory of Type 2 inflammation leads to persistent dysfunction in CRSwNP patients (Ordovas-Montanes et al., 2018).

Multi-gene expression-based biomarkers of asthma were studied in a Network-identified Transcription Factor – framework (Ahsen et al., 2019). ETS translocation variant (ETV4) and Peroxisome proliferator-activated receptor gamma (PPARG) were identified as being the most significant transcription factors. The group further performed validation studies using a nasal epithelium cell line in which both transcription factors were knocked down by siRNA. The respective cell lines produced a significantly decreased amount of IL-8 and IL-6, before poly (I:C) stimulation and before (Ahsen et al., 2019).

EPITHELIAL DYNAMICS IN UPPER AIRWAY DISEASES

Airway barriers likely have a key role in the development of CRS and AR. In AR, mucosal inflammation is triggered by allergens, together with other environmental factors, leading to IgE-mediated mucosal inflammation. In CRS, mucosal inflammation is triggered by a dysfunctional interaction between exogenous agents and the immune system (Liao et al., 2014; Lam et al., 2015; Hoggard et al., 2017). Compared to AR, there is larger inter- and intra-individual variation in the causal factors behind CRS (**Figure 1**).

Environmental Factors and Upper Airway Epithelium

Allergen entry into airway mucosa is facilitated by several allergen-, host-, and environment-dependent mechanisms. Allergens are able to penetrate airway mucosa by their proteolytic, lipid-binding, and microbial-mimicking properties (Toppila-Salmi et al., 2015). Mite allergen entry has shown to be facilitated by altered pattern recognition pathways (Lam et al., 2015). Our study group has shown that birch pollen allergens (Bet v1) are able to bind plasma membrane lipid rafts (Toppila-Salmi et al., 2015) and to be transported in caveolar vesicles through the nasal epithelium until they reach mast cells. However, this was detected in allergic patients only (Joenvaara et al., 2009). Moreover, natural birch exposure causes transcriptomics alterations in controls; the fold changes were detected in the nasal epithelial transcripts belonging to Gene Ontology (GO) –category "Immunology". In contrast, transcripts of the AR group were enriched in "Response to virus" and "Cellular transportation" –categories (Mattila et al., 2010). Similar categories have been demonstrated in grass pollen allergy (Roschmann et al., 2011). We performed whole transcriptomic sequencing of nasal epithelial brush samples and demonstrated that birch pollen allergen immunotherapy alters the transcript profile during pollen exposure season toward that of control samples (Hanif et al., 2019). Moreover, the data showed that in AR patients who started with subcutaneous birch pollen immunotherapy, microbiome diversity of nasal epithelium shifted toward that of controls (Hanif et al., 2019).

AR has been shown to associated with active/passive smoking (Saulyte et al., 2014). Taken together, in AR, airborne allergens, as well as tobacco smoke, microbes, and air pollutants are related to the development and aggravation of the disease (Saulyte et al., 2014; Wiksten et al., 2018).

In prevention of CRS development and its progression, commensal organisms likely play a protective role by preventing mucosal colonization by pathogens and putatively providing metabolites improving mucosal health. It has been assumed that microbial agents and microbiome dysbiosis are some of the most important drivers of CRS pathogenesis. *S. aureus* can directly affect mucosal barrier function and drive Type 2 inflammation (Ryu and Kim, 2020). Molecular sequencing techniques are evolving in power and enable studies on total and relative microbial abundance and their functional activity within the sinonasal tract (Earl et al., 2018). Yet it is important to note intra- and inter-individual variation of the samples and sites of sampling (Copeland et al., 2018). In a cross-sectional study involving sequencing of the V3-V4 region of the 16S ribosomal RNA (rRNA) gene in nasal samples, decreased middle meatal microbiome diversity was detected in AR, CRSsNP, and CRSwNP groups compared to controls, with high variability in microbe profiles and even within subjects (Lal et al., 2017). Hence, recent studies support the hypothesis that dysbiosis of the microbiome may trigger mucosal inflammation both in AR and in CRS (Knight et al., 2018). Airway viral infections have been implicated in development and exacerbations of AR and asthma, however, their role in CRS pathogenesis remains unclear. In addition, the role of fungi has been shown in at least two CRS phenotypes, fungal balls and allergic fungal rhinosinusitis (AFRS).

Cigarette smoke extract has been shown to disrupt epithelial junction proteins and transepithelial resistance in an epithelial sinonasal model derived from control patient samples (Tharakan et al., 2016). In addition, cigarette smoke and viral infection might contribute to polyp remodeling (Yamin et al., 2015). Decreased levels of airway epithelial clara cell protein 16 (CC16) are found in the nasal secretions and plasma of patients with CRS and in subjects exposed to high levels of air pollutants (Peric et al., 2018). There is some evidence that heavy metal exposure is related to CRS (Khlifi et al., 2015).

Upper Airway Epithelial Functions During AR and CRS

The upper airway barrier provides several important innate immunity functions, blockage of microbe entry, and recruitment of leukocytes. Airway epithelium plays a critical role in conducting and humidifying air, responding to trigeminal and olfactory stimuli, and in host defense. The airway epithelial barrier comprises ciliated cells, olfactory cells (in olfactory

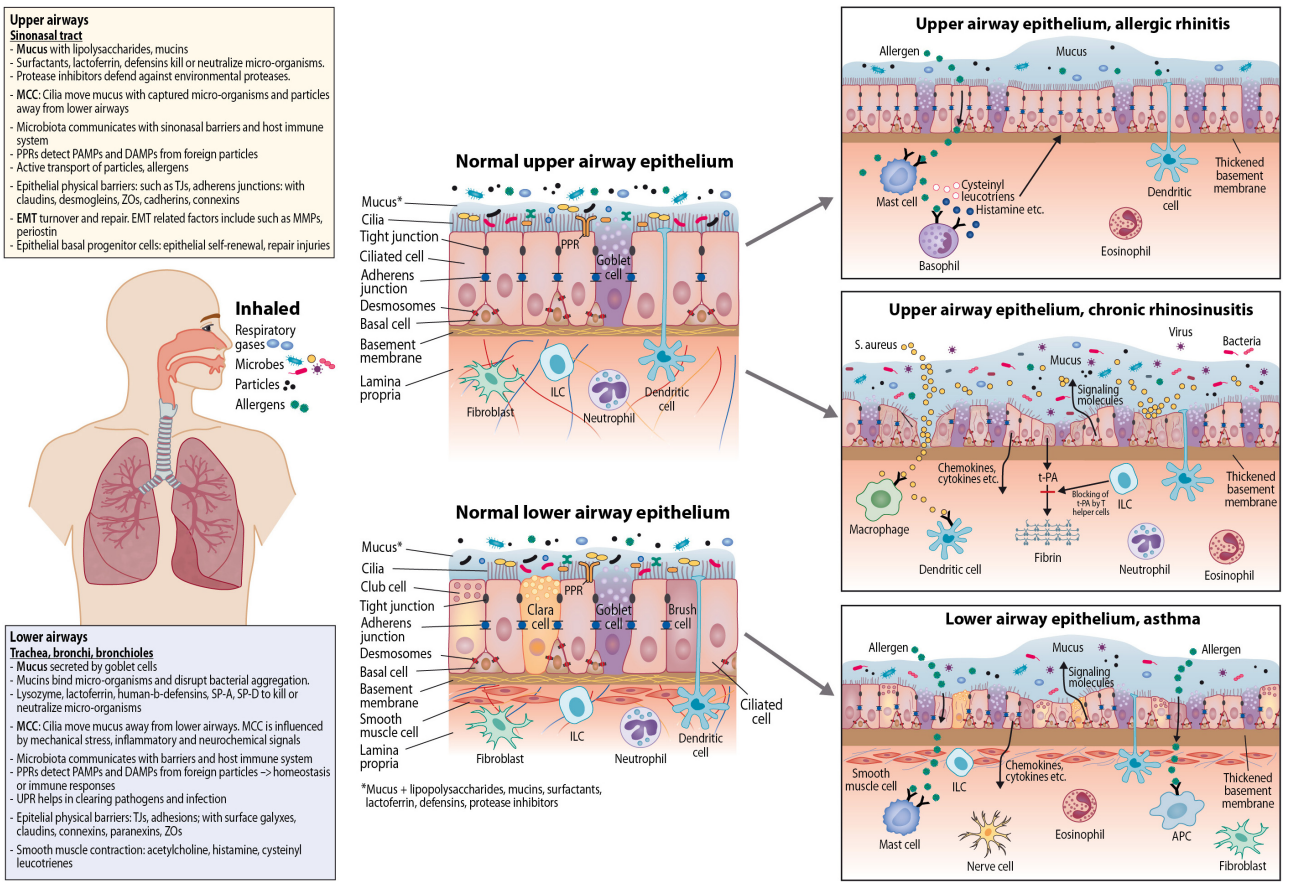

FIGURE 1 | Epithelial dynamics in upper and lower airway epithelium; normal epithelium and epithelium in allergic rhinitis, chronic rhinosinusitis, and asthma. APC, Antigen-presenting cell; ILC, innate lymphoid cell; PPR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern; MCC, mucociliary clearance; TJ, tight junction; t-PA, tissue plasminogen activator; ZO, zonula occludin; EMT, epithelial-mesenchymal transition; MMP, matrix metalloproteinase; SP, surfactant protein; UPR, unfolded protein response.

epithelium), mucus-secreting goblet cells, basal cells with progenitor capacity (Bravo et al., 2013) and few chemosensory cells (Kohanski et al., 2018).

Motile cilia consist of microtubules and dynein arms, powered by adenosine triphosphate (ATP) (Knowles et al., 2013; Popatia et al., 2014). Coordinated ciliary beating transports debris-laden mucus from respiratory passages toward the oropharynx (Toppila-Salmi et al., 2015). Genetic and acquired defects in mucociliary flow with increased mucus viscosity are associated with a high incidence of CRS (Cutting, 2005; Gudis et al., 2012). Cilia are coated with mucins and tethered mucopolysaccharides (Toppila-Salmi et al., 2015). Li Y. Y. et al. (2014) detected cilia abnormalities in CRSwNP (Jiao et al., 2015), and that impaired ciliated epithelial differentiation may be mediated via decreased interferon gamma (IFNγ) and IL-13 levels leading to secondary declines in ciliary beat frequency (Jiao et al., 2015). Reduced expression of WDPCP, a ciliogenesis protein, due to inflammatory cytokines resulted in impaired ciliogenesis and cilia function in CRS patients (Ma et al., 2017). Secretory cells produce polymeric gel-forming mucins, such as MUC5AC and MUC5B (Toppila-Salmi et al., 2015). Gel-forming mucins are secreted into the airway lumen and are responsible for the characteristic viscoelastic properties of the mucus gel layer. Tipirneni et al. (2018) identified that dynamic mucus strand velocities from submucosal glands, a major component of mucociliary clearance, were significantly decreased in CRS. Surfactant protein A gene expression has been shown to be increased in CRS and decreased in primary atrophic rhinitis (El-Anwar et al., 2015). In overview, CRS is commonly associated with mucociliary dysfunction.

Both epithelial specification and terminal differentiation are critical to epithelial homeostasis in airway diseases (Ordovas-Montanes et al., 2018). Environmental and mucosal signals regulate epithelial stem-cell self-renewal under normal conditions (Yilmaz et al., 2012) and in CRS (Ordovas-Montanes et al., 2018). Under physiological conditions, environmental and intrinsic signals are able to rapidly alter the composition and function of the epithelium. A study group performed single cell transcriptomics of polyps/scraping epithelium of twelve CRSwNP and nine control subjects. They detected differences in expression of antimicrobial genes by secretory cells, a loss of glandular cell heterogeneity, and that polyp basal progenitor cells were locked to a Type 2 memory (Ordovas-Montanes et al., 2018). Epithelial basal progenitor cells

are able to migrate and proliferate into ciliated and goblet cells in injured regions.

Upper airway epithelium secretes several cytokines including TSLP, IL-33, and IL-25. These cytokines are released by tissue damage, pathogen recognition or allergen exposure. They effect Th2 cell function either directly or via innate lymphoid cells (ILCs), which in turn produce IL-5, IL-9, and IL-13 (Licona-Limon et al., 2013; Scadding, 2014), related to Th2-type CRS, asthma and AR (Toppila-Salmi et al., 2015). *S. aureus* colonization is more common in patients with CRSwNP than controls (Tomassen et al., 2016). *S. aureus* produces serine protease-like protein (Spl), which causes Th2- biased inflammatory responses via TSLP and IL-33 (Stentzel et al., 2017; Ryu and Kim, 2020). *S. aureus* specific IgE has been associated with both CRSwNP and asthma. *S. aureus* enterotoxins and *S. aureus* Spls have been found to have allergenic properties (Stentzel et al., 2017; Ryu and Kim, 2020). Type 2 cytokines inhibit t-PA (tissue plasminogen activator) resulting in the deposition of fibrin mesh to form the tissue matrix of NPs (Takabayashi et al., 2013).

Pattern recognition receptors (PRRs) rapidly detect microbial and other foreign molecular patterns and either maintain homoeostasis or induce immune responses. Luukkainen et al. (2018) stimulated a co-culture of peripheral blood mononuclear cells and nasal epithelium, differentiated from stem/progenitor cells, with H3N2-virus and detected rapid activation of monocytes, natural killer (NK)-cells and innate T-cells (MAIT and γδ T cells). We demonstrated high baseline nasal epithelial expression of Toll Like receptor (TLR) proteins (TLR1-7, TLR9-10) and MyD88 both in AR and in controls (Renkonen et al., 2015). After off-seasonal intranasal birch pollen challenge, a negative change in the expression score of TLR1 and TLR6 proteins was detected in the atopic group. Tengroth et al. (2014b) demonstrated abundant TLR3, TLR7, TLR9, RIG-I, and MDA-5 in nasal epithelium. The group detected defects in TLR9-mediated microbial defense in CRSwNP (Tengroth et al., 2014a). Studies on polyp and control tissue show that increased epithelial TLR2 (Sun et al., 2012) and TLR4 may be related with CRSwNP (Sun et al., 2012; Shimizu et al., 2016; Hu and Li, 2018). Nasal polyp fibroblast activation may occur via TLR2 (Shin et al., 2016a,b; Tsai et al., 2018), TLR4 (Cho et al., 2014, 2016), TLR5 (Shin et al., 2016b), and TLR9 (Park et al., 2018) might be related to polyp B-cell activation (Xu et al., 2014). NOD family PRRs form a major component of the inflammasome, and are related in programmed pro-inflammatory cell death distinct from apoptosis. Jardeleza et al. (2013) detected involvement of inflammasome complexes and their signaling pathways in *Staphylococcus aureus* -biofilm positive CRSwNP. Lin et al. (2016) detected increased NLRP3 and caspase-1 in eosinophilic CRSwNP, and augmented inflammasome signaling pathway by lipopolysaccharides (LPS). Bitter taste receptors (T2Rs) are G protein-coupled receptors that function as non-classical PRRs. Bacterial quinolones and acyl-homoserine lactones, secreted by gram-negative bacteria, can activate airway T2R-mediated immune responses (Lee and Cohen, 2014; Freund et al., 2018). Linkage studies have demonstrated associations between taste receptor genetics with CRS (Cohen, 2017).

Airway epithelium secretes defense molecules such as surfactant, lactoferrin, and defensins, which kill or neutralize microorganisms. Some evidence suggests that decreased secretion of host defense molecules is associated with CRS (Tieu et al., 2010). Decreased expression levels of palate, lung, and nasal epithelium clone protein (PLUNC), and increased surfactant-B and alpha-defensin levels have been observed in CRSwNP, possibly secondary to loss of glands (Seshadri et al., 2012; Jardeleza et al., 2013; Lin et al., 2016; Tsybikov et al., 2016).

Protease inhibitors regulate environmental proteases that might compromise barrier integrity. Blocking protease allergens with inhibitors reduces allergic responses in AR (Suzuki et al., 2006). Fukuoka et al. (2019) showed that human cystatin SN, an endogenous protease inhibitor, suppresses AR symptoms by inhibiting allergen protease activities and by allergen-specifically protecting nasal TJ barrier. Kouzaki et al. (2016) showed lower host expression of two protease inhibitors (cystatin A and SPINK5) in nasal epithelial cells extracted from patients with eosinophilic CRS compared with control and non-eosinophilic CRS groups. This suggests that an imbalance of proteases and protease inhibitors within the epithelial barrier may contribute to the pathogenesis of Type 2 diseases in general (Wu et al., 2018).

Epithelial cells undergo turnover and repair after injury through epithelial to mesenchymal transition (EMT), with a rapid and normally reversible modulation of the epithelial phenotype toward mesenchymal cells (Toppila-Salmi et al., 2015). During EMT, epithelial cells lose cell-cell polarity and adhesion to become migratory. They get mesenchymal features such as alpha-smooth muscle actin, vimentin, matrix metalloproteinases (MMPs), and transcription factors. In CRS, aberrant epithelial structure and function may lead to increased permeability to foreign material suggesting this as an early factor in CRS pathogenesis (Pothoven et al., 2015; Ramezanpour et al., 2016; Suzuki et al., 2016; Jiao et al., 2019). Inflammation leads to remodeling with cytokines, mediators, enzymes, and other factors determining the remodeling pattern, not fully depending on the CRS phenotype. The duration and type of inflammation affect mucosal structure and function, and clinical severity of inflammation. Remodeling changes of CRS include fibrosis, basement membrane thickening (BMT), goblet cell hyperplasia, epithelial barrier abnormalities and polyp formation, osteitis, and angiogenesis (Barham et al., 2015; Kuhar et al., 2017).

Periostin promotes adhesion and migration of epithelial cells and is associated with CRSwNP (Ishida et al., 2012; Ohta et al., 2014; Laury et al., 2015; Milonski et al., 2015; Shiono et al., 2015; Wang et al., 2015; Ebenezer et al., 2017; Xu et al., 2017; Wei et al., 2018; Yang et al., 2018; Lehmann et al., 2019) and asthma (Carpagnano et al., 2018; Wei et al., 2018). The EMT process is driven by an array of factors such as WNT, reactive oxygen species, proteases, HIF1, IL-13 Epiregulin, Oncostatin M, and IL-1 (Oyer et al., 2013; Batzakakis et al., 2014; Schleimer, 2017; Aazami et al., 2018; Yang et al., 2018; Tomaszewska et al., 2019). CRSwNP patients have altered expression levels of EMT related factors, such as MMP-1 (Malinsky et al., 2013; Homma et al., 2017), MMP-2 (Malinsky et al., 2013), TIMP-1 (Wang et al., 2012; Muluk et al., 2015), MMP-7 (Yang et al., 2017; Chen et al., 2018), MMP-9 (Wang et al., 2012; Malinsky et al., 2013;

Yeo et al., 2013; Li et al., 2015; Muluk et al., 2015; Chen et al., 2018; Suzuki et al., 2018; Xiang et al., 2019), TIMP-2 (Li et al., 2015) E-cadherin (Hupin et al., 2014; Kim et al., 2018; Deng et al., 2019). BMT is associated with duration of inflammation independent of tissue eosinophilia (Kountakis et al., 2004; Kuhar et al., 2017). TGF-β has been most closely linked to fibrosis, but IL-13 and osteopontin have also been implicated in BMT and fibrosis (Rehl et al., 2007; Van Bruaene and Bachert, 2011; Shi et al., 2013; Schleimer and Berdnikovs, 2017). Abnormalities of the coagulation cascade have also been associated with polyp formation including Factor X, tissue factor and thrombin (Shimizu et al., 2015; Shimizu et al., 2017; Takabayashi et al., 2019). There is some evidence that stem cells in the epithelium maintain a memory for the chronic immature EMT state in severe Type 2 CRS (Lehmann et al., 2019), promoting barrier failure, antigen access, and this inflammation (Pothoven and Schleimer, 2017). Type 2 cytokines inhibit t-PA activity so in the presence of high levels of Type 2 inflammation, the matrix will be retained and grow (Peterson et al., 2012).

Compared to the CRS phenotype, remodeling has been less studied in patients with AR. Li and Li (2019) performed transmission electron microscopy, western blot, and qPCR to nasal epithelial samples of patients with AR and detected increased autophagosomes, Beclin-1, LC3-II, and Collagen III, along with increased symptom scores, suggesting a link between autophagy and airway remodeling in AR. Taken together, several epithelial phenomena take part in the development and chronicity of the inflammation during CRS and AR.

Epithelial physical barriers are maintained by intercellular junctions. Tight junctions (TJ) are located the most apically, are linked to the cytoskeleton and inhibit solute and water movement through the paracellular space, thus establishing cell polarity (Toppila-Salmi et al., 2015). Inhaled allergens, microbial or viral infections, cytokines, hypoxia, and zinc deficiency are able to affect TJ molecules and epithelial barrier function in the airways (Roscioli et al., 2017; Jiao et al., 2019). Several genes/molecules, such as SPINK5, S100A7, S100A8/9, PCDH1, NDRG1, SPRR, and p63 are involved in modulating the physical barrier function in CRS (Jiao et al., 2019). Yu et al. (2013) demonstrated decreased expression of Epithelium membrane protein 1 (EMP1), a TJ protein, in nasal polyp epithelium compared to control nasal mucosa. Soyka et al. (2012) detected a decreased trans-tissue resistance in biopsy specimens from patients with CRSwNP and also decreased TJ proteins. Suzuki et al. (2016) detected that nasal polyp had a higher expression of claudin (Cld)1 but lower expression of tricellulin compared with the turbinate. Integrity of the nasal epithelial TJ barrier has been shown to be compromised in Chinese patients with eosinophilic and non-eosinophilic CRSwNP transforming growth factor (TGF)-β1 seems to plays an important role in inducing TJ barrier defects (Jiao et al., 2018). Li et al. showed decreased expression of epithelial zonula occludin (ZO)-1, Cld1, desmoglein (DSG)1, and DSG2 in CRSwNP and decreased expression of Cld1, DSG1, and DSG2 in CRSsNP (Li Y. et al., 2014).

Adherens junctions are located more basally than TJs. Epithelial cadherin (E-cadherin) creates intercellular interactions. Together, these junction proteins function to limit intercellular passage of fluid and protect the underlying tissue from exposure to noxious and allergenic stimuli (London and Ramanathan, 2017). Epithelial cell communication is mediated via Gap junction channels, which are formed by connexin proteins enabling cell communication (Kim et al., 2016). Expression of connexins have been demonstrated to be increased in CRS compared to controls (Kim et al., 2016). Hence, epithelial barrier dysfunction may contribute to AR and CRS through allowing increased passage of antigens and exposure of underlying tissue to these stimuli (Toppila-Salmi et al., 2015; London and Ramanathan, 2017).

EPITHELIAL DYNAMICS IN LOWER AIRWAY DISEASES

Ventilation moves air through the conducting airways to and from the alveoli. The inhaled air contains respiratory gases, particles, microbes, and toxins. Defense mechanisms preventing the entry of unwanted substances into the lung tissue and circulatory system include the branching structure of the conducting airways, the layers of mucus covering the airways, mucociliary clearance, contraction of the airway smooth muscle, the tight adhesions of airway epithelial cells (AEC) and their underlying stroma, and the production of host-defense molecules regulated by exposure to toxins, pathogens and cytokines (Lambrecht and Hammad, 2012; Whitsett and Alenghat, 2015).

Asthma is a chronic pulmonary disease characterized by airway inflammation, airway hyperreactivity, and recurrent, reversible airway obstruction. Several asthma phenotypes related to disease mechanisms exist; these include childhood-onset allergic asthma, adult-onset eosinophilic asthma, obesity-related asthma, and neutrophilic asthma (Wenzel, 2012). Airway inflammation is initiated by AECs as a defense against inhaled pathogens and particles, e.g., allergens. In asthma these defense mechanisms are hyperreactive. Excessive mucus production contributes to airway obstruction, which is mainly caused by contraction of airway smooth muscle (Erle and Sheppard, 2014; **Figure 1**).

Environmental Factors, Epithelium, and Asthma

The risk factors for asthma include AR and allergic conjunctivitis, atopic dermatitis, exposure to air pollution, cigarette smoke, occupational risk factors, obesity, and genetic factors (Polosa and Thomson, 2013; Ilmarinen et al., 2015; Toskala and Kennedy, 2015; Willis-Owen et al., 2018; Toppila-Salmi et al., 2019). Childhood-onset asthma has a stronger genetic component than adult asthma and is triggered due to dysregulated allergy and epithelial barrier function (Pividori et al., 2019; Schoettler et al., 2019). The development of adult asthma is more likely in patients with an accumulation of several risk factors and with allergic multimorbidities (Hallit et al., 2019; Pividori et al., 2019; Toppila-Salmi et al., 2019). Asthma, rhinitis, and chronic rhinosinusitis often co-exist (Jarvis et al., 2012).

The airway microbiome communicates with the respiratory epithelium and has an important role in maintaining airway health (Hansel et al., 2013; Morris et al., 2013; Huang et al., 2015). Disruption of normal mucociliary clearance in smokers, and in patients with asthma or cystic fibrosis (CF) affects airway microbiome homeostasis and may lead to disease (Dickson et al., 2013; Hansel et al., 2013; Morris et al., 2013). Early childhood respiratory tract microbial exposure influences immune responses, regulating Th1 and Th2 immunity and affecting future asthmatic responses (Busse et al., 2010; Ege et al., 2011). In children with a family history of asthma, the risk of asthma is increased by severe respiratory syncytial virus (RSV) infections (Sigurs et al., 2000). Virus infections of the respiratory tract, especially caused by rhinoviruses, associate with asthma exacerbations (Busse et al., 2010). Patients with asthma may have deficient IFNγ response leading to prolonged and more severe viral infections, deficient IFNγ response has also been linked to Th2 type immune reaction (Lisspers et al., 2018).

Epithelial Functions in Asthma

The trachea, bronchi, and bronchioles are mainly lined by ciliated pseudostratified epithelium. Also serous, club, goblet and neuroendocrine cells, and smooth muscle cells are found in the airways.

In patients with asthma and bronchiectasis, excessive goblet cell differentiation, and mucus production are common (Whitsett and Alenghat, 2015).

Submucosal glands in the trachea and bronchi are lined by basal, ciliated, myoepithelial, serous and goblet cells, and secrete fluids and host-defense molecules such as lysozyme, lactoferrin, human-b-defensins, and surfactant proteins A and D. Mucins are large glycoproteins mainly produced by goblet cells (mucin granules), they have important functions in cell-cell interaction, EGFR signaling and airway protection (Thai et al., 2008; Voynow and Rubin, 2009). Mucins tied to AECs (MUC4, MUC13, MUC16, MUC21) create a barrier that by pathogen or host-associated proteases can shed, enabling the unwanted microbe to be removed by mucociliary clearance. Secreted airway mucins (MUC5B, MUC5AC, and MUC2) form a microbe binding, bacterial aggregation disrupting gel. The expression of secreted mucins is induced by transcription factors e.g., MAPK, STAT6, and inhibited by transcription factors FOXa2 and TTF-1 (Maeda et al., 2011; Alevy et al., 2012; Whitsett and Alenghat, 2015).

Dysfunctional movement of the cilia leads to impaired mucociliary clearance, accumulation of thick mucus and recurrent infections. Primary ciliary dyskinesia (PCD) patients have structurally abnormal cilia, decreased production of nitric oxide by AECs, bronchiectasis, and chronic bacterial infections of both the upper and lower airways (Knowles et al., 2013; Popatia et al., 2014). Secondary impairment of ciliary movement, e.g., due to smoking or CF, leads to abnormalities in airway hydration and mucus production. Ciliary function is influenced by mechanical stress, inflammatory and neurochemical signals, including paracrine signals of AECs. The gap junctions between cells exchange responses induced by these stimuli, connexin Cnx43 plays an important role in this (Martin and Prince, 2008; Bou Saab et al., 2014).

The respiratory epithelium recognizes pathogen-associated molecular patterns (PAMP), from commensal microbes or pathogens, and danger-associated molecular patterns (DAMP), from cell stress or cell death. Membrane associated or cytosolic PRRs expressed in AECs recognize PAMPs and DAMPs, resulting in signaling via TLRs, mitogen-activated protein kinase (MAPK), IRF and nuclear factor-κB (NF-κB) family transcription factors, reactive oxygen species (ROS), and Janus kinase – signal transducer and activator of transcription (JAK-STAT) signaling pathway, some of which also interact (Athari, 2019). The resulting cytokine and chemokine signaling and production of antimicrobial proteins leads to recruitment and activation of innate and adaptive immune system cells, and regulation of barrier function (Voynow and Rubin, 2009; Lambrecht and Hammad, 2012; Erle and Sheppard, 2014; Whitsett and Alenghat, 2015). Also unfolded protein response (UPR) helps in clearing pathogens and infection by linking the synthesis of misfolded proteins encoded by pathogens to inflammatory signaling, activating apoptosis and necrosis to remove the pathogens and infected epithelial cells (Osorio et al., 2013). Alveolar macrophages, but also AECs ingest infected and apoptotic cells by phagocytosis (Juncadella et al., 2013).

TLR4 can be activated by LPS, cigarette smoke, RSV, and inflammatory cytokines (Monick et al., 2003; Armstrong et al., 2004; Pace et al., 2008). Allergens cause TLR4-dependent activation of NF-κBs, resulting in secretion of chemokines and cytokines, such as IL-33, TSLP, and IL-25, and activation and recruitment of pulmonary dendritic cells, Type 2 innate lymphoid cells (ILC2 cells) and Th2 lymphocytes (Hammad et al., 2009; Tan et al., 2010; Lambrecht and Hammad, 2014). Interferons are secreted to defend against viral infections, and in patients with asthma, deficient IFNγ response may lead to prolonged and more severe viral infections (Lisspers et al., 2018). A study group performed expression profiling of airway epithelium that demonstrated similar cytokine profiles of EGR1, DUSP1, FOSL1, JUN, MYC, and IL-6 after stimulation of AECs with either dsRNA or with house dust mite, however, both triggers also induced a specific response (e.g., ATF3, FOS, and NF-κB1) (Golebski et al., 2014). It could thus be possible that microbial infection and its underlying immune dysfunction might be a phenotypic or clinical feature of both atopic and non-atopic chronic conditions in the airways rather than only a secondary effect (Juhn, 2014).

Epithelial extracellular vesicles (EVs) can transfer microRNAs (miRNAs). In particular miR-34a, miR-92b, and miR-210 may have a role in the development of the Th 2 response in asthma (Bartel et al., 2019). As a sign of epithelial dysfunction in asthma, AEC DNA methylation is different in asthmatics vs. non-asthmatics (Clifford et al., 2019).

A recent study shows that asthmatic Type 2 inflammation and airway hyperresponsiveness are related to mast cell infiltration into the airway epithelium and that AECs and mast cells communicate through IL-33 signaling that regulates

inflammation (Altman et al., 2019). In allergic asthma, the inflammatory process is initiated by Th2 cells that produce cytokines, such as IL-4, IL-5, IL-9, IL-13, leading to the production of IgE, eosinophilia, and goblet cell hyperplasia (Whitsett and Alenghat, 2015; Athari, 2019). Pathways to regulate goblet cell hyperplasia are also activated by epidermal growth factor receptor (EGFR) (Tyner et al., 2006). Contraction of the airway smooth muscle, resulting in airway narrowing, is induced by acetylcholine released from efferent parasympathetic nerves, or by histamine and cysteinyl leukotrienes released from mast cells and basophils (Erle and Sheppard, 2014). Asthma is modulated also by Th9 and Th17 cells, that produce IL-17F, IL-22, and IL-17A inducing airway inflammation and enhancing smooth muscle contractility (Kudo et al., 2013). Airway epithelium and smooth muscle cells communicate also via epithelial cell-derived endothelin-1 (Lan et al., 2018). Airway hyperresponsiveness is linked to IL-4 (Athari, 2019), and also to IL-13 and IL-17 by inducing RhoA expression, and Ca^{2+} sensitization of bronchial smooth muscle (Erle and Sheppard, 2014). Airway smooth muscle contractibility is also increased by TNFα (Goto et al., 2009).

The barriers created by AECs are important for the health and normal function of the airways. Increased epithelial permeability and inflammation with disruptions in the TJ complexes may affect the pathogenesis of asthma and other pulmonary diseases (Georas and Rezaee, 2014). Barriers of AECs include secretory products, surface glycocalyces, membranes and intracellular junction proteins consisting of claudins, connexins, paranexins, adhesions, and ZOs. Pulmonary cells claudins, such as claudin 3 (Cld3), Cld4, and Cld18 create interlocking structures forming these TJs (Whitsett and Alenghat, 2015).

The alveoli are lined by only two cell types, simple squamous alveolar squamous epithelium type I (90%) or cuboidal type II cells. The type II alveolar cells are the main progenitor cells for alveolar cells. The type I alveolar cells are in close interaction with the pulmonary capillary endothelial cells to ensure exchange of respiratory gases. Surfactant lipids and proteins secreted by type II epithelial cells diminish alveolar surface tension. They ensure optimal surface tension, prevention of alveolar collapse but ensuring elasticity and gas exchange. The type II alveolar cells have many lipid-rich lamellar bodies, microvilli on the apical surfaces and express surfactant homeostasis mediating lipids and proteins, such as surfactant protein (SP)-A, SP-B, SP-C, SP-D5. SPs bind, aggregate and/or directly kill microbial pathogens, enhancing their clearance by immune system cells (Numata et al., 2010; Whitsett et al., 2010). SP-A and SP-D belong to collectins (innate host-defense proteins); they bind with PAMPs. SP-A and SP-B form tubular myelin that together with lipids forms a pool of surfactant and host-defense proteins (e.g., lysozymes and SP-C). This enhances opsonization and killing of pathogens by alveolar macrophages and regulates macrophage, neutrophil, and lymphocyte activity (Hartshorn, 2010; Whitsett and Alenghat, 2015). Loss of SP-B or SP-C production impairs both alveolar barrier and macrophage functions and causes tissue injury and inflammation (Akei et al., 2006). The volume and composition of surfactant pools are maintained by *de novo* synthesis, reuptake and recycling by alveolar type II cells, also by the catabolic activity of alveolar macrophages, in processes partly regulated by granulocyte-macrophage colony-stimulating factors (GM-CSF) (Whitsett et al., 2010; Whitsett and Alenghat, 2015). Impairment of GM-CSF signaling has been associated with susceptibility to bacterial and viral infections (Tourdot et al., 2008).

Two types of macrophages can be found in the lungs, alveolar macrophages and interstitial macrophages (Jiang and Zhu, 2016). Macrophages can roughly be classified to classically activated (M1) and alternatively activated (M2) macrophages, mirroring Th1 and Th2 polarization of T cells. M1 macrophages are induced by INFγ and LPS, they are involved in pathogen clearance. M2 macrophages are induced by IL-4 and IL-13, and are involved in wound healing and anti-inflammatory responses (Sica and Mantovani, 2012; Liu et al., 2013; Shapouri-Moghaddam et al., 2018). It is possible that M1-mediated inflammation in the adipose tissue of obese patients enhances M2-mediated asthmatic inflammation of the lungs (Sharma et al., 2017). CD163 is a known marker for M2 macrophages, it seems to have a role in airway hyperresponsiveness and asthma (Jiang and Zhu, 2016; Tokunaga et al., 2019). Alveolar macrophages respond to pathogens, sense antigens and activate innate and acquired immunity. During infection, they clear apoptotic cells and inhibit inflammation (Westphalen et al., 2014). Alveolar epithelial cells and macrophages directly communicate via Cnx43 channels to modify inflammatory signals and regulate cytokine and chemokine expression in response to pathogens (Westphalen et al., 2014; Whitsett and Alenghat, 2015). Interventions to modulate phenotypes of alveolar macrophages may have therapeutic potential in the treatment of asthma (Jiang and Zhu, 2016).

Epithelial Functions in Bronchiectasis, Primary Ciliary Dyskinesia, and Cystic Fibrosis

Bronchiectasis results from severe airway infection and inflammation. Its pathomechanisms are in part unknown. Patients with bronchiectasis have irreversible bronchial dilatation with excessive goblet cell differentiation and mucus production (Whitsett and Alenghat, 2015). Defective airway host-defense, infections and inflammation contribute to the development of bronchiectasis (Guan et al., 2018).

In PCD patients, the dysfunctional movement of cilia leads to impaired mucociliary clearance, accumulation of thick mucus, recurrent infections, and may lead to the development of bronchiectasis with chronic bacterial infections (Knowles et al., 2013; Popatia et al., 2014). Chronic infections occur both in the lower and upper airways. CRS and CRSwNP are common in patients with bronchiectasis according to a Spanish study (Guilemany et al., 2009). However, Chinese adults with bronchiectasis seem to have less CRS than in western populations (Guan et al., 2015).

Bronchiectasis is also a common manifestation of cystic fibrosis (CF) (Guan et al., 2018). Patients with CF have mutations in the gene encoding cystic fibrosis transmembrane

conductance regulator (CFTR), leading to inhibition of Cl^- and HCO_3^- transport by airway and submucosal gland epithelial cells. This results in thickening of mucus, which leads to flawed mucociliary clearance, secondary impairment in ciliary movement and chronic bacterial infection (Whitsett and Alenghat, 2015), both in lungs and paranasal sinuses (Hamilos, 2016). CRSwNP occurs with increased prevalence in CF patients (Hamilos, 2016). There is evidence that increased intracellular levels of Cl^- may be associated with chronic inflammation in bronchiectasis and CF (Zhang et al., 2018).

There is a strong association of a homozygous mutation of the chloride transport gene (CFTR) with childhood-onset CRS (Kim and Ober, 2019). Heterozygous CFTR mutations are also associated with CRS signals, albeit less than in clinical CF, with disease usually presenting in adulthood (Hsu et al., 2013; Yoo and Suh, 2017). The impaired mucociliary flow seen with CFTR mutations is presumed to intensify microbial exposure, also affecting disease course (Wang et al., 2000). The importance of CFTR in childhood-onset CRS suggests that also other barrier-related genes might play a role in CRS initiation.

Much of the non-protein encoding DNA encodes functional RNAs, important in gene regulation. A microarray analysis of bronchial brushings showed that 1,063 out of over 30,000 long non-coding RNA transcripts had different expression between CF and non-CF individuals, the pathologic processes in CF patients' airway epithelium are thus possibly partly driven by non-coding RNAs, possibly altering gene expression regulation (McKiernan et al., 2014).

EPITHELIUM AND UNITED AIRWAY CONCEPT

Most genes related to viral responses were similarly induced in upper and lower airways, in a study involving poly (I:C) -stimulated intra-individual primary nasal and bronchial epithelial cells, however, asthma patients had impaired induction of several interferon-related genes (Wagener et al., 2014). In a cross-sectional study of asthmatic children; transcriptomic sequencing was performed in 10 children and targeted sequencing in 40 children. Expression profiles reassembled in nasal and bronchial brushings, were specific to asthma independently of atopic status, and clustering analysis identified Th2-high and low subjects differentiated by expression of 70 genes (such as IL-13, IL-5, periostin, CLCA1, SERPINB2) (Poole et al., 2014). These Th2-high subjects more likely had atopy, atopic asthma, eosinophilia, and rhinitis. Hence, using less invasive nasal brushing samples and epithelial profiling may be clinically applicable when assessing asthma endotype and specific treatment. There is evidence that nasal epithelial cells could act as surrogates to bronchial epithelial cells in studies investigating airway inflammation (McDougall et al., 2008; Roberts et al., 2018). This is, however, not always feasible, bronchial epithelial cells are more susceptible to rhinovirus infection than nasal epithelial cells (Lopez-Souza et al., 2009).

CONCLUSION AND FUTURE NEEDS

Airway epithelium has important innate immune functions, all these functions seem to be essentially involved in the development of AR, CRS, and asthma.

Under normal conditions, several airborne factors are inhaled through the respiratory system and they interact with the airway barriers. In healthy individuals, the airway barrier limits the entry of pathogens and allergens, regulates the interaction with the host immune system promoting homeostasis. In AR, CRS, and asthma, barrier penetration results in inflammatory responses that are dynamic and heterogeneous and not clearly matched to the inciting agents. Asthma is not a single disease entity, and childhood-onset and adult-onset asthma have different backgrounds regarding their genetics, association with AR and CRS, possibly also differences in microbe-host interactions. Currently, only triggering factors of AR and other allergic diseases are known. Of note, allergens have an impact in pathogenesis and exacerbation of atopic asthma, both childhood-onset and adult-onset. Development of CRS and asthma seem to involve several triggering factors, such as microbiome dysbiosis, which together with host barrier immunity leads to development and aggravation of the disease. However, it is not fully understood whether microbiome dysbiosis is a primary or secondary event. Microbiota changes during development, aging, sporadic events, treatment, between anatomic compartments, and between individuals. Well-controlled studies, and the latest methodology are mandatory to identify putative causal relationships between functionally active microbiota and chronic airway diseases. Bronchial epithelial sampling is more complicated, whereas nasal epithelial sampling can be performed easily, and with little harm. Genome-scale experiments and genome-environmental interaction analyses are important approaches when searching for pathways of chronic upper airway diseases.

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REVIEW
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FGF2, an Immunomodulatory Factor in Asthma and Chronic Obstructive Pulmonary Disease (COPD)

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The fibroblast growth factor 2 (FGF2) is a potent mitogenic factor belonging to the FGF family. It plays a role in airway remodeling associated with chronic inflammatory airway diseases, including asthma and chronic obstructive pulmonary disease (COPD). Recently, research interest has been raised in the immunomodulatory function of FGF2 in asthma and COPD, through its involvement in not only the regulation of inflammatory cells but also its participation as a mediator between immune cells and airway structural cells. Herein, this review provides the current knowledge on the biology of FGF2, its expression pattern in asthma and COPD patients, and its role as an immunomodulatory factor. The potential that FGF2 is involved in regulating inflammation indicates that FGF2 could be a therapeutic target for chronic inflammatory diseases.

Keywords: asthma, chronic obstructive pulmonary disease, fibroblast growth factor 2, immunomodulation therapeutics, airway structural cells

INTRODUCTION

Chronic inflammatory airways disease is a major economic burden and public health challenge worldwide. Asthma and chronic obstructive pulmonary disease (COPD) are the most prevalent among them, with 235 million people suffering from asthma (Whalen and Massidda, 2015) and 250 million from COPD (Al-Haddad et al., 2014). Though differing in etiology, asthma, and COPD share pathological features, which include chronic inflammation, airflow limitation, and airway wall remodeling (Holgate et al., 2015; Barnes, 2018). Chronic airway inflammation is central to disease pathophysiology, contributing to airway dysfunction and remodeling through the release of inflammatory mediators and interaction with airway structural cells, and therefore, is the primary therapeutic target (Holgate, 2012; Barnes, 2016). For a long time, anti-inflammatory drugs have been applied as first-line treatments for chronic airway diseases. Nevertheless, evidence suggests that the current drugs are not always effective in inhibiting chronic airway inflammation. For example, some asthmatic patients who respond poorly to corticosteroids also do not respond to anti-IL5/anti-IL13 treatments, leaving severe asthma a disease that has no effective cure (Astrazeneca, 2017; Drick et al., 2018). As for COPD patients, corticosteroids are inefficient partly because cigarette smoking impairs histone deacetylase 2 activity resulting in a lack of suppression of

anti-inflammatory effects of corticosteroids (Barnes, 2004; Barnes et al., 2004). Moreover, anti-IL-5 treatments failed to show efficacy in severe COPD patients, and treatment with kinase inhibitors could impair innate immunity through inhibiting cellular components of inflammation, which evoke concerns about their usage (Gross and Barnes, 2017; Narendra and Hanania, 2019). In these situations, alternative treatments for chronic airway diseases, particularly asthma and COPD, are urgently needed. Recently, the immunomodulatory function of airway structural cells, e.g., airway epithelial cells (AECs), airway smooth muscle cells (ASMCs), and endothelial cells (ECs), has been attracting much interest. A myriad of articles showed that airway structural cells, regarded as immunomodulatory cells, are the targets for, and source of, various inflammatory mediators, in the perpetuation of the chronic inflammation via autocrine or paracrine pathways (Hahn et al., 2006; Tliba and Amrani, 2008; Al-Soudi et al., 2017). For instance, in the presence of inflammatory mediators, airway structural cells express adhesion molecules that are essential to the recruitment of inflammatory cells. This would trigger the production and secretion of multiple cytokines, chemokines, and growth factors, which in turn contributes to airway inflammatory states. Of note, the complex network between inflammation and airway structural cells has been rarely targeted by the current drugs and thus may open new avenues for potential anti-inflammatory therapies.

Fibroblast growth factor 2 (FGF2, also known as basic fibroblast growth factor, bFGF), a potent mitogen for fibroblasts, has been reported to be overexpressed in severe asthma and COPD, particularly patients with exacerbated COPD and smokers with chronic bronchitis (Redington et al., 2001; Kranenburg et al., 2005; Guddo et al., 2006; Pavlisa et al., 2010). Moreover, increased sputum FGF2 was reported to be a biomarker of airway remodeling and associated with lung function (Bissonnette et al., 2014). It is also evident that FGF2 plays a critical role in cell proliferation, differentiation, migration, and apoptosis in airway structural cells (Bosse et al., 2006; Yi et al., 2006; Zhang et al., 2012; Schuliga et al., 2013), contributing to epithelial repair, ASMCs hyperplasia, and vascular remodeling. Interestingly, some studies suggest that FGF2 may be associated with airway inflammation, and is a potential target for inflammatory diseases (Jeon et al., 2007; Kim et al., 2018; Laddha and Kulkarni, 2019). In this review, we will provide an overview of the immunomodulatory function of FGF2, particularly through airway structural cells-driven processes, identifying raising questions, and proposing future research directions for targeting FGF2 in asthma and COPD.

FGF2 DISCOVERY AND SIGNALING

Discovery of FGF2 and Its Isoforms

FGF was first discovered in pituitary extracts as a potent mitogen for fibroblasts in 1973 (Armelin, 1973). Then, FGF2 was isolated at basic pH and named "basic fibroblast growth factor," with FGF1 (also known as an acidic fibroblast growth factor, aFGF) purified with acidic extraction condition. Since then, 22 FGF family members have been identified, with diverse physiological functions. FGF2 is encoded by the *FGF2* gene, a 3-exon gene localized on human chromosome 4q26–27 (Ornitz and Itoh, 2001). It has low (18-kDa) and high (22-, 22.5-, 24-, and 34-kDa) molecular weight isoforms, which are translated from a single transcript by starting from alternative, in-frame start codons. These isoforms have different expression patterns. Generally, the low molecular weight (LMW) FGF2 is considered cytoplasmic or/and nuclear and can be secreted. Of note, unlike most of FGF family members, LMW FGF2 lacks a classical amino-terminal signal peptide that directs secretion (Mignatti et al., 1992). However, it can be found anchored to extracellular matrix (ECM) components at the extracellular surface of the plasmalemma and within the basement membrane of different tissues (Folkman et al., 1988; Shute et al., 2004). More recent evidence suggests that LMW FGF2 can be released not only from damaged cells but also via an unconventional secretory pathway that is based upon direct protein translocation across plasma membranes as opposed to the traditional endoplasmic reticulum/Golgi apparatus-dependent protein secretion pathway (La Venuta et al., 2015). By contrast, The HMW FGF2 has been identified in the nucleus, with its additional amino-terminal sequences providing the nucleus-localization signal. Whilst several studies have identified that HMW FGF2 signaling is FGF receptor (FGFR)-independent, and the physiological function of HMW FGF2 remains unclear. Therefore, in this review, we will focus on LMW FGF2 (identified as FGF2, unless stated otherwise), for which FGF2 usually signal either in the cytoplasm without secretion or via representative membrane receptor activation to modulate subsequent downstream signaling events in an autocrine or paracrine pattern.

FGF2 Signaling and Basic Function

Four high-affinity receptor tyrosine kinases have been identified as FGFs receptors, comprising FGFR1 through FGFR4. Of note, FGFR5, recently discovered to interact with FGFs, has been proposed to act as a negative regulator of FGFs signaling in the light of lacking the tyrosine kinase domain (Sleeman et al., 2001). Once binding with FGFs, FGFRs undergo conformational changes leading to tyrosine kinase activation and subsequent the activation of intracellular signalings including mitogen-activated protein kinases (MAPKs) (Maher, 1999; Willems-Widyastuti et al., 2013), phosphatidylinositol 3-kinase (PI3K)/Akt (Lin et al., 2011), signal transducer and activator of transcription (STAT) (Deo et al., 2002), and phospholipase (PL) Cγ (Sufen et al., 2011) (summarized in **Figure 1**). Correspondingly, the activation of these pathways serves to modulate diverse cell functions, including proliferation (Sulpice et al., 2002; Fernandes et al., 2004), differentiation (Klint et al., 1999; Dolivo et al., 2017), migration (Sufen et al., 2011), and apoptosis (Sahni et al., 2001).

Interestingly, beyond the direct effect of FGF2 in modulating cell function, recent evidence suggests that FGF2 can also function as an immune-modulatory factor that might play a role in immune homeostasis and dysfunction as well. In the following text, we will review FGF2 as an immunomodulatory

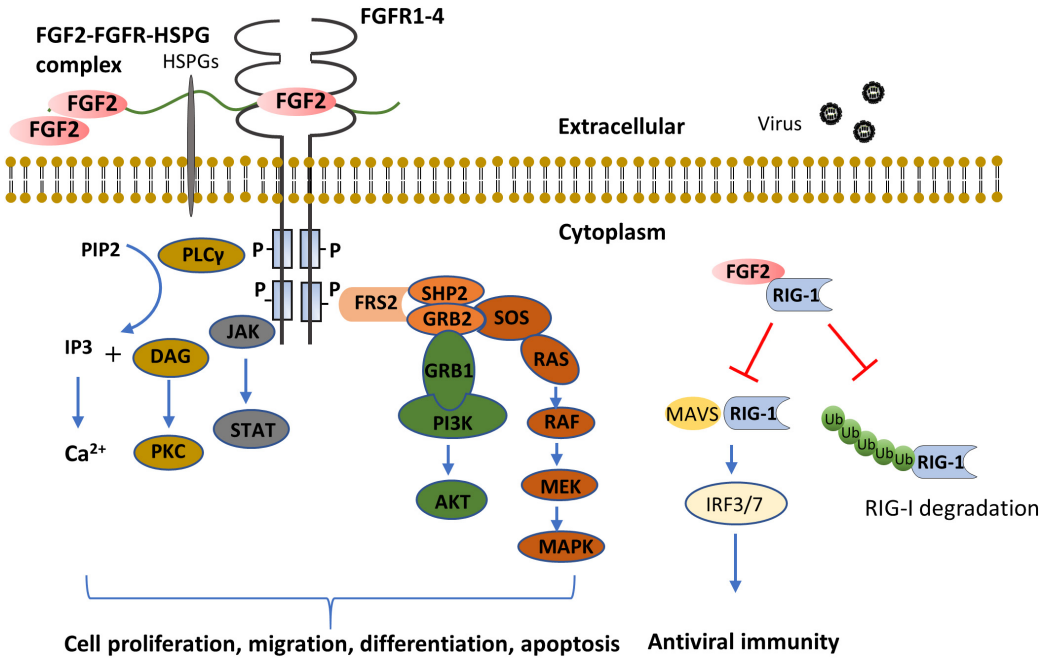

FIGURE 1 | FGF2 functions through FGFR dependent or independent pathways. The binding of FGF2 to FGFR induces the formation of FGF2-FGFR-HSPG complex, which leads to receptor dimerization and transphosphorylation of tyrosine kinase domains. The major FGFR kinase substrate, FRS2α, is phosphorylated by the activated FGFR kinase and recruits the adaptor protein, GRB2 and SHP2. This results in subsequent activation of MAPK and PI3K-AKT pathways. In addition, the combination of FGF2 and FGFR also activates JAK and PLCγ, the former activates the STAT pathway, the latter hydrolyzes PIP2 into IP3 and DAG, and activates Ca^{2+} and PKC signaling, respectively. As compared to extracellular FGF2 signaling, cytosol FGF2 binds to RIG-1 to prevent RIG-1 degradation. While in viral infection, the binding of FGF2 with RIG-1 tends to prevent the binding of MAVS with RIG-1, and thus inhibit anti-viral innate immunity. (AKT, protein kinase B, also known as PKB; DAG, diacylglycerol; FGF2, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRS2α, FGF receptor substrate 2α; GRB1, growth factor receptor-bound protein 1; GRB2, growth factor receptor-bound protein 2; HSPG, heparan sulfate proteoglycan; IP3, inositol trisphosphate; IRF, interferon regulatory transcription factor; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral-signaling protein; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PKC, protein kinase C; PLC, phosphoinositide phospholipase C; RIG-1, retinoic acid-inducible gene 1; SHP2, src homology 2-containing phosphotyrosine phosphatase; SOS, son of sevenless; STAT, signal transducer and activator of transcription). (Klint et al., 1999; Maher, 1999; Sahni et al., 2001; Deo et al., 2002; Sulpice et al., 2002; Fernandes et al., 2004; Lin et al., 2011; Sufen et al., 2011; Willems-Widyastuti et al., 2013; Liu et al., 2015; Dolivo et al., 2017).

factor in airway diseases, particularly asthma and COPD, which hopefully will provide new insight into the inflammation processes in these diseases.

IMPLICATIONS OF FGF2 IN AIRWAY DISEASES

The Physiological Role of FGF2 in the Lungs

FGF2 plays critical roles in all five stages of lung development: embryonic, pseudoglandular, canalicular, saccular, and alveolar. The alveolar formation in mice starts at birth and lasts for about a month; whereas in humans, it starts in the womb and lasts until the age of five or longer to mature (Burri, 2006). During the lung development process, FGF2 expression pattern is dynamic in different pulmonary cell types, indicating its differential role during the lung development stages. In mouse embryogenesis, mesoderm-derived Fgf1 and Fgf2 are involved in commiting the lung fate at the ventral foregut endoderm on embryonic day 8 (E8) (El Agha et al., 2017). An FGF2 immunolocalization study in developing rat fetal lung demonstrated that FGF2 protein localizes to AECs, the basement membrane, mesenchymal and mesothelial cells, as well as the extracellular matrix during pseudoglandular stage (Han et al., 1992). Also increasing is FGFR in AECs, suggesting that FGF2 may be involved in the control of AECs' proliferation. Besides, FGF2 also localized to the branch points of airway buds, ASMCs, and putative VSMCs, indicating the role of FGF2 in airway system development. In contrast to early development stages, FGF2 is not detectable in airway epithelium in late fetal rat lung. This persisted at early postnatal rat lung, and the amount of FGF2 in ASMCs and many other cells in the alveolar region is also lesser (Powell et al., 1998). The physiological significance of this changing expression pattern of FGF2 is far from elucidated but could be linked to its regulation on alveolar development. For example, Yi et al. (2006) suggest that FGF2 and the FGFR1(IIIc) might contribute to the physiologic pulmonary cell apoptosis that is normally seen shortly after birth. This process may help to get rid of excess fibroblasts and epithelial cells so as to increase the gas exchange surface area. Although numerous studies have

suggested the role of FGF2 in lung development, *Fgf2* knockout mice ($Fgf2^{-/-}$) develop near normally, have no reported pulmonary abnormalities, and are fertile, indicating that other growth factors may compensate the function of FGF2 in lung development (Zhou et al., 1998).

Comparatively, in the mature lung, FGF2 can be expressed in AECs, ASMCs, VSMCs, ECs, and mesenchymal cell populations. Also expressed in the same cell types are the representative receptors of FGF2 (e.g., FGFR1, see below for more details (Gonzalez et al., 1996; Kranenburg et al., 2002, 2005). In addition, extracellular FGF2 has also been found in the epithelial basement membrane and pericellular matrix of ECs (Shute et al., 2004). Indeed, FGF2 is the main growth factor that is anchored to the ECM and basement membrane through preferential binding to glycosaminoglycan (GAG) side chains of cell-associated or ECM-associated heparan sulfate proteoglycans (HSPG) (Nugent and Iozzo, 2000). This interaction offers protection from proteolysis and serves as a reservoir of stable, but inactive FGF2, requiring release for biological activity (Bosse and Rola-Pleszczynski, 2008). Furthermore, its storage presumably leads to rapid cellular signaling in the face of sudden changes in local environment conditions (Redington et al., 2001). A number of mechanisms for the release of FGF2 from ECM and basement have been identified. These include proteolytic cleavage of HSPG core protein by heparinases, the action of GAG degrading enzymes, and the ability of heparin or endoglycosidase to elute FGF2 from HSPG-binding sites (Bosse and Rola-Pleszczynski, 2008). The release of FGF2 then enables it to bind to FGFRs with varying affinities on the plasma membrane.

FGF2 Expression Pattern in Airway Diseases

There is great interest in the potential role of FGFs in the pathophysiology of chronic inflammatory airway diseases. Studies suggest that FGF2 expression is clinically relevant in these diseases. For example, FGF2 levels in the bronchoalveolar lavage fluid (BALF) of atopic asthma patients are significantly higher than healthy controls. Importantly, it can be further elevated in response to allergen exposure 10 min after segmental bronchoprovocation (Redington et al., 2001). This could be attributed to the activation of mast cells and AECs, which releases FGF2 and the desequestration of FGF2 from the ECM mediated by mast cell-produced heparin (Redington et al., 2001). Moreover, FGF2 levels in the sputum could be a biomarker for asthma severity in light of the correlation of its levels with lung function, implicating its role in airway hyperresponsiveness, the main feature of asthma (Bissonnette et al., 2014). The significance of FGF2 in disease severity also lies in COPD conditions, as FGF2 levels are elevated in the serum of patients with exacerbated COPD as compared to patients with stable COPD and healthy subjects (Pavlisa et al., 2010). In support, immunohistochemistry analysis has shown that FGF2 expression increased in the airways of asthmatic and COPD patients. For example, Kranenburg and colleagues demonstrated that the expression of FGF2 and FGFR1 were increased significantly in the bronchial epithelium in COPD patients as compared with non-COPD. Likewise, another study suggested that epithelial FGF2 was significantly more abundant in patients with mild asthma than in healthy subjects (Shute et al., 2004). Moreover, the overexpression of FGF2 in VSMCs and ECs of the small pulmonary vessels and FGFR1 in both large and small vessel types in COPD may be involved in regulating the process of pulmonary vascular remodeling (Kranenburg et al., 2002, 2005). Altogether, these data suggest that FGF2 expression is elevated in multiple pulmonary cell types in asthma and COPD, and may be involved in the pathogenesis of chronic inflammatory airway diseases. Besides its effect in tissue remodeling, there is an emerging role of FGF2 in airway inflammation, which has placed this molecule as a common link between airway inflammation and airway structural cells. Therefore, in the next section, we will review the immunomodulatory function of FGF2 in both inflammatory cells and airway structural cells (summarized in **Figure 2**), and discuss the possibility of targeting FGF2 so as to inhibit airway chronic inflammation in asthma and COPD.

FGF2 in Inflammatory Cells

Asthma and COPD are chronic inflammatory airway diseases that are characterized by the infiltration of inflammatory cells. They can be recruited from circulation within minutes to hours when the body responds to stimuli, such as allergens. Interestingly, it has been shown that FGF2 can be released from those inflammatory cells including T lymphocytes, eosinophils (Hoshino et al., 2001), mast cells (Kearley et al., 2011), macrophages (Yum et al., 2011), and myeloid dendritic cells (Sozzani et al., 2007), which might partly explain the elevated FGF2 expression in the BALF of asthma patients after allergen stimulation. More importantly, elevated FGF2 modulates inflammatory cell recruitment and activation as well. In this scenario, Takagi et al. (2000) showed that FGF2 regulates the expression of adhesion molecules, i.e., L-selectin and CD11b, on the surface of blood neutrophils, the most abundant inflammatory cells present in the airway of COPD patients (Pesci et al., 1998). These adhesion molecules mediate the adherence of circulating neutrophils to vascular endothelial cells and thus facilitate the recruitment of neutrophils into the airways (Takagi et al., 2000). Moreover, two studies have demonstrated that FGF2 amplifies the inflammatory cells' function in bacterial clearance through increasing the complement receptors (CRs), such as CR3 on blood monocytes and CR1 on neutrophils, and oxidative product formation (Takagi et al., 2000; Ohsaka et al., 2001). Interestingly, the latter is not a direct effect of FGF2, for FGF2 itself does not enhance the production of intercellular H_2O_2 in neutrophils, indicating that FGF2 might coordinate with other factors in modulating inflammatory cell function.

While the present evidence suggests FGF2 as an immunomodulatory factor in regulating inflammatory cell recruitment, adhesion, and cell function, unfortunately, there is not much evidence documenting the effect of FGF2 on inflammatory cells in asthma and COPD. Therefore, further studies will be required to determine the direct effect of FGF2 and, subsequently, the underlying mechanisms.

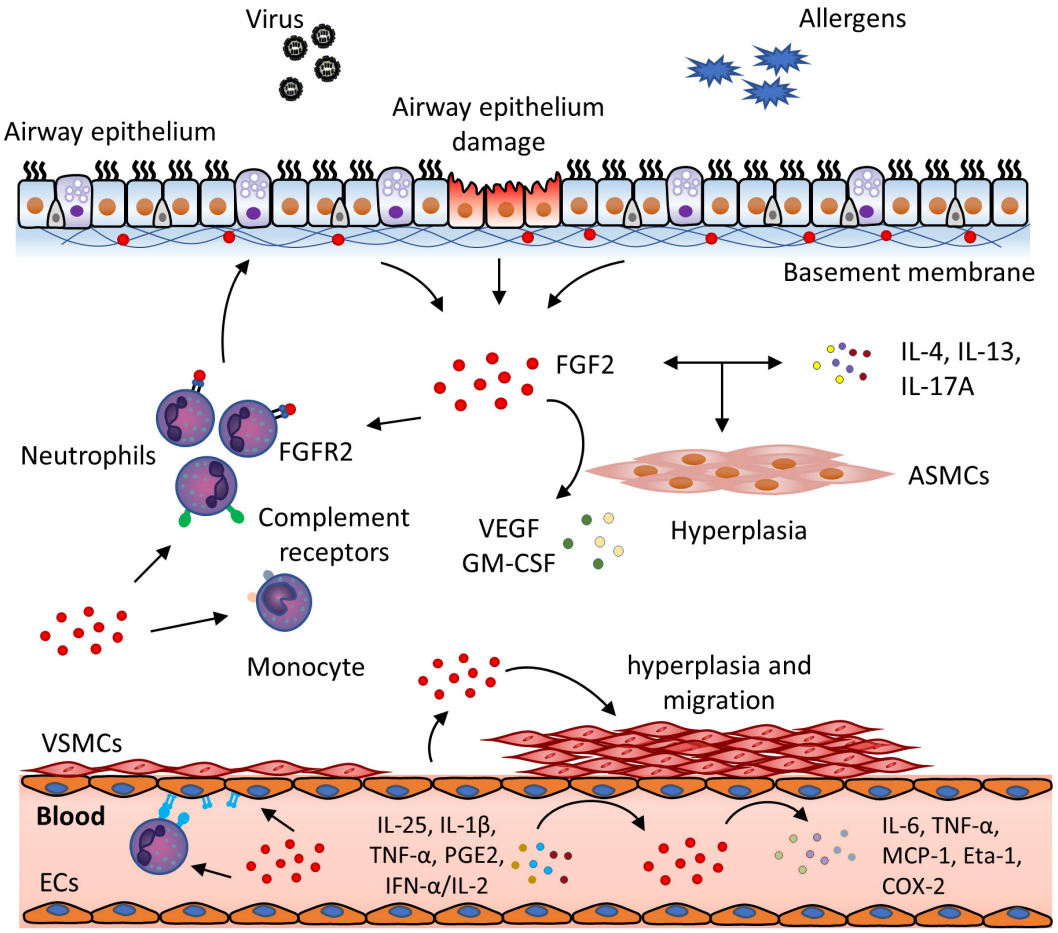

FIGURE 2 | FGF2 exerts immunomodulatory function in viral infection and chronic airway inflammation. In the presence of viral infection and allergen exposure, FGF2 is secreted from damaged airway epithelial cells and released from extracellular storage. FGF2 then plays a role in the interplay of inflammatory cells and airway structural cells. In the airways, FGF2 could (1) promote neutrophils recruitment and activation through FGFR2 and complement receptors; (2) activate monocytes via upregulation of complement receptors; (3) cooperate with inflammatory cytokines, e.g., IL-4, IL-13, and IL-17A, to induce ASM hyperplasia, and (4) promote ASM to release VEGF and GMSF. In the blood vessel, excess FGF2 can be secreted by ECs in presense of inflammatory mediators, such as IL-25, IL-1β, TNF-α, PGE2 and IFN-α/IL-2. FGF2 could then (1) upregulate the expression of adhesion molecules on ECs and neutrophils, which promotes neutrophil infiltration and transmigration; (2) further activate ECs to produce inflammatory mediators, e.g., IL-6, TNF-α, MCP-1, Eta-1 and COX-2, and (3) promote VSMCs hyperplasia and migration to contribute to vascular remodeling. (ASMC, airway smooth muscle cell; COX-2, cyclooxygenase-2; EC, endothelial cell, Eta-1, osteopontin; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; MCP-1, monocyte chemoattractant protein-1; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell). (Okamura et al., 1991; Cozzolino et al., 1993; Barleon et al., 1996; Wempe et al., 1997; Kage et al., 1999; Takagi et al., 2000; Ohsaka et al., 2001; Kranenburg et al., 2002; Santos et al., 2002; Bonacci et al., 2003; Lee et al., 2004; Mor et al., 2004; Shute et al., 2004; Bosse et al., 2006, 2008; Andres et al., 2009; Finetti et al., 2009; Wang et al., 2012; Ogawa et al., 2018).

FGF2 in Airway Structural Cells

Airway structural cells, including AECs, ASMCs, and ECs, are the central effector cell types in asthma and COPD. Beyond their effect in tissue remodeling and impairing lung function; increasing studies have focused on their role in modulating airway inflammation. This is related not only to the immunomodulatory function of these cells, but also their crosstalk with inflammatory cells that shapes the immune network. Strikingly, FGF2, and its specific receptor, FGFR1, are overexpressed in airway structural cells under diseased conditions, as mentioned above. In this context, FGFs might be the key to orchestrate the immunomodulatory function of typical airway structural cells and their crosstalk with inflammatory cells, which therefore could serve as an attractive therapeutic target for chronic airway inflammation. For the next sections, we will further discuss the role of FGF2 in different airway structural cell types, by which the role of FGF2 as an orchestrator in airway inflammation will be delineated.

Airway Epithelial Cells

AECs play a pivotal role in host defense against a wide range of environmental insults by acting as the first barrier. It is critical in innate immunity against pathogens and allergens, which trigger the following inflammatory processes. In some

studies, this is critical for the onset and exacerbation of asthma and COPD (Gohy et al., 2015; Ladjemi et al., 2018). By interacting with AECs, FGF family members, mostly FGF7 and FGF10, are involved in innate immunity directly and indirectly (Wu et al., 2011; Gardner et al., 2016; Yuan et al., 2019). Comparatively, the role of FGF2 in AECs mediated innate immunity is also attracting research interest. For example, in Liu's study, cytosolic FGF2 stabilizes inactivated retinoic-acid inducible gene-1 (RIG-1), the primary pathogen recognition receptor (PRR) against virus infection, via preventing proteasome-mediated RIG-1 degradation in physiological conditions. Whereas upon viral infection, FGF2 suppresses antiviral signaling by inhibiting the interaction of activated RIG-1 with downstream mitochondrial antiviral-signaling protein (MAVS) and type I interferon production (Liu et al., 2015) (**Figure 1**). Of note, the above-mentioned study suggests FGF2 as a negative regulator for innate immunity, and Wang et al. reported contradicting results. They showed that the increased level of secreted FGF2, which is predominately derived from AECs induced by H1N1 infection, protects recruiting, and activating neutrophils (Wang et al., 2018). This inconsistency might be due to the difference between cytosolic and secreted FGF2 protein in AECs upon viral infection.

AECs mediated innate immunity plays a crucial role in initiating allergen-induced inflammatory pathways in asthma and COPD. In addition to the function of AECs in recruiting immune cells, which subsequently link to adaptive immunity and inflammation in asthma and COPD, AECs themselves serve to be the reservoir for inflammatory cytokines in responding to stimulations. A study showed that FGF23, another FGF family member, enhances IL-1β production in primary bronchial epithelial cells from COPD patients through activating FGFR4/PLCγ/calcineurin/nuclear factor of activated T-cells (NFAT) pathway (Krick et al., 2018). Furthermore, FGF23 and TGF-β could increase the transcripts of IL-8, an essential chemokine in neutrophils recruitment (Krick et al., 2017), which further supports the role of FGF family members in epithelial-mediated inflammation. In-depth studies need to be conducted to fully elucidate the role of FGF2 in innate immunity in the different context of conditions.

Whilst FGF2 modulates AECs-mediated innate immunity and contributes to airway inflammation, not to ignore is that FGF2 might also play a role in epithelial repair and barrier function. Indeed, FGF2 has been characterized as a protective factor in repairing epithelium. As to bleomycin-induced lung injury and pulmonary fibrosis, more *Fgf2* knockout mice succumb to death due to compromised epithelial repair process and barrier function recovery (Guzy et al., 2015). This indicates that FGF2 provides a protective endogenous epithelial reparative signal in the setting of lung injury. However, the mechanism underlying the protective effects of Fgf2 signaling during airway injury has not yet been fully elucidated. Also, FGF2 may be involved in epithelial repair not only by exerting its intrinsic role but also cooperating with other inflammatory cytokines or mediators. This was supported by Song's study that FGF2 cooperates with IL-17 to repair damaged intestinal epithelium through the Act1-mediated signaling pathway (Song et al., 2015). Nevertheless, whether this is the case in the lung remains to be elucidated.

Airway Smooth Muscle Cells

ASMCs are recognized as the main effector cell type in asthma and COPD. Both clinical and animal model studies suggest that the ASM layer is significantly thicker in chronic inflammatory diseases, thereby contributinge to airflow limitation and impairment of lung function. Importantly, on top of its contractile properties to induce airway constriction, there is an increasing research interest for the immunomodulatory role of that ASM played in perpetuating chronic airway inflammation (Damera and Panettieri, 2011). FGF2 has been shown to contribute to ASM hyperplasia, in light of the mitogenic property of FGF2 in ASMCs, which might play an indirect role in the pathogenesis of chronic airway inflammation. This mitogenic effect of FGF2 also lies at the interplay between FGF2 and remodeling associated molecules, typically TGF-β (Bosse et al., 2006), through the autocrine platelet-derived growth factor (PDGF) loop. In this context, FGF2 increases the expression of PDGF receptor (PDGFR), as well as tissue plasminogen activator (tPA) that is a protease known to activate PDGF ligands. This is accompanied by the increased production of PDGFR ligands (PDGF-AA and PDGF-CC) induced by TGF-β (Bosse et al., 2006). Other than this, FGF2 and TGF-β are coexpressed in the remodeled airway *in vivo*, by peribronchial mononuclear cells. In parallel, FGF2 induces TGF-β overexpression in macrophages *in vitro*, which may amplify the interaction of FGF2 and TGF-β in inflammation status (Yum et al., 2011). In addition to TGF-β, the inflammatory cytokines including IL-4, IL-13, and IL-17, the pivotal mediators driving the pathogenesis of asthma and COPD, enhance FGF2-mediated ASMCs' proliferation in different experiment settings (Bosse et al., 2008; Ogawa et al., 2018), which further support the interaction of FGF2 with inflammation on tissue remodeling in disease status.

On the other hand, there is evidence that FGF2 might be a direct immunomodulatory factor by inducing the secretion of pro-inflammatory mediators, including growth factors, cytokines, and chemokines in ASMCs. Reported by Willems-Widyastuti, FGF2 regulates the production of vascular endothelial growth factor (VEGF) in ASMCs, which may induce T cell differentiation and monocytes recruitment (Barleon et al., 1996; Mor et al., 2004), through MAPK pathway (Willems-Widyastuti et al., 2011). In another example, FGF2 significantly increases the levels of macrophage colony-stimulating factor (GM-CSF) in ASMCs (Bonacci et al., 2003), which is critical to activate macrophages in asthma and COPD, indicating a role for FGF2 in immune cell activation. Except for this, FGF2 also plays a role in the contact-dependent communication between immune cells and ASMCs, which is featured in chronic airway diseases and being critical for ASM functional changes and immune cell survival (Ramos-Barbon et al., 2005). In this scenario, FGF2/FGFR signaling in ASMCs is thought to trigger the formation of lymphocyte-derived membrane conduits, which are a continuum of cell membrane

extensions and connect ASMCs and activated $CD4^+$ T cells (Farahnak et al., 2017).

Endothelial Cells

Despite the limited research on pathological changes of ECs in asthma and COPD compared to AECs and ASMCs; ECs have proven to be an indispensable participant in shaping the immune and inflammatory network in airway diseases (Asosingh et al., 2018). ECs play critical roles in regulating vessel tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation through production of multiple factors in response to different stimulis. In asthma and COPD, endothelial dysfunction, along with vessel inflammation, has been observed (Green and Turner, 2017). Moreover, in asthmatic children, the extent of endothelial dysfunction, as manifested by excess production of circulating vascular cell adhesion molecule-1 (sVCAM-I), was found to be associated with asthma severity (Butov et al., 2019), indicating the critical role of ECs in asthma pathogenesis. Indeed, in light that ECs serves as the gateway of inflammatory cells' transendothelial migration into the lung parenchyma, the activation of ECs, e.g., expression of adhesion molecules and pro-inflammatory mediators, would be crucial in modulating inflammatory cell recruitment. In support of this, Oelsner et al. (2013) demonstrated that the expression levels of adhesion molecules on the surface of ECs are inversely related to lung function. In another study, the eotaxin mRNA was shown to be increased in ECs from asthma patients, and the level was associated with airway hyperresponsiveness (Ying et al., 1997), which further supports the role of ECs in modulating airway inflammation in chronic inflammatory diseases such as asthma and COPD.

ECs are key immunomodulatory cells in airway diseases, but the involvement of FGF2 in the dysfunction of ECs in asthma and COPD patients has not been well studied yet. However, FGF2 has been proven as an effective regulator of ECs-mediated angiogenesis and inflammation in different experimental settings. It has been well established as a potent inducer of angiogenesis that exert its effect on EC proliferation (Sahni and Francis, 2004), differentiation (Klint et al., 1999), and migration (Pintucci et al., 2002), directly or indirectly by inducing other angiogenetic factors, such as Heparin-binding EGF-like growth factor (HBEGF) and platelet-derived growth factor, B polypeptide (PDGFB) (Andres et al., 2009). FGF2 production and release from ECs can be triggered by inflammatory mediators, e.g., IL-25, IL-1β, TNF-α, prostaglandin E2 (PGE2) and IFN-α/IL-2 (Okamura et al., 1991; Cozzolino et al., 1993; Lee et al., 2004; Finetti et al., 2009; Wang et al., 2012), as well as cell damage and hypoxia (Pintucci et al., 1999; Luo et al., 2011). These findings suggest that inflammation might cooperate with FGF2 to orchestrate the amplification loop of the angiogenic response in ECs. Of note, there are also negative feedback mechanisms that contradict the angiogenetic effect of FGF2. Such examples are C-X-C Motif Chemokine Ligand 13 (CXCL13), CXCL4/CXCL4L1, and long-pentraxin 3 (PTX3) (Spinetti et al., 2001; Rusnati et al., 2004; Struyf et al., 2004; Presta et al., 2018), which need to be carefully evaluated in different diseases and inflammation types.

In addition to its angiogenetic effect, FGF2 may amplify the EC-mediated inflammation. According to recent *in vitro* studies, FGF2 may stimulate the production of various pro-inflammatory factors and chemoattractants, including IL-6, TNF-α, and monocyte chemoattractant protein 1 (MCP-1) in ECs (Wempe et al., 1997; Andres et al., 2009). These factors are essential for immune cell proliferation, survival, activation, and trafficking (Wempe et al., 1997; Li et al., 2018; Mehta et al., 2018). In another two studies, FGF2 could also induce the expression of extracellular matrix protein osteopontin (OPN/Eta-1) and cyclooxygenase-2 (COX-2) in ECs. The former is an important component of cellular immunity and inflammation (Leali et al., 2003), whereas the latter is responsible for the production of prostanoids, a key factor associated with the pathological processes of inflammatory diseases (Kage et al., 1999). Furthermore, FGF2 itself is involved in inflammatory response as well. In Okamura's study, FGF2 inhibition completely blocked TNF-α-induced IL-6 production (Okamura et al., 1991). This suggests to us that FGF2 could be critical in inflammatory processes, and thus might be a key modulator in chronic inflammatory diseases, such as asthma and COPD.

Finally, FGF2 potentiates inflammatory cell recruitment by enhancing EC surface adhesion molecules expression. FGF2 increased the expression of ICAM-1, VCAM-1, *E*-selectin, and *P*-selectin on ECs, resulting in more efficient leukocyte migration into the surrounding tissues in an acute dermal inflammation model (Zittermann and Issekutz, 2006). Interestingly, in another aspect, FGF signaling is critical to maintaining vascular integrity and endothelial barrier function (Gillis et al., 1999; Komarova and Malik, 2008; Murakami et al., 2008; Hatanaka et al., 2012). In this regard, the recruitment of inflammatory cells induced by FGF2 may be independent of increasing vascular permeability, but rather the induction of adhesion molecules, as stated above.

Of note, whilst numerous studies support the pro-inflammatory effect of FGF2 in ECs, the anti-inflammatory activity of FGF2 has also been observed. For example, by pretreating human umbilical vein ECs with recombinant FGF2 protein for 3 days, the reduced leukocyte adhesion and transendothelial migration induced by stimulation can be observed (Griffioen et al., 1996). Further studies will be required to determine how FGF2 exert its immunomodulatory effect in chronic inflamed diseases.

Other Cell Types

In addition to AECs, ASMCs, and ECs, other pulmonary cells also exhibit pathological changes in asthma and COPD. Examples of these cell types include VSMCs and fibroblasts, which are critical components of vascular and airway remodeling as a result of dysregulated cell proliferation, differentiation, activation, and migration (Kranenburg et al., 2002; Santos et al., 2002). It is noteworthy that these cell types may also play active roles in chronic inflammation (Kendall and Feghali-Bostwick, 2014; Stenmark et al., 2018). In consideration of the potential of FGF2 to function as an immunomodulatory factor in AECs, ASMCs, and ECs, FGF2 may play also play similar roles in VSMCs and fibroblasts as well. In support of this, evidence showed

that FGF2 is overexpressed in VSMCs from COPD patients; whereas in another study, FGF2 promotes a proinflammatory phenotype of VSMCs through increasing cellular IL-1α secretion (Schultz et al., 2007). As for fibroblast, FGF2 was shown to be a potent pulmonary fibroblast mitogen *in vitro* (Khalil et al., 2005). Moreover, FGF2 was also shown to upregulate the transcription of S100A8, a marker of inflammation, in murine fibroblast, through the MAPK pathway (Rahimi et al., 2005). These studies thus warrant future investigations into FGF2's role in these cells.

FGF2 AS A POTENTIAL THERAPEUTIC TARGET IN ASTHMA AND COPD

As outlined in the previous sections, uncontrolled airway inflammation is shown to be the key driving force for the pathogenesis of asthma and COPD. Therefore, much effort has been devoted to the development of anti-inflammatory drugs to control the disease. These drugs include both broad-spectrum anti-inflammatory medicine, typically corticosteroids, and more recently targeted drugs such as anti-IL5 and anti-IL13 were added to the repertoire of treatments. However, due to the heterogeneity of asthma and COPD, these drugs are often not applicable to certain phenotypes and endotypes in some group of patients (Brightling et al., 2015; Narendra and Hanania, 2019). Additionally, these drugs are also not targeting the crosstalk between inflammation and airway structural cells, which has proven to be the critical component in the network of sustained airway inflammation. In this scenario, we highlighted FGF2 as an important immunomodulatory factor that mediates the interplay between inflammation and airway structural cells and emphasize FGF2 as a potential therapeutic target for asthma and COPD.

Targeting FGF2 and their receptors has already been done in the drug development for cancer and other diseases, where they can potentially be applied to asthma and COPD. These drugs include chemicals to suppress FGF2 expression levels, anti-FGF2 antibody, FGFR inhibitors and recombinant FGF2 (Fu et al., 1998; De Aguiar et al., 2016; Lv et al., 2018; Loriot et al., 2019). Some drugs have been approved by the FDA, while others are in clinical trials and have been shown to have mild side effects. Encouragingly, the application of FGF2 targeted therapy has recently been expanded to pulmonary diseases. For example, neotuberostemonine, a natural alkaloid isolated from *Stemona tuberosa*, was applied to decrease the levels of FGF2 in bleomycin-induced pulmonary fibrosis mice (Lv et al., 2018). In another early report, colchicine inhibited FGF release from alveolar macrophages *in vitro* (Peters et al., 1993). These reports hence highlighted the feasibility of targeting FGF2 in asthma and COPD. Nevertheless, prior to developing these drugs for clinical use, extensive research is needed to study the role of FGF2 in different cell types and disease states. For instance, FGF2 may function as an inflammatory enhancer by regulating the functions of inflammatory cells and airway structural cells. Conversely, FGF2 may also serve protective effects on minimizing lung injury and maintaining tissue integrity. The latter was supported in a study of bleomycin-induced lung injury model (Guzy et al., 2015). It is also interesting to note that there were two studies of *in vivo* mouse models of acute asthma and COPD which showed that recombinant FGF2 (rFGF2) is protective against airway inflammation and lung function, rather than enhancing inflammation (Jeon et al., 2007; Kim et al., 2018). The inconsistencies between *in vivo* and *in vitro* studies might be due to the nature of different experiment setups, as well as the limitations of *in vivo* and *in vitro* models in reproducing clinical characteristics. For example, the mouse model of asthma used was acute inflammation model in which chronic airway inflammation and remodeling is usually absent. This might lead to different inflammatory components, wherein airway remodeling and angiogenesis are not involved. In such acute models, FGF2 treatment might help to repair acute lung injury and maintain tissue integrity, and thereby is protective by counteracting acute inflammation-induced tissue damage and increased permeability (Guzy et al., 2015). In contrast, in chronic inflammation, FGF2 might tend toward an inflammatory amplifier via interaction with inflammatory cells and airway structural cells. Moreover, rFGF2 treatment may exacerbate angiogenesis, as demonstrated in the clinical usage of FGF2 in chronic coronary artery disease and critical limb ischemia (Udelson et al., 2000; Ono et al., 2018). Therefore, more clinical studies and chronic disease models are required to fully elucidate the immunomodulatory and repair functions of FGF2 in different experimental settings prior to its clinical application.

FUTURE DIRECTIONS AND CONCLUSION

In this review, we provide a comprehensive analysis of FGF2's function as an immunomodulatory factor in chronic airway diseases, with emphasis on asthma and COPD. Nevertheless, questions remain to be addressed regarding the effect of FGF2 in pulmonary cell types and diverse disease endotypes. Therefore, future studies need to be conducted. These could include (1) FGF2 expression patterns in the presence of acute and chronic inflammation status; (2) the interaction of FGF2 with typical immune cell types in asthma and COPD; (3) role of FGF2 in modulating the function of airway structural cells in airway remodeling, inflammation, and lung function; (4) role of FGF2 in *in vivo* disease models with acute or chronic inflammation; and (5) clinical studies in asthma and COPD patients with varying disease severity. With these studies being performed, a better understanding of FGF2 biology and its immunomodulatory role in asthma and COPD could provide potential alternative options for patients that are unresponsive to current anti-inflammatory treatments.

AUTHOR CONTRIBUTIONS

YY and D-YW conceived of this review. YT, YQ, and YY drafted this manuscript. ZC, JL, and YG designed the figures. TT and KT provided critical feedback.

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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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Disruption of AKAP-PKA Interaction Induces Hypercontractility With Concomitant Increase in Proliferation Markers in Human Airway Smooth Muscle

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With the ability to switch between proliferative and contractile phenotype, airway smooth muscle (ASM) cells can contribute to the progression of airway diseases such as asthma and chronic obstructive pulmonary disease (COPD), in which airway obstruction is associated with ASM hypertrophy and hypercontractility. A-kinase anchoring proteins (AKAPs) have emerged as important regulatory molecules in various tissues, including ASM cells. AKAPs can anchor the regulatory subunits of protein kinase A (PKA), and guide cellular localization via various targeting domains. Here we investigated whether disruption of the AKAP-PKA interaction, by the cell permeable peptide stearated (st)-Ht31, alters human ASM proliferation and contractility. Treatment of human ASM with st-Ht31 enhanced the expression of protein markers associated with cell proliferation in both cultured cells and intact tissue, although this was not accompanied by an increase in cell viability or cell-cycle progression, suggesting that disruption of AKAP-PKA interaction on its own is not sufficient to drive ASM cell proliferation. Strikingly, st-Ht31 enhanced contractile force generation in human ASM tissue with concomitant upregulation of the contractile protein α-sm-actin. This upregulation of α-sm-actin was independent of mRNA stability, transcription or translation, but was dependent on proteasome function, as the proteasome inhibitor MG-132 prevented the st-Ht31 effect. Collectively, the AKAP-PKA interaction appears to regulate markers of the multi-functional capabilities of ASM, and this alter the physiological function, such as contractility, suggesting potential to contribute to the pathophysiology of airway diseases.

Keywords: airway smooth muscle, A-kinase anchoring proteins (AKAP), protein kinase A (PKA), asthma, chronic obstructive pulmonary disease (COPD)

INTRODUCTION

Airway smooth muscle (ASM) cells are phenotypic plastic, being able to switch between a more proliferative (and less contractile) or a more contractile (and less proliferative) state in response to mitogens, growth factors (e.g., transforming growth factor-β), and/or alterations in extracellular matrix composition (Halayko et al., 2008). Phenotypic plasticity of ASM plays an important role in the pathophysiology of obstructive pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, by enabling airway hypercontractility and ASM hypertrophy and hyperplasia (Halayko et al., 2008), which both contribute to airway narrowing and airflow limitation (Amrani and Panettieri, 2003; Chung, 2005). The hypercontractile phenotype is characterized by an increased expression of contractile proteins, including α-smooth muscle actin (SMA) and calponin (Halayko et al., 2008). We have previously shown that cyclic adenosine monophosphate (cAMP) regulates mitogen-induced phenotypic plasticity (Roscioni et al., 2011a,c). Furthermore, activation of the cAMP effector protein kinase A (PKA) prevents mitogen-induced proliferation by blunting the activation of extracellular regulated kinase 1/2 (ERK1/2) and p70 ribosomal protein S6 kinase (p70S6K), as well as inhibits mitogen-induced reduction in contractile protein expression in ASM strips (Roscioni et al., 2011a,c).

A-kinase anchoring proteins (AKAPs) are scaffolding proteins that provide a molecular platform for other proteins to ensure that signaling effectors are appropriately targeted to different domains, thereby specifying and facilitating intracellular signal transduction (Wong and Scott, 2004). Several AKAP isoforms have been identified that can differ in their cellular localization and interaction partners (Skroblin et al., 2010). A unifying feature of AKAPs is their ability to anchor the regulatory subunits of PKA in proximity to substrates via a conserved short α-helical structure. A cell permeable inhibitor peptide, stearated (st)-Ht31, was developed based on this short α-helical structure to block the interaction between all members of the AKAP family and PKA. St-Ht31 is designed in such a way that it specifically inhibits the interaction between the RII subunits of cAMP-dependent PKA and AKAPs. The sequence of st-Ht31 (24 amino acids) is derived from endogenously expressed, human thyroid RII anchoring protein Ht31 (Pawson and Scott, 1997; Vijayaraghavan et al., 1997; Esseltine and Scott, 2013; Poppinga et al., 2014).

AKAPs are important in compartmentalizing cAMP within the cellular context, thus providing spatio-temporal regulation of the cAMP pathway (Wong and Scott, 2004). Due to the ability of AKAPs to interact with the β2-adrenoceptor and phosphodiesterases, these are important considerations, as β2-agonists and phosphodiesterase inhibitors are commonly used cAMP-elevating drugs for the treatment of respiratory disease (Poppinga et al., 2014).

On this basis, AKAPs regulate a number of cellular responses including intracellular actin dynamics and cell cycle progression (Kim et al., 2013; Han et al., 2015). Cell cycle kinetics is controlled by various effectors, including p70S6K, ERK1/2, proliferating cell nuclear antigen (PCNA), cyclins, and retinoblastoma protein (Rb) (Leonardi et al., 1992; Karpova et al., 1997; Grewe et al., 1999; Bertoli et al., 2013; Dick and Rubin, 2013). Most of these effectors can interact with AKAPs and dysfunction of AKAPs is associated with cell cycle dysregulation (Akakura et al., 2008; Pellagatti et al., 2010; Kong et al., 2013; Han et al., 2015; Petrilli and Fernández-Valle, 2016). For instance, AKAP8 (also known as AKAP95), which is expressed in human ASM, can interact with the cell cycle regulator cyclin D1 (Arsenijevic et al., 2004; Horvat et al., 2012; Poppinga et al., 2015). However, the overall role for AKAPs in regulating cell cycle transition and contractility in human ASM is yet to be defined. Using the specific AKAP-PKA interaction disruptor, st-Ht31 (Skroblin et al., 2010), the current study explores the role of AKAPs in regulating human ASM phenotypic modulation. Here we demonstrate that disruption of AKAP-PKA interactions specifically alters the phosphorylation status or expression of a subset of cell cycle regulatory proteins in human ASM cells, which is unexpectedly paralleled by hypercontractility in human bronchial strips.

MATERIALS AND METHODS

Cell Culture

Human ASM cell cultures were generated from macroscopically healthy 3rd–5th generation bronchial segments obtained from three different donors undergoing lung resection surgery, and thereafter low passage number primary cultures (P2–P3) were made senescence-resistant by stable ectopic expression of human telomerase reverse transcriptase (hTERT) as described previously (Gosens et al., 2006; Burgess et al., 2018). Primary human ASM cells were isolated from human tracheal sections from anonymized lung transplantation donors (obtained from the Department of Cardiothoracic Surgery, University Medical Center Groningen) as previously described (Roscioni et al., 2011b). Cell cultures were maintained in DMEM (Life technologies, 11965-092) containing heat-inactivated fetal bovine serum (10% vol/vol), streptomycin (50 U/ml) and penicillin (50 mg/ml) in a humidified atmosphere at 37°C in air/CO_2 (95%:5% vol/vol). hTERT ASM cultures up to passage 30 were used.

[^{3}H]-Thymidine Incorporation Assay

hTERT ASM cells were plated in 24-well plates at 20,000 cells/well. After growing to confluence, cells were serum deprived for 3 days and subsequently incubated with 50 μM st-Ht31 (V8211, Promega) or vehicle (i.e., water) for 4 h, then incubated in the presence of [^{3}H]-thymidine (0.25 μCi/ml) for 24 h. After incubation, cells were washed twice with PBS at room temperature and subsequently with ice-cold 5% trichloroacetic acid on ice for 30 min and the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]-thymidine was quantified by liquid-scintillation counting using a Beckam LS1701 β-counter as described previously (Roscioni et al., 2011a).

Cell Viability Assay

hTERT ASM cells were plated in 24-well plates at 20,000 cells/well and serum deprived for 3 days. Subsequently the cells were incubated with st-Ht31 (50 μM) or vehicle for 24 h, after which

the cells were washed twice with PBS and incubated with 5% vol/vol AlamarBlue® (DAL1100, Thermo Fisher Scientific) in HBSS for 45 min. AlamarBlue® is converted into its fluorescent form by mitochondrial cytochromes in viable cells. Therefore, the amount of fluorescence is proportional to the number of living cells. Cell viability was assessed by measuring fluorescence emission using a Wallac 1420 Victor 2TM (excitation: 570 nm, emission: 590 nm).

Fluorescence-Activated Cell Sorting Analysis

To study the effect of st-Ht31 on cell cycle distribution, fluorescence-activated cell sorting (FACS) analysis was performed in the hTERT ASM cells. hTERT ASM cells were plated in 6-well plates at 200,000 cells/well. After growing to confluence, cells were serum deprived for 3 days and subsequently treated with st-Ht31 (50 μM) or vehicle for 24 h. After incubation, cells were detached by trypsin treatment and washed twice with warm PBS (37°C). Cells were then resuspended in ice-cold PBS and subsequently fixed in ice-cold 70% ethanol. After centrifugation, the cell pellet was resuspended in PBS containing 10 μg/ml propidium iodide, 20 mM EDTA, 0.05% Tween 20 and 50 μg/ml RNAse, and incubated overnight at 4°C. Cell cycle analysis was performed on a BD FACSCalibur (Becton, Dickinson and Company, BD, Franklin Lakes, NJ, United States). Fluorescence histograms were collected for at least 10,000 cells. The cell cycle distribution was analyzed using ModFit LT flow cytometry modeling software (ModFit LT, version 4.0.5).

Western Blot

hTERT ASM cells were plated in 6-well plates at 200,000 cells/well. After serum deprivation for 3 days, cells were treated with st-Ht31 (50 μM) for 24 h. To study the possible mechanisms underlying the effect of st-Ht31 on α-SMA and calponin protein expressions, inhibitors (MG-132: 5 μM, ab141003, Abcam; cycloheximide: 5 mg/ml, 44189, BDH Biochemicals; actinomycin D: 1 μg/ml, A1410, Sigma; chloroquine diphosphate salt: 50 μM, C6628, Sigma) were added for the final 24 h. After washing twice with ice-cold PBS, cells were lysed using 100 μl of RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% nonyl phenoxypolyethoxylethanol), supplemented with 1 mM Na_3VO_4, 1 mM NaF, 1,06 mg/ml β-glycerolphosphate, 1 μg/ml apoprotein, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The cell lysate was homogenized by passing through a 25-gauge needle for 10 times. Protein content was determined using the Pierce BCA protein assay.

Equal amounts of protein were prepared for SDS-PAGE by adding 4X SDS loading buffer and ultrapure water, and separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, followed by blocking with 1x Roti®-Block (A151, Carl Roth), and incubated overnight with primary antibodies (see **Table 1**). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (see **Table 1**). Protein bands were visualized using Western Lightning® Plus-ECL (NEL104001EA, PerkinElmer) and quantified using ImageJ J 1.48v. Proteins were normalized to GAPDH, lamin A/C, total ERK1/2 (p44/42 MAPK) or caveolin-1, as appropriate.

TABLE 1 | Antibodies used for Western blot analysis.

Protein of interest	Primary antibody	Secondary antibody
α-sm-actin	1:1000, A2547, Sigma	1:2000, A9044, Sigma
Calponin	1:1000, C2687, Sigma	1:5000, A9044, Sigma
PCNA	1:1000, sc-7907, Santa-Cruz	1:2000, A0545, Sigma
Pan-ubiquitin	1:1000, ab7780, Abcam	1:3000, A0545, Sigma
Cyclin D1	1:1000, #2926, Cell Signaling	1:1000, A9044, Sigma
Phosphorylated (p)-Rb	1:500, #9308, Cell Signaling	1:1000, A0545, Sigma
p-p70S6K	1:500, sc-7984-R, Santa-Cruz	1:2000, A0545, Sigma
p-ERK1/2 (p-p44/42 MAPK)	1:2000, #9101S, Cell Signaling	1:5000, A0545, Sigma
Total ERK1/2 (p44/42 MAPK)	1:2000, #9102, Cell Signaling	1:5000, A0545, Sigma
Caveolin-1	1:1000, sc-894 (HRP conjugated), Santa-Cruz	Directly labeled
Lamin A/C	1:1000, sc-7292, Santa-Cruz	1:2000, A9044, Sigma
GAPDH	1:2000, sc-47724, Santa-Cruz	1:8000, A9044, Sigma

Immunofluorescence

Primary human ASM cells or immortalized hTERT ASM cells were plated at a density of 20,000 cells per well on a 24-well plate with cover slips placed at the bottom of each well. Cells were serum deprived for 3 days and subsequently treated with st-Ht31 (50 μM) or vehicle for 24 h. After stimulation, cells were washed twice with PBS and fixed with 4% paraformaldehyde + 4% sucrose for 15 min, followed by 0.3% Triton X100 for 2 min at room temperature. Cells were blocked with a blocking buffer containing 5% bovine serum albumin (BSA) and 2% donkey serum at room temperature for 1 h. After fixing, cells were incubated overnight at 4°C with the primary antibody (α-sm-actin: 1:1000, A2547, Sigma; cyclin D1: 1:100, #2926, Cell Signaling; AKAP8: 1:50, sc-10766, Santa-Cruz) diluted in 1% BSA. The next day, after thorough wash, cells were incubated with the secondary antibody (anti-rabbit FITC, Green, 1:500, 65-6111, Thermo Fisher Scientific) for 1 h at room temperature in a dark chamber. After a thorough wash, nuclei were stained with Hoechst (1:10,000, H3570, Invitrogen) for 5–10 s, immediately followed by two quick and four 10 min washing steps with dd-H_2O. Finally, cover slips were placed and attached on microscope slides using ProLong Gold antifade reagent (Invitrogen). Images were taken and analyzed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens, Olympus Corporation, Tokyo, Japan). The background corrected fluorescence measurements were performed with Image J 1.48v as previously described (Burgess et al., 2010).

mRNA Isolation and Real Time PCR

hTERT ASM cells were plated in 6-well plates at a concentration of 200,000 cells/well. After serum deprivation (1 day for actin

alpha 2 (Acta2), calponin 1 (Cnn1), and 3 days for AKAP8), cells were treated with st-Ht31 (50 μM) for 10 h. To determine the half-life of ACTA2 mRNA, hTERT ASM cells were pretreated with Actinomycin D (4 μM) 30 min prior to treatment with st-Ht31 (50 μM) or vehicle for various time-points up to 24 h. mRNA from hTERT ASM cells was extracted using a Nucleospin RNA II kit (Machery Nagel) and quantified using spectrophotometry (NanoDrop, Thermo Fisher Scientific). 1 μg of mRNA was converted into cDNA by reverse transcriptase using Promega tools (Madison). cDNA was subjected to real-time PCR (RT-PCR) using a MyiQ^{TM} Single-Color detection system (Bio-Rad Laboratories Inc. Life Science Group) and the specific primers (see **Table 2**). RT-qPCR was performed in duplicate using SYBR Green (Roche) with denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 30 s for 40 cycles followed by 10 min at 72°C. The amount of target gene was normalized to ribosomal subunit 18 S (designated as ΔCT). Relative differences were determined using the equation $2^{-(\Delta\Delta Ct)}$.

Human Tracheal Smooth Muscle Strips

Human tracheal tissue from anonymized lung transplantation donors was obtained from the Department of Cardiothoracic Surgery, University Medical Center Groningen. All tissue was collected according to the Research Code of the University Medical Center Groningen[1] and national ethical and professional guidelines ("Code of conduct," Dutch federation of biomedical scientific societies[2]). After dissection of the smooth muscle layer and careful removal of the mucosa and connective tissue, human tracheal smooth muscle strips of identical length and width were prepared as described previously (Roscioni et al., 2011c). The average weight of the bronchial strips is 13.9 ± 5.9 mg (average ± standard deviation, $n = 55$). The main difference in weight is due to interindividual differences between tissue of the donors, rather than between bronchial strips derived from the same donor. For each experiment, we randomize the prepared bronchial strips before subsequent treatment is started. To limit the possibility of variations between tissue preparations of the same donor, we perform each experiment at least in duplicate and the average value for the contractility of both tissue strips together is considered as one independent data-point. Tissue strips were transferred to serum-free DMEM supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), apo-transferrin (human, 5 μg/ml) and ascorbic acid (100 μM). The strips were incubated with st-Ht31 (50 μM) or vehicle for 24 h in an Innova 4000 incubator shaker (37°C, 55 rpm). After culture, strips were thoroughly washed and mounted in an organ bath for isometric tension measurements.

[1]https://www.umcg.nl/SiteCollectionDocuments/Englis/Researchcode/UMCG-Researchcode,%20basic%20principles%202013.pdfh

[2]http://www.federa.org

TABLE 2 | primers used for qRT-PCR.

Gene of interest	Primers
Acta2	Forward 5′-CTTTCATTGGGATGGAGTCAGC-3′
	Reverse 5′-ACAGGACGTTGTTAGCATAGAGA-3′
Cnn2	Forward 5′-TCTTTGAGGCCAACGACCTG-3′
	Reverse 5′-GGGATCATAGAGGTGACGCC-3′
AKAP8	Forward 5′-ATGCACCGACAATTCCGACT-3′
	Reverse 5′-CATAGGACTCGAACGGCTGG-3′
18S	Forward 5′-CGCCGCTAGAGGTGAAATTC-3′
	Reverse 5′-TTGGCAAATGCTTTCGCTC-3′

Isometric Contraction Measurement

Isometric contraction experiments were performed essentially as described previously (Roscioni et al., 2011c). Briefly, ASM strips were mounted for isometric recording in 20 ml organ-baths, containing Krebs-Henseleit (composition in mM: NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, and glucose 5.50) buffer at 37°C. During a 90 min equilibration period with wash-outs every 30 min, resting tension was adjusted to 1 g, followed by pre-contractions with 10 μM methacholine. Following wash-out, maximal relaxation was established by the addition of 0.1 μM (-)-isoprenaline. Tension was readjusted to 1 g, followed by refreshing of the Krebs-Henseleit buffer twice. After another equilibration period of 30 min, cumulative concentration–response curves were constructed with methacholine (0.1 nM – 1 mM). When maximal tension was reached, strips were washed several times and maximal relaxation was established using 10 μM (-)-isoproterenol. Contractions were corrected for tissue weight and expressed as percentage of the maximal methacholine-induced contraction in vehicle-treated strips. Curves were fitted using Prism 5.0. After the contraction protocol, strips were collected and tissue homogenates were prepared as previously described (Roscioni et al., 2011c) for western blot measurement of α-sm-actin, calponin and PCNA.

Statistics

Data are expressed as means ± SEM of n individual experiments. Statistical significance of differences was evaluated using Prism 5.0 software by performing One-sample T-tests. For the isometric contraction experiments: non-linear curve fit was performed and subsequent statistical curve comparison was done using the extra sum-of-squares F-test using Prism 5.0 software. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Role of AKAPs in Proliferation of Human ASM Cells

Treatment with st-Ht31 significantly increased [^{3}H]-thymidine incorporation in hTERT ASM cells (**Figure 1A**), indicating enhanced DNA synthesis. However, st-Ht31 treatment for 4 days did not affect cell viability (**Figure 1B**). We further assessed cell cycle distribution of propidium iodide stained hTERT ASM cells by flow cytometry and found that st-Ht31 exposure had little effect (**Figure 1C**).

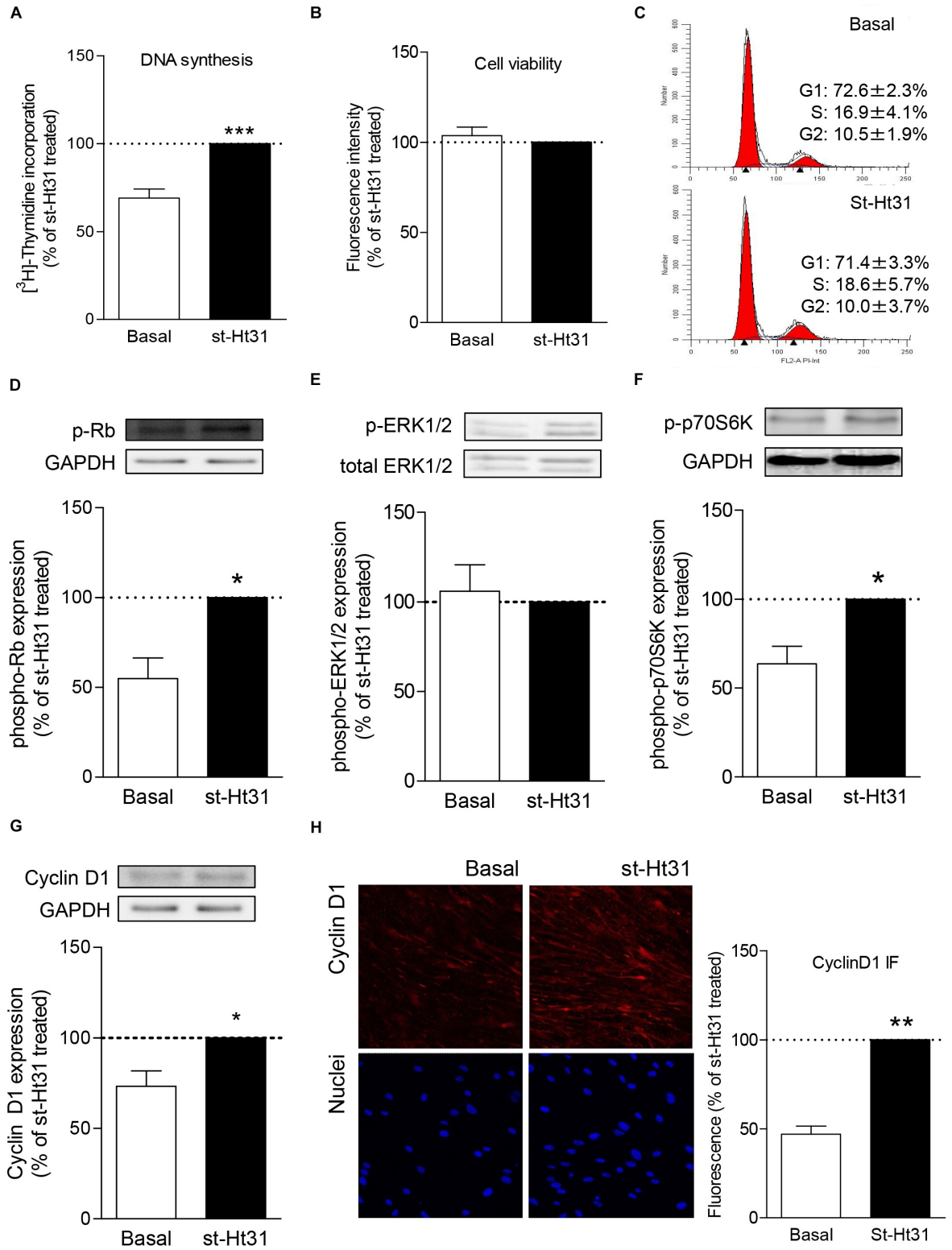

FIGURE 1 | The effects of st-Ht31 on proliferation markers in human airway smooth muscle cells. hTERT ASM cells were serum-deprived for 3 days and treated with st-Ht31 (50 μM). **(A)** [^{3}H]-thymidine was added 4h after st-Ht31 and incorporated [^{3}H]-thymidine was quantified 2 4h later. $n = 15$. **(B)** After 24h of treatment with st-Ht31, cell viability was assessed using AlamarBlue®. $n = 5$. **(C)** FACS analysis was performed 24 h after st-Ht31 treatment. $n = 3$. **(D–H)** Protein expression of the indicated proteins was measured 24 h after st-Ht31 treatment using Western blot **(D–G)** or immunofluorescence (IF, **H**). $n = 4–9$. $^*p < 0.05$, $^{**}p < 0.05$, and $^{***}p < 0.001$ compared to basal.

To further understand the paradoxical increase in DNA synthesis without cell cycle induction with st-Ht31 treatment, we investigated the abundance and phosphorylation status of cell cycle regulator proteins. Treatment of hTERT ASM cells with st-Ht31 enhanced the phosphorylation status of the cell-cycle regulators Rb and p70S60K, without affecting ERK1/2 (p42/44 MAPK) phosphorylation (**Figures 1D–F**). Moreover, st-Ht31 treatment increased cyclin D1 abundance in hTERT ASM cells (**Figures 1G,H**).

AKAP8 Expression and Localization in Human ASM Cells

Previous studies by us and others demonstrated protein and/or mRNA expression of a subset of AKAPs in human ASM, including AKAP1, AKAP2, AKAP3, AKAP5 (also known as AKAP79), AKAP8, AKAP9, AKAP10, AKAP11, AKAP12, AKAP13, Ezrin, and MAP2B (Horvat et al., 2012; Poppinga et al., 2015). We currently investigated the effect of st-Ht31 on the expression of AKAP8, as this particular AKAP is associated with cell cycle progression (Han et al., 2015). Treatment of hTERT ASM cells with st-Ht31 decreased the fluorescent intensity of total AKAP8 compared to vehicle treated cells (**Figure 2A**). The ratio of the cellular distribution of AKAP8 between nuclei and non-nuclear compartments was not altered by st-Ht31, indicating that st-Ht31 causes a general decrease in AKAP8 abundance. In line with the changes in protein level, we found that st-Ht31 also significantly decreased AKAP8 mRNA expression (**Figure 2B**). Besides AKAP8, we also measured the expression of AKAP5, AKAP12, and Ezrin, but none were significantly altered by st-Ht31 (data not shown). Primary human ASM cells also showed decreased AKAP8 expression, without an effect on its cellular distribution, in response to st-Ht31 (**Figure 2C**).

Role of AKAPs in Contractile Protein Expression in Human ASM Cells

Treatment of hTERT ASM cells with st-Ht31 resulted in a significant increase in the abundance of the contractile proteins α-sm-actin and calponin (**Figures 3A,B**). Remarkably, the mRNA abundance for *ACTA2* and *CNN1* (encoding α-sm-actin and calponin, respectively) was decreased (i.e., *ACTA2*) or unaltered (i.e., *CNN1*) in response to st-Ht31 (**Figure 3C**). Next, we investigated if disruption of the AKAP-PKA interaction in human ASM cells by st-Ht31 affected mRNA stability of *ACTA2*. Expression of *ACTA2* decreased over time in human ASM cells and this process was unaffected by st-Ht31 (**Figure 3D**).

To investigate the potential mechanism(s) for the differential effects of st-Ht31 on mRNA and protein abundance of contractile proteins α-sm-actin and calponin, we independently inhibited gene transcription, translation, and protein degradation using pharmacological tools. Inhibition of RNA synthesis by actinomycin D decreased basal and st-Ht31-induced α-sm-actin and calponin protein abundance in human ASM cells (**Figures 4A,B**). However, compared directly to time-matched actinomycin D treated cultures, the addition of st-Ht31 still increased sm-α-actin and calponin abundance in human ASM cells (**Figures 4A,B**). Cycloheximide, an inhibitor of protein synthesis, did not affect (α-sm-actin) or even enhanced (calponin) contractile protein abundance in the presence or absence of st-Ht31 (**Figures 4A,B**). Pharmacological suppression of protein degradation with the proteasome inhibitor MG-132, was without major effect on basal abundance of sm-α-actin and calponin, but prevented the induction of α-sm-actin by st-Ht31 (**Figures 4A,B**). Concomitant, we observed a transient increase in ubiquitination of a variety of proteins in response to short-term st-Ht31 treatment (**Figure 4C**). Finally, inhibition of protein turnover by targeting lysosomal enzymes with chloroquine did not affect contractile protein abundance at baseline or after treatment with st-Ht31 (**Figures 4A,B**).

Role of AKAPs in Regulating Contractility and Proliferation in Human Bronchial Strips

To investigate the functional consequence of increased contractile protein abundance that we observed in cultured ASM cells, we incubated human ASM tissue strips with st-Ht31 and subsequently assessed methacholine-induced isometric contraction. Treatment with st-Ht31 significantly increased contraction by 1.3-fold (E_{max}; **Figure 5A**), without affecting the sensitivity to methacholine (pD_2-values: 5.28 ± 0.18 and 5.36 ± 0.18 for basal and st-Ht31 treated, respectively). The increased capacity to generate (maximum) force was accompanied by a concomitant increase in α-sm-actin protein abundance (**Figure 5B**), whereas calponin expression showed more variation and was not significantly altered (data not shown). Furthermore, treatment of human ASM strips with st-Ht31 resulted in an increase in the S-phase marker *proliferating cell nuclear antigen* (PCNA) (**Figure 5C**).

DISCUSSION

We demonstrate that disruption of AKAP-PKA interactions using st-Ht31 increases expression of the contractile proteins α-sm-actin and calponin in human ASM cells, and development of hypercontractile human ASM in bronchial strips. In parallel, st-Ht31 enhances DNA synthesis and expression of markers for ASM cell proliferation, although neither cell viability nor cell cycle distribution are affected directly. Collectively, these findings indicate that AKAP-PKA mediated signaling regulates ASM phenotype and function.

AKAPs are a group of structurally diverse proteins, which act as scaffolds for a variety of structural and signaling molecules to facilitate targeting of different cellular microdomains (Wong and Scott, 2004; Poppinga et al., 2014). We recently demonstrated that the expression of several AKAPs in ASM and bronchial epithelial cells is differentially affected by cigarette smoke (Oldenburger et al., 2014; Poppinga et al., 2015). Moreover, we observed differential expression of AKAPs in lung tissue of COPD patients (Oldenburger et al., 2014; Poppinga et al., 2015). All AKAPs combine with the regulatory subunits of PKA through a short α-helical structure to guide activity in different sub-cellular locales (Esseltine and Scott, 2013). This functional feature was exploited in the development of the st-Ht31 peptide, as it

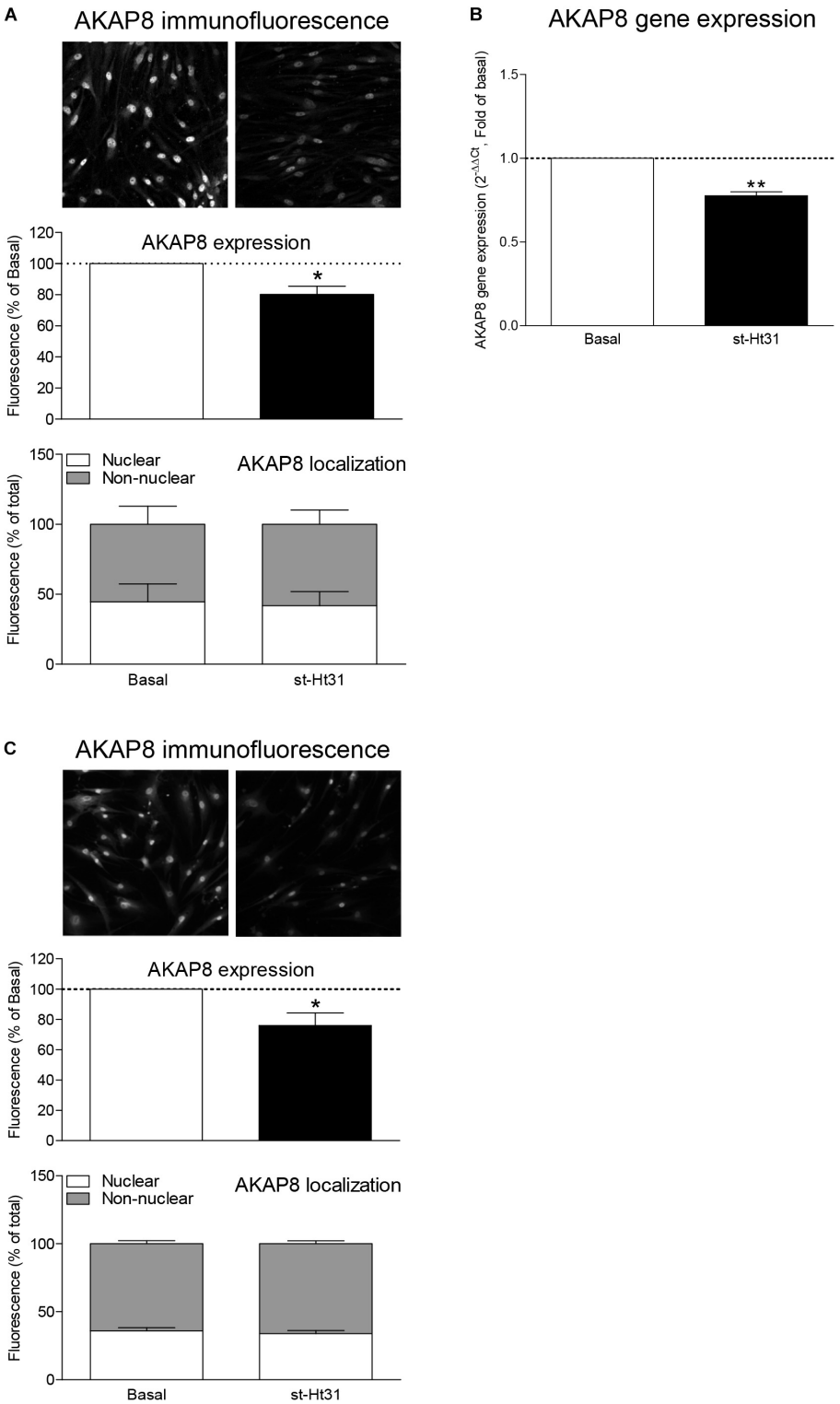

FIGURE 2 | The effects of st-Ht31 on AKAP8 expression and cellular localization in human airway smooth muscle cells. **(A)** hTERT ASM cells were serum deprived for 3 days and treated with st-Ht31 (50 μM) for 24 h. The expression and localization of AKAP8 was measured by immunofluorescence. Representative images are shown. Images were quantified by Image J 1.48v. $n = 4$. **(B)** AKAP8 mRNA expression was measured in hTERT ASM cells using RT-PCR and normalized to ribosomal subunit 18 S (ΔCT). Relative differences were determined using the equation $2^{-(\Delta\Delta Ct)}$. $n = 4$. **(C)** Primary human ASM cells were serum deprived for 3 days and treated with st-Ht31 (50 μM) for 24 h. The expression and localization of AKAP8 were measured by immunofluorescence. Representative images are shown. Images were quantified by Image J 1.48v. $N = 5$, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to basal.

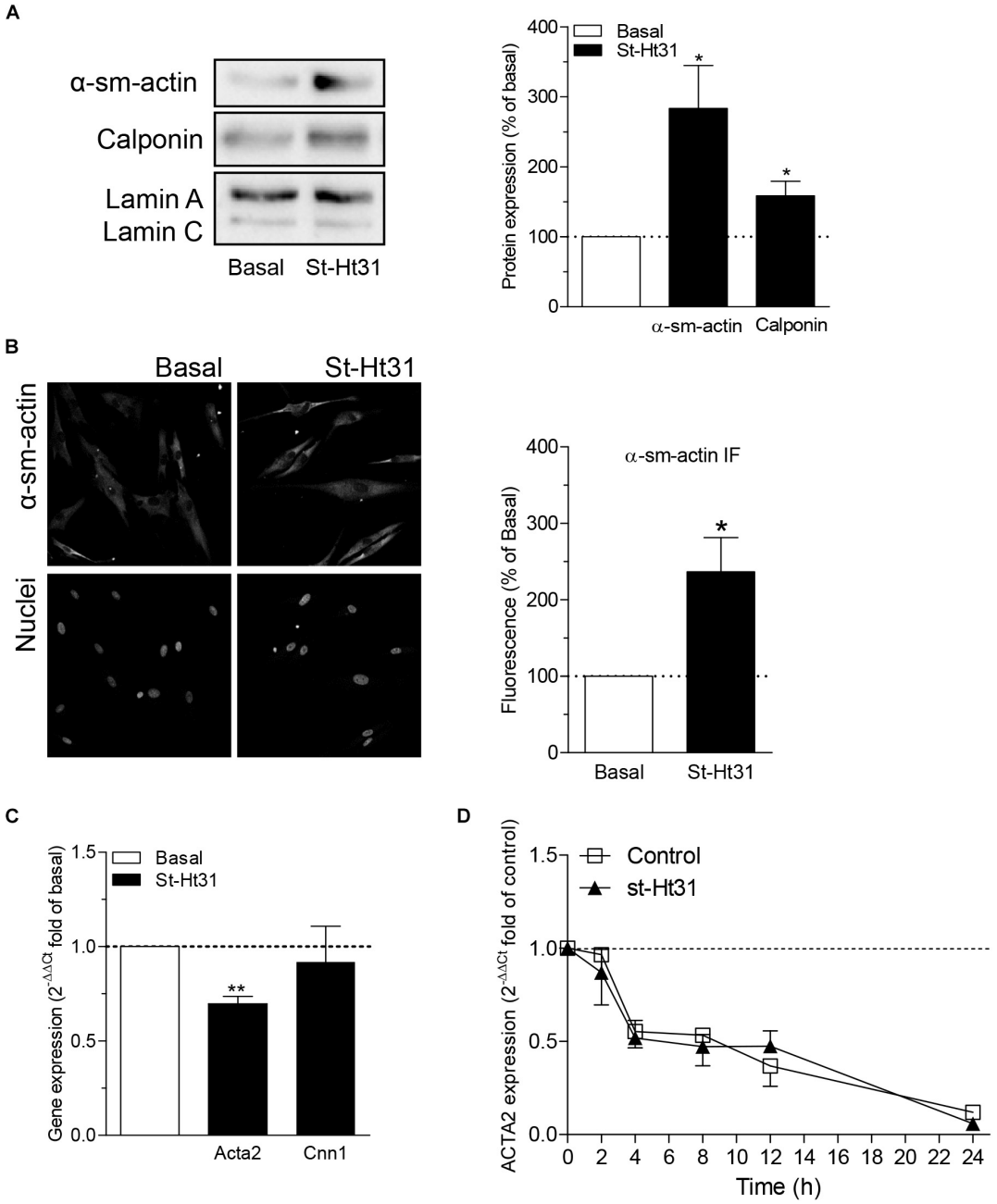

FIGURE 3 | The effect of st-Ht3 on contractile markers in human airway smooth muscle cells. **(A,B)** hTERT ASM cells were serum deprived for 3 days and treated with st-Ht31 (50 μM) for 24 h. Protein expression of α-sm-actin **(A,B)** and calponin **(A)** were measured by western blot **(A)** or immunofluorescence (IF, **B**). Data expressed as means ± SEM of $n = 5$–8. **(C)** hTERT ASM cells were serum deprived for 1 day and treated with st-Ht31 (50 μM) for 1 h. *ACTA2* and *CNN1* expression was measured using RT-PCR and normalized to ribosomal subunit 18 S (ΔCT). Relative differences were determined using the equation $2^{-(\Delta\Delta Ct)}$. $n = 4$. **(D)** hTERT ASM cells were pretreated with actinomycin D (4 μM) and subsequently treated without (control) or with st-Ht31 (50 μM) for the indicated time-points. *ACTA2* expression was measured using RT-PCR and normalized to ribosomal subunit 18 S (ΔCT). Relative differences were determined using the equation $2^{-(\Delta\Delta Ct)}$. $n = 6$. *$p < 0.05$ and **$p < 0.01$ compared to basal.

mimics the short α helical structure in AKAPs, to competitively block AKAP interaction with PKA (Vijayaraghavan et al., 1997). However, stimulus-induced PKA activation in human ASM is not affected by st-Ht31 (Poppinga et al., 2015). Since its development, st-Ht31 has been shown to alter a variety of cellular functions, including cardiac muscle contraction and cell cycle progression (McConnell et al., 2009; Gao et al., 2012).

ASM cells exhibit phenotype plasticity, which allows modulation of contractile and proliferative functional capacity in response to changes in the surrounding microenvironment

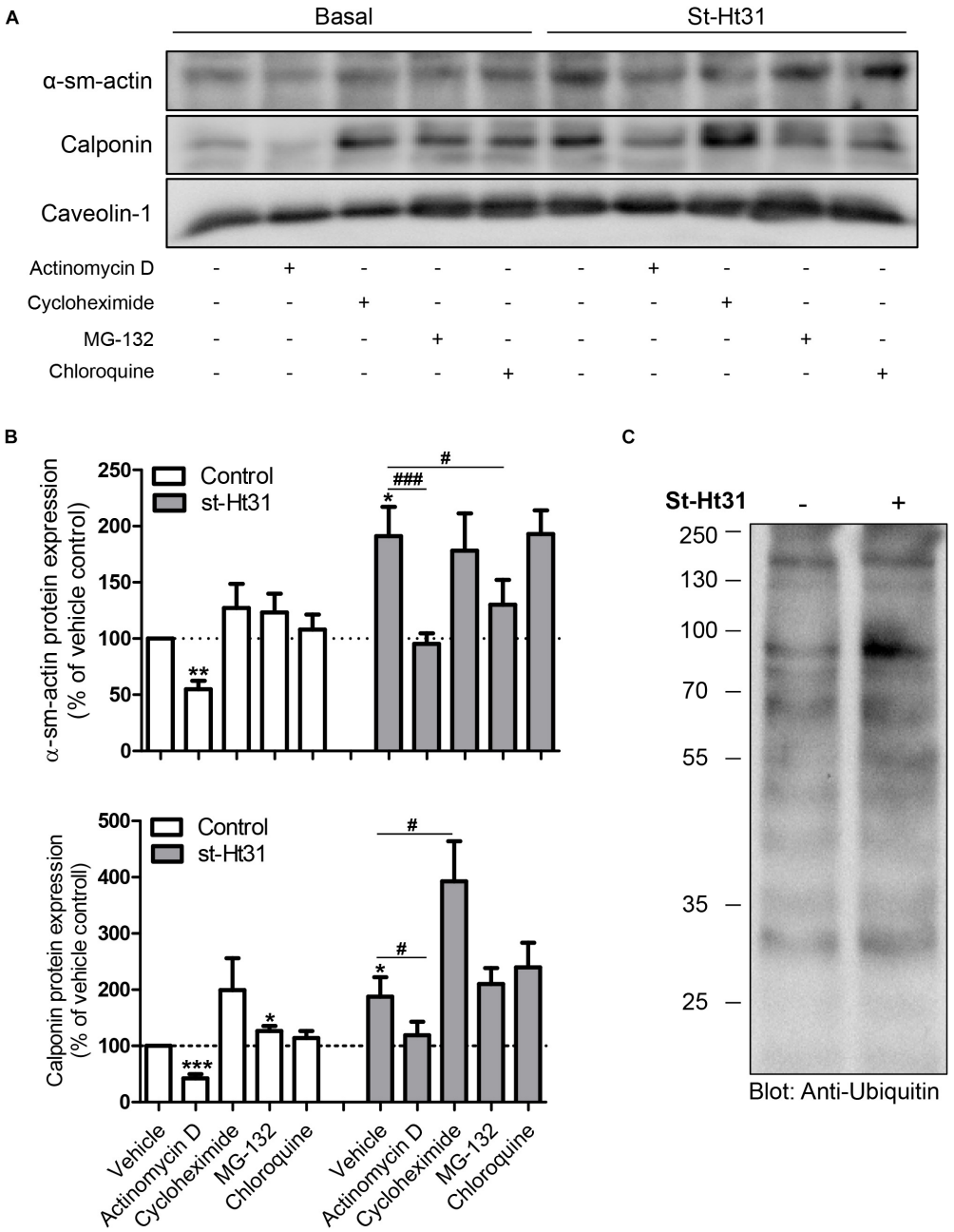

FIGURE 4 | Potential mechanisms underlying st-Ht31-induced contractile protein expression in human airway smooth muscle cells. **(A,B)** hTERT ASM cells were treated with st-Ht31 (50 μM) in the absence or presence of actinomycin D (1 μg/ml), cycloheximide (5 mg/ml), MG-132 (5 μM) and chloroquine (50 μM). Protein expression of α-sm-actin and calponin was analyzed and quantified. n = 5–8 experiments. **(C)** hTERT ASM cells were treated with vehicle or st-Ht31 (50 μM) for 1h. The degree of protein ubiquitination in whole cell lysate was detected using an anti-ubiquitin antibody. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to vehicle without st-Ht31, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to vehicle with st-Ht31.

(Halayko et al., 2008). Prolonged exposure to mitogens induces a hyperproliferative, hypocontractile ASM phenotype, characterized by increased DNA synthesis and mitosis, as well as reduced contractile protein abundance (Halayko et al., 2008; Simeone-Penney et al., 2008; Roscioni et al., 2011a,c). On the other hand, other stimuli such as transforming growth factor-β1 (TGF-β1), insulin or changes in extracellular laminin composition can induce a hypoproliferative, hypercontractile ASM phenotype (Ma et al., 1998; Gosens et al., 2003; Schaafsma et al., 2007; Halayko et al., 2008; Dekkers et al., 2009; Tran et al., 2013, Ojiaku et al., 2018). Interestingly, we now demonstrate that interrupting the interaction between PKA and AKAPs simultaneously increases markers of a hyperproliferative (e.g., DNA synthesis, activation of cell cycle proteins and

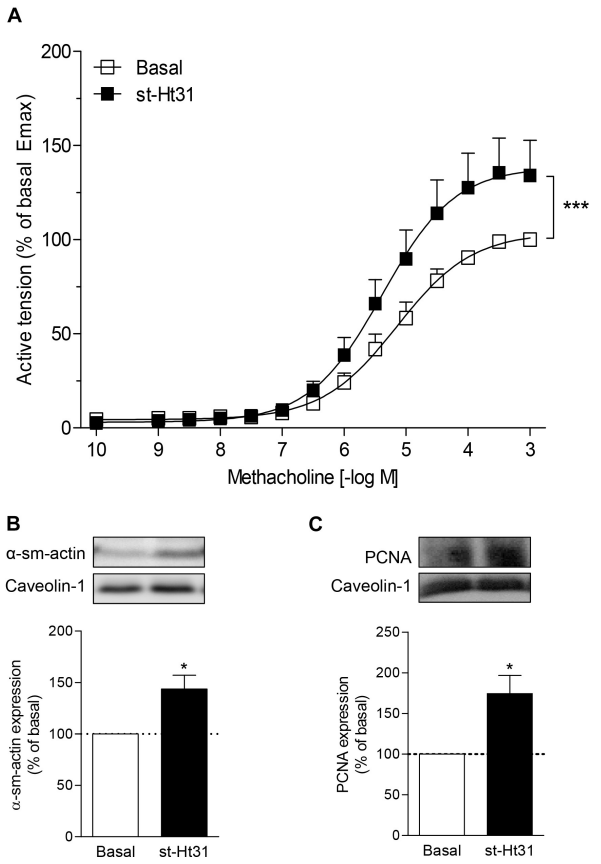

FIGURE 5 | The effects of st-Ht31 on contraction of human airway smooth muscle strips. Isolated human tracheal smooth muscle strips were incubated without or with st-Ht31 (50 μM). **(A)** Methacholine-induced isometric contraction was measured. $N = 7–8$. $^{***}p < 0.001$ compared to basal as determined by an extra sum-of-squares F-test. **(B,C)** The expression of α-sm-actin **(B)** and PCNA **(C)** was determined in the tracheal strips by immunoblotting. $N = 4–5$, $^{*}p < 0.05$ compared to basal.

increased expression of the proliferative marker PCNA) and a hypercontractile phenotype (e.g., increased expression of contractile proteins and increased contractility) in cultured human ASM cells and in intact human ASM strips.

A potential modulator of ASM phenotype is p70 ribosomal S6 kinase (p70S6K) (Halayko et al., 2004; Roscioni et al., 2011a). Mitogen exposure of human ASM cells leads to phosphorylation and activation of p70 ribosomal S6 kinase (p70S6K), which promotes cell proliferation via upregulation of various proteins, including the cell-cycle checkpoint determinant cyclin D1 (Scott et al., 1996; Karpova et al., 1997; Grewe et al., 1999; Takuwa et al., 1999; Ravenhall et al., 2000; Chambard et al., 2007; Roscioni et al., 2011b). We have previously demonstrated that p70S6K phosphorylation reduced by activation of PKA (Roscioni et al., 2011a). Disruption of the AKAP-PKA interaction by st-Ht31 leads to a loss of intracellular targeting of PKA, which, however, is not accompanied by a loss of PKA activity (Poppinga et al., 2015). Although PKA can still be activated, it cannot signal properly in a spatiotemporal manner in the presence of st-Ht31. In the current study, we observed an enhanced p70S6K phosphorylation and increased Cyclin D1 expression in human ASM in response to st-Ht31, without an effect on ERK1/2 activity. Cyclin D1 associates with pre-existing cyclin-dependent kinases to phosphorylate target proteins, such as Rb, that further enable and modulate cell cycle progress into S phase (Lundberg and Weinberg, 1998). In agreement, st-HT31 concomitantly increases phosphorylation of Rb and DNA synthesis (Withers et al., 1997; Takuwa et al., 1999). Moreover, in intact human bronchial strips st-Ht31 treatment resulted in increased PCNA expression, a protein that is expressed during the S phase of the cell cycle (Leonardi et al., 1992). Nonetheless, the st-HT31-induced DNA synthesis was not accompanied by a change in cell cycle distribution nor by an alteration of cell viability, suggesting that disruption of the AKAP-PKA interaction is not sufficient on its own to induce cells to traverse the S phase of the cell cycle. Our FACS analysis in human ASM cells confirm that st-Ht31 has no significant impact on the fraction of cells in the S phase. Interestingly, AKAPs, particularly AKAP8, interact with cyclins, including cyclin D1 (Eide et al., 1998; Arsenijevic et al., 2004; Han et al., 2015; Qi et al., 2015). AKAP8 can reside in the nucleus, as confirmed by our study, and is thought to be involved in DNA replication and expression of several genes associated with cell cycle regulation (Coghlan et al., 1994; Eide et al., 1998; Han et al., 2015). An AKAP8-cyclin D binding site has been identified that overlaps with a CDK4 binding site, suggesting that nuclear AKAP8 may compete with CDK4 for cyclin D1 (Arsenijevic et al., 2006). Therefore, the observed downregulation of AKAP8 by st-Ht31 in ASM cells, without alterations in cellular distribution, could be permissive for interaction of cyclin D1 with CDK4, thereby supporting Rb phosphorylation and early S phase activities. On the other hand, AKAP8 has been found to regulate M phase events of the cell cycle, such as chromatin condensation, by interacting with DNA and associated proteins including a condensin complex component, Eg7 and histone deacetylase 3 (Collas et al., 1999; Steen et al., 2000; Li et al., 2006). Based on this, and in light of our observations, we hypothesize that, in addition to disrupting the interactions between AKAPs and PKA, st-Ht31-induced AKAP8 downregulation could contribute to disturbed cell cycle kinetics, that in our studies are revealed by increased proliferative markers without an increase in cell number.

Another interesting observation from our study is that disruption of the AKAP-PKA interaction by st-HT31 increases contractile protein expression in human ASM. However, st-Ht31 had little or no effect on CNN1 expression and even decreased ACTA2 expression. Our results also demonstrate that st-Ht31 does not affect mRNA stability of ACTA2. Furthermore, blocking of RNA synthesis by actinomycin D decreased both basal and st-HT31-induced protein abundance of α-sm-actin and calponin. However, compared to solely actinomycin D treatment addition of st-Ht31 still induced an increase in contractile protein expression in human ASM cell. This suggests that the effects of st-Ht31 are, in part, due to post-translational effects. This is confirmed by our observation that inhibition of protein translation using cycloheximide did not affect st-Ht31-induced contractile protein accumulation. Recently, AKAPs

have been identified as factors involved in protein turnover, specifically involved with ubiquitin-proteasome systems that tag substrate proteins for subsequent degradation by the covalent attachment of ubiquitin (Rinaldi et al., 2015). This system involves modification of substrate proteins by the covalent attachment of multiple ubiquitin molecules. Ubiquitination is a post-translational modification that generally directs proteins for degradation by the proteasome. However, ubiquitination has also been implicated in many other cellular processes, including transcriptional regulation (Ciechanover, 2005; Stringer and Piper, 2011). We show that treatment of human ASM with the proteasome inhibitor MG-132 prevents st-Ht31-induced α-sm-actin accumulation. In agreement, in vascular smooth muscle cells MG-132 reduces contractile protein expression by reducing myocardin activation (Yin et al., 2011). Myocardin and myocardin related transcription factors (MRTFs) are transcriptional co-activators of serum response factor (SRF), a master regulator of α-smooth muscle actin. In cardiac myofibroblasts, α-sm-actin expression is linked to AKAP-Lbc (also known as AKAP13) by its regulatory effects on myocardin related transcription factors (MRTFs) (Cavin et al., 2014). Clearly, future studies are necessary to investigate the effects of st-HT31 as a potential regulator of myocardin and/or MRTF activation and subsequent contractile protein expression in human ASM cells.

Most importantly, our functional analyses demonstrated increased contraction of human bronchial strips after st-Ht31 treatment. This hypercontractile ASM phenotype is accompanied by increased abundance of α-sm-actin. Of note, the involvement of AKAPs in muscle contraction has previously been shown in the heart (McConnell et al., 2009). Similar to the findings in cultured cells, st-Ht31 also increased a marker of proliferation, i.e., PCNA, in *ex vivo* human bronchial strips. Thus, markers of proliferation and contractility are simultaneously increased in ASM tissue and cultured cells. However, st-Ht31 does not alter cell viability in cultured cells, but does increase contractility of intact human ASM tissue. These findings demonstrate the importance of studying functional readouts for the different phenotypes: cell proliferation and contractility. Thus, st-Ht31 may initially induce markers that could underpin modulation to a proliferative phenotype. However, since it is insufficient to fully induce cell cycle progression and proliferation, this appears to trigger a response, either indirectly or in a mutually exclusive manner, that leads to a later induction of a hypercontractile phenotype. This hypothesis is supported by the finding that insulin acutely induces DNA synthesis in cultured ASM cells, but long-term treatment with insulin induces a hypercontractile phenotype in intact ASM tissue (Gosens et al., 2003).

In summary, we show that st-Ht31 leads to a simultaneous increase in markers of a hypercontractile phenotype and those of a hyperproliferative phenotype in both ASM cells and intact ASM strips. However, increased DNA synthesis and the activation of early cell cycle regulators are not sufficient to complete proliferation and increase cell number. In contrast, the increase in contractile protein expression (marker of a hypercontractile phenotype) did lead to an increase in contractility, indicating the overall effect of disruption of AKAP-PKA interaction is the induction of a hypercontractile phenotype. Previous studies from our group and others, have reported on the expression of at least 12 AKAP family members in human ASM, which might be of interest to target independently or all together by silencing and/or Crisp/Cas9 technologies in future studies (Horvat et al., 2012; Poppinga et al., 2015). In conclusion, AKAP-PKA interactions in ASM cells and tissue limits the contractile function of ASM, likely by restricting the expression of contractile proteins and the development of a hyperproliferative phenotype. Our observations have biological and drug development implications in obstructive respiratory disease, such as asthma and COPD, where there is both an increase in ASM mass and ASM contractility (Lambert et al., 1993; Chung, 2005, 2008; Bentley and Hershenson, 2008). The mechanisms of drugs used in the treatment of respiratory disease, such as β_2-agonists and phosphodiesterase inhibitors, most likely encompass proper AKAP-PKA interactions.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All tissue was collected according to the Research Code of the University Medical Center Groningen (https://www.umcg.nl/SiteCollectionDocuments/English/Researchcode/UMCG-Researchcode,%20basic%20principles%202013.pdf) and national ethical and professional guidelines ("Code of conduct," Dutch federation of biomedical scientific societies, http://www.federa.org). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BH, WP, SD, and CE designed and performed the experiments and performed the data analyses. HB, HMe, HMa, and MS designed the experiments and oversaw all data analyses. AH provided immortalized human airway smooth muscle cells. HB, BH, and MS drafted the figures and manuscript. All authors have critically revised the manuscript, reviewed, and approved the final manuscript as submitted to take public responsibility for it.

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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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