Advances in Experimental Medicine and Biology 1303

Yong-Xiao Wang *Editor*

Lung Inflammation in Health and Disease, Volume I

Springer

Advances in Experimental Medicine and Biology

Volume 1303

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2019 Impact Factor: 2.450 5 Year Impact Factor: 2.324

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-63045-4 ISBN 978-3-030-63046-1 (eBook)
https://doi.org/10.1007/978-3-030-63046-1

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Inflammation is a ubiquitous natural cellular process in virtually all types tissues, organs, or systems of the human body. This process can be acute and chronic. Acute inflammation is an immediate healthy response to protect and repair the body from harmful stimuli. Usually it occurs within a couple of hours. Chronic inflammation is a lengthy cellular process that is not part of natural healing and thus may lead to diseases such as arthritis, asthma, pulmonary hypertension, etc.

Inflammation can also be classified as systemic or localized. The former affects the entire human body, which is a pathogenetic component in numerous acute and chronic diseases including atherosclerosis, diabetes, sepsis, trauma, and others with a significant morbidity and mortality. The latter is localized as in a specific organ. For example, inflammation caused by asthma or pulmonary hypertension is localized in the lungs.

Lung diseases are very common and can also be very severe. It is well known that lung infections are the greatest single contributor to the overall global health burden. For instance, lung diseases are the most common causes of death among children under 5 years of age – more than 9 million annually. Indeed, pneumonia is the leading killer of children worldwide. Asthma is the most common chronic disease, affecting about 14% of children globally and continuing to rise. Likewise, COPD is recognized to be the fourth leading cause of death in the world and the numbers are rising. The lung is not only the largest internal organ in the human body, but also the only internal organ that is exposed constantly to the external environment; as such, no other organ is more vital and vulnerable than the lung. This may explain the common morbidity and mortality of lung diseases.

Systemic inflammation may induce and even exacerbate local inflammatory diseases. Likewise, local inflammation can cause systemic inflammation. Indeed, there is increasing evidence of coexistence of systemic and local inflammation in patients with asthma, COPD, and other lung diseases. Moreover, the comorbidity of two and even multiple local inflammatory diseases occurs often. For instance, rheumatoid arthritis not only occurs frequently together with pulmonary hypertension, but also promotes development of the latter. The local and systemic comorbidity as well as two or more inflammatory diseases significantly deteriorate the quality of life and may even exacerbate death in patients.

The current treatment options for lung diseases are neither always effective nor specific at all. Development of new therapeutics is earnestly needed.

Equally desperately, the molecular mechanisms and physiological significance of lung diseases are still not fully understood. Apparently, this despondent fact is a major encumberment to creating new efficacious drugs in the treatment of lung diseases. This scenario is even worse in two and more lung diseases accompanied with other inflammatory diseases due to their complexity and diversity.

Despite the current state being unsatisfactory, great advancements have been made in many aspects of lung diseases from the molecular geneses to regulatory mechanisms, signaling pathways, cellular processes, basic and clinical technologies, new drug discoveries, clinical manifestations, laboratory and clinical diagnoses, treatment options, and predictive prognosis. To the best of our knowledge, however, no one cohesive book is available to present these state-of-the-art advances in the field. Thus, as one of the major aims, we compile this timely and much-needed book to provide a high-quality platform in which well-known scientists and emerging pioneers in basic, translational, and clinical settings can present their latest, exciting findings in the studies of lung inflammation in health and disease. The contents from multiple outstanding authors with unique expertise and skills in molecular and cell biology, biochemistry, physiology, pharmacology, biophysics, biotechnology, translational biomedicine, and medicine will provide new knowledge, concepts, and discoveries in the field. The second major aim is to help direct future research in lung diseases and other inflammatory diseases. The scope of this book includes nearly all new and important findings from very recent basic, translational, and clinical research in the studies of the molecular genesis, networks, microdomains, regulation, functions, elimination, and drug discoveries of inflammation in lung health and disease, which are involved in animal and human lung epithelial cells, smooth muscle cells, *endothelial* cells, adventitial cells, fibroblasts, neutrophils, *macrophages*, *lymphocytes*, and stem/progenitor cells. Lastly, but importantly, the book will offer the latest and most promising results from clinical trials in terms of exploring interventions of local and systemic inflammation in the treatment of lung diseases.

This book features contributions from numerous basic, translational, and physician scientists in the field of pulmonary vasculature redox signaling in health and disease, and as a result offers a widespread and comprehensive overview for academic and industrial scientists, postdoctoral fellows, and graduate students who are interested in redox signaling in health and disease and/or normal and pathological functions of the pulmonary vasculature. The book may also be valuable for clinicians, medical students, and allied health professionals.

We are sincerely grateful for the overwhelming support from leading scientists and experts who responded to our request to contribute chapter articles. Due to their contributions, we are now pleased to be able to share Volumes I and II. Volume I includes 20 chapters that report the latest and most important findings on the molecular genesis, networks, microdomains, regulations, functions, and drug discoveries of inflammation from basic, translational, and clinical research.

I want to express my wholehearted gratitude to all of the authors for their dedication and diligence in contributing book chapters, particularly during the challenging and unprecedented times of the global COVID-19 pandemic. Many of the authors in this book have not only performed exceptional roles as writer, but also reviewer. Their selfless contributions are sincerely appreciated. I also want to thank Ms. Alison Ball and Mr. Arjun Narayanan at Springer Nature for their assistance, patience, and enthusiasm in seeing this book to fruition.

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1 Potential Role of Mast Cells in Regulating Corticosteroid Insensitivity in Severe Asthma

Abdulrahman Alzahrani, Aamir Hussain, Fahad Alhadian, Jameel Hakeem, Sana Douaoui, Omar Tliba, Peter Bradding, and Yassine Amrani

Abstract

The mechanisms driving corticosteroid insensitivity in asthma are still unclear although evidence points toward a potential role of lung mast cells. Indeed, a number of in vitro studies using various cell types showed that different mediators produced by activated mast cells, including cytokines, have the capacity to interfere with the therapeutic action of corticosteroids. In patients with severe allergic refractory asthma, the anti-IgE monoclonal antibody (mAb), Omalizumab, has been shown to be associated with a marked reduction in inhaled and systemic use of corticosteroids, further suggesting a key role of mast cells in the poor response of patients to these drugs. The present chapter will discuss the possible underlying mechanisms by which mast cells could contribute to reducing corticosteroid sensitivity seen in patients with severe asthma.

Keywords

Mast cells · IgE · Airway inflammation · Receptor · Airway smooth muscle · Cytokines · Growth factors · Alarmins

1.1 Introduction

Mast cells are playing a key role in asthma pathogenesis via their ability to initiate and perpetuate the type2 (or Th2) cytokine-dependent allergic inflammation in the lung. This occurs via the secretion of various key cytokines such as interleukin 4 (IL-)4 and IL-13 which induce Th2 cell proliferation and the production of allergen-specific IgE by B-cells, and IL-5 which promotes eosinophilic inflammation [1]. Mast cells in asthma are activated through many mechanisms including the high-affinity IgE receptor FcεRI, Toll-like receptors, in response to the secretion of alarmins (TSLP, IL-33, IL-25) by airway epithelium. There is evidence of ongoing mast cell activation in severe asthma, irrespective of the clinical phenotype [2]. Different studies have shown infiltration of mast cells within the epithelium, submucosa layer, and airway smooth muscle and the ability of various mast cells mediators to induce key structural/clinical features of asthma such as mucus hypersecretion, epithelium permeability,

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_1

airway hyper-responsiveness (AHR), bronchoconstriction and airway remodeling [3]. This book chapter will *discuss the capacity of mast cells to modulate the response of asthmatic patients to corticosteroid therapy*. Evidence from our group showed that mast cells can blunt the response to bronchodilator agonists in airway smooth muscle via the paracrine action of secreted TGFβ [4] or following cell–cell physical interaction [5, 6]. Dysfunction of ß2-agonists in mast cells can also be induced following the autocrine action of secreted SCF [7]. These studies clearly suggest that mast cells can alter the therapeutic response of lung structural cells to current therapies.

Here, we will not describe the biology of mast cells nor its role in asthma pathogenesis (summarized in Fig. 1.1) as these have been extensively discussed in our last review [3]. Rather, we will briefly summarize the evidence from clinical studies that have linked mast cells to corticosteroid therapy and focus most of the discussion around the mechanisms that explain how mast cells contribute to the reduced corticosteroid responses in severe asthma and the latest inhibitory strategies targeting mast cells.

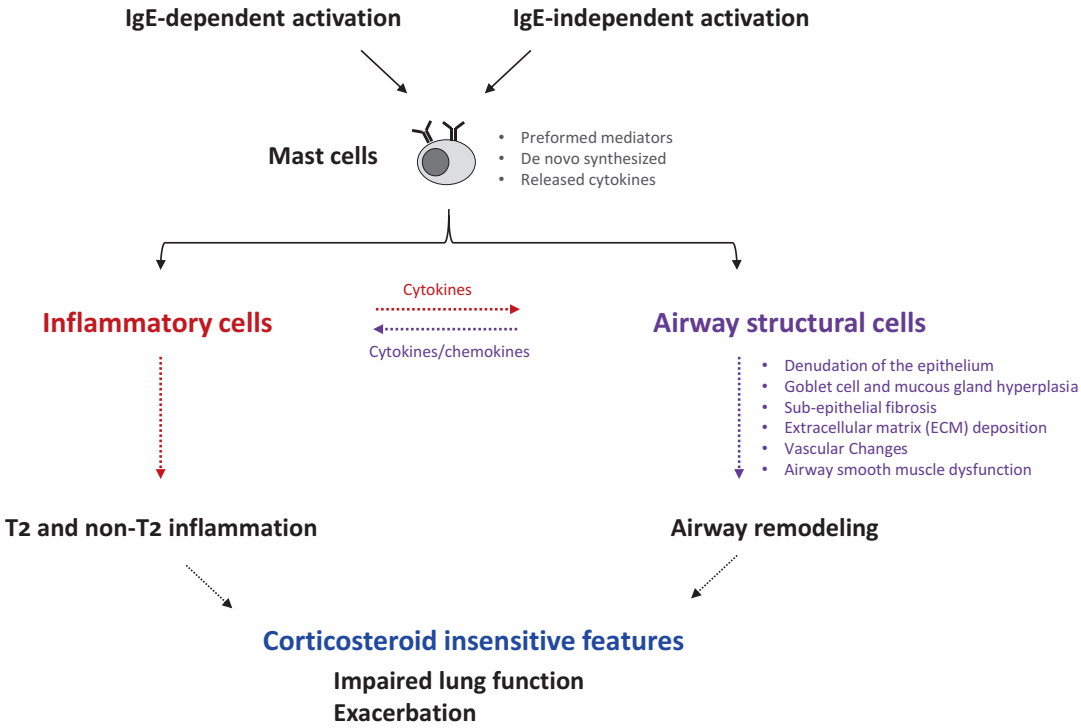

Fig. 1.1 Role of mast cell in asthma pathogenesis. Activation of mast cells via either IgE (allergen) or non-IgE mechanisms (such as TLR ligands, IgG, cytokines, complement components, neuropeptides, chemokines) can lead to the production of **preformed mediators** (chymase, tryptase, histamine), **de novo synthesized mediators** (leukotriene and prostaglandin lipid mediators), and **released cytokines** (Th2-Th1 cytokines, alarmins, growth factors). These mediators can regulate key features of asthma such as airway inflammation and airway remodeling by two main mechanisms: (i) their capacity to recruit and/or activate inflammatory cells (eosinophils, innate lymphoid cells, and lymphocytes) into the lungs and (ii) to alter the function of airway structural tissues associated with increased mast cell infiltration (epithelium, airway smooth muscle, goblet cells, vasculature). In addition, a bidirectional interaction between recruited inflammatory cells within the lung and airway structural tissues via the secretion of inflammatory and chemoattractant mediators or cell-cell interactions can also indirectly contribute to airway inflammation and airway remodeling. The overall activation of mast cells within the lung leads to impaired lung function and increase rate of exacerbation, features that are clearly insensitive to corticosteroids and improved with Omalizumab therapy

1.2 Increased Airway Infiltration of Mast Cells Is a Key Feature in Asthma Pathogenesis

The key contribution of mast cells in asthma pathogenesis can be explained by their high abundance within dysfunctional airway subcompartments of patients as reported by different studies. The authors that have stained mast cells in endobronchial biopsies using antibodies against tryptase have found that infiltration of mast cells in the airways often correlates with various clinical aspects of the disease (Table 1.1). Pesci and colleagues were among the first to demonstrate a greater infiltration of activated mast cells within human bronchial mucosa compared to healthy subjects [8]. Carroll and colleagues later extended these findings in patients with fatal asthma by reporting increased degranulated mast cells in various locations including submucosal mucous glands [9, 10] as well as in the airway smooth muscle [9, 10]. This unexpected infiltration of mast cells within the airway smooth muscle tissue was reported to strongly correlate with disease severity (as mostly seen in fatal cases) [9, 10], abnormal lung function (when assessing PC_{20} for methacholine) [11], or levels of TGF-β1 expression in the airway smooth muscle itself [12]. Other studies confirmed the greater number of mast cells in different structural compartments including epithelium [13], as well as in airway smooth muscle in both allergic and nonallergic asthmatics, with their number and activation state (assessed by extracellular deposition of mast cell products) to be significantly higher in smooth muscle bundle in patients with allergic asthma [14]. Brightling and colleagues found that the number of mast cells (positively stained for both CXCR3 and tryptase) was greater in the airway smooth muscle compared to that in bronchial submucosa in patients with asthma [15]. The concept of infiltration of tryptase-positive mast cells within the airway smooth muscle tissue has since been validated in different cohorts of asthma patients [12, 16–18].

The mechanisms leading to mast cells infiltration within the airways have not been completely

Table 1.1 Studies describing features of mast cell infiltration within the airways in asthma patients

References	Mast cell infiltration in the lung	Features of mast cells in asthma
[8] n = 13 stable asthma n = 8 healthy controls	Airway epithelium and Lamina propria	Degranulation greater in asthmatics
[9, 10] n = 8 fatal asthma n = 8 non-fatal asthma n = 8 healthy controls	ASM bundle and mucous glands	Degranulation related to disease severity
[11] n = 17 asthma n = 13 eosinophilic bronchitis (EB) n = 11 healthy controls	ASM bundle	Greater number in asthma vs EB and controls Correlation with impaired lung function (PC_{20}) in asthma
[12] n = 9 controls n = 10 intermittent asthma n = 9 persistent asthma	ASM bundle	Greater number in asthma Number related to TGFβ expression in ASM
[14] n = 29 allergic and non-allergic asthma	Epithelium, lamina propria and ASM bundle	Greater number in ASM of allergic asthma vs non-allergic asthma
[15] n = 16 asthma n = 14 controls	ASM bundle and submucosa	Greater number in asthma Greater number of CXCR3+ mast cells in ASM versus submucosa
[16] n = 5 controls n = 9 persistent asthma	ASM bundle	Greater number in asthma Correlation with vasoactive intestinal peptide (VIP) staining in ASM
[17] n = 10 controls n = 16 asthma	ASM bundle and submucosa	Greater number in asthma Greater degranulation in muscle vs submocusa No correlation with asthma severity

(continued)

Table 1.1 (continued)

References	Mast cell infiltration in the lung	Features of mast cells in asthma
[18] n = 18 controls n = 12 asthma	ASM bundle	Correlation with α-smooth muscle actin staining in ASM
[19] n = 34 controls n = 53 mild asthma n = 21 moderate asthma n = 57 severe asthma	Airway epithelium and submucosa	Greater number of double positive (chymase and tryptase) in the epithelium in severe asthma

elucidated. In vitro studies have shown that various factors produced by the epithelium or airway smooth muscle are capable of exerting chemoattractive effects toward mast cells including fractalkine/vasoactive intestinal peptide axis [16], CXCL10 via CXCR3 receptor [12], and TGFß/SCF [15]. The observation that all these mediators were found to be produced in vivo by ASM bundles strongly support the essential role of structural lung cells in the recruitment of mast cells within the lungs in patients with asthma.

As patients with severe asthma (5–10%) poorly respond to the current asthma management guideline therapies including corticosteroids [20], it is important to understand the mechanisms driving this poor response to corticosteroids. A number of different clinical trials conducted in severe asthmatics have shown that the monoclonal antibody omalizumab, that targets specifically circulating IgE, leads to improved asthma symptoms, pulmonary function (%FEV1 predicted), morning peak expiratory flow, rates of exacerbations, and a reduction in markers of inflammation and airway remodeling [21]. These observations reinforce the concept that IgE-dependent release of mast cell mediators contributes to the pathogenesis of severe asthma. Therefore, a legitimate question that remains to be answered is whether mast cells can play a role in asthma severity by interfering with corticosteroid therapy via secreted (stored and newly synthesized) mediators. In the following sections, we will summarize the growing literature that links mast cells to the impaired corticosteroid sensitivity seen in asthma.

1.3 Clinical Evidence Suggesting a Role of Mast Cells in Corticosteroid Insensitivity

Many clinical studies have confirmed that Omalizumab was effective in improving asthma control [22–24]. Omalizumab led to improved asthma symptoms, reduced rates of exacerbations, and improved features of airway remodeling as evidenced by the reduction in airway wall thickness seen in CT scans [22, 25, 26]. In addition, omalizumab treatment improved pulmonary function such as forced expiratory volume in 1 s (FEV1) and morning peak expiratory flow [22, 25, 27]. The question was whether omalizumab was associated with changes in patients' response to either inhaled (ICS) or oral (OCS) corticosteroids. Previous reports have revealed a strong association between IgE levels and a high usage of ICS [28]. Most studies focusing in severe asthma have demonstrated that the marked reduction in peripheral blood IgE levels induced by omalizumab therapy was also associated with a reduction in the use of both ICS and OCS [22, 27, 28]. An elegant review by MacDonald colleagues has recently summarized the overall clinical impact of omalizumab observed from 42 different studies [29]. The authors concluded that omalizumab therapy for >2 months or longer led patients to either reduce or stop their ICS/OCS usage suggesting a role of mast cells in mediating corticosteroid insensitivity in severe allergic asthma. The underlying mechanisms by which mast cells could drive corticosteroid insensitivity have not been elucidated. A number of mediators produced by activated mast cells have been reported to interfere with corticosteroid responses in various cell types associated with asthma.

1.4 Mediators Produced by Mast Cells and Associated Mechanisms Shown to Blunt Corticosteroid Sensitivity

Numerous clinical and preclinical reports have supported the critical implication of mast cells in the pathogenesis of severe asthma [3]. Not only the number of mast cells increased in the airways of severe asthmatics but their number correlated with markers of disease severity [30]. As stated before, severe allergic asthma patients treated with omalizumab, the anti-IgE monoclonal antibody, show clear improvement of various clinical outcomes [21]. A number of studies have demonstrated the clinical values of targeting various mast cell mediators in severe asthmatics including pro-inflammatory cytokines such as TLSP, IL-4, IL-5, and IL-13 known to regulate eosinophilic inflammation [31], or IL-17 reported to drive neutrophilic inflammation [32]. Interestingly, several in vitro studies have reported some of these mast cell cytokines have the capacity to induce corticosteroid insensitivity in various cells associated with asthma (summarized in Table 1.2).

Table 1.2 Mediators produced by activated mast cells that are capable of altering corticosteroid response in various cell types involved in asthma pathogenesis

Mast cell mediators	Target cells	Mechanisms of steroid insensitivity
TNFα/ IFNγ	Airway smooth muscle cells	Dominant negative effect of GRβ Competition for the transcriptional co-activator GRIP-1 PP5-dependent GRα dephosphorylation
IL-2/ IL-4	PBMCs T lymphocytes (CD4+ and CD8+ T cells) PBMCs PBMCs Eosinophils	Reduced nuclear GRα ligand binding activity Reduced GRα nuclear translocation and dependent gene expression (MKP-1) Downregulation of GRα levels p38MAPK-γ dependent GRα phosphorylation PP5-dependent GRα dephosphorylation
IL-2	Th2 lymphocytes Murine cell line (HT-2)	Downregulation of GRα levels (mRNA) Reduced GRα binding to FKBP5 promoter STAT5-dependent pathways
IL-13	Human bronchial epithelial cells PBMCs (monocyte fraction)	Not investigated Decreased GRα binding activity
IL-17A IL-17/ IL-23	Airway epithelial cells PBMCs	PI3K-dependent reduction in HDAC2 activity GRβ upregulation
TGFβ	Airway epithelial cells	ALK5-dependent inhibition of GRα dependent gene expression
IFNγ	Airway epithelial cells	Activation of JAK/ STAT1 pathways
TSLP	Natural helper cells Innate lymphoid cells (ILC2)	STAT5 pathways and expression of Bcl-xL MEK- and STAT5-dependent pathways

1.4.1 Interleukin 2 and 4 (IL-2/IL-4)

A number of original studies carried out in isolated peripheral blood mononuclear cells (PBMCs) or alveolar macrophages were the first to support the existence of corticosteroid insensitive features in immune cells in patients within steroid-resistant asthma (defined by their FEV1% changes following a course of corticosteroids) and with severe asthma (defined based on the GINA guidelines) [33–39]. A more recent report showed that neutrophils derived from steroid-resistant asthmatics exhibited a blunted ex vivo response to dexamethasone [40]. IL-2 and IL-4 were among the first cytokines known to be produced by mast cells (at least from mouse work for IL-2) to have been tested for their ability to modulate corticosteroid responses in asthma. Although IL-2 is typically produced by activated T-lymphocytes, evidence have suggested a critical role of IL-2-derived from mast cells in the suppression of allergic dermatitis [41], in part via the ability of IL-2 to regulate the expansion of regulatory cells [42]. Most studies focusing on

PBMCs and T-cells have reported IL-2 and IL-4 exposure for 48 h can blunt the anti-inflammatory actions of dexamethasone [43–45]. The precise mechanisms underlying cytokine-induced steroid insensitivity in these cells involved mostly changes in GRα function occurring at multiple levels: (i) a reduction in nuclear GRα translocation [44], (ii) decreased in GRα expression [45], or (iii) reduction in nuclear GRα binding affinity in T-cells [43]. Similar effects of IL-2 and IL-4 were as well seen in eosinophils treated for shorter time (16 h) which led to reduced GRα expected responses to dexamethasone such as receptor phosphorylation and the ability to stimulate the expression of anti-inflammatory proteins such as GILZ and MKP-1 [46]. Only one report showed that IL-2 on its own could reduce the pro-apoptotic effect of dexamethasone in human Th2 cells, an effect possibly due to the decreased levels of GRα and interaction with FKBP5 [47].

1.4.2 TNFα

Activated mast cells represent a crucial source of TNFα in asthma [48, 49]. A number of preclinical studies using blocking strategies have indeed confirmed the contribution of TNFα in driving some corticosteroid resistance features seen in severe asthmatics including infiltration of various inflammatory cells [50], or neutrophilic inflammation [51]. The mechanisms by which TNFα promotes corticosteroid resistance have not been completely elucidated but in vitro studies performed on structural cells isolated from the lungs have led to some interesting observations. Studies conducted in human ASM cells, for example, have demonstrated that the production of fluticasone-resistant chemokines/cytokines (i.e., CXCL10, CCL5, and CXCL8) can be induced by TNFα when associated with IFNγ [52]. The underlying mechanisms likely result from the modulation of GRα transactivation function caused by three different inhibitory pathways: (i) the antagonistic action of GRβ, dominant negative isoform of GRα, (ii) the competition for GRα essential transcriptional co-activator GRIP-1 and, (iii) protein phosphatase PP5-dependent dephosphorylation of GRα (reviewed in [52]). The "GRβ" hypothesis has been investigated in asthma, although its role remains still controversial [53–55]. The ability of IFNγ to render lung structural cells refractory to fluticasone when combined to TNFα may likely related to the synergistic activation of IFNγ-associated steroid insensitive pathways. We showed that activation of the transcription factor IRF-1 became resistant to fluticasone when induced by TNFα in the presence of IFNγ [56]. Similarly, we also reported that in lung epithelial cells, the ability of fluticasone to inhibit steroid-insensitive genes induced by IFNγ could be restored when JAK pathways were blocked using siRNA strategy aimed at the downstream signaling molecule STAT-1 [57]. Targeting the JAK/STAT axis may therefore represent a novel therapeutic option for reversing corticosteroid insensitivity in asthma.

1.4.3 TGFβ

Growth factors produced by mast cells have been also associated with steroid insensitivity in asthma. Elegant studies from Stewart's group in Melbourne provided the first evidence that TGFβ is able to reduce dexamethasone-induced GRE-dependent gene expression not only in A549 lung adenocarcinoma-derived epithelial cell line [58] but also in differentiated primary air–liquid interface human bronchial epithelial cells, via a mechanism involving the TGFβ type I receptor kinase (ALK5) [59]. A more recent study identified cofilin1, an intracellular actin-modulating protein, as the main downstream pathway driving TGFβ-induced corticosteroid insensitivity in lung epithelial cells [60]. The same group demonstrated that infection of human airway epithelial cells with different respiratory viruses including respiratory syncytial virus, rhinovirus, and influenza A virus led to corticosteroid insensitivity in part via autocrine action of TGFβ and associated ALK5 pathways [61]. We recently reported a role of mast cell-derived TGFβ in the inhibition of β2-receptor function in airway smooth muscle cells [4]. Whether TGFβ regulates corticosteroid responses in other lung structural cells via similar ALK5 mechanisms remains to be further investigated.

1.4.4 Interleukin 17 (IL-17)

IL-17 is also another mast cell-derived cytokine involved in asthma that has been associated with steroid insensitivity in severe asthma. McKinley and colleagues were the first to suggest a role of Th-17 cells in driving steroid resistance in asthma in a mouse of allergic asthma [62]. The authors found that both airway inflammation and airway hyper-responsiveness were resistant to dexamethasone in allergen-challenged mice following adoptive transfer of Th17 cells. Another study using of neutralizing antibody clearly indicated that some of corticosteroid insensitive features following ozone exposure in mice, such as neutrophilic inflammation and BALF cytokine levels, were mediated by IL-17 [63]. In vitro work in 16HBE14o human bronchial epithelial cells (16HBE) confirmed the capacity of IL-17 to markedly reduce the inhibitory action of budesonide on TNFα-induced IL-8 production. Mechanisms driving IL-17-induced steroid resistance involved a reduction of HDAC2 expression via phosphoinositide-3-kinase (PI3K) pathways [64]. In PBMCs, IL-17/IL-23 combination reduced dexamethasone-induced suppression of cell proliferation via the inhibition of GRα transactivation and transrepression properties [65].

1.4.5 Interleukin 13 (IL-13)

IL-13 has been considered as one of the essential cytokines involved in asthma pathophysiology which can originate from Th2 lymphocytes, innate lymphoid cells, and mast cells. Although elevated IL-13 levels have been correlated with typical asthma features including airway hyperresponsiveness, mucus hypersecretion, and airway remodeling, there is also evidence for a role in steroid resistance [66]. Administering IL-13 directly in mouse airways using an adenoviral vector resulted in airway inflammatory changes that are unresponsive to dexamethasone including neutrophils and macrophages lung accumulation [67]. In primary human bronchial epithelial cells, IL-13 stimulated the production of the profibrotic factor TGFβ2 that was unaffected by dexamethasone [68]. In PBMCs treated with IL-13, GRα binding activity was found to be impaired in the monocyte population and associated with a reduced inhibitory effect of hydrocortisone on LPS-induced IL-6 production [69]. Interestingly, none of the other cytokines tested (IL-1, IL-3, IL-5, IL-7, IL-8, IL-12, or granulocyte-macrophage-CSF) had any effect of steroid sensitivity in these cells. These studies reinforce the concept that IL-13 is an important driver of steroid-insensitive pro-remodeling and pro-inflammatory responses in the airways.

1.4.6 Alarmins (TLSP)

An elegant report combining a mixture of in vitro and in vivo studies was the first to suggest the implication of TSLP in driving corticosteroid refractory responses in one family member of type 2 innate lymphoid cells (ILC2) called natural helper (NH) cells [70]. The TSLP-induced steroid resistance was mediated via the activation of STAT5 signaling pathways, through mechanisms that remain to be further explored. A more recent study performed in blood and lung ILC2s revealed that the ability of dexamethasone to reduce the production of type 2 cytokines was greatly impaired by TSLP or IL-7 [71]. This study suggests the involvement of common signaling pathways downstream to the IL-7 receptor α in the regulation of steroid insensitivity. Interestingly, as reported in NH cells, corticosteroid resistance in ILC2s was mediated via both MEK- and STAT5-dependent pathways. Activated mast cells are a source of TSLP in asthmatic airways, and might therefore promote steroid resistance through this mechanism [72, 73].

1.5 Potential Mast Cell Inhibitors for the Treatment of Allergic Diseases

Recent reports have uncovered a number of different strategies that are capable to inhibiting mast cells and their contribution to lung

diseases. One elegant report provided the first evidence that Imatinib, a KIT inhibitor, decreased airway hyper-responsiveness (methacholine PC_{20}), mast-cell counts, and a marker of mast cell activation (serum levels of tryptase) in severe asthmatics [74]. Inhibiting another tyrosine kinase (Spleen tyrosine kinase Syk) by SYKi has been reported to inhibit IgE-mediated contraction and production of mast cell mediators in precision cut lung slice (PCLS) model [75]. Similarly, RN983, an inhibitor of bruton's tyrosine kinase (Btk) required for mast cell activation has proven to be effective in reducing the early asthmatic response in mouse model of allergic asthma when given by inhalation [76]. More recently, a study using FDA-approved BTK inhibitors (BTKi's) demonstrated promising therapeutic actions both in vitro (allergen-induced contraction) and in vivo (IgE-mediated anaphylaxis), supporting the key role played by Btk in FcεRI-mediated mast cell degranulation [77]. The use of the pharmacological inhibitor by AGK2 allowed to demonstrate the central contribution of NAD+ (nicotinamide adenine dinucleotide)-dependent deacetylase SIRT2 pathways in mediating mast cell degranulation and allergic airway inflammation in a murine model [78]. The clinical benefit of noncompetitive inhibitory antibody against human β-tryptase in both mouse and primate models of allergic response has been described as a promising treatment of severe asthma [79]. The mitochondrial STAT3 appears to be another target as inhibitors called Mitocur-1 and Mitocur-3 significantly suppressed degranulation of cultured rodent and human mast cells and reduce key allergic features in a OVA murine model such as blood histamine and eosinophilia [80]. Activating specific pathways could also serve as a potential strategy to suppress mast cell function. Levels of Raf kinase inhibitor protein (RKIP), which has been described as a negative regulator of IgE-mediated allergic response [81], are decreased in peripheral blood of asthma patients. This suggests a possible defect of RKIP as a new mechanism underlying allergic responses in asthma.

1.6 Conclusions

Clinical trials as well as real-life studies have demonstrated that anti-IgE therapy (omalizumab) is associated with a corticosteroid-sparing effect in moderate to severe asthma. This reduction in corticosteroid usage/dependence was associated with marked improvements in different clinical outcomes including the rate of exacerbations and asthma symptoms. Unfortunately, not all severe asthmatics respond to omalizumab. It is likely that mediators released by activated mast cells via both IgE-dependent and IgE-independent pathways may play a key role in driving patients' reduced sensitivity to corticosteroid therapy. Indeed, a number of in vitro studies conducted in immune cells and lung structural cells have shown that different mediators (Th1 and Th2 cytokines, growth factors, alarmins) produced by mast cells can blunt the response to corticosteroids via multiple mechanisms. This include effects on the function of GRα ranging from impaired receptor phosphorylation, receptor DNA-binding activity, and receptor competition for transcriptional co-activator. These studies further support the capacity of mast cells to contribute to the overall mechanisms blunting corticosteroid therapy in severe asthma. Identifying how mast cells regulate corticosteroid insensitive features could led to novel therapeutic interventions for the treatment of refractory severe asthma. Potential therapeutic interventions targeting mast cells besides current anti-IgE omalizumab include soluble inhibitors of pathways to prevent mast cell degranulation (see Sect. 1.4.4 above), monoclonal antibodies against key mast cell mediators and pharmacological inhibition of signaling pathways interfering with corticosteroid receptor function (summarized in Fig. 1.2).

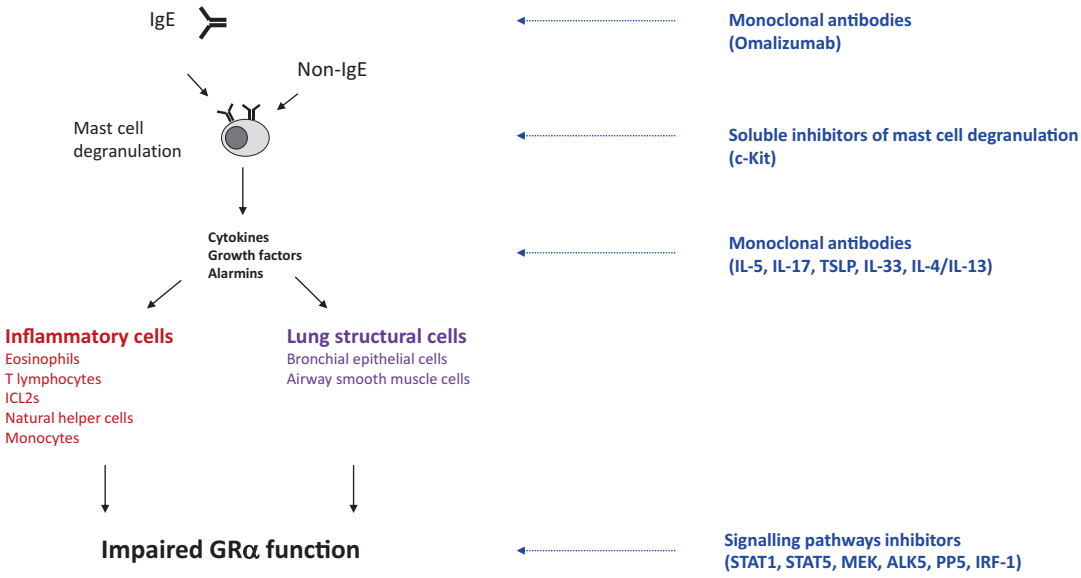

Fig. 1.2 The proposed therapeutic strategies to restore patients' response to corticosteroids in severe asthmatics. One current therapy targeting mast cells include omalizumab which has clearly demonstrated clinical benefits in patients with severe allergic asthma including the need for high doses of corticosteroids. Considering that not all patients respond to omalizumab and the fact that mast cells can be activated via IgE-independent mechanisms, other potential strategies need to be developed. These therapies are based on their ability to target mast cell activation (Kit inhibitors such as Dasatinib or Imatinib) or prevent the action of its produced mediators known to impair corticosteroid therapy in various cell types using monoclonal antibodies. Targeting also specific signaling pathways using soluble pharmacological inhibitors may also contribute in preventing the action of these mast cell mediators

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2 Galectin-3 Promotes ROS, Inflammation, and Vascular Fibrosis in Pulmonary Arterial Hypertension

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Abstract

Pulmonary Arterial Hypertension (PAH) is a progressive vascular disease arising from the narrowing of pulmonary arteries (PA) resulting in high pulmonary arterial blood pressure and ultimately right ventricular (RV) failure. A defining characteristic of PAH is the excessive remodeling of PA that includes increased proliferation, inflammation, and fibrosis. There is no cure for PAH nor interventions that effectively impede or reverse PA remodeling, and research over the past several decades has sought to identify novel molecular mechanisms of therapeutic benefit. Galectin-3 (Gal-3; Mac-2) is a carbohydrate-binding lectin that is remarkable for its chimeric structure, comprised of an N-terminal oligomerization domain and a C-terminal carbohydrate-recognition domain. Gal-3 is a regulator of changes in cell behavior that contribute to aberrant PA remodeling including cell proliferation, inflammation, and fibrosis, but its role in PAH is poorly understood. Herein, we summarize the recent literature on the role of Gal-3 in the development of PAH and provide experimental evidence supporting the ability of Gal-3 to influence reactive oxygen species (ROS) production, NOX enzyme expression, inflammation, and fibrosis, which contributes to PA remodeling. Finally, we address the clinical significance of Gal-3 as a target in the development of therapeutic agents as a treatment for PAH.

Keywords

Pulmonary · Galectin-3 · ROS · Vascular remodeling · Inflammation · Fibrosis

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2.1 Pulmonary Arterial Hypertension (PAH)

Pulmonary Arterial Hypertension (PAH) is a progressive disease of the lung vasculature that is characterized by a sustained elevation of pulmonary arterial pressure [1]. PAH is currently defined as a mean pulmonary artery pressure at rest ≥ 20 mmHg [2], which can result in increased

Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_2

pulmonary vascular resistance subsequently leading to compensatory right ventricular hypertrophy [1, 3]. Medial hypertrophy of pulmonary artery (PA) smooth muscle cells is a hallmark feature of PAH [4], which elicits vessel luminal occlusion [5]. In most forms of PAH, muscularization of small distal PA occurs [6], and is further characterized by excessive arterial cell proliferation, fibrosis, and inflammation, leading to medial remodeling, rarefaction, and a loss of compliance of the pulmonary blood vessels [5, 7–9]. Increased resistance to blood flow via loss of PA compliance contributes to the failure of the right ventricle (RV) [10, 11]. In addition, the response of the RV to the increased afterload associated with PAH increases end-diastolic volume, hypertrophy, alters contractility, induces dilation, cardiac fibrosis, and eventual decompensation [12]. Ultimately, increased RV volume (diastolic and systolic) combined with increased intraluminal cardiac pressure leads to an unsustainable increase in wall stress that culminates in right heart failure and ultimately death [13–15].

In PAH, within the vessel wall, endothelial cells become dysfunctional and fail to maintain homeostasis, and vascular smooth muscle cells undergo a phenotypic switch from a quiescent contractile phenotype to a "synthetic" phenotype that is characterized by a decrease in contractile smooth muscle genes, increased secretion of matrix and proteases, and increased proliferation [16, 17]. The subsequent increase in pro-inflammatory and pro-fibrotic molecules increases fibrosis, inflammation, and the deposition of extracellular matrix [18–22]. The signaling moieties that modify cellular properties in PAH remain ill-defined, but endothelin, PDGF, TGF-β, BMPs, hypoxia, altered metabolism, reactive oxygen species (ROS), and nitrogen species (RNS) have all been identified as contributing factors [19, 23–25].

2.2 Evidence for ROS Signaling in PAH

Previous evidence shows increased levels of ROS in both human and experimental models of PAH [26–32]. The major ROS that are produced in the pulmonary vasculature are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and hydroperoxyl radical ($HO_2^{\cdot}$) [33]. Numerous mechanisms have been proposed to account for increases in ROS including altered eNOS activity and increased NOX enzyme expression and activation. Steady-state levels of ROS reflect the balance between ROS generation and eradication/scavenging, and evidence supports alterations in both pathways in PAH [25]. We have previously shown that ROS contributes to the development of PAH [34] and Fig. 2.1a shows that PA contraction to angiotensin II is enhanced from rats with monocrotaline (MCT)-induced PAH. In addition, treatment of pre-contracted vessels with the antioxidant Tempol, elicits a greater relaxation of induced tone in MCT-treated vessels compared to control conditions (Fig. 2.1b), suggesting that increased oxidant tone in PA from MCT-induced PAH augments vascular contraction. Of the ROS produced, O_2^- and H_2O_2 activate multiple signaling pathways promoting cell proliferation and apoptosis, elevated vascular tone, fibrosis, and inflammation,

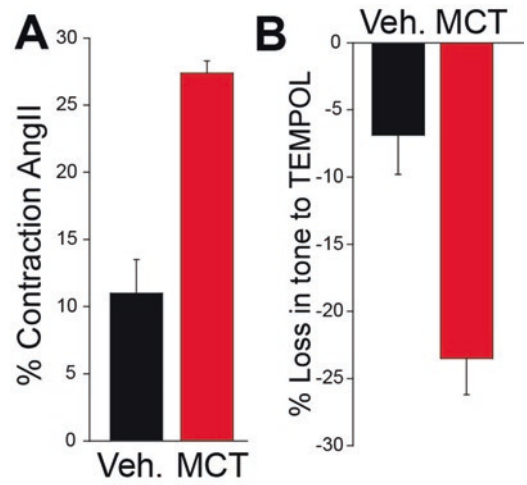

Fig. 2.1 Hypertensive PA produces greater contractile force dependent on ROS. (**a**) PA from control (vehicle) and MCT-rats were mounted on a myograph (1 g passive tension) and contracted with low dose Angiotensin (Ang) II (100 nM). (**b**) Drop in tension following addition of ROS scavenger TEMPOL (100 mM). n = 3–4 per group. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

which are all hallmark signs of PAH [33]. However, the cellular basis and functional significance of ROS in PAH remain poorly described. The human genome encodes five NOX isoforms and four of these isoforms, NOX1, NOX2, NOX4, and NOX5 are expressed in pulmonary vascular cells, and NOX4 is regarded as a constitutively active enzyme that produces levels of H_2O_2 that are primarily controlled by changes in gene expression [35, 36]. Increased expression of NOX4 has been reported in human PAH [37], and several lines of evidence support an important role for NOX4 in the pathogenesis of PAH in rat and human [34, 37] but this premise is less well-defined in mice [38–40]. NOX4 has been reported to be a major NADPH oxidase homolog expressed in human pulmonary arterial smooth muscle cells (PASMCs) [41], and its expression at the mRNA and protein level is significantly increased in lungs from patients with idiopathic pulmonary arterial hypertension (IPAH) compared to healthy lungs [37], which suggests a correlation between NOX4 and the onset of PAH. NOX4 mediates the hypoxia-induced growth of human PASMCs [42], and other studies report that NOX4 expression is highest in endothelial cells and perivascular fibroblasts [34, 43, 44]. Endothelial cell NOX4 is thought to be protective, and supports endothelial nitric oxide synthase function [45, 46], whereas fibroblast NOX4 is highly upregulated by TGFβ and is pro-fibrotic [34, 47]. Collectively, these findings support the argument for NOX4 expression being integrally involved in pulmonary vascular remodeling by promoting arterial medial smooth muscle proliferation, endothelial proliferation, and adventitial fibroblast-activation in PAH. NOX4 is also upregulated in cardiac hypertrophy and myocardial remodeling [48].

Epidemiologically, PAH is more frequent in women than men, and left untreated has a survival time of 5–7 years post-diagnosis [49]. From a therapeutic standpoint, there are a number of vasodilator drugs that are indicated for the treatment of PAH, but none of the current therapeutics offers long-term success for survival due to limited effectiveness and unwanted side effects [50]. More importantly, focus is being increasingly placed on the underlying causes of the vascular remodeling that is a hallmark of the disease [51].

2.3 Galectin-3

Galectin-3 (Gal-3; *LGALS3*, Mac-2) is a member of the lectin family of proteins, which recognize and bind to specific carbohydrate motifs on glycosylated proteins as well as lipids [52]. Gal-3 protein was first identified in the 3 T3 mouse fibroblast cell line [53] and is robustly expressed in the lung [54]. The gene encoding Gal-3 was cloned in 1987 and changes in mRNA expression in fibroblasts were observed in response to growth factors [55]. Gal-3 protein is present in both the cytoplasm and nucleus of cells, with higher expression in the nucleus of proliferating cells [56]. Gal-3 cellular expression appears to be age-dependent with robust expression induced by growth factors in young cells, which deteriorates in aged cells or those with replicative senescence [57]. Approximately 30 years ago, the macrophage surface antigen, Mac-2 was determined to be identical to Gal-3 and shown to be expressed in high concentrations by specific subpopulations of pro-inflammatory macrophages and secreted into the extracellular space [58, 59]. As the name implies, Mac-2 expression was extensively used to identify or mark macrophages [60]. It is now known that Gal-3 expression is also expressed in fibroblasts (where it was originally discovered), smooth muscle cells [61], endothelial cells [62], activated T-cells [63], epithelial cells [64, 65], and select types of tumor cells [66].

Gal-3 belongs to a family of 16 related members that all share an evolutionarily conserved carbohydrate recognition domain (CRD) that can bind β-galactosides and lactose but they differ in their ability to bind more complex saccharides. Gal-3 "family" members can be broadly classified into three types: the prototypes which contain one CRD and are monomers or homodimers (includes galectins- 1, 2, 5, 7, 10, 11, 13, 14, 15, and 16), the chimeras (Gal-3 is the only member) which contain one CRD and a self-association domain, and the tandem-repeat galectins (galectin- 4, 6, 8, 9, and 12) which have two CRDs connected by a linker peptide. As the only chimeric galectin, Gal-3 is comprised of a C-terminal CRD that is present in all members of the galectin family and a unique N-terminal domain that contains glycine and proline-rich domains that enable

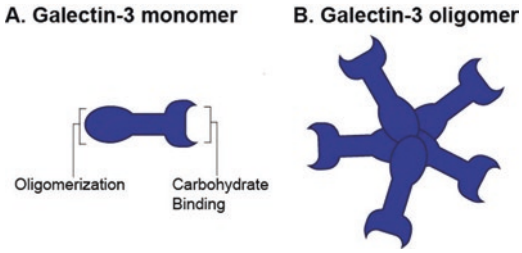

Fig. 2.2 Schematic illustration of Gal-3 monomer (a) and oligomer (b). Gal-3 is understood to be initially expressed as a monomer that assembles into a larger multimer in response to carbohydrate-binding and other post-translational modifications. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

Gal-3 to oligomerize with other Gal-3 molecules (Fig. 2.2a) or to engage in protein–protein interactions with other proteins. Gal-3 is initially expressed as a monomer but can self-assemble into dimers and higher-order structures in response to diverse stimuli. Cysteine 173 (previously referred to as cysteine 186) has been proposed as a critical residue that enables disulfide bonds between homodimers [67]. Carbohydrate binding to the C-terminal CRD of Gal-3 triggers a structural change in the N-terminus to enable oligomerization into pentamers (Fig. 2.2b) [68, 69], and specific monoclonal antibodies targeting the N-terminus of Gal-3 facilitate the multimerization of Gal-3 highlighting the important role of the N-terminus in regulating the formation of Gal-3 oligomers [70]. It has also been reported that the C-terminal CRD can initiate self-assembly within the CRD [69, 71], and this phenomenon can modify the N-terminal domain and also impact oligomerization and substrate binding [72]. Tissue transglutaminase can also directly promote Gal-3 oligomerization, which may increase and stabilize interactions with substrates [73, 74].

Gal-3 is found in the cell cytosol, nucleus, and the extracellular space, but how Gal-3 traffics to these different intracellular locations remains poorly understood although it may involve post-translational modifications, protein binding, or vesicular traffic. Cytosolic Gal-3 can regulate intracellular signaling and apoptosis/cell survival [75], and in the nucleus Gal-3 affects RNA processing, and in the extracellular space, Gal-3 binds to numerous ligands including receptors and integrins to influence signaling, including cell to cell and cell to matrix interactions. Gal-3 does not contain a signal peptide and its secretion to the extracellular space, while polarized, appears to be unaffected by chemical inhibition of the "classical secretory" pathway [76, 77]. Instead, Gal-3 secretion is inhibited by methylamine and increased by heat shock and calcium mobilizing agents, which suggests that exocytosis is a major export pathway [78]. In spite of this knowledge, several important questions remain as to whether this pathway accounts for the export of both free and encapsulated Gal-3, as secreted Gal-3 is reported to be predominantly free from being packaged into extracellular vesicles [79]. Based on a CRISPR-Cas9 genomic screen, another proposed mechanism for secretion is that Gal-3 may bind to N-linked glycosylated proteins with signal peptides that are en route to the plasma membrane, and that N-linked glycosylation is not required for secretion but essential for extracellular membrane binding [79]. An alternative mechanism for secretion is the reported ability of Gal-3 to penetrate lipid bilayers allowing the moiety to enter/exit cells, as well as traffic to the nucleus and other intracellular organelles [80].

Galectin-3 is also subject to several post-translation modifications. For example, it is cleaved by matrix metalloproteinases 2 and 9 between Ala62 and Tyr63 to yield an intact CRD and N-terminal peptides, which results in increased carbohydrate binding and reduced oligomerization [81]. Gal-3 is also a substrate for other proteases including MMP-7, MMP-13, MT1-MMP, PSA, and proteases encoded by parasites [82], and is primarily phosphorylated on Ser6, Ser12 [64], and Tyr107 [83, 84] although these phosphorylations may be signal-dependent. Phosphorylation can impact the subcellular localization of Gal-3 by promoting translocation from the nucleus to the cytoplasm [85], thus influencing its ability to regulate apoptosis in the cytoplasm [86]. Ser6 phosphorylation can impact the ability of Gal-3 to recognize carbohydrate motifs, and the phosphorylation of Tyr107 may impair protease-dependent cleavage [82].

2.4 Galectin-3 Ligands

Ligand-binding specificity is encoded by the CRD of Gal-3 and while there are overlapping substrates, it has been shown to bind to distinct subsets of glycoproteins [87]. Gal-3 binds to numerous substrates including (but not limited to) signaling molecules (Ras, TGFβ), transcriptional regulators (β-catenin), ribonucleoproteins (RNA splicing), cell surface receptors (integrins (β1), TGFβ, DMBT1, VEGF, EGFR), lysosomal proteins, and matrix proteins (fibronectin, collagen, laminin) [88–94]. In addition to glycosylated proteins, Gal-3 can also bind to glycosphingolipids present on mammalian cells, which may enable interaction with ABO blood group antigens and the HNK-1 antigen in neurons and leukocytes [95].

Gal-3 influences a variety of processes including RNA splicing proliferation, altered signaling, migration, apoptosis, fibrosis, and inflammation [75, 96–100]. As a result, a pathogenic role for Gal-3 has been proposed in numerous diseases such as cancer [101, 102], inflammatory [103, 104], and fibroproliferative disorders such as pulmonary, cardiac, and hepatic fibrosis [98, 105–109]. How Gal-3 alters signaling depends on its intracellular location and the ability of its CRD to recognize specific glycosylation motifs on substrates. Gal-3 binds to the epidermal growth factor receptor (EGFR) in mesenchymal cells, which results in a mitogenic response and increased collagen [110], and also binds with high avidity to advanced glycosylation end-product binding proteins in a variety of cell types including macrophages and endothelial cells [111, 112]. A notable marker for cell adhesion, CD98 has also been reported to be a receptor for Gal-3 [113], and there is support for a mechanistic link between CD98, interleukin-4 (IL-4), and Gal-3 to elicit macrophage activation and drive both inflammatory and fibrotic diseases [114]. Further studies show that Gal-3 can bind to CD45, which induces cellular apoptosis [115].

With regard to ligands, ECM glycoproteins (laminins and integrins) have been identified as matrices that interact with Gal-3 [116, 117]. Specifically, Gal-3 expression increases β_1 integrin-mediated cell adhesion to both laminin and fibronectin [117], and LGALS3BP (Galectin-3 binding protein) mediates induction of VEGF to promote angiogenesis [118], as well as cell and antiviral response/innate immunity [119, 120]. LGALS3BP forms ring-shaped oligomers (mostly decamers) that interact with Gal-3 and also fibronectin, collagens, and integrins [121]. In the nucleus, Gal-3 has been detected as part of the spliceosome complex where it is important for pre-mRNA splicing, which is mediated via a specific interaction with the U1 small nuclear RiboNucleoProtein snRNP which facilitates pre-mRNA splicing [122]. Lysosome membrane permeabilization (LMP) occurs in response to excessive lipids, osmotic changes, and increased ROS [123]. Following lysosomal damage, β-galactoside containing proteins normally protected within the lumen of the lysosomes are exposed to the cytosol where they are recognized by galectins including Gal-3 [124]. Elevated ROS which can induce LMP have been shown to increase the lysosomal localization of Gal-3 [125].

2.5 Galectin-3 in Inflammation

Vascularized tissue and individual cells respond to injury, infection, and irritation by initiating an inflammatory response. While acute (early) inflammation usually resolves itself to enable the transition to the process of healing, chronic inflammation is the failure of acute inflammation to resolve itself, resulting in harmful or deleterious conditions usually through persistence of an inflammatory stimulus [126]. Gal-3 is an important regulator of the immune system, and is highly expressed in myeloid cells including monocytes, macrophages, dendritic cells, and neutrophils, and contributes to both acute and chronic inflammation. Gal-3 binds directly to CD11b on macrophages [114], and CD66 on neutrophils to regulate inflammatory cell extravasation [127], and regulates immune cell differentiation as well as the binding to numerous pathogens including LPS (the endotoxin from gram-negative bacteria) [128], *H. pylori* [129], pathogenic fungi, and *Trypanosoma cruzi* [130].

Gal-3 can also function as a pattern-recognition receptor (PRR) and a danger-associated molecular pattern (DAMP) [131] that can promote the assembly of inflammasomes to produce IL-1β and IL-18 thus activating the unfolded protein response, which amplifies inflammatory responses by potentiating NFκB among other pathways. Gal-3 is generally regarded as a pro-inflammatory molecule, and has been reported to activate T- and B-lymphocytes [132], mast cells [133], monocytes and macrophages [134], and neutrophils [135]. Gal-3 is expressed on the surface of human monocytes and differentiation to macrophages is accompanied by increased expression levels. In addition, Gal-3 is important in regulating macrophage polarization toward the M2 phenotype, and macrophages lacking Gal-3 show an impaired ability to express M2 gene sets in response to IL-4 [136]. Gal-3 is also important for phagocytosis, and macrophages lacking Gal-3 exhibit reduced ability to remodel actin fibers, suggesting that intracellular Gal-3 contributes to macrophage phagocytosis [137]. Gal-3 can function as a chemoattractant, and high levels promote the inward migration of monocytes and macrophages [134]. Gal-3 can also recognize the carbohydrate modifications on the luminal side of vacuolar membranes, which enables the delivery of antimicrobial GTPases to pathogen-containing vacuoles [138]. While these actions of Gal-3 are important in defending cells from pathogens and maintaining the health of an organism, not all are beneficial. For example, Gal-3 expression is increased by influenza and while helpful for an antiviral response, it has been shown to facilitate the binding of *Streptococcus pneumoniae* to the pulmonary epithelium resulting in increased susceptibility of influenza patients to pneumonia [139]. Similarly, Gal-3 has numerous roles in inflammatory diseases such as atherosclerosis, sepsis, arthritis, asthma, and systemic sclerosis, which are reviewed in more detail elsewhere [140]. Lastly, another mechanism by which Gal-3 regulates the function of the immune system is through the regulation of ROS production (discussed below).

2.6 The Role of Galectin-3 in Fibrosis

Fibrosis refers to the deposition of excessive amounts of connective tissue as part of a reparative process, often secondary to inflammation, that results in the scarring of hardening of a tissue or organ impairing the ability to function efficiently. Gal-3 has long been identified as a mediator of tissue and organ fibrosis [141]. By activating fibroblasts, Gal-3 induces secretion of collagen leading to fibrosis [98, 99]. In PAH, fibrosis occurs in both the lung vasculature and the right ventricle [142], and vascular fibrosis results from a diverse range of stimuli including oxidative stress, inflammatory cell signaling, release of inflammatory cytokines, compromised endothelial function, and the production of endothelium-derived vasoactive substances including the renin-angiotensin-aldosterone system [143]. Collagen I expression is increased by Gal-3 in rat vascular smooth muscle cells, and in hypertensive aldosterone treated rats, Gal-3 expression is increased along with the onset of vascular hypertrophy, inflammation, and fibrosis, which is reversed in the presence of pharmacological Gal-3 inhibition and absent in Gal-3 KO mice [100]. Wang and colleagues investigated the effect of Gal-3 on pulmonary vascular fibrosis in the MCT-treated rat model of PAH and observed increased vascular fibrosis and Gal-3 mediated TGF-β1-induced vascular fibrosis via the STAT3 and MMP9 signaling pathways [144]. In the setting of PAH, the right ventricle (RV), under conditions of prolonged increases in volume overload and excessive afterload, undergoes a plethora of compensatory and eventually decompensatory pathophysiological and morphological remodeling changes that eventually lead to right heart failure. Among these alterations in cardiac morphology is the development of fibrosis [145], and recent studies show increased circulating levels of Gal-3 in cardiac fibrosis, which may provide utility as a clinical biomarker providing diagnostic information for the potential onset of HF [146, 147]. Increased levels of Gal-3 are seen in fibrotic hearts and multiple lines of evidence suggest it contributes to myocardial fibrosis. In mice,

knockout or pharmacological inhibition of Gal-3 reduces cardiac fibrosis and improves function [148], while in rats, infusion of recombinant Gal-3 for 4 weeks promotes cardiac fibroblast proliferation, collagen production, and cyclin D1 expression leading to ventricular dysfunction [149]. Mechanistically, hyaluronic acid has been reported to be a major component of myocardial fibrosis, and Gal-3 upregulates CD44, which increases levels of hyaluronic acid [150, 151].

2.7 PAH Is Associated with Increased Levels of Galectin-3

Increasing evidence supports a role for Gal-3 in the development of PAH. In humans with PAH, circulating Gal-3 are elevated and correlate with RV ejection fraction, and end diastolic and systolic volumes [12], which is supported by other studies showing that Gal-3 levels correlate with the severity of PAH, is a biomarker of disease progression [143], and a strong predictor of mortality in PAH [152]. Circulating levels of Gal-3 correlate with RV dysfunction [153], which is in agreement with a recognized role for Gal-3 as a reliable indicator of left-sided cardiac failure [154, 155]. Gal-3 expression has also been shown to be upregulated in different established experimental rat models of PAH. Luo et al. [156] have reported that Gal-3 is upregulated in lung tissue from the hypoxia-induced rat model of PAH, and we have shown increased Gal-3 expression in the MCT-induced rat model and the Sugen 5416/Hypoxia rat model of PAH [157]. Elevated Gal-3 expression has also been reported in the hypoxia-induced mouse model of PAH [158].

2.8 Galectin-3 Induces the Functional Development of PAH

Hao et al. reported that chronic hypoxia increased both RV hypertrophy and RVSP in wild-type mice [158]; however, in Gal-3 KO mice, both of these indices were not elevated by hypoxia suggesting an attenuation of PAH. Similarly, in the hypoxia-induced rat model of PAH, both mPAP and RVSP, as well as the Fulton Index (RV/LV + S, an index of RV hypertrophy) were increased by hypoxia but inhibited by N-Lac, a nonselective galectin inhibitor [156]. In addition, Luo and colleagues found that Gal-3 inhibition by N-Lac attenuated medial hypertrophy as well as collagen deposition in the PA, suggesting that Gal-3 expression is involved in both PA proliferation and fibrosis, possibly via a TGF-β1 signaling pathway [156]. The MCT and Sugen/hypoxia rat models of PAH are irreversible leading to *cor pulmonale* and are more inflammatory and fibrotic [6, 159–161]. Barman and colleagues observed an increase in Gal-3 expression in isolated PA from the MCT rat model of PAH, Sugen/hypoxia rat model of PAH, and human PAH, which was found primarily within the medial smooth muscle layer [157]. Toward this end, inhibitors of Gal-3 which have been used in animal models of fibrosis and human NASH [105–107] were used to assess a functional role of Gal-3 in these experimental models. It was found that Gal-3 inhibition in prevention studies reduced PA vascular remodeling and ameliorated in vivo indices of PAH, and inhibition of Gal-3 in reversal studies also showed significant efficacy at slowing disease progression [157]. To provide a complementary genetic approach that is more selective, Gal-3 was knocked out in the Sprague-Dawley (SD) rat using CRISPR Cas9 technology, and noninvasive indices of PAH were assessed in vivo using high-resolution digital ultrasound in both wild-type (WT) and Gal-3 KO rats treated with or without MCT. Data showed that MCT-treated WT rats exhibited a time-dependent increase in PAH that was absent in Gal-3 KO rats [157]. In addition, while RSVP was significantly increased in WT rats exposed to Sugen/Hypoxia, there was no difference in RVSP between control WT rats and Sugen/Hypoxia exposed Gal-3 KO rats [157]. Collectively, these results advance the hypothesis that Gal-3 expression is increased in PAH from rodent models and human PAH, which contributes to the vascular remodeling of PA and the development of PAH.

2.9 Galectin-3 Promotes PAH Through Numerous Mechanisms

The ability of Gal-3 to regulate cell proliferation has been well-documented [90, 97, 162], and Gal-3 levels are higher in some proliferating cancer cells [102, 140, 163, 164]. In PA from rodents and humans with PAH, Gal-3 expression was detected within the hyperproliferative medial smooth muscle layer, which correlated with increases in numerous cellular markers of proliferation in isolated PA. In contrast, proliferation was significantly decreased in PA isolated from MCT-treated rats in which Gal-3 function was suppressed through pharmacological inhibition or absent in Gal-3 KO rats. In addition, isolated PASMC from KO rats have the reduced capacity to proliferate, and this deficit is rescued by recombinant Gal-3, and increased expression of Gal-3 via adenoviral mediate gene transfer, which stimulates human PASMC proliferation [157]. The ability of PDGF to stimulate PASMC proliferation has been shown to be dependent on increased expression of Gal-3 and is reduced by silencing Gal-3 [165]. Further, inhibition of Gal-3 in human (H)PASMCs reduces proliferation by decreasing cyclin D1 expression and increasing p27 expression, thereby promoting a contractile phenotype [158]. Gal-3 has also been shown to mediate the ability of TGFβ to increase the proliferation of pulmonary fibroblasts [156].

As discussed above, ROS contributes to the development of PAH. Gal-3 has been shown to promote ROS generation in a range of cells. For example, recombinant Gal-3 stimulates dose-dependent ROS production in neutrophils [135, 166], and monocytes [167], while in mast cells, extracellular Gal-3 induces apoptosis via release of superoxide [168]. Whereas the ability of Gal-3 to induce ROS production appears to be mediated by its extracellular actions, the mechanisms by which Gal-3 promotes ROS production are not completely understood. In the MCT-model of PAH, we found increased expression of NOX1, NOX2, and NOX4 mRNA in isolated PA (Fig. 2.3a–c), and pretreatment with a specific inhibitor of Gal-3 that ameliorates PAH [157] lead to significant reductions in NOX1, NOX2, and NOX4 expression (Fig. 2.3a–c). Increased intracellular and extracellular Gal-3 can contribute to superoxide production, and transduction of mouse peritoneal macrophages with a Gal-3 adenovirus resulted in increased phorbol myristate acetate (PMA)-stimulated superoxide production (Fig. 2.3d). Alternatively, extracellular recombinant Gal-3 increased superoxide production in mouse peritoneal macrophages, which was accompanied by increased expression of NOX2, the major oxidoreductase in immune cells (Fig. 2.3e). To assess whether Gal-3 contributes to vascular ROS production in PAH, we measured the expression levels of 8-hydroxy deoxyguanosine, a molecular footprint of DNA damage due to ROS, in lungs from control rats, rats treated with MCT and MCT in the presence of a Gal-3 inhibitor. We found that MCT increased the levels of ROS as estimated by 8-hydroxy deoxyguanosine, and that pre-treatment with the Gal-3 inhibitor reduced ROS levels to control values (Fig. 2.3f). Collectively these results suggest that in vivo Gal-3 contributes to the elevation of ROS via upregulation of multiple NOX isoforms that promote aberrant vascular remodeling. Others have shown a relationship between Gal-3 and oxidative stress in blood vessels [169]. For example, monocytes treated with PMA, which induces NADPH-oxidase-dependent ROS, increased both Gal-3 mRNA and protein expression, which was inhibited by the putative NADPH inhibitor, apocynin [170]. Evidence also shows a possible relationship between Gal-3 and NOX4 to promote RV remodeling. He et al. found that a positive correlation exists between serum NOX4 and Gal-3 levels in PAH patients [171]. Further, in the MCT-induced rat model of PAH, both Gal-3 and NOX4 expression were upregulated in the RV myocardium with specific staining of both moieties in the intracellular myocardial matrix [171]. In specific cell types, it has been proposed that Gal-3 stimulates cardiac fibroblasts to promote RV fibrosis via interacting with NOX4, and it has been observed that knockdown of Gal-3 can inhibit NOX4 protein expression and NOX4- derived production of ROS, which is greatly increased in cardiac fibrosis [171]. While

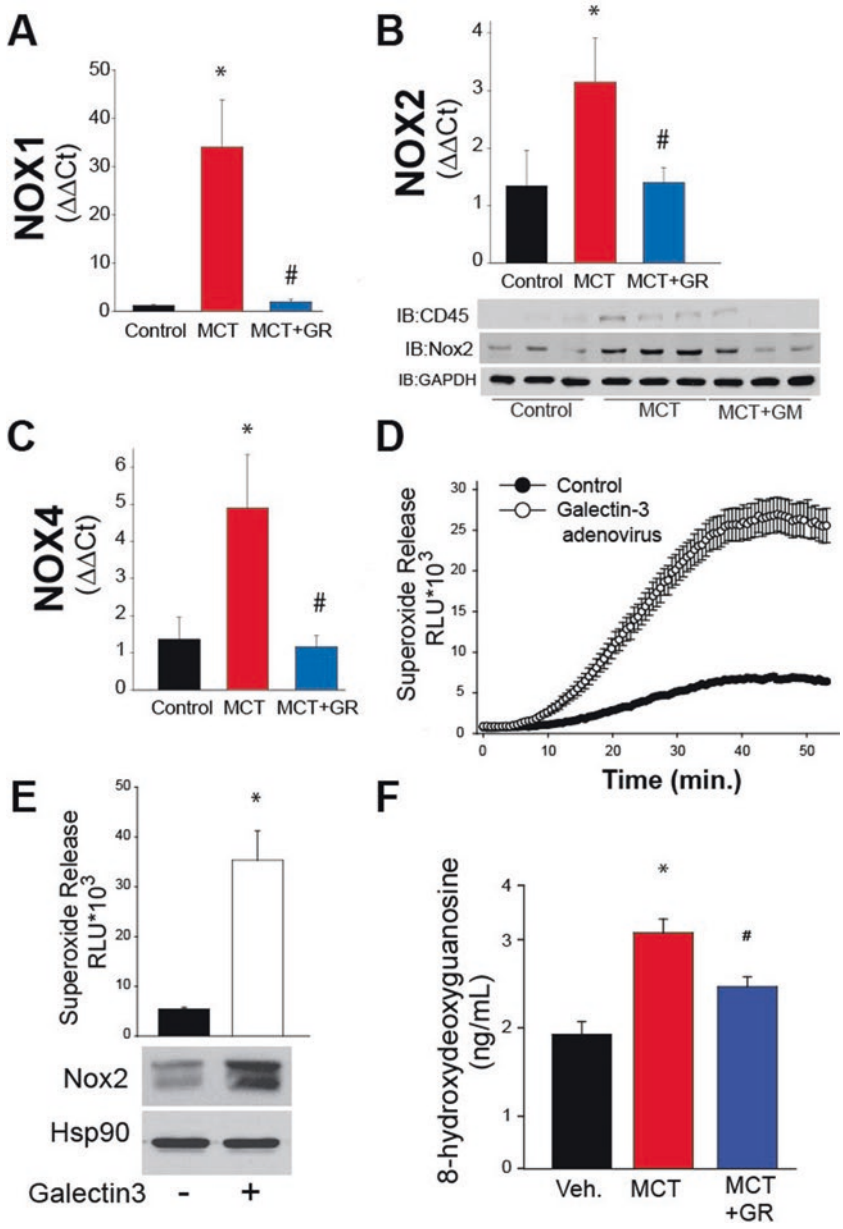

Fig. 2.3 Galectin-3 increases the expression of NOX enzymes and ROS production in pulmonary arteries from a rat model of PAH. The expression of NOX enzymes was determined in pulmonary arteries (PA) isolated from rats treated with MCT for 4 weeks. Relative expression of (**a**) NOX1 mRNA, (**b**) NOX2 mRNA and protein, and (**c**) NOX4 mRNA was determined in PA isolated from control, MCT, and MCT-treated with the Gal-3 inhibitor, GR by real-time PCR. In (**d**), mouse peritoneal macrophages were transduced with control (GFP) or Gal-3 adenovirus and the ability to generate reactive oxygen species was determined using enhanced L-012 chemiluminescence. In (**e**), mouse peritoneal macrophages were incubated with recombinant Gal-3 (10 μg/ml) and 24 h later basal superoxide production was determined using L-012 versus NOX2 expression. In (**f**), the levels of 8-Hydroxydeoxyguanosine, a molecular footprint of ROS production in vivo was measured by ELISA in lung tissue isolated from control, MCT, and MCT-treated rats plus the Gal-3 inhibitor GR. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

we found that inhibition of Gal-3 robustly decreased the expression level of NOX4 (Fig. 2.3c), we did not observe an ability of Gal-3 to upregulate NOX4 expression in fibroblasts (Fig. 2.5d) suggesting other cell types and/or mechanisms are involved.

Pulmonary hypertension is accompanied by increased vascular inflammation [7, 172, 173] and recruitment of inflammatory cells [174]. As discussed above, Gal-3 is intimately involved in the function of immune cells. To assess the role of Gal-3 in regulating vascular inflammation we measured the expression level of inflammatory markers in isolated PA from control, MCT, and MCT plus Gal-3 inhibitor-treated rats. We found that MCT-induced PAH was associated with increased expression of IL-6 (pro-inflammatory cytokine), CD45 (pan leukocyte marker), CD68 (monocytic cell marker), and CD4 (T-cell marker) in isolated PA, and inhibition of Gal-3 significantly attenuated MCT-induced vascular inflammation (Fig. 2.4a–d). In addition, silencing Gal-3 resulted in reduced expression of IL-6 (Fig. 2.4e). To determine the mechanism by which Gal-3 impacts vascular inflammation, we treated HPASMC with LPS with and without recombinant Gal-3. LPS induced the phosphorylation of p65, a transcription factor that orchestrates many aspects of inflammatory signaling. As shown, in cells pretreated with recombinant Gal-3, phosphorylation of p65 was increased in unstimulated cells, suggesting priming and subsequent response to LPS were enhanced (Fig. 2.4f).

As stated earlier, fibrosis contributes to the stiffening and compromised function of organs and blood vessels, and PAH is accompanied by increased pulmonary artery stiffness [175, 176], increased deposition of matrix [8], and increased numbers of vascular fibroblasts [177]. Gal-3 is a potent regulator of fibrosis and has been identified as a contributing factor to idiopathic pulmonary fibrosis [109], liver fibrosis [107], renal fibrosis [99], cardiac fibrosis [147], and vascular fibrosis [100]. To investigate a possible pathogenic role of Gal-3 in regulating vascular fibrosis in a model of PAH, we measured indices of fibrosis in PA from control, MCT-treated, and MCT with a Gal-3 inhibitor. We found that MCT-induced PAH resulted in increased expression of CD90 (a marker of fibroblasts) and Grem1 (a marker of fibrosis). Specifically, pre-treatment with the Gal-3 inhibitor significantly reduced markers of vascular fibrosis (Fig. 2.5a–b), and in isolated lung fibroblasts, recombinant Gal-3 and TGFβ increased collagen expression. However, there was no significant interaction between Gal-3 and the actions of TGFβ (Fig. 2.5c) as recombinant Gal-3 failed to increase the expression of fibroblast NOX4 and ACTA2 (a marker of myofibroblasts), which were robustly increased by TGFβ. These data suggest that Gal-3 contributes to the vascular fibrosis seen in hypertensive pulmonary arteries, but that its actions on fibroblasts are distinct from those of TGFβ.

Gal-3 is expressed to varying degrees in a number of cell types and as discussed above, Gal-3 can have a variety of effects, depending on the cell type involved. Our group has shown that the majority of Gal-3 protein expression is found in the smooth muscle rich PA media where it regulates proliferation [157], while other studies show that Gal-3 is expressed in perivascular fibroblasts [156] in experimental models of PAH. However, given the prominent roles of macrophage Gal-3 in atherosclerosis [61, 178, 179], and fibrosis [109] it would not be surprising if this particular cell type is also a source of Gal-3 in either the PA or the RV. The relative ability of Gal-3 to influence ROS, inflammation, and fibrosis (as shown in Figs. 2.3, 2.4, and 2.5) may depend heavily on the cell type expressing Gal-3. Functional delineation of the various autocrine versus paracrine actions of Gal-3 in different cell types awaits further investigation.

2.10 Summary and Clinical Perspectives

Numerous studies thus far support the premise that Gal-3 expression is increased in both rodent and human PAH. Although circulating levels of Gal-3 likely originate from increased expression in the right ventricle, increases in expression in

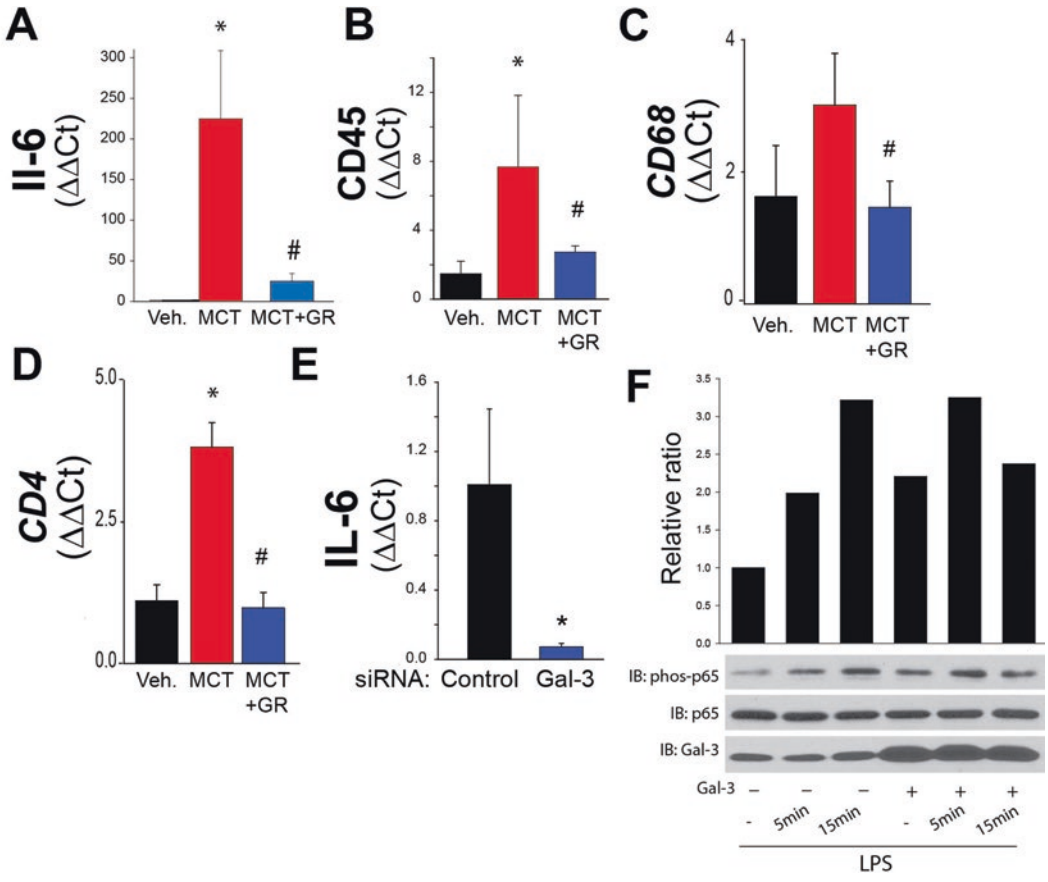

Fig. 2.4 Galectin-3 promotes inflammation in hypertensive pulmonary arteries. The expression of pro-inflammatory genes was determined in pulmonary arteries (PA) isolated from rats treated with MCT for 4 weeks. Relative expression of (**a**) Il-6 mRNA, (**b**) CD45 mRNA (**c**) CD68 mRNA, and (**d**) CD4 mRNA was determined in PA isolated from control, MCT, and MCT-treated with the Gal-3 inhibitor, GR by real-time PCR. In (**e**) silencing Gal-3 in human pulmonary artery smooth muscle cells (HPASMC) reduced IL-6 mRNA expression. In (**f**), Gal-3 regulates NF-κB activity. HPASMC were pretreated with recombinant Gal-3 (10 μg/ml) and then exposed to vehicle or LPS and time-dependent changes in the levels of phosphorylated p65, total p65, and Gal-3 determined by Western blot. n = 3–4 per group. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

isolated PA suggest local mediated-effects of Gal-3 to promote PA remodeling through changes in cell proliferation, increased ROS, inflammation, and fibrosis (Fig. 2.6). Gal-3 is expressed in many cell types and influences a variety of mechanisms to alter cell function, which contributes to the changes in cellularity as well as pulmonary vascular and RV function seen in PAH. Given that PAH is a complex disease originating from diverse mechanisms in multiple cell types, the experimental evidence insinuates that targeting Gal-3 may be a useful therapeutic approach. As recent studies suggest that Gal-3 serves as a circulating biomarker in humans that tracks PAH severity and progression, the ability of Gal-3 inhibitors to impact multiple pathways may be advantageous in the approach to treating complex diseases like PAH. In addition, these specific inhibitors may also have utility in combinatorial strategies that have significantly greater potential to delay the progression of pulmonary vascular disease [180].

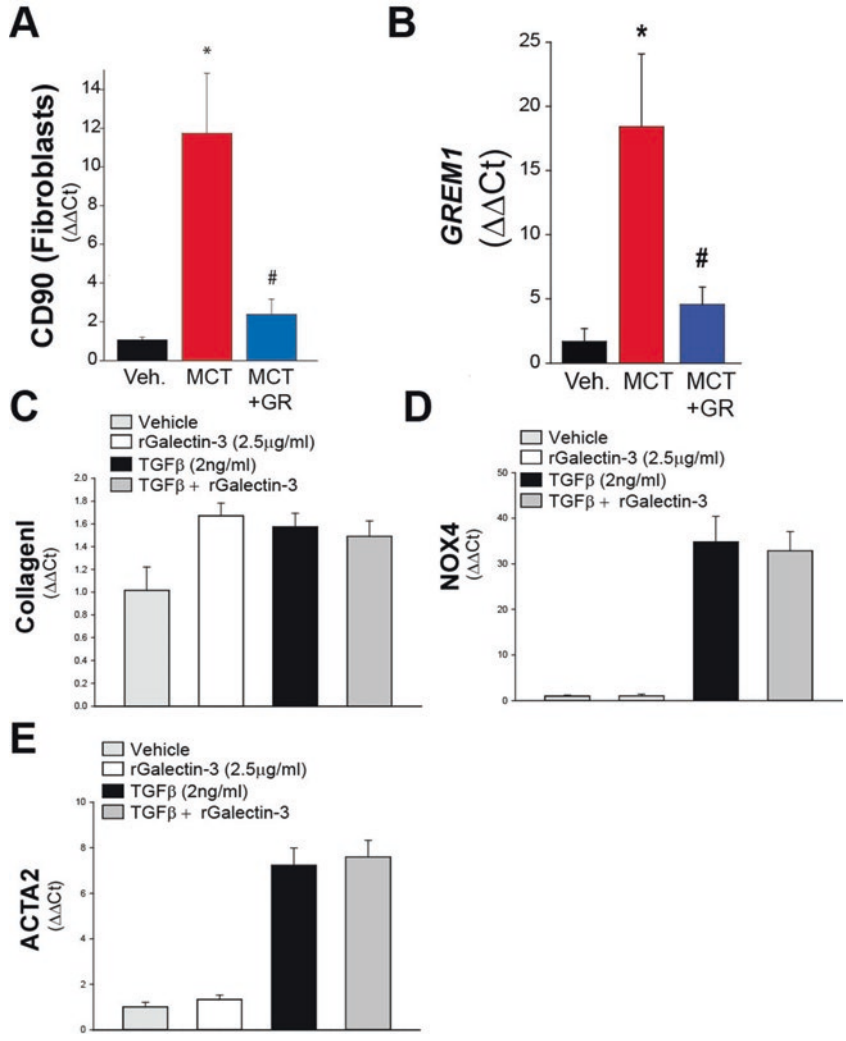

Fig. 2.5 Galectin-3 promotes vascular fibrosis in hypertensive pulmonary arteries. The expression of pro-fibrotic markers was determined in pulmonary arteries (PA) isolated from rats treated with MCT for 4 weeks. Relative expression of (**a**) CD90 (Thy1, fibroblast marker) and (**b**) GREM1 mRNA was determined in PA isolated from control, MCT, and MCT-treated with fibroblasts was determined. In (**c**), recombinant Gal-3 (10 μg/ml) increased collagen expression in fibroblasts but did not modify the ability of TGF-β1. In (**d**) recombinant Gal-3 did not increase NOX4 expression or alter the ability of TGF-β1 to robustly increase NOX4 expression. In (**e**), recombinant Gal-3 did not increase the expression or smooth muscle actin or alter the ability of TGF-β1 to robustly increase expression. n = 3–4 per group. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

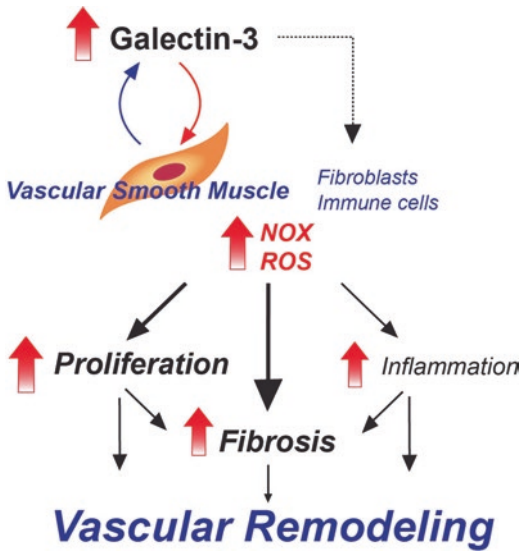

Fig. 2.6 Summary of the proposed mechanisms by which Gal-3 promotes NOX and ROS-mediated vascular remodeling in PA to induce PAH. Gal-3 increases cell proliferation, inflammation, and fibrosis (matrix deposition) via paracrine and autocrine functions via different cell types. (Reprinted with copyright permission from *Antioxidants and Redox Signaling*, Volume 31, Issue 14, Mary Ann Liebert, Inc., New Rochelle, NY (Publisher))

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3 Anti-inflammatory Effects of Statins in Lung Vascular Pathology: From Basic Science to Clinical Trials

Reem Faraj, Danyelle Paine, Stephen M. Black, and Ting Wang

Abstract

HMG-CoA reductase inhibitors (or statins) are cholesterol-lowering drugs and are among the most widely prescribed medications in the United States. Statins exhibit pleiotropic effects that extend beyond cholesterol reduction including anti-atherosclerotic, antiproliferative, anti-inflammatory, and antithrombotic effects. Over the last 20 years, statins have been studied and examined in pulmonary vascular disorders, including both chronic pulmonary vascular disease such as pulmonary hypertension, and acute pulmonary vascular endothelial injury such as acute lung injury. In both research and clinical settings, statins have demonstrated promising vascular protection through modulation of the endothelium, attenuation of vascular leak, and promotion of endothelial repair following lung inflammation. This chapter provides a summary of the rapidly changing literature, summarizes the anti-inflammatory mechanism of statins on pulmonary vascular disorders, and explores clinical evidence for statins as a potential therapeutic approach to modulation of the endothelium as well as a means to broaden our understanding of pulmonary vasculopathy pathophysiology.

Keywords

Acute lung injury · Pulmonary hypertension · Acute respiratory distress syndrome · Mortality · Statin · HMG-CoA reductase inhibitors · Vascular leak · Endothelial

Abbreviations

ITGB4	Integrin β4
3'UTR	3′ untranslated region
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
CAD	Coronary artery disease
COPD	Chronic obstructive pulmonary disorder
CpG	CG dinucleotides
CRAC	Ca^{2+}-release-activated Ca^{2+}
CVD	Cardiovascular disease
DMNT	DNA Methyltransferases
ECM	Extracellular matrix

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_3

EMP	Circulating endothelial microparticle
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ET-1	Endothelin-1
FA	Focal adhesion
HARP trail	Hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in Acute lung injury to Reduce Pulmonary dysfunction trial
HAT	Histone acetylase transferase
HDAC	Histone deacetylases
ICU	Intensive care unit
LBI	Laminin-binding integrins
LCA	Latent class analyses
MAPK	Mitogen-activated protein kinase
miRNA	microRNAs
MMP	Matrix metalloproteases
MMP-9	Matrix metallopeptidase 9
MPO	Myeloperoxidase
PAK 4	P21-activated kinase 4
PASMC	Pulmonary arterial smooth muscle cell
PHD-3	HIF1α-prolyl-4-hydroxylase 3
PVEC	Pulmonary vascular endothelial cell
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
SAILS trail	Statins for acutely injured lungs from Sepsis trial
SIRT1	Silent information regulator 1
Treg	Regulatory T-cell
VILI	Ventilator-induced lung injury

3.1 Introduction

Acute lung injury (ALI) and its more severe form acute respiratory distress syndrome (ARDS) are devastating disorders of the pulmonary endothelium affecting over 200,000 US patients/year with a mortality of 30–35%. Despite over four decades of research, few pharmacological interventions have become available. Effective treatments are limited to minimization of harm and prolonging survival time rather than the prevention of lung damage. Pulmonary arterial hypertension (PAH) is a group of progressive lung disorders also characterized by vascular endothelial dysfunction. Despite recent advancements in the management of PAH and the advent of targeted therapies, it is still associated with unacceptable morbidity and mortality-prompting a need for novel avenues of therapeutics. HMG-CoA reductase inhibitors (or statins) are cholesterol-lowering drugs and are among the most widely prescribed medications in the United States. Statins exhibit pleiotropic effects that extend beyond cholesterol reduction including anti-atherosclerotic, antiproliferative, anti-inflammatory, and antithrombotic effects. In pulmonary vasculopathy, statins have demonstrated vascular protection through modulation of the endothelium, attenuation of vascular leak, and promotion of endothelial repair following lung inflammation. This chapter provides a summary of the rapidly changing literature and explores clinical evidence for statins as a potential therapeutic approach to modulation of the endothelium as well as a means to broaden our understanding of pulmonary vasculopathy pathophysiology.

3.2 Part I. Mechanisms of Statin Modulation on Vasculature

Statins may play a beneficial role in the treatment of pulmonary hypertension, acute lung injury, and other disorders of the pulmonary vasculature by several different mechanisms identified in vitro and in experimental studies [1]. Statins have demonstrated improvement in the mechanistic progression of pulmonary vascular pathologies by targeting vascular remodeling, inflammatory pathways, endothelial barrier dysregulation, and the increased migration and proliferation of apoptosis-resistant vascular cells.

3.2.1 Pulmonary Vascular Remodeling Effects

Pulmonary vascular remodeling is the process of structural change in pulmonary arteries and is an unfortunate consequence of both direct and indirect insults. The change in pulmonary vascular architecture can occur as a result of a primary injury or secondary to chronic changes in intravascular pressure [2]. In the latter, vessel walls become thickened in order to withstand the sustained changes in intraluminal pressure overtime. While collagen deposition is imperative under physiologic conditions for the formation of the extracellular matrix (ECM), the excessive accumulation associated with pathology combined with additional smooth muscle deposition compromises vasodilatory activity and leads to increased pulmonary vascular resistance. These maladaptive responses are common in pulmonary hypertension and other diseases that compromise pulmonary vasculature.

3.2.1.1 Decreased Muscularization of Small Vessels

Uninhibited proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) in the medial layer of the arterioles leads to a common histopathological observation seen in all forms of pulmonary hypertension. Medial hypertrophy occurs as a result of increased migration and proliferation of PASMCs coupled with fibroblast-induced formation of an ECM. Ultimately, there is an extension of smooth muscle to normally unmuscularized pulmonary arteries resulting in vasculopathy [3]. In rats exposed to cigarette smoke for 16 weeks, simvastatin ameliorated lung parenchymal destruction and pulmonary hypertension by decreasing expression of MMP-9 (matrix metallopeptidase 9), a proteinase upregulated in patients with emphysema that is responsible for the degradation of ECM [4]. Additionally, treatment with simvastatin inhibits double lamina formation and medial thickening [4]. Kim et al. also found that simvastatin-mediated MMP-9 downregulation is likely caused by the inhibition of Ras prenylation in alveolar macrophages [5].

3.2.1.2 Attenuation of Vascular Proliferation

Members of the Rho family of GTPases (RhoA, RhoC, and Rac1) play a pivotal role in cellular regulation of apoptosis and are necessary in order to prevent the survival and accumulation of damaged cells that contribute to pathology. Kaneta et al. demonstrated that hydrophobic statins induce apoptotic cell death in endothelial cells thereby preventing their continued proliferation [6]. Hydrophobic statins can enter the cell and cause localization of RhoA from the membrane to the cytosol thereby inactivating it [6]. It is likely that hydrophobic statins increase phosphorylation of proteins involved in the process of apoptotic cell death by increasing the expression of Rac1. Induction of apoptosis by Rac1 and other Rho GTPases requires activation of caspase-3, an endoprotease that plays a role in the destruction of DNA fragmentation and cytoskeletal protein degradation [7]. Rats treated with simvastatin had increased activation of caspase-3 and fewer obliterated pulmonary blood vessels in a severe pulmonary hypertension rodent model. Investigators believe that the mechanism by which simvastatin reduced pulmonary hypertension was through apoptosis of obliterated vessels, allowing for the reopening of those vessels. Simvastatin inhibited proliferation and induced apoptosis of rat primary PVECs (pulmonary vascular endothelial cells) at a concentration as low as 1 micromolar. Additionally, they found that treatment with simvastatin partially restored caveolin-1, caveolin-2, and phosphor-caveolin expression. Downregulation of caveolin-1 has been suggested to play a role in the angiogenic response [8].

3.2.2 Epigenetic Modification Effects

There is debate regarding statins' ability to induce epigenetic modifications, i.e., reversible DNA modifications that alter gene expression. Evidence suggests that statins are capable of inhibiting histone deacetylase activity and increasing histone acetylation [9–11]. One spec-

ulation is that statins increase the quantity of available acetyl-CoA to be used in addition to histone tails by HATs (histone acetylase transferase) while also inhibiting HDACs (histone deacetylases) from removing those acetyl groups. Computational stimulations confirmed the direct inhibition of HDAC activity through binding of lovastatin to the active site of HDAC2 [12]. Recently, further complex mechanisms have been characterized to reveal the modification of HDAC2 function by statins. Statins inhibit prelamin A processing, therefore reducing protein interaction between HDAC2 and lamin A/C, a docking molecule and cherapron of HDAC2 [13].

In addition to histone modification, statins can promote DNA demethylation, a process in which methyl groups are added to cytosines by DMNTs (DNA methyltransferases) [14, 15]. Promoter regions of genes often contain a high density of CG dinucleotides (CpG) that are susceptible to hypermethylation. Hypermethylation of these promoter regions prevents transcription factor binding and can ultimately inhibit gene expression [15]. Demethylation of gene promoter regions has been reported following treatment with statins [16–18]. Statin treatment has also been linked with reduced expression of DMNT mRNA and protein [16, 17]. Regulatory T-cells (Tregs) are a subset of T-cells involved in the maintenance of immunological homeostasis through suppression of autoreactivity and constitutively express a master gene, Foxp3 [18]. Epigenetic modifications regulate the induction of Foxp3 expression via histone acetylation and DNA demethylation. Simvastatin increases Foxp3+ Treg expression in vitro likely through reduction of DNA methylation and this has important implications in inflammatory disease states such as atherosclerosis [18]. Likewise, altered Treg expression is likely to play a role in the statin-mediated attenuation of immunopathology associated with lung disease.

microRNAs (miRNAs) are noncoding RNAs capable of regulating genes by incorporating themselves into the RNA-induced silencing complex (RISC) and binding to 3′ untranslated region (3'UTR) of target mRNAs [19]. Statins have also demonstrated posttranscriptional regulation of gene expression by influencing the levels of miRNAs responsible for the translation of proteins involved in vasculature function [20]. Further, Statins have been implicated in modulation of miRNAs that are believed to influence endothelial progenitor cells (EPCs) [20]. EPCs are circulating cells that adhere to sites of hypoxic or ischemic damage on the endothelium and play an important role in vessel formation and repair through cytokine release and ultimately their differentiation into endothelial cells. Endothelial cells are somatic cells and have limitation proliferation potential. ECs that have exhausted this proliferative potential enter a terminal phase known as "endothelial cell senescence" and are no longer capable of division. While endothelial senescence is implicated in the vascular aging process, ECs subject to external cellular insults may also enter cellular senescence. Endothelial senescence has been implicated in vasculopathies such as coronary artery disease (CAD) and endothelial dysfunction of pulmonary vasculature in PAH [21, 22]. Silent information regulator 1 (SIRT1)-related miRNAs target SIRT1 with the subsequent reduction in SIRT1 expression, ultimately leading to endothelial senescence. Tabuchi et al. observed that in a trial of patients with CAD, 8 months of oral treatment with atorvastatin resulted in the downregulation of miR34a in combination with an elevation in peripheral EPC counts [21]. Additionally, intensive lipid-lowering statin therapy increases the number of circulating EPCs and prevents EPC telomere shortening, ultimately accelerating reendothelialization in rats [23].

3.2.3 Immune Modulation Effects

PAH is associated with perivascular inflammation as a result of alterations in vascular inflammatory cell metabolism and is thought to have an immunological component in its pathogenesis [24]. This altered cell metabolism impairs the vasculature's ability to resolve inflammation and propagates vascular remodeling. In rodent models of PH, intravascular accumulation of immune cells and elevation of cytokines and chemokine

levels often precedes pulmonary vascular remodeling, suggesting that alterations in immunological responses are part of the pathogenesis of PAH [25]. Similarly, the ARDS is characterized by an impairment in the balance of inflammatory cells and mediators involved in both the innate and adaptive immune responses. This clinical syndrome occurs most commonly as a result of respiratory infection by viral or bacterial pathogens. Clinical and experimental data have shown that key players of the immune system are involved in the pathogenesis of ARDS, regardless of whether the etiology is pathogenic or as a result of indirect systemic inflammation.

There is a growing body of literature to support lipid-lowering independent or pleiotropic anti-inflammatory properties of HMG-CoA reductase inhibitors in disease states with an immunomodulatory component through mechanisms such as reduction in the production of pro-inflammatory mediators, suppression of inflammatory cytokines, inhibition of molecules necessary for proper antigen presentation, inhibition of inflammatory gene transcription, and activation of anti-inflammatory transcription factors. Several potential mechanisms have been postulated for the abrogation of inflammation associated with statin use in respiratory diseases such as asthma and chronic obstructive pulmonary disorder (COPD). One such mechanism includes the reduction of inflammatory cytokines and chemokines that occur as a result of altered prenylation. Another mechanism is interference with antigen presentation. For example, statins can alter T-cell and antigen-presenting cell functionality and are capable of suppressing Th17 cells' ability to secrete inflammatory cytokine IL-17 [26].

The immunomodulatory effects of statins have also been demonstrated in a number of autoimmune and immune-related disease states which contain a high T-cell property [27] such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [28–30]. One of the most detrimental events in the inflammatory cascade is leukocyte infiltration into target organs. Statins have been shown to decrease the expression of cellular adhesion molecules on leukocytes as well as endothelial cells. Statins are, therefore, capable of attenuating leukocyte migration and adhesion to the intended organ. Specifically, statins can downregulate intercellular adhesion molecule-1 and endothelial vascular cell adhesion molecule-1 as well as decreasing the expression of matrix metalloproteases (MMPs) that enable leukocyte migration through the ECM [31–33]. Additionally, the cholesterol modulation normally associated with statins results in the instability of lipid rafts, the small membranous structures necessary for stabilization of the actin cytoskeleton. In addition to their role in the stabilization of the cellular membrane, lipid rafts act as bridges between molecules involved in immune activation. Improperly formed lipid rafts can interfere with interactions between inflammatory cells and subsequent activation of the immune system [34]. There is also evidence that statins may have benefits in lung vascular pathology. Statins may play a role in abrogating the innate and adaptive immune responses in pulmonary vascular diseases through mechanisms from instability of lipid rafts preventing proper antigen presentation and PAH to decreased expression of MMPs and subsequent inhibition of leukocyte migration through the ECM in ARDS [30–33]. Together these findings implicate statins as potential novel targets in autoimmune disease.

3.2.4 Effects on GTPase Isoprenylation Signaling

HMG-CoA reductase inhibitors prevent the formation of the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) through the mevalonate pathway. Isoprenoids are biologically active lipid intermediates required for the posttranslational modification of several small GTP-binding proteins that influence inflammatory pathways involved in adhesion, migration, proliferation, apoptosis, matrix degradation, and coagulation. For example, RhoA is involved in T-cell migration and has been linked to the attenuation of autoimmune damage in inflammatory disease states such as encephalomyelitis [35]. Posttranslational modifications of these proteins

is also required for membrane and protein–protein interactions [36]. Kim et al. demonstrated the simvastatin mediated increase in Foxp3+ T_{reg} expression is caused by inhibition of protein geranylgeranylation, and this pharmacological regulation of Foxp3+ Treg plays a role in attenuation of immunopathological pathways in vascular disease [18].

3.2.5 Transcription Factor Effects

Statins are well-known for their atheroprotective effects through reduction in mortality and cardiovascular events. The last two decades have seen a growing interest in understanding the mechanisms by which statins combat vascular inflammation in a cholesterol independent manner. One pleiotropic effect of statins that has been extensively studied is modulation of vascular gene transcription. Modulation of transcription factors that regulate inflammation in vascular disease has been well characterized with statins. Statins extensively interact with transcription factors which may explain some of the protective effects unrelated to cholesterol reduction. Simvastatin, atorvastatin, and lovastatin strongly inhibit the activation of transcription factors nuclear factor κB (NF-κB), activator protein-1 (AP-1), and hypoxia-inducible transcription factor 1a (HIF1α) in human aortic endothelial cells and well as aortic smooth muscle cells [37]. NF-κB regulates gene expression in vascular disease which promotes inflammation and its activation has been linked to endothelial dysfunction in vascular disease and has also been identified in atherosclerotic plaques [38]. Statins prevent TNFα-induced NF-κB binding activity, nuclear translocation of the NF-κB p65 subunit, as well as NF-kappaB controlled tissue factor gene transcription in cultured endothelial cells, without interfering with IκBα phosphorylation and degradation [39]. AP-1 is a transcription factor that regulates genes including matrixes metalloproteinases, cytokines, adhesion molecules, and inducible nitric oxide synthase [40–42]. The effects of statins on AP-1 DNA binding may occur through the inhibition of prenylation as Ras activates the mitogen-activated protein kinase (MAPK) cascade which is upstream of AP-1 [43]. Thus, inhibition of Ras activity likely inhibits downstream phosphorylation and subsequent activation of AP-1, leading to reduction of MMP expression [44]. HIF-1α is well-known for regulation of gene transcription in the vasculature. It forms heterodimers with HIF-1β and is regulated by oxygen concentration. In a hypoxic state, HIF-1α activates the transcription of several genes involved in preservation of vascular function (such as by modulating vascular tone and inflammation) and adaption to hypoxia [45]. This transcription factor is imperative for the maintenance of pulmonary vasculature in acute and chronic hypoxic situations where the prior leads to immediate pulmonary vasoconstriction and the latter results in remodeling of the vasculature wall [45, 46]. Simvastatin increases HIF-1α expression [47] in endothelial cells, possibly via the direct inhibition of HIF-1α -prolyl-4-hydroxylase 3 (PHD-3) [48], which negatively regulates the protein level of HIF-1α.

Kv1.3 is the dominantly expressed potassium channel and human T-cells and provides a driving force for calcium influx [49]. Kv1.3 channels are expressed on T-cells and play a regulatory role in resting membrane calcium influx through Ca^{2+}-release-activated Ca^{2+} (CRAC) channels in T-cells. Kv1.3 channel expression in T-cells is upregulated in many animal models of autoimmune disorders. Studies have shown that Lovastatin blocked Kv1.3 channels in human T-cells in a concentration- and voltage-dependent manner [50]. Additionally, investigators found that Lovastatin downregulated calcium-dependent transcription factors NF-κB p65/50 and NFAT1 and in a dose-dependent manner [50]. Functional immunosuppression was also confirmed as Lovastatin inhibited T-cell proliferation and IL-2 secretion [50]. Lovastatin mediated attenuation of IL-2 secretion was partially neutralized following injection with Mevalonate [50], further supporting that immune-modulating effects of Lovastatin extend beyond its cholesterol-lowering abilities.

3.2.6 Endothelial Barrier Protection

The pulmonary vascular endothelium is highly distensible under physiologic conditions and remains a semipermeable barrier between the vascular space and the alveoli. Insult to the vasculature from ALI-related and intensive care unit (ICU)-related injurious stimulation including LPS and ventilation associated excessive mechanical stress, a syndrome defined as ventilator-induced lung injury (VILI) causes increased permeability of capillaries and subsequent lung edema. Similarly, ALI secondary to sepsis is exudative and results in the efflux of proteinaceous material into alveolar spaces as a result of increased capillary permeability [51]. Attempts to identify therapeutics to minimize or attenuate the progression of vascular leak in ALI or VILI have been unsuccessful. However, statins have emerged as a promising class of drugs capable of modulating the endothelial barrier in ALI. In a mouse model of ALI, animals pretreated with 20 mg/kg of simvastatin exhibited decreased levels of protein and inflammatory cytokines in bronchoalveolar lavage (BAL) fluid as well as significant reductions in myeloperoxidase (MPO), an inflammatory enzyme marker found in neutrophil granulocytes [52]. Additionally, leakage of Evans blue dye bound tightly to albumin was significantly attenuated in LPS-challenged mice that were pretreated with simvastatin, demonstrating that simvastatin confers endothelial cell barrier protection [52]. Endothelial cell barrier function relies on cytoskeletal components and their contractile mechanisms [53]. Simvastatin induces actin cytoskeletal rearrangement and confers protection of the barrier integrity following treatment with edemagenic agents such as thrombin [54] and following exposure to LPS [54]. Cytoskeletal rearrangement following simvastatin treatment occurs through the inhibition of Rac1 membrane localization, which leads to NADPH oxidase assembly and reactive oxygen species (ROS) generation, and elevation in cytosolic Rac1, which antagonizes RhoA-mediated barrier disruption downstream of LPS or thrombin [54]. Modulation of Rac1 is dependent on simvastatin-induced geranylgeranylation [54], a posttranslational modification required for several anti-inflammatory pathways involved in cell proliferation and apoptosis, leukocyte adhesion, and eNOS production [55].

3.2.6.1 Integrin β4

Simvastatin's protective effect on endothelial cell barrier function in the setting of LPS-induced ALI also appears to be dependent upon the functional role of Integrin β4 (gene code ITGB4) [54]. ITGB4 is the most upregulated gene in endothelial cells (ECs) following treatment with simvastatin [56]. Laminin-binding integrins (LBI) such as ITGB4 are important adhesion receptors in cell migration, invasion, and morphogenesis [57]. In endothelial cells, ITGB4 is an essential adhesion molecule within EC focal adhesions (FAs), structures that are required for bidirectional signal transduction between the EC cytoskeleton and the cell-matrix interface [58]. ITGB4 is a known adhesion and signaling protein in human microvessels [59] and has been implicated as a mediator of endothelial cell barrier regulation, the key feature of acute injury, via peripheral cytoskeletal remodeling and lamellipodia formation following excessive lung stretch [52, 54]. Unlike other laminin-binding integrins, Integrin β4 contains an unusually long cytoplasmic domain capable of mediating protein interactions and signaling pathways. Previously, it was thought that Integrin β4 is pro-inflammatory and that its pro-inflammatory effects were due to phosphorylation at several tyrosine sites located along its cytoplasmic domain [56]. Chen et al. developed several constructs of Integrin β4 protein containing deleted or mutated portions of its cytoplasmic tail in order to characterize its function in the attenuation of lung inflammatory responses. They found that the expression of inflammatory cytokines (IL-6, IL-8, MCP-1, and RANTES) in media was attenuated in ECs transfected with Integrin β4 protein mutants [56]. These results are consistent with in vivo studies in which genetically engineered mice expressing mutated Integrin β4 (lacking the cytoplasmic domain) have significantly attenuated inflammatory indices after exposure to high tidal mechanical ventilation [60]. Further, transfection of a

mutant lacking the cytoplasmic domain confers the same protective effects as wild type mice pretreated with simvastatin [56]. In mice mutated to express a truncated Integrin β4, without the intracellular domain [56], pretreatment with simvastatin does not confer any additional protection, suggesting that simvastatin's effects are influenced by Integrin β4. ITGB4 exists physiologically as multiple different splicing variants. One of these variants, ITGB4E, lacks most of the cytoplasmic domain. Human pulmonary endothelial cells pretreated with simvastatin and exposed to pathological cyclic stress increase ITGB4 transcription with enhanced generation of the ITGB4E splice variant. Thus, Elevated ITGB4E expression following simvastatin treatment could be a potential mechanism by which statins confer lung protection in murine models of ALI/VILI and offers a novel avenue for biomarker and drug discovery.

3.2.6.2 PAK4-Cdc42 Pathway

Cdc42 is a member of the Rho GTPase family whose activation is dependent on posttranslational modification by geranylgeranyl pyrophosphate, an isoprenoid intermediate which is decreased in the presence of HMG-CoA reductase inhibitors. Inhibition of Cdc42 has also been implicated in simvastatin's protective effects against lung injury. However, silencing Cdc42 was found to have no significant impact on thrombin-induced endothelial cell permeability and thus failed to support a significant role for Cdc42 in simvastatin-mediated regulation of endothelial cell integrity [54]. Conversely, a more recent study found that simvastatin attenuated LPS-induced ALI via cytoskeleton stabilization and that this was mediated by regulating the pulmonary Cdc42-PAK4 pathway and altering levels of circulating endothelial microparticles (EMPs) [61]. EMPs are released from cells and have been used to assess the magnitude of injury to intercellular junctions in response to LPS [61]. P21 activated kinase 4 (PAK 4) is a Cdc42 effector protein and plays a role in cytoskeletal rearrangements following LPS-induced lung injury and the simvastatin-induced cytoskeletal stabilization appears to be mediated through alterations of PAK 4 [61]. In addition, the administration of an oral PAK4 inhibitor induces pathological changes consistent with lung injury such as alveolar wall thickening and increased wet-to-dry (W/D) in rats [62]. EMPs were also elevated and simvastatin attenuated this elevation of EMP release [62].

3.2.7 Attenuation of Oxidative Stress

Oxidative stress has been implicated in several vasculopathies including atherosclerosis, coronary artery disease, and pulmonary hypertension. Statins have been shown to be of benefit in these pathologies and patients on high-intensity statin therapy have a reduced risk of mortality in CVD. The mechanisms by which statins confer protection in these pathologies, however, remains to be fully elucidated.

3.2.7.1 Effects on Endothelial Senescence

Reactive oxygen species are detrimental to endothelial cells and can ultimately lead to endothelial senescence. Statins have been shown to inhibit oxidative stress-induced endothelial senescence [63]. SIRT1 regulates the production of mitochondrial ROS in arterial endothelial cells [64]. Statin treatment enhances SIRT1-dependent mitochondria biogenesis in human umbilical vein endothelial cells stimulated with hydrogen peroxide [63] and could be a mechanism by which statins confer protection in vasculopathy. In a clinical study in which patients with CAD were randomized to treatment with rosuvastatin or atorvastatin, the atorvastatin group had markedly increased SIRT1 levels, whereas the rosuvastatin group showed no change in SIRT1 levels, suggesting that increases in SIRT1 may contribute to atorvastatin's benefit on endothelial function in vascular disease [21]. However, it is not known whether the lack of benefit seen with rosuvastatin may also be due to its hydrophilic properties. The differences in utility between statins based on hydrophilicity will be further explored below.

3.2.7.2 Inhibition of RhoA-Rho Kinase Signaling

Statins do not exclusively inhibit the cholesterol biosynthetic pathway. In addition to inhibiting the formation of mevalonic acid, statins also inhibit synthesis of isoprenoids intermediates such as farnesylpyrophosphate and geranylgeranyl pyrophosphate, both of which are responsible for isoprenylation and subsequent activation of Rho. Activated Rho (RhoA) participates in a variety of cellular functions ranging from cell migration to apoptosis. Additionally, RhoA and Rho-kinase are involved in regulating the expression of endothelial nitric oxide synthase (eNOS) and proliferation of vascular smooth muscle cells and evidence suggests that RhoA and Rho-kinase pathways are involved in the development of pulmonary hypertension (PH). Inhibitors of Rho-kinase have been shown to attenuate mean pulmonary arterial pressures in monocrotaline induced-PH in rats [65] and to reduce pulmonary cardiovascular remodeling in rats with chronic hypoxia-induced PH [66]. Statins essentially act as RhoA/Rho-kinase inhibitors and can therefore exert beneficial effects on the endothelium through the RhoA/Rho-kinase signaling pathway [67], suggesting that statin regulation of this pathway may be an important mechanism of treating PH. This, however, remains to be fully elucidated and only offers a potential avenue in the search for novel therapeutics in pulmonary hypertension [67, 68].

3.2.7.3 Effects on Vasoconstrictive and Vasodilatory Balance

Nitric Oxide (NO) is a vasoactive substance that plays a role in the regulation of the vascular endothelium. Decreased NO is associated with endothelial dysfunction and an increase in reactive oxygen species. Endothelin-1 (ET-1) is a vasoconstrictive agent that works in opposition to NO. In addition to its vasoconstrictive properties, ET-1 also participates in the proliferation of smooth muscle cells which ultimately propagates pulmonary vascular remodeling. Statins confer endothelial barrier protection through increased expression of eNOS which also corrects the imbalance between NO and endothelin 1 [69]. Lee et al. offered potential mechanism for the inhibition of smoking-induced increase in mean pulmonary arterial pressures seen in rats pretreated with simvastatin. They demonstrated that simvastatin abrogated the decrease in eNOS expression in human lung microvascular cells following exposure to cigarette smoke. Although there were no differences seen in the expression of ET-1 protein expression, the restoration of eNOS protein expression could explain the positive effects on pulmonary hypertension [4] (Figs. 3.1, 3.2, and 3.3).

3.3 Part II. Statins and Lung Disease: The Landscape of Clinical Data in Endothelial Dysfunction

ALI and its more severe form ARDS are devastating disorders affecting over 200,000 US patients/year with a mortality of 30–35% [70]. The pathobiology of ALI/ARDS features increased lung vascular permeability due to loss of endothelial cell barrier integrity resulting in alveolar flooding, which ultimately leads to respiratory failure and death. Unfortunately, insights into ALI pathobiology have been incremental with no viable therapies realized. As ALI/ARDS is often a direct consequence of severe respiratory or systemic inflammatory disease (e.g., severe pneumonia and sepsis), the clinical need for a precise diagnostic/predictive biomarker or effective therapy is desperately needed.

Preclinical data from in vitro and animal studies has demonstrated the potential role of statins in ALI through their ability to modulate underlying mechanisms involved in the development and progression of ALI. Naturally, the next step was to identify whether this beneficial role of statins in ALI could be demonstrated in human subjects. In 2011, the first randomized, placebo-controlled clinical trial was conducted in patients with ALI and provided proof of concept. The Hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in Acute lung injury to Reduce Pulmonary dysfunction (HARP) trial was a single-center, randomized, double-blind,

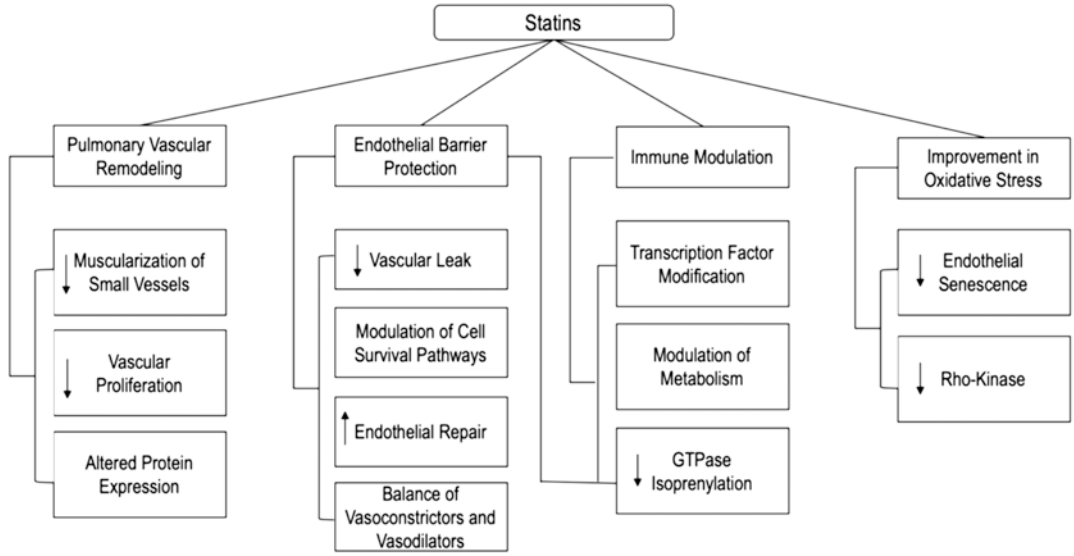


Fig. 3.1 Statins modulate pulmonary endothelium in vascular disease. HMG CoA Reductase Inhibitors (statins) are primarily used in clinical practice for cholesterol reduction and the prevention of cardiovascular disease. In recent years, research has identified a potential new utility for this class of drugs in the treatment of lung disease through pleiotropic or extrahepatic effects. Statins may play a beneficial role in the treatment of pulmonary hypertension, acute lung injury, and other disorders of the pulmonary vasculature by several different mechanisms identified in vitro and in experimental studies. Statins have demonstrated improvement in the mechanistic progression of pulmonary vasculopathies by targeting vascular remodeling, endothelial barrier dysregulation, and the increased migration and proliferation of apoptosis-resistant vascular cells

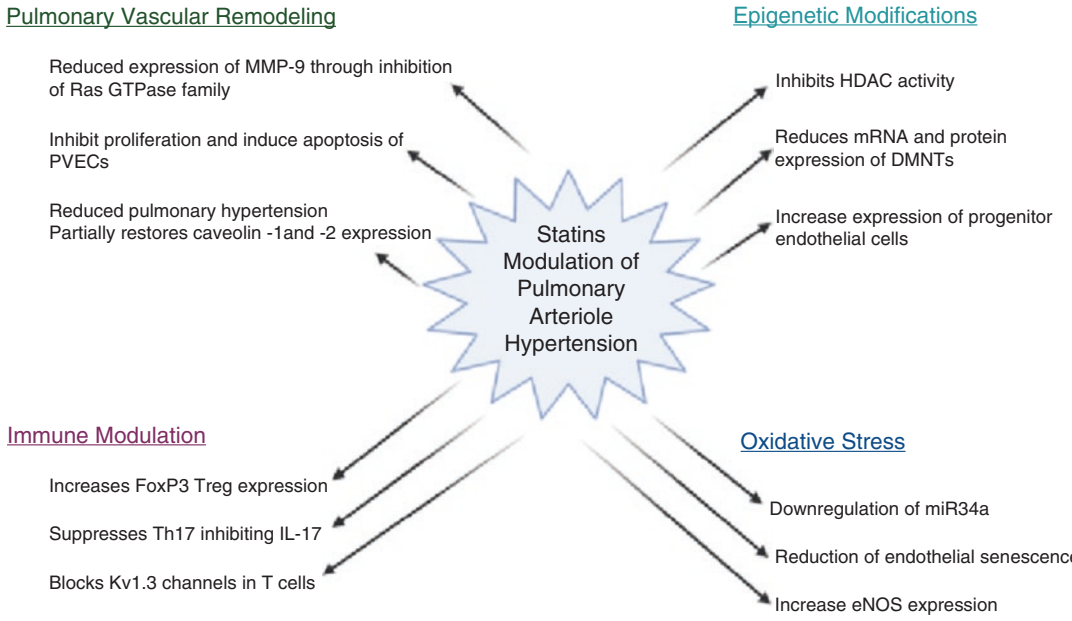


Fig. 3.2 Statin modulation of PAH. This figure summarizes the various functions of statins in pulmonary vascular remodeling, Epigenetic modifications, immune modulation, and oxidative stress

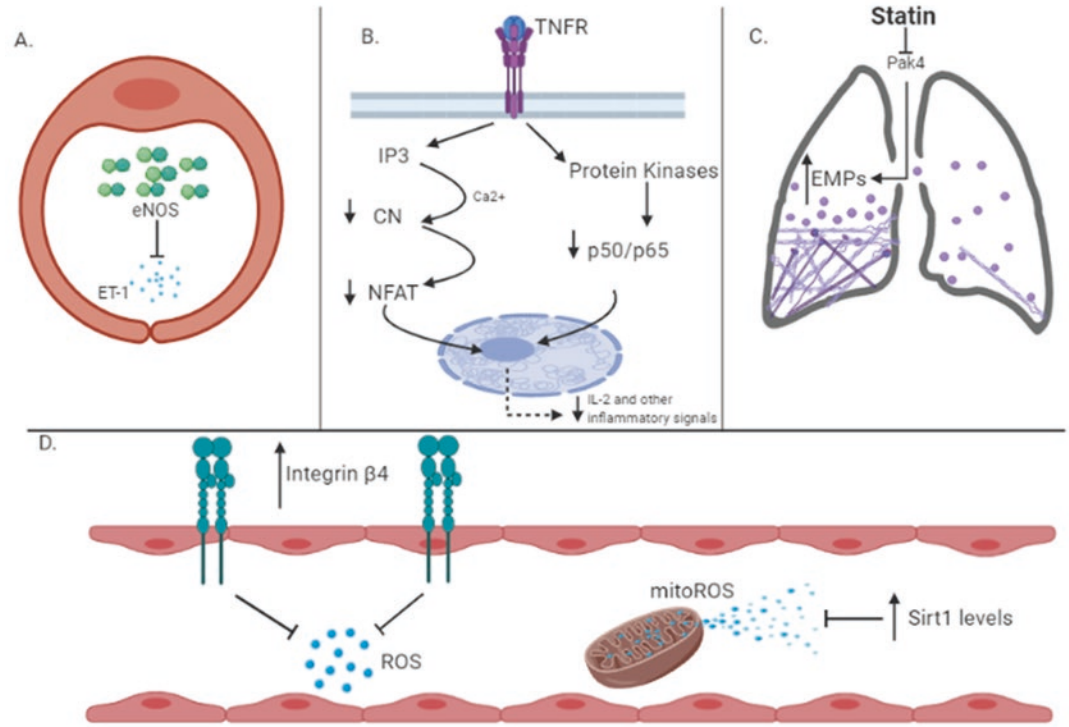
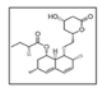
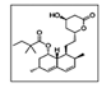
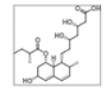
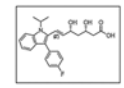
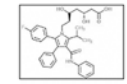
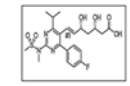
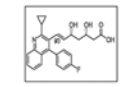


Fig. 3.3 Statins modulation of acute lung injury**.** Panels A thru D represents the various ways that statins role in acute lung injury. (**a**) *An endothelial cell with ET-1 production.* The increased expression of eNOS leads to the reduction in ET-1 expression. (**b**) *NFAT and NFKβ pathways are modulated through statins by downregulating the expression of downstream signaling molecules*. NFAT proteins are activated by the phosphatase calcineurin leading to the rapid entry of NFAT into the nucleus. On the contrary, the NFKβ Pathway consists of protein kinases such as IkB kinase that are activated and released for the initiation of the transcription factors p50/p65 translocation into the nucleus. Both pathways lead to the expression of pro-inflammatory cytokines and complement factors, however, statins produce a reduction in signaling cascade. C. *Statins leads to the inhibition of PAK4.* The downregulation of PAK4 pathway leads to increased expression of EMPs permitting cytoskeletal rearrangement. D. *ROS-induced oxidative stress is reduced by statin expression.* ROS is inhibited due to the increased expression of ITGβ4 on endothelial cells. ITGβ4 has been shown to provide oxidative protection. SIRT1 reduces the production of mitochondrial ROS (mitoROS) that can be produced in dysfunctional endothelial cells

placebo-controlled study that investigated simvastatin 80 mg for up to 14 days in a population of 60 patients with ALI [71]. At day 14, the simvastatin-treated group has an improvement in non-pulmonary organ dysfunction, improvement in pulmonary dysfunction, and was well tolerated [71]. This proof of concept clinical trial established that treatment with simvastatin was safe as there were no differences in the rate of adverse events between both groups and that simvastatin may be associated with improvements in organ dysfunction secondary to ALI [71]. Additionally, individuals pretreated with simvastatin had lower levels of pro-inflammatory markers IL-8 and CRP, a finding consistent with those observed in animal studies [71].

Completed clinical trials involving human subjects using FDA-approved statins available on the market. Only nine studies have demonstrated a clear benefit in humans with the majority belonging to patients with pulmonary hypertension. Only two studies have found a clear demonstrated benefit for the use of statins in ALI/ARDS, although post hoc analyses and latent class analyses may now offer a new perspective on the outcomes of these trials as well as an explanation for the discrepancy seen between preclinical and clinical studies (Tables 3.1 and 3.2).

Table 3.1 Comparison of FDA-approved statins

							
Statin (Generic Name)	Lovastatin	Simvastatin	Pravastatin	Fluvastatin	Atorvastatin	Rosuvastatin	Pitavastatin
Statin (US Trade Name)	Mevacor	Zocor	Pravachol	Lescol	Lipitor	Crestor	Livalo
Molecular Formula	C24H38O6	C25H40O6	C23H36O7	C24H26FNO4	C33H35FN2O5	C22H28CaFN3O6S+2	C50H46CaF2N2O8
Bioavailability	5%	5%	17%	6%	14%	20%	51%
Clinically Approved Dose	10-40mg	10-80mg	10-80mg	20-40mg	10-80mg	5-40mg	1-4mg
Lipophilicity	Lipophilic	Lipophilic	Hydrophilic	Lipophilic	Lipophilic	Hydrophilic	Lipophilic
Half-Life (Hours)	2-4	2-3	1-3	4.7	15-30	19	12
CYP Metabolism	3A4	3A4	ND	2C9	3A4	Limited	Limited
Clinical Studies							
ARDS	NO	YES	NO	NO	NO	YES	NO
Airway Disease	YES	YES	YES	NO	YES	YES	NO
PH/PAH	NO	NO	NO	NO	YES	NO	YES

Pharmacokinetic and pharmacodynamic properties of statins currently available on the market in the United States. Despite demonstrating their ability to produce cholesterol-independent effects, statins exhibit differences in their pharmacodynamic and pharmacokinetic profiles with regard to lipophilicity, bioavailability, tissue permeability, and metabolism. Only a handful of statins have been studied in human subjects with pulmonary endothelial vasculopathy

The promising results from the proof of concept HARP trial prompted two more large, multicenter, randomized, placebo-controlled trials in ALI patients [85, 94]. The Hydroxymethylglutaryl-CoA reductase inhibition with simvastatin in Acute lung injury to Reduce Pulmonary dysfunction (HARP-2) was a multicenter, prospective, controlled clinical trial that randomized patients in a 1:1 fashion to receive either simvastatin 80 mg or placebo once a day for up to 28 days and were subsequently followed at 3, 6, and 12 months post-randomization. Patients fulfilled the criteria for ALI based on the American-European Consensus Conference Definition [95]. This study included 540 patients within 48 h of ARDS onset and was intubated and mechanically ventilated. However, no differences were seen between groups in ventilator-free days even after baseline adjustments. Further, no differences were observed in the number of days free of nonpulmonary organ failure or mortality at 28 days. Additionally, unlike the HARP trial, adverse events related to simvastatin were significantly more common in the treatment group. Ultimately, the HARP-2 trial failed to demonstrate improvement in clinical outcomes following simvastatin treatment in ARDS. A subgroup analysis also failed to identify effect modification by age, CRP level, requirement of vasopressor treatment, and presence or absence of sepsis. The Statins for Acutely Injured Lungs from Sepsis (SAILS) trial was also conducted parallel to HARP-2 and aimed to identify whether rosuvastatin, a hydrophilic statin, would reduce mortality or improve secondary outcomes in patients with sepsis-associated ARDS. The study was stopped early due to futility and investigators concluded that rosuvastatin therapy did not improve clinical outcomes in these patients. Additionally, it appeared that rosuvastatin may have ultimately contributed to hepatic and renal organ dysfunction [96]. Following the disappointing results from HARP-2 and SAILS, researchers have speculated reasons for the failure of statins to demonstrate benefit in ARDS/ALI and the inconsistency seen between preclinical and clinical studies. These are discussed below.

3.3.1 Heterogeneity of ARDS: Failure of Statins or Trial Design?

In 2012, the diagnosis of ARDS was updated to the Berlin definition, which characterized ARDS

Table 3.2 Clinical trials of statins in pulmonary vascular pathology

	Author	Year	Disease	Type of study	Dosage	Size	Outcomes	Statin benefit
All statins								
	Kor et al. [72]	2009	ARDS/ALI	Cohort	NA	45	13, 14, 17, 18,19	–
	O'Neal et al. [73]	2011	ARDS/ALI	Cohort	NA	149/575	13, 14, 18	+/–
	Terblanche et al. [74]	2011	ARDS/ALI	Cohort	NA	219/1397	17	–
	Bajwa et al. [75]	2012	ARDS/ALI	Cohort	NA	413/2743	15, 16	–
	Bruyere et al. [76]	2014	ARDS/ALI	Cohort	NA	93/349	6	+
	Yadav et al. [77]	2014	ARDS/ALI	Cohort	NA	722/1845	16	–
	Mansur et al. [78]	2015	ARDS/ALI	Cohort	NA	108/404	6	++
	Holzhauser et al. [79]	2017	PH	Cohort	NA	138/762	6	++
Simvastatin								
	Kao et al. [80]	2005	PAH	Cohort	20-80 mg	16	1, 2, 5, 10	+
	Shyamsundar et al. [81]	2009	ARDS/ALI	RCT	40-80 mg	30	20	++
	Wilkins et al. [82]	2010	PAH	RCT	40-80 mg	19/42	1, 8, 11	–
	Reed et al. [83]	2011	PAH + COPD	Cohort	N/A	34/112	2, 5	++
	Kawut et al. (ASA-STAT) [84]	2011	PAH	RCT	40 mg	32/65	1, 8	–
	Craig et al. (HARP) [71]	2011	ARDS/ALI	RCT	80 mg	60	17	+/–
	McAuley et al. (HARP-2) [85]	2014	ARDS/ALI	RCT	80 mg	259/540	6, 17, 18	+/–
Rosuvastatin								
	Barreto et al. [86]	2008	PAH	RCT		30/60	20	++
	Dinglas et al. (SAILS) [87]	2016	ARDS/ALI	RCT		379	6, 18, 19	–
	Chogtu et al. [88]	2016	PH + COPD	RCT	10 mg	32/62	1, 7, 9	++
Pravastatin								
	Lee et al. [89]	2009	PAH + COPD	RCT	40 mg	27/53	2, 8, 12	++
Atorvastatin								
	Reed et al. [83]	2011	PAH + COPD	Cohort	N/A	34/112	2, 5	++
	Zeng et al. (APATH) [90]	2012	PAH	RCT	10 mg	112/220	1, 8, 10	–
	Liu et al. [91]	2013	PAH + COPD	RCT	20 mg	33/68	2	++

(continued)

Table 3.2 (continued)

	Author	Year	Disease	Type of study	Dosage	Size	Outcomes	Statin benefit
	Moosavi et al. [92]	2013	PAH + COPD	RCT	20 mg BID	24/45	1, 2, 10	−
	Arian et al. [93]	2018	PH + COPD	RCT	40 mg	21/42	2	+

1. 6MWD (6 min walk distance)
2. Pulmonary arterial pressures
3. Cardiac index
4. Right atrial pressures
5. Pulmonary vascular resistance
6. All-cause mortality
7. QOL (Quality of life)
8. Borg dyspnea score
9. Pulmonary function tests (FVC, FEV_1, FEV_1/FVC)
10. Hemodynamic parameters
11. Right ventricular (RV) mass
12. Exercise time
13. Hospital length of stay (LOS)
14. ICU LOS
15. Mechanical ventilation
16. ARDS
17. Organ function (pulmonary)
18. Ventilator free days
19. ICU mortality
20. Inflammatory markers (NF-kB, CRP, MMP-7, −8, −9)

Statin benefit:
+ Some benefit
++ Clear benefit
− No benefit
+/− Mix

based on the severity of hypoxemia in addition to onset, origin, and radiographic findings. This update reflected the heterogeneous nature of ARDS as a clinical syndrome [97]. ARDS is a disease of heterogeneity that has been described based on etiology (insult), pathophysiology (indirect vs. direct), and degree of inflammatory response. High mortality rates in trials of ARDS patients pose a specific challenge for clinical trials: unfeasibly high recruitment numbers in order to detect a small reduction in mortality. One paper estimated that detection of 5% reduction in mortality would require a cohort of at least 2200 patients [98]. To combat this, many strategies have been proposed to classify subgroups in ARDS clinical trials. Traditionally, ARDS severity was physiologically derived and patients were enrolled in clinical trials based on objective measures such as PaO2/FiO2, pulmonary dead space, and ventilatory pressures. ARDS patients have also been stratified based on etiology and the onset of symptoms. There is no consensus on what objective measures should be used to categorize ARDS patients in clinical trials and it remains a heavily debated topic. Recent papers present evidence for the use of biologically derived phenotypes as a method to classify ARDS patients in clinical trials aimed at identifying therapeutics.

Failure to show benefit in clinical studies may be overcome by identifying biologically derived phenotypes in ARDS, allowing them to target specific underlying biological pathways and developing targeted therapies [98]. In accordance, many investigators have performed latent class analyses (LCA) in order to identify a cohort (subset) of ARDS patients in whom statin treatment may be beneficial [98, 99]. A latent class analysis is a mixture model that identifies subgroups in a heterogeneous population based on prespecified variables. An LCA on the population of ARDS patients studied in the HARP-2 trial identified that 90% of patients could be classified within one of two latent class phenotypes based on the onset of ARDS following severe trauma [100]. Specifically, patients with the early-onset phenotype experienced an increased severity in early hypotension and thoracic trauma and were more likely to receive a blood transfusion during resuscitation. In addition, this early-onset phenotype exhibited significantly higher levels of biomarkers of lung damage (sRAGE, Ang-2), confirming the existence of distinct molecular phenotypes between patients in the ARDS cohort studied in HARP-2 [100]. Although the investigators did not find a difference in mortality between the early- and late-onset phenotypes, the difference in expression of biomarkers of endothelial function could potentially explain the lack of benefit seen in the HARP-2 cohort. A meta-analysis of ARDS patients identified higher 90-day mortality in patients with higher baseline plasma sRAGE levels, illustrating epithelial barrier injury as a prognostic marker in ARDS [101]. This further supports the idea that statins could still play a role in ARDS treatment through promotion of alveolar epithelial barrier integrity. Two subphenotypes of ARDS were also identified following a latent class analysis on two randomized controlled trials of ARDS (ARMA and ALVEOLI trials). These were classified as: "hyper-inflammatory" and "hypo-inflammatory." The "hyper-inflammatory" phenotype, also known as Phenotype 2, is characterized by high levels of inflammatory biomarkers, severe shock, and metabolic derangement. In contrast to Phenotype 2, the severity of inflammation and shock is significantly less in the "hypo-inflammatory" phenotype, or Phenotype 1 [99]. These two subphenotypes display an association with clinical outcomes and, even more interestingly, differences in response to treatment. In the cohort of patients from the ALVEOLI trial, subjects classified as Phenotype 2 displayed worse clinical outcomes relative to those classified as Phenotype 1 including higher mortality and less ventilator-free days [99]. Latent class assignment of inflammatory phenotypes also displayed differential effects of high versus low PEEP strategies. Patients randomized to receive low PEEP had a higher mortality if they displayed Phenotype 2 and a lower mortality for Phenotype 1 [99, 102]. Conversely, those randomized to receive high PEEP had a higher mortality if they displayed Phenotype 1, highlighting the differences in effect of high versus low PEEP on mortality and clinical outcomes between the two phenotypes

[99, 102]. Additionally, these two ARDS phenotypes respond differently to fluid management [103]. These findings bring up an important question, if clinical outcomes and response to fluid management strategies differ between phenotypes, could phenotypic profiles also respond differently to statin therapy? In 2018, a latent class analysis was performed on patients from the HARP-2 trial which found a difference in clinical outcomes and differences in survival between the subphenotypes [104]. Phenotype 2, characterized by a "hyper-inflammatory" profile, demonstrated improved 28- and 90-day survival with simvastatin relative to placebo [104]. The results of this post hoc analysis challenge the original conclusion that simvastatin treatment did not improve outcomes in ARDS patients [85]. Stratification based on specific phenotypes of ARDS appears to demonstrate the clinical benefit of statins that more closely reflects observations seen in preclinical studies. This has led to the school of thought that "phenotype-dependent treatment response" in ARDS promotes a potential new strategy for the treatment of ARDS but requires further validation and standardization [105].

So far the only prospective observational study of patients with ARDS on statin therapy that has stratified patients was based on the severity of ARDS as either mild, moderate, or severe [78]. In this study. there was a positive effect of statin therapy on 28-day survival among patients with severe sepsis-associated ARDS, suggesting that patients with severe ARDS may present with the "hyperinflammatory" phenotype [104]. Moreover, patients in the severe ARDS subgroup receiving statin therapy had more vasopressor-free days and required less ECMO therapy relative to those who received placebo. It would be interesting to identify whether the two ARDS subphenotypes are present in this cohort and whether severity correlates with Phenotype 2. In addition to the beneficial impact on survival, statin therapy was also found to be accompanied by significantly lower SOFA scores in patients with severe ARDS [78]. Lower cardiovascular SOFA scores observed in the study are consistent with the preclinical data on statins and their pleiotropic effects which attenuate anti-inflammatory responses in cardiovascular disease.

3.3.2 Statins: Lone Wolf or Class Effect?

All statins have pleiotropic effects in a variety of different disease states from autoimmune disorders such as multiple sclerosis and rheumatoid arthritis to the neurological and cognitive deficits of neurofibromatosis and Alzheimer's. The magnitude of pleiotropy, as well as the degree to which they exert these extra-hepatic effects, is dependent upon their individual pharmacokinetic properties. Despite demonstrating their ability to produce cholesterol-independent effects, statins exhibit differences in their pharmacodynamic and pharmacokinetic profiles with regard to lipophilicity, bioavailability, tissue permeability, and metabolism [106].

Although preclinical studies suggest that statin protection in pulmonary vascular disease is a class effect, it is becoming more evident that not all statins are the same. A post hoc analysis of the SAILS trial involving ARDS patients treated with rosuvastatin did not demonstrate differences in outcome between the two ARDS subphenotypes in the same manner as simvastatin in HARP-2 [107]. This suggests that there may be subtle differences and interclass variability within the statins. The differences in clinical outcome between the two trials may be explained by the variation of inclusion criteria since the benefits of statin therapy in ARDS seems to be dependent upon ARDS severity. For instance, patients in the HARP-2 cohort had higher baseline PaO2/FiO scores relative to the SAILS cohort [96]. If the effect of statins is dependent upon the magnitude of severity, this could explain why a benefit was seen in Phenotype 2 with simvastatin but not rosuvastatin.

The variability between statins' gene modulatory activity may be involved in the discrepancies observed in clinical trials. An in-depth examination of the variability in gene expression profiles of pancreatic cancer following treatment with different statins demonstrated a modulating effect

on genes encoding regulators of growth factors as well as genes responsible for the formation of focal adhesions and integrin activation [108]. It has also been reported that a correlation exists between the lipophilicity of a statin and its ability to induce gene expression change [108]. Hydrophilic statins such as pravastatin and rosuvastatin demonstrate poor intracellular penetration as evidenced by the observation that more hydrophobic statins (simvastatin) reach considerably higher intracellular concentrations by comparison. Changes in the expression of cytoskeletal proteins also display variability among the statins. Simvastatin upregulates several genes involved in cell-to-cell adhesion while rosuvastatin and pravastatin do not. The variability of gene modulatory activity between simvastatin and rosuvastatin may also provide an explanation for the discrepancies seen between the post hoc analyses conducted on the HARP-2 and SAILS trials [99, 107]. Perhaps simvastatin may be more effective as a pleiotropic agent in lung disease such as ARDS because of its ability to modulate genes involved in the arrangement of cytoskeletal proteins and ultimately enhances barrier function through this pathway. As discussed above, the ITGB4E variant upregulation following simvastatin treatment and its role in the protection of endothelial barrier function [52]. However, whether this splicing event and subsequent upregulation of E variant is a class effect remains to be discovered. Furthermore, simvastatin exhibits better tissue penetration due to it hydrophobic nature, allowing it to reach higher intracellular concentrations compared with hydrophilic rosuvastatin [108]. More comprehensive studies on the gene expression profiles of endothelial cells could further elucidate the mechanisms by which statins confer lung protection in pulmonary vascular disease.

3.3.3 Nanomedicine-Based Drug Delivery Systems: The Solution to the Statin Problem?

The use of statins in pulmonary vascular disease requires consideration of the administered dose and drug concentration. Currently, there is no consensus or established statin concentration for use in cell experiments demonstrating pleiotropy. In clinical trials, the choice of dose that is administered has fallen within the scope of what is approved by the drug's FDA labeling. The HARP-2 study chose to use simvastatin at a dose of 80 mg because this is the dose used in the proof of concept study that had preceded it. Simvastatin 80 mg is considered high-intensity cholesterol-lowering therapy and is the maximally tolerated dose of this statin approved by FDA labeling [71, 85]. At the conclusion of HARP-2, investigators were unsure if adverse events due to high dose statin use had masked any potential benefit in the study [85]. Similarly, investigators of the SAILS study suspected that futility may have occurred as a result of inadequate plasma levels of rosuvastatin (7.3 ng vs. 10-70 ng/mL desired) [96]. Thus, the discrepancy seen between statin concentration in preclinical studies and human trials could also be due to the fact that clinically relevant doses of statins have not traditionally been used in animal experiments [109]. For example, in animals, high doses of systemic statins demonstrated attenuation in the progression of PAH [110, 111]. In humans, however, maximally tolerated doses of statins (simvastatin 80 mg/day) did not demonstrate the same benefit [82]. Thus, it is possible that clinically approved doses of statins in humans is a limitation to the protective benefit seen in animal studies. If this is the case, then the question becomes what plasma concentration of statins is required to induce pleiotropic effects, and are they clinically relevant? A systematic literature search conducted by Bjorkhem-Bergman et al. found that the pleiotropic effects of statins were detected at maximum concentrations between 1 and 50 μmol/L in human serum [109]. Traditionally, statin doses that have been used in rodent studies range between 1 and 100 mg/kg of body weight. For comparison, doses used in human studies vary between approximately 0.1–1 mg/kg of body weight. In pulmonary vascular disease, the ranges studied are even more defined. Only one study compared two doses of simvastatin in a mouse model of ALI. Mice were pretreated with 5 mg/kg or 20 mg/kg of simvastatin intraperitoneally

24 h before and concomitantly with LPS to observe whether there were differences in the response to lung injury [112]. Only mice that received 20 mg/kg of simvastatin demonstrated a statistically significant reduction in myeloperoxidase (MPO) and neutrophils in BAL fluid relative to vehicle control [112]. Additionally, only the higher dose group demonstrated an attenuation of LPS-induced lung vascular leak [112]. In an average weight of an American male, this would be equivalent to approximately 1.8 grams of simvastatin over 20-times the maximally accepted clinical dose that can be safely administered to humans. Thus, the use of high dose statins in animals is problematic because they are not clinically relevant. Many pharmaceutical agents on the market today are FDA approved for doses that fall within a therapeutic index window: the doses are large enough to produce the intended effects of the drug without the risk of causing serious harm or toxicity. For many therapeutic agents, including statins, a large enough concentration in the body can cause additional effects with one caveat-elevated risk of toxicity and off-target effects. Therefore, the high doses used in animals may be misleading and misinforming. Further, only orally administered statins have been studied in clinical trials for ARDS/ALI and PH/PAH. One reason for the inconsistency seen between preclinical and clinical studies of statins in pulmonary vascular disease may be lack of delivery to specific tissues and/or monocytes/macrophages in orally administered statins. Statins are notorious for their neuromuscular side effects including rhabdomyolysis, axonal neuropathy, and myopathy. Statins are also associated with an elevation in hepatic enzymes, without clinically significant hepatotoxicity, and possibly cancer and dementia [113, 114]. The incidence of musculoskeletal perturbations with statins is both dose-dependent and independent of LDL cholesterol reduction [115]. Higher doses of statins are imperative in order to elicit the Rho-dependent effects involved in modulation of pulmonary vascular pathology. Unfortunately, higher doses of statins are limited by the likelihood of polyneuromyopathy and neuromuscular adverse effects since they occur in a dose-dependent manner.

This leads to the question; can the beneficial effects of statins be amplified while keeping overall doses within a safe range? Further, could this be achieved with selective and targeted drug delivery, allowing statins to accumulate at the necessary concentrations but in such a manner that would allow for the beneficial effects without lending the risk of off-target damage and adverse events. Thus, an alternative drug delivery system may offer a solution by bypassing dose-limiting toxicities while enhancing the efficacy of statins. One such approach utilized a bioabsorbable polylactic/glycolic acid (PLGA)-nanoparticle (NP)-mediated drug delivery system for pitavastatin delivered by intratracheal instillation attenuated PAH development in a rodent model of PAH [111]. Site-specific delivery to tissues with enhanced vascular permeability and inflammation when injected intravenously was also achieved in a mouse model of atherosclerosis [116]. Pitavastatin-NP also demonstrated 30 times higher efficacy relative to pitavastatin alone in the attenuation of PAH and improved overall survival in a monocrotaline-rodent model [110]. Pitavastatin-NP also attenuated the small pulmonary artery stenosis and obstruction relative to pitavastatin alone [110]. Finally, unlike traditional PDE5 inhibitors used to treat PAH, pitavastatin-NP did not produce alterations in hemodynamic parameters which could also be an additional advantage as a potential therapeutic option in PH. A phase I clinical trial using a single, intravenously administered pitavastatin-NP regimen has been undertaken in 40 healthy subjects [117]. The administration of nanotechnology-based medicine pitavastatin-NP (NK-104-NP) was well tolerated in healthy subjects and exhibited dose-dependent pharmacokinetics [117].

There are advantages in the development of novel drug delivery systems based on nanoparticle technology. Firstly, the delivery to a specific tissue decreases off-target and adverse-effects.

Secondly, following release into the targeted cells, the nanoparticle delivery system is degraded into CO_2 through a hydrolytic mechanism, preventing the formation of any toxic metabolic products [110, 117]. Thus, this type of novel drug delivery system could have major implications for the use of statins in pulmonary vascular disease. A phase II clinical trial is currently underway to study the in vivo distribution of pitavastatin (NK-104-NP). Based on the results, further clinical trials will be conducted using this novel nanoparticle drug delivery system of a statin drug for the treatment of PAH. The results of which will be compelling.

3.4 Part II. Conclusions

Statins have been shown to have beneficial effects in the treatment of a number of lung diseases including PH, ARDS, VILI, and other pulmonary vasculopathies at the level of experimental animal models. Statins have also consistently demonstrated positive clinical outcomes in PH patients while the data in ARDS and ALI is mixed. However, updated statistical methods and new categorization of heterogeneous disease states are offering new perspectives on the outcomes of clinical trials that may explain these inconsistencies. Certainly, there is a need for continued investigation to increase our mechanistic understanding of the roles plated by statins in pulmonary vascular pathology as our knowledge is still very limited in relation to class effects of the statins. Additionally, advances in drug delivery systems and gene expression technology are continuing to provide new avenues for research that did not exist before. Thus, it is still too early to write off the statins as therapeutics for the treatment of lung diseases (Table 3.3).

Table 3.3 Utility of statins in lung disease

ARDS	Early reduction in IL-6, IL-8, and plasma CRP	Safe, modest improvement in pulmonary function in patients ventilated at 14 days
ALI	Simvastatin attenuates thrombin-induced human lung endothelial barrier dysfunction	
VILI		No improvement in clinical outcomes
		Simvastatin did not improve clinical outcomes in ARDS vs. placebo
		Rosuvastatin did not reduce mortality or improve secondary outcomes
PAH/PH	Reduction of mean pulmonary arterial, and right ventricular systolic pressure	Improvement in 6 min walk test
		Improvement in cardiac output
	Reduction in right ventricular, left ventricular, intraventricular septal weight	Decreased right ventricular mass
		Decreased BNP
	Improvement in pulmonary vascular remodeling	Increases in exercise time
	Reduction of proliferation	Decreased in systolic pulmonary arterial pressure
	Apoptosis of smooth cells and endothelial cells	Decrease in urinary endothelin-1 levels
	Inhibition of adventitial fibroblast proliferation	
	Improvement in oxidative stress	
	Increased survival rate in animals with PE induced PH treated with atorvastatin	

Summary of conclusions from preclinical versus clinical studies of FDA-approved statins. Statins demonstrate consistently positive effects on clinical outcomes in PH while the data in ALI/ARDS is mixed. However, in recent years, post hoc analyses and an improved understanding of the heterogeneity of ALI/ARDS pathophysiology may shed light on why clinical outcomes do not reflect the benefit seen in in vitro studies

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4 Evolving Schema for Employing Network Biology Approaches to Understand Pulmonary Hypertension

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Abstract

Reductionist approaches have served as the cornerstone for traditional mechanistic endeavors in biomedical research. However, for pulmonary hypertension (PH), a relatively rare but deadly vascular disease of the lungs, the use of traditional reductionist approaches has failed to define the complexities of pathogenesis. With the development of new -omics platforms (i.e., genomics, transcriptomics, proteomics, and metabolomics, among others), network biology approaches have offered new pipelines for discovery of human disease pathogenesis. Human disease processes are driven by multiple genes that are dysregulated which are affected by regulatory networks. Network theory allows for the identification of such gene clusters which are dysregulated in various disease states. This framework may in part explain why current therapeutics that seek to target a single part of a dysregulated cluster may fail to provide clinically significant improvements. Correspondingly, network biology could further the development of novel therapeutics which target clusters of "disease genes" so that a disease phenotype can be more robustly addressed. In this chapter, we seek to explain the theory behind network biology approaches to identify drivers of disease as well as how network biology approaches have been used in the field of PH. Furthermore, we discuss an example of in silico methodology using network pharmacology in conjunction with gene networks tools to identify drugs and drug targets. We discuss similarities between the pathogenesis of PH and other disease states, specifically cancer, and how tools developed for cancer may be repurposed to fill the gaps in research in PH. Finally, we discuss new approaches which seek to integrate clinical health record data into networks so that correlations between disease genes and clinical parameters can be explored in the context of this disease.

Keywords

Pulmonary hypertension · Systems biology · Network biology · Omics

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_4

4.1 Introduction

Molecular breakthroughs in medical research have historically resulted from singular focus on individual genes, cellular components, or organ systems as drivers of diseases. Risk factors for disease were considered in isolation rather than as co-occurring factors where their summation could ultimately lead to disease. In the modern, post-genomic era of medicine, there is a shift to a network-based approach in discerning disease processes. This approach integrates genomic, biochemical, physiological, and cellular data into a network to model disease progression and response to treatment [1]. The hope is that this approach will provide better insight into the connection between genotype and phenotype of diseases by taking into consideration gene–gene interactions as well as environmental impacts and regulatory systems on each person's genetic code [2]. Advances in whole-genome sequencing, single-cell -omics techniques, and development of bioinformatics algorithms increasingly make this network-based analysis now feasible.

While the impact of network medicine has increasingly been obvious in human diseases such as cancer where molecular profiling is common, the value of such an approach in rarer diseases is increasing. Pulmonary hypertension (PH) is a rare, complex, and often fatal vascular disease of the blood vessels of the lung with undefined molecular origins that have been difficult to understand by traditional reductionist methods of discovery. Clinically, PH is defined by specific pressure and resistance measures of the pulmonary vasculature obtained by invasive right heart catheterization. However, a substantial degree of heterogeneity and interindividual variability in disease manifestation makes PH an extremely challenging disease to manage. The current clinical classification of PH defines five separate groups of PH (I-V) as defined by the World Symposium on PH (WSPH). Group I PH, or pulmonary arterial hypertension (PAH), includes a wide array of subtypes where pulmonary arteriolar remodeling is thought to predominate pathogenesis and thus are more responsive to pulmonary vasodilator therapy. Other more prevalent PH subtypes (Groups II-V) display substantial heterogeneity in both disease manifestation and response to vasodilators [3–5]. The causes of PH are broad, encompassing disparate genetic and acquired pathobiological triggers [6–8]. Advancing genetic studies and whole-genome sequencing increasingly have implicated more factors as genetically associated with PAH risk and/or severity [9, 10]. Furthermore, current vasodilatory therapies for PH do not appear to target the molecular origins of disease and neither regress, prevent, or cure the disease. Because of the sheer disease complexity, it has become increasingly important to consider alternative methods, such as advanced network biology approaches, to make important insights into PH pathogenesis [11].

4.2 In Silico Network Theory

In general, the goal of in silico network-based analysis is to discern the overarching relationships governing network integrity. In molecular biology, the most common application to date is to apply such network theory to the study of the molecular interactions among genes functionally connected to one another. In such an interactome, each node represents a single gene while links, or edges, between nodes represent interactions between genes. Networks in biology do not have a random distribution where each element of the network has an equal number of interactions with other members in the network [1]. These networks contain clusters of genes that are highly interconnected. Often these networks contain highly interconnected clusters, defined as hubs which hold the entire network together. Hubs are defined as the top 20% of nodes with the highest number of connections [1]. Each hub is considered to represent a biological process and connections between hubs connect various biological processes needed for cellular function. Most networks display a "small world" phenomenon. That is, most proteins are connected by few interactions implying that most biological processes are interconnected and altering the behavior or one such node will alter the behaviors of directly

connected nodes as well as neighboring clusters. Essential genes, genes that are necessary for cellular and organ function, have the most interactions with other members in a central location. Essential genes will also have the shortest number of paths to various biological processes demonstrating betweenness centrality [1]. Compared to essential genes, the disease module hypothesis dictates that disease genes are often peripherally located and demonstrate a high level of interconnectedness with other disease components. Disease components that are located near one another most likely affect the same dysregulated pathways. These interconnected disease genes create a disease module [12]. Various tools have been developed to analyze the connectedness of nodes and map interactions to distinguish central and peripheral processes (Fig. 4.1).

To build a disease gene regulatory network, one common approach entails analyzing sequencing data from experimental and control conditions to identify genes that are either upregulated or downregulated. These differentially expressed genes can be used to construct a network through functional analysis of gene–gene interactions. Once constructed, the regulatory components of the network can be mapped onto existing gene–gene interactions (Fig. 4.2). Once a given disease network is built, researchers can identify relevant disease modules that may offer novel and often unanticipated information about disease pathogenesis, in general. Neighboring clusters of peripherally located disease genes can identify interactions between dysregulated pathways. For example, pro-inflammatory genes may cluster together while hypoxia inducing genes may cluster together. The interactions between genes in the pro-inflammatory cluster and the hypoxia cluster may elucidate new connections between hypoxia and inflammation. Genes of interest can relate to several modules

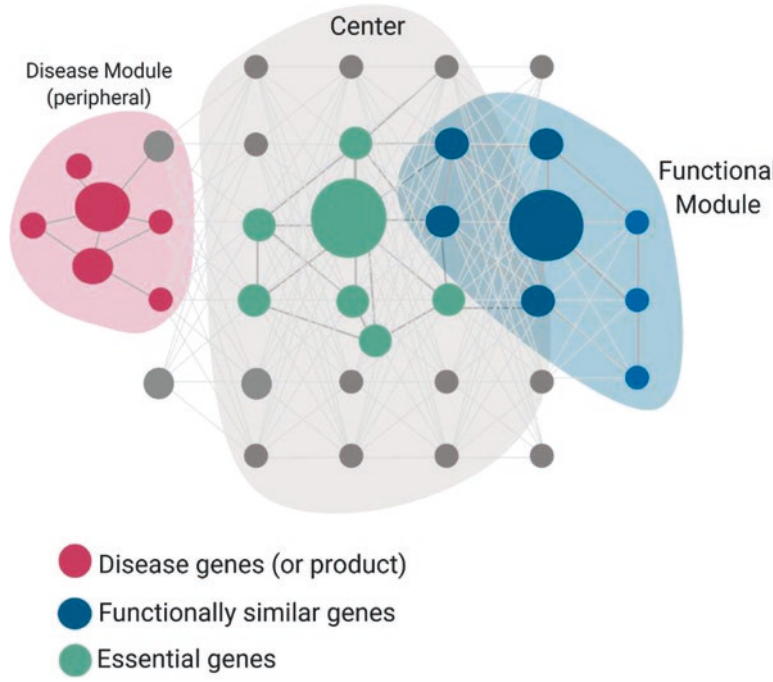

Fig. 4.1 Topological features of gene networks. In this schematic representation of networks of gene-gene interactions, nodes are depicted as circles and size of the circle depicts density of connections. Dark gray lines depict statistically significant connections between genes or products. Disease modules are located on the periphery of the network and are not densely connected to other essential components of the network. In contrast, essential genes are located in the center of the network and are heavily connected to each other as well as to functional components. The functional module is a cluster of statistically significant connections between functionally related components. (Adapted from [1])

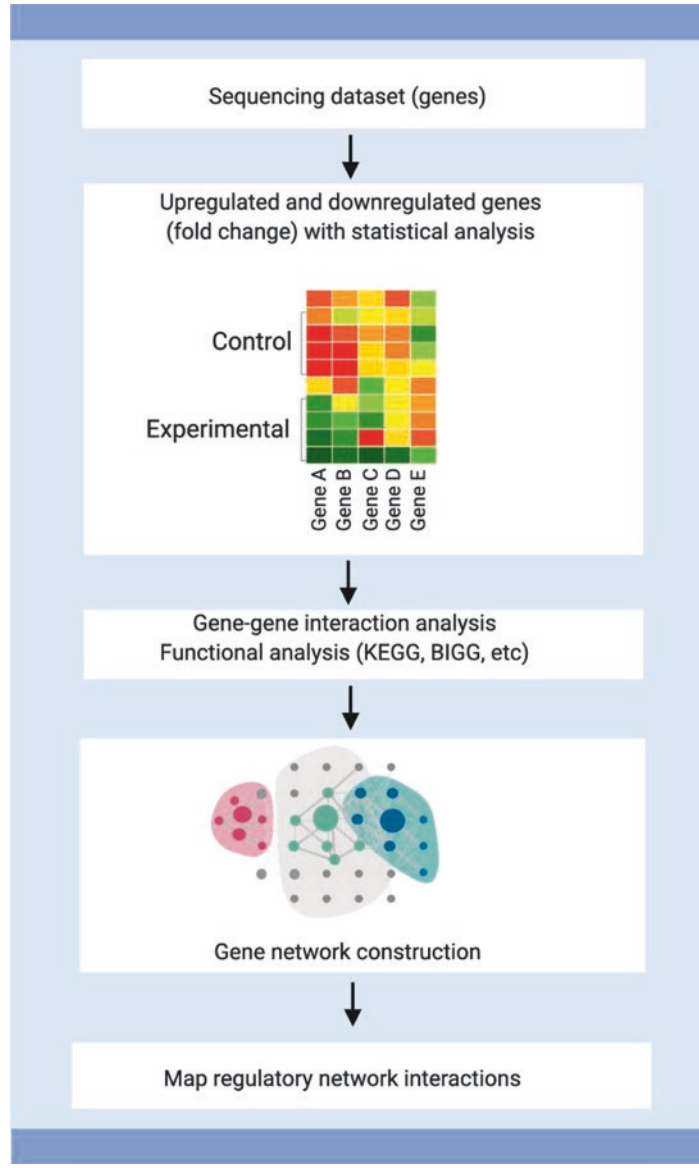

Fig. 4.2 Example of the workflow to construct gene networks from RNA sequencing data. Starting with RNA sequencing datasets between control and disease conditions, fold changes in genes are calculated and statistically significant changes are considered for network construction. Gene-gene interactions and pathways analyses can then be mapped from databases of known interactions, yielding a gene-gene interaction network. Short-RNA sequencing separately can reveal miRNAs that are dysregulated. Regulatory interactions (microRNA-gene interactions based on microRNA seed sequence-gene pairing) can be mapped onto the existing gene-gene interaction network to construct the final network using sequence-based prediction algorithms

showing interconnectedness of dysregulated pathways that contribute to the disease [13]. In most networks, challenging a single component of the system, such as one disease-causing gene, does not change the overall architecture of the network [14]. Other than essential genes, most other genes show redundancy. Therefore, it can be fruitful to consider the connectedness of nodes that create a disease module and to rank targets of interest based on their degree of interaction with other members of this disease module.

More recently, computational analysis methods have moved toward constructing networks based on differential dependencies rather than interactome maps based on differential gene expression. EDDY, Evaluation of Dependency DifferentialitY, is a tool that was developed to create interactomes based on differential dependencies between a set of genes based on different conditions [15]. Previous work into PH gene networks was based on gene expression where an experimental condition and a control condition

were both sequenced to correlate genes that were either upregulated or downregulated in the experimental condition compared to the control condition. EDDY was developed to identify dysregulated pathways by analyzing dependencies that were altered between the experimental and control groups (Fig. 4.3) [16]. Analysis of differential dependencies requires more statistical power; therefore, EDDY is only applicable with larger datasets that carry multiple samples representing each condition, (i.e., single-cell RNA sequencing of multiple cells from the same tissue; sequencing from multiple patients with the same disease, etc.) [15].

For the field of PH, attempts have been made to construct PH disease gene networks, based on curated, or known, genes implicated in PH pathogenesis [10, 13, 17–19]. Construction of PH net-

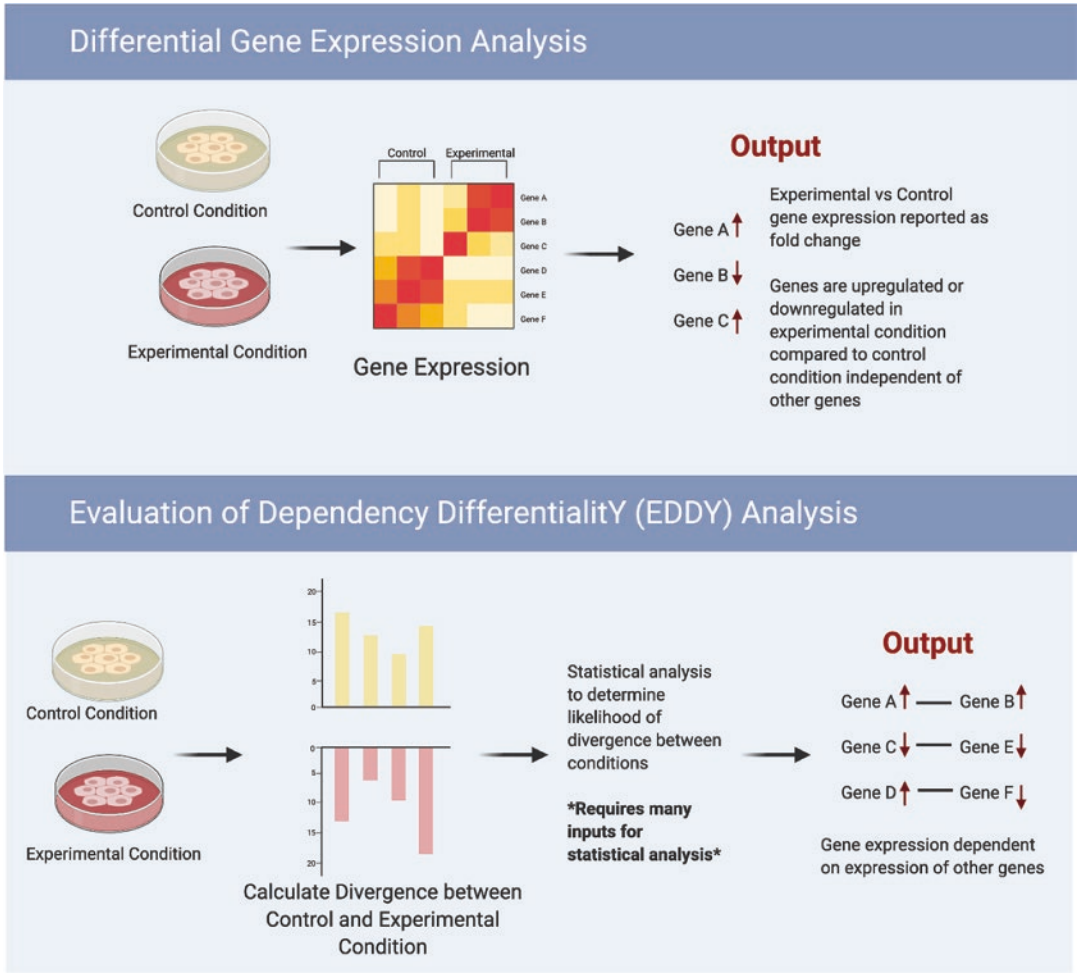

Fig. 4.3 Differential Gene Expression Analysis vs. Evaluation of Dependency DifferentialitY (EDDY). Traditional differential gene expression analysis requires sequencing data from control and experimental conditions (top panel). The difference in gene expression is expressed as fold change of gene expression in the experimental vs control condition. Statistical analysis validates whether gene expression results are significantly changed between the experimental and control groups. EDDY allows for differential dependency analysis where divergence between the sequencing results of the control and experimental sets are calculated (bottom panel). EDDY requires more statistical power and therefore is only applicable to larger datasets (single-cell RNA sequencing). Analysis with EDDY determines how expression of genes are dependent on others and how these dependencies change from the control to experimental conditions. (Adapted from [15])

works that catalog more indices than merely genes alone is now possible and could offer insights into multidimensional gene relationships not available to date. There has been increasing interest in the role of metabolites in PH and how altered bioenergetics are implicated in disease [20, 21]. This work is driving the development of databases that encompass -omics strategies beyond genotypes and gene regulatory networks. Furthermore, network construction with nodes that represent diseases or organ systems has also offered the ability to discern associations at more physiologic rather than simply molecular levels. Most recently, EDDY was employed to study the differential dependencies between control and PAH samples provided by the Pulmonary Hypertension Breakthrough Initiative (PHBLI) [22]. This study reconstructed networks previously based on differential gene expression through differential dependency analysis. In this study, pathways were identified that rewired the previous PH network based on differential dependency analysis. These pathways represented a rewiring of 40–100% of the original PH network and provided new targets of interest for mechanistic follow-up [22].

4.2.1 MicroRNA Regulatory Networks in PH

One example of how network theory can predict the activity of complex regulatory gene systems includes the study of microRNAs (miRNAs) that carry overlapping functions. MiRNAs are noncoding RNAs that are transcribed in the intron regions of the protein-coding genes [23]. MiRNAs are transcribed to pri-miRNAs by RNA Pol II. The pri-miRNAs are then cleaved within the nucleus by Dorsha, the RNAse III microprocessor, to pre-miRNAs. Pre-miRNAs are transported to the cytosol as dsRNA constructs. In the cytosol, Dicer, the RNAse III enzyme, produces the double-stranded miRNA (ds-miR). This ds-miR is then loaded onto the Argonaute (AGO) protein family (RISC loading) which then selects one strand of the miRNA to become the mature miRNA and discards the other. The RISC complex containing the miRNA functions to repress translation [24]. The miRNA binds to the 3′ UTR region of the target mRNA, and the AGO protein recruits factors to degrade the mRNA through deadenylation and decay. The 5′ region of the miRNA contains a "seed" sequence of 2–7 nucleotides that is specific for target recognition of the mRNA transcript to be degraded. This seed sequence is used in several validated bioinformatics algorithms to predict mRNA targets of the miRNAs [24].

To decipher the most meaningful miRNA targets within the system, our group has used hypergeometric analysis to rank miRNAs according to those with the most system-wide effects on a predefined PH disease gene network. Since miRNAs are known to negatively regulate gene expression, a directionality of information flow was taken into consideration along with the size and density of connections of miRNAs (miRNA spanning score) with gene nodes in the PH system [25]. These approaches combined with statistical analysis can then predict meaningful relationships between key players in a disease module. These predictions must then be validated in vitro and in vivo.

To create a miRNA regulatory network for PH, our group first started by consolidating a list of genes implicated in the development of PH using PubMed and the search term "pulmonary hypertension." Annotation of protein-protein interactions, protein-DNA interactions, kinase-substrate interactions, and metabolic interactions created the PH interactome [13, 26]. Thereafter, TargetScan5 was used to predict targets of miRNAs that regulated this network of genes using miRNA seed sequences. This method of overlaying miRNAs with genes implicated in PH ranked miRNAs that regulated different aspects of the PH network. This model revealed miR-21 as a master regulator of BMP and Rho/Rho kinase activity and the miR-130/301 family as coordinators of vasomotor tone through control of PPAR-γ [26]. Mutations in the BMP pathway have been implicated in PH; however, these mutations have shown variable phenotypes. To validate the in silico model, our group went on to show that miR-21 null mice showed an exaggerated PH

phenotype. In the same vein, Bertero et al. identified miR-130/301 as a miRNA family that interacted with genes in the PH expanded network. MiR-130/301 directly controlled PPAR-γ expression which in turn regulated proliferation and a plethora of vasoactive modifiers in vascular smooth muscle and endothelial cells [26].

Our group expanded this exploration into the miR-130/301 family with studies of ECM remodeling. Transcriptomic analysis revealed that ECM modeling genes were differentially upregulated in the diseased lung and liver tissues. Previous network analysis had revealed that miR-130/301 targetted ECM remodeling proteins. To further assess, the role of this miRNA family on fibrosis, we constructed a fibrosis network with genes involved in ECM remodeling [27]. As expected, this network showed similarity with the original PH network, and, correspondingly, the miR-130/301 family was highly ranked by miRNA spanning score, thus indicating the overlap with fibrosis and PH. From this initial fibrosis network, our group demonstrated that a YAP/TAZ-miR-130/301 circuit caused ECM remodeling in PH via a PPAR-γ-APOE-LOX axis [27]. Pharmacological inhibition of the YAP/TAZ pathway or downstream effects (ApoE, LOX) ameliorated these effects, illustrating the utility of network biology to identify new pharmacological targets [11, 26–29].

4.2.2 Long Non-coding RNA Regulatory Networks

Other than miRNA regulatory networks, long non-coding RNAs (lncRNAs) have been implicated in the development of various pathological states in PH. The use of network biology to predict lncRNAs that interact with gene networks has much to be explored. In 2015, profiling of chronic thromboembolic PH patients revealed 185 lncRNAs that were differentially expressed. Co-expression networks revealed dysregulation of lncRNAs, NR_036693, NR_027783, NR_033766, and NR_001284 implicated in inflammatory response and antigen presentation [30]. More recent work has shown lncRNAs and miRNAs dysregulated in hypoxic PH; lncRNA MEG3 dysregulation triggering pulmonary artery smooth muscle cell (PASMC) proliferation via the p53 pathway; and the lncRNA-linked proliferation of PASMCs in response to abnormal PDGF treatment [31–33]. These works have shown that, similar to miRNAs, lncRNAs are dysregulated by hypoxic and inflammatory pathways. Interestingly, recent findings have shown that miRNAs may be responsible for the stability of lncRNAs; and conversely, lncRNAs may bind to miRNAs to sequester miRNAs and allow mRNA gene transcription and translation [34]. These reciprocal interactions allow for non-coding RNAs to regulate gene expression in various conditions. These findings add a layer of complexity to the existing model of post-transcriptional gene regulatory systems, but also provide an opportunity to use network biology to analyze such interactions. To date, a network biology approach has not modeled lncRNA regulatory systems with the known PH interactome.

4.3 Network Pharmacology

The same philosophy that mapped miRNA interactions with genes to build a regulatory network can be translated to mapping drug interactions onto gene interaction networks. To date, network pharmacology approaches have been employed in the drug development field to predict possible drug targets and adverse effects. Currently, only 400–600 of the total 100,000 functional proteins are being targeted by current FDA approved drugs [35]. Mapping known FDA approved drugs onto gene interaction networks provides significant evidence against the reductionist approach to drug development as most drugs target proteins that have more interactions than the average protein [14, 36]. The scale-free architecture of most networks allows the deletion of single nodes without disruption of the entire network, except for the deletion of essential genes.

As shown in the work with the miR-130/301 family, drug targets can be predicted using a systems biology approach. Current research goals using network pharmacology include polyphar-

macologic targets where one molecule binds to multiple targets [37]. Network pharmacology can also be used to predict whether drugs will synergistically show improvement over single-drug regimens. Cassas et al. used a network-based approach to determine synergistic drug targets for ischemic stroke. The authors focused on finding functionally similar druggable target proteins to NOX4, a preclinically validated target. The authors predicted that a NOS inhibitor worked synergistically to a NOX4 inhibitor to treat ischemic stroke and consequently proved this finding in vitro and in vivo [38].

This field provides various opportunities for the discovery of new drug targets and for the repurposing of current FDA approved drugs for use in PH [39]. Network pharmacology approaches allow for the screening of multiple drugs to predict the effect on multiple targets before validating these predictions through more reductionist approaches. For example, the Loscalzo group created a network of FDA approved drugs and quantified their interactions with proteins implicated in cardiovascular specific disease modules [40]. Through this initial bioinformatics screen, the authors identified that hydroxychloroquine, an immune-modulatory treatment, has a cardioprotective effect. Conversely, the model also predicted that carbamazepine, an antiepileptic drug, has a cardiotoxic effect. The current shift to a more personalized medicine-centered patient care model calls for taking individual patient genetic and genomic data into account to predict possible drug interactions before prescribing therapies. Using network pharmacology, both novel beneficial effects and adverse reactions in a variety of disease contexts can be predicted.

A network pharmacology approach may also be possible to define novel putative targets of known compounds. Zuo et al. used network pharmacology to predict potential targets of traditional Chinese herbal medicine formulas [41]. Interestingly, in this paper, the authors predicted targets of the herbal compound Yu Ping Feng (YPF). Through target prediction, the authors build a target-pathway interaction network. The authors were able to overlay diseases onto this network based on which diseases corresponded to the dysregulated pathways. Similar to the process by which ranks were assigned to miRNAs that interacted with genes implicated in the known PH network, the authors in this paper calculated a contribution score for the disease modules that were targeted by YPF [41]. The disease modules that contributed the most to a certain disease were scored higher. Although the methodology of screening every known FDA approved drug through such a method could prove laborious, the concept of a contribution score would, in theory, allow one to map dysregulated disease modules to known diseases with more efficiency. More recently, the Loscalzo group expanded upon their previous work and created a Genome-wide Positioning Systems network (GPSnet) algorithm that predicts potential drugs to target disease modules from an individual patient's DNA/RNA sequencing results [42]. This algorithm predicted that ouabain, an anti-arrhythmic drug, targets the HIF1α/LEO1 pathway and can be used in the treatment of lung cancer [42]. These algorithms that use patient sequencing data to predict drugs to target disease modules are be key in the push for more personalized medicine approaches. Especially, with the variability in drug response in PH, these algorithms can be used to repurpose FDA approved drugs for individual patients who are unresponsive to traditional therapies.

4.4 Intersection Between PH and Other Diseases

One of the challenges to developing network-based models for PH is the availability of tissue samples to develop such a network [43]. In other disease models, such as cancer, the plethora of sequencing data from these disease states has helped to build robust networks to predict dysregulated processes. One of the possibilities to overcome this challenge in the field of PH is to correlate similarities between PH and other disease states so that those networks can be used to predict outcomes for PH. To assess the overlap between distinct diseases, the Barabasi group created the "human disease network" where two different diseases, representing two separate

nodes, are connected by an edge if there is one gene that is mutated in both of the diseases [44, 45]. This approach can be translated to the field of PH to reveal other diseases with overlapping genetic associations. Cancer, specifically, provides such an opportunity. It is known that there is overlap between pathological characteristics of cancer and PH including escape from apoptosis, dysregulated anaerobic metabolism (Warburg effect) as well as an increase in glutaminolysis, sustained proliferation, evading growth suppressors, evading immune surveillance, and the inflammatory microenvironment [46, 47]. More recently, dysregulation of similar miRNAs has been implicated in both the development of cancer and PH [48–54]. These similarities between the dysregulated systems in cancer and PH provide the opportunity to translate predicted dysregulated pathways in cancer as targets for this relatively rare vascular disease.

4.5 Use of Electronic Medical Records to Build Disease-Specific Networks for PH

Since PH is a rare disease, recruiting patients for an adequately powered clinical trial, or amassing a repository of clinical samples for research requires ample funding and time [25]. Using electronic medical records for research purposes may address some of these problems. Electronic medical records allow researchers to amass both patients and appropriate controls for studies. Furthermore, the move toward electronic medical records connected to biobanks would expand their usefulness in research. Since 2007, the NIH has funded a move toward integrating biorepositories with clinical data in the electronic medical records. The electronic Medical Records and Genomics Network (eMERGE Network) includes 9 sites as of 2011. The goal of eEMERGE was to allow the connection between genotype and phenotype by building a patient's genotype data into a system that contained their clinical data [24]. This move to integrate sequencing data with clinical data would move EMR data from epidemiologic research only and tie its use into understanding disease pathology.

Recently, groups have started to build networks with EMR data that integrate EMR data with molecular data. Since networks have been used to understand protein-protein interactions, groups have translated this model to analyzing gene associations with clinical symptoms. Hanaeur et al. used the Molecular Concept Map that was used to initially detect associations between genes. The authors built the network with clinical symptoms from clinical problem summary entries of 327,000 patients. The authors used an odds-ratio and p-value analysis to narrow down the list of 750,000 associations and explored a subset that yielded expected and previously unknown associations between diseases [55]. One such association of relevance was the association between non-insulin-dependent diabetes mellitus and hypertension. This method of analysis can be expanded to correlate clinical data with gene maps as was employed by Park et al. The authors constructed a network of Medicare data that mapped disease patterns and overlaid this data with known cellular networks. The authors used this method to identify comorbidities between dysregulated cellular networks and diseases in the Medicare data network [56]. This method expands the network analysis from one institution EMR data to a nationwide clinical database. More importantly, this model allows the association of genes that were implicated in one disease to be linked to other diseases. These studies could provide the basis of exploring the associations of PH with other diseases through both clinical associations and pathological associations. There are, of course, challenges to handling EMR data as there is clinician to clinician variability within how this data is entered as well as institution to institution variability. Working with EMR data and integrating its use into research also warrants discussion on patient privacy, opt-in/opt-out approaches, and the longitudinal nature of the research proposed.

Building networks that integrate both EMR data and molecular genomics provides an exciting opportunity to create connections between clinical phenotypes of disease and pathological genetic changes. To initially develop such a system, a systematic approach to collecting both EMR data and -omics data must be set in place.

One such system is in place at Vanderbilt University Medical Center, one of the institutions participating in the eMERGE study [57]. There, a patient, who is consented for the study at the hospital has his/her blood collected, stored, and later sequenced. This allows for a correlation with that patient's EMR and -omics data with the goal of finding personalized genomic strategies to treatment. Once a large enough database has been amassed correlations between molecular changes and clinical EMR data can be analyzed through associations between the datasets. In this way, clinical parameters such as lab values, hemodynamics measures (increased MAP, increased RV pressure, increased pulmonary pressure gradient, PVR, etc.), pulmonary function testing results, exercise testing measures can be correlated to physiologic disturbances that contribute to the changes in hemodynamic values (intimal hypertrophy, hypoxic vasoconstriction, altered cellular metabolism, etc.). A network approach using this method of mapping EMR data to -omics data from individual patients would be predicted to show clusters according to disease pathologies. For example, hemodynamics altered in PH should cluster with lab values altered in PH. Novel interactions between EMR parameters and sequencing results can unveil unexplored relationships between clinical and molecular data. Likewise, quantitative measures emerging from imaging data corresponding to MRI, CT, and X-ray can be similarly correlated to molecular sequencing data from corresponding tissues. If a systematic approach is taken to quantify radiological data, any relationship between the molecular data and imaging data would provide valuable insight into the phenotypic drivers of disease.

4.6 Conclusion

The development of network biology models for human disease and specifically PH has led to advancements in the field in understanding the dysregulated pathways that contribute to vascular remodeling. Previous work looking at miRNA regulatory networks into PH has yielded new information about how single miRNAs and miRNA families are dysregulated in PH. Network pharmacology approaches have yet to be explored in PH. Such approaches can be used to screen current FDA-approved drugs predicted to work on pathways that are dysregulated in PH such as hypoxia and inflammation in the vasculature bed. Additionally, polypharmacology approaches can be used to screen for a drug regimen, rather than one stand-alone compound, to combat the myriad of dysregulated pathways in PH. So far, research using EMR-based networks have correlated known risk factors to multiple disease pathologies. This can be expanded to include gene maps such as the model that used the Molecular Concept Map to correlate genes implicated in one disease to the pathobiology of PH (Fig. 4.4).

Network biology approaches can fill the gaps that currently exist in research in PH, such as predicting disease development from limited access to tissue samples. The integration of multiple-omics platforms with EMR data will elucidate the process of how these disease processes lead to the development of PH. Current endeavors such as PVDomics, Pulmonary Hypertension Breakthrough Initiative (PHBI), and PAH Biobank are amassing collections of clinical data and patient samples for -omics profiling in PH. PVDomics seeks to create a thorough molecular endotype (genomics, proteomics, metabolomics, etc.) across all pulmonary vascular diseases and correlate these results to WHO classification of PH [58]. The PHBI collects and preserves tissue and blood samples from lung transplant patients with and without PH. Finally, the PAH Biobank archives blood samples from patients specifically with Group I PAH. To further, the progress of cataloging data of dysregulated pathways, cancer databases could also be integrated with PH databases in the future. Ultimately, the time is rapidly approaching when these data sets could offer enough statistical power for appropriate network analysis – thus overcoming the traditional obstacles to PH research and transforming our understanding of the pathogenesis of this disease in years to come.

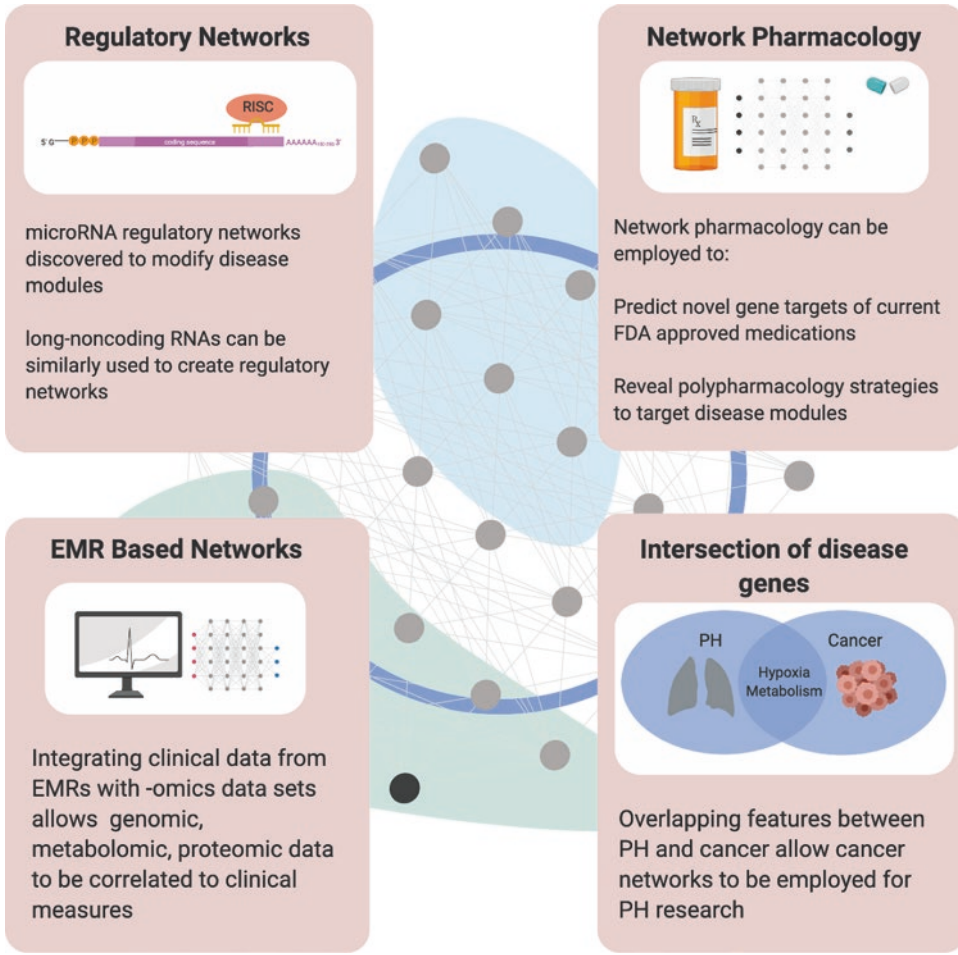

Fig. 4.4 Applications of network biology relevant to PH. (1) Most of the work on network biology applications thus far has focused on gene-gene interactions and miRNA-gene interaction and regulatory networks. (2) Network pharmacology models allow for current FDA-approved therapies to be repurposed to target pathways dysregulated in PH. (3) EMR-based networks facilitate researchers to draw connections between clinical parameters and molecular factors (genes, metabolites, etc.). (4) Network biology approaches to overlap pathways dysregulated in several disease processes, such as cancer and PH, could allow cancer databases to be repurposed for PH research

Acknowledgments Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number T32GM008208. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosures SYC has served as a consultant for Zogenix, Aerpio, and United Therapeutics; SYC is a director, officer, and shareholder in Numa Therapeutics; SYC holds research grants from Actelion and Pfizer. SYC has filed patent applications regarding the targeting of metabolism in pulmonary hypertension. SG-C has no disclosures.

Funding Information National Institutes of Health (R01 HL124021, HL 122596, HL 138437, and UH2 TR002073); American Heart Association (18EIA33900027) (S.Y.C.).

National Institutes of Health (T32GM008208) (SG-C).

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Pulmonary Inflammation and KRAS Mutation in Lung Cancer

5

Phouthone Keohavong and Y. Peter Di

Abstract

Chronic lung infection and lung cancer are two of the most important pulmonary diseases. Respiratory infection and its associated inflammation have been increasingly investigated for their role in increasing the risk of respiratory diseases including chronic obstructive pulmonary disease (COPD) and lung cancer. Kirsten rat sarcoma viral oncogene (KRAS) is one of the most important regulators of cell proliferation, differentiation, and survival. KRAS mutations are among the most common drivers of cancer. Lung cancer harboring KRAS mutations accounted for ~25% of the incidence but the relationship between KRAS mutation and inflammation remains unclear. In this chapter, we will describe the roles of KRAS mutation in lung cancer and how elevated inflammatory responses may increase KRAS mutation rate and create a vicious cycle of chronic inflammation and KRAS mutation that likely results in persistent potentiation for KRAS-associated lung tumorigenesis. We will discuss in this chapter regarding the studies of KRAS gene mutations in specimens from lung cancer patients and in animal models for investigating the role of inflammation in increasing the risk of lung tumorigenesis driven primarily by oncogenic KRAS.

Keywords

Inflammation · KRAS mutation · Tumor microenvironment · COPD · Lung cancer

Abbreviations

AKT	protein kinase B
ALK	anaplastic lymphoma receptor tyrosine kinase genes
BALF	bronchoalveolar lavage fluid
BHT	butylated hydroxytoluene
CCSP	club cell secretory protein, aka CC10
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CXCL5	C-X-C motif chemokine 5
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FOXP3	forkhead box P3
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_5

GTP	guanosine triphosphate
HIF-1α	Hypoxia-inducible factor 1-alpha, aka HIF-1-alpha
ICB	immune checkpoint blockade
IDO1	indoleamine 2,3-dioxygenase
IFN	interferon-γ
IL	Interleukin
KC	keratinocyte chemoattractant
KRAS	Kirsten rat sarcoma viral oncogene
LAG3	lymphocyte-activation gene 3
MAPK	mitogen-activated protein kinase
MCA	3-methylcholanthrene, aka 3-MC
MCP-1	monocyte chemotactic protein 1
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1 alpha
MIP-2	macrophage inflammatory protein 2
mTOR	mammalian target of rapamycin
NDMA	N-nitrosodimethylamine
NNK	nitrosamine4-(methylnitrosamino)-1-(3- pyridyl)-l-butanone
NSCLC	non-small cell lung cancer
NTHi	nontypeable *Haemophilus influenzae*
PAH	polycyclic aromatic hydrocarbons
PD-1	programmed cell death protein 1
PI3K	phosphatidylinositol-3 kinase
PTEN	phosphatase and tensin homologue deleted from chromosome 10
ROS	reactive oxygen species
SCLC	small cell lung carcinoma
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TNM	tumor (T), node (N), metastasis (M)
Treg	regulatory T cell

5.1 Lung Cancer Overview

Lung cancer is the leading cause of cancer mortality in the United States with estimated 228,150 newly diagnosed cases and 142,670 deaths in 2019 [1]. Epidemiological data strongly associate exposure to exogenous factors, chiefly from tobacco smoking, to the increased risk of lung cancer [2–5]. Public education to promote abstinence from tobacco smoking and smoking cessation has gained some momentum in the United States, although tobacco use has continued. Therefore, since lung cancer, like many other cancers, takes many years to develop, smokers and ex-smokers still represent individuals with a high risk of developing lung cancer in the years to come [6, 7].

Lung cancer is a heterogeneous disease that comprises multiple histologic subtypes and mainly includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell lung carcinoma (SCLC). The first three subtypes are termed collectively non-small cell lung carcinoma and have different clinical features from SCLC. About 85% of lung cancer is non-small cell lung cancer (NSCLC), of which lung adenocarcinoma and lung squamous cell carcinoma are the most common histological subtypes [8, 9]. Although tobacco smoking is the most common etiology for lung cancer and accounts for most lung cancer-related deaths [2–5], environmental and occupational exposure to agents such as arsenic, chromium, asbestos, nickel, cadmium, beryllium, silica, diesel fumes, and coal-burning smoke are also known to cause lung cancer [10–12]. In addition, other possible risk factors include acquired lung diseases, infections, family history of lung cancer, hormonal and reproductive factors, and radon gas seems to also increase lung cancer [3]. Regardless of the identification of well-established causal risk factors, cigarette smoking remains the primary risk factor of the global epidemic of lung cancer.

An extensive effort has been made for lung cancer in regard to screening, minimally invasive techniques for diagnosis, and advancement in therapeutics. However, the 5-year survival rate remains low at only 18% [1], as the majority of patients are diagnosed with locally advanced or metastatic disease, in which the curative surgery is no longer feasible [13]. Regardless of curative surgery for early-stage lung cancer, 20–40% of stage I patients will have tumor recurrence, which remains the main cause of cancer-related death [14–17]. Patients with stage I lung adenocarcinoma, which is the most common histological

subtype, vary in survival outcome. It indicates that the current tumor (T), node (N), metastasis (M) staging system fails to distinguish patients with a higher risk of recurrence for stage I disease following surgical resection [18].

Adjuvant chemotherapy has been shown to decrease disease recurrence and prolonged overall survival in patients with stage II-III disease [19–22], but its role in stage I remains controversial and lacks biomarkers for the indication of treatments. In addition, most patients with advanced or metastatic disease are typically treated with cytotoxic chemotherapy with a modest increase in survival. During the last two decades, the discovery of small molecular inhibitors targeting genetic alternations has improved the survival rates for the subsets of cancer patients. Patients with the mutated epidermal growth factor receptor (EGFR) responded to erlotinib or gefitinib, and those with altered anaplastic lymphoma receptor tyrosine kinase genes (ALK) responded to crizotinib [23, 24]. A study showed that the frequency of *EGFR* and *ALK* mutation in lung adenocarcinoma is 27% and < 8%, respectively, although the frequencies vary by region and ethnicity and the majority of lung cancer patients do not contain these genetic alternations [25]. Even though the subsets of patients with these mutations are treated with targeted therapies, they eventually developed resistance within 1–2 years of starting therapy [26]. Immunotherapy such as immune checkpoint blockade (ICB) has been used recently for lung cancer treatment with promising clinical responses, but the response rate is low and only a small subset of patients benefited from the treatment [27] while most patients who responded to initial ICB treatment finally developed resistance. Several mechanisms for acquired resistance to ICBs have been identified including the defects in interferon-γ (IFN) signaling or major histocompatibility complex (MHC) presentation, and the increased levels of the enzyme indoleamine 2,3-dioxygenase (IDO1), which impaired T cell function by the deprivation of tryptophan [28–30].

Overall, major challenges still remain in lung cancer detection and treatment. Extensive efforts have been made during the past decades to better understand the molecular etiology of the initiation and progression of lung tumors and factors that affect the risk of lung tumorigenesis.

5.2 Pathogenesis of Lung Cancer

The development of carcinoma of the lung follows a latent period that spans several decades as the normal respiratory epithelium is exposed to various carcinogens. The response of the normal mucosa to these stresses is believed to be a predictable progression from high-grade dysplasia to carcinoma in situ and eventually resulting in invasive carcinoma [31, 32]. There is an average period of 4–5 years during which time individuals exfoliate markedly atypical cells (that actually represent carcinoma in situ) into the bronchial secretions before the progression to an invasive carcinoma [33, 34].

The progression from normal to initiated cells to invasive tumor is a long and multiple stage process which takes multiple years and proceeds presumably through a series of molecular events leading to an accumulation of genetic variation including mutational, chromosomal, and epigenetic changes [35–39]. In this paradigm, one major pathway to malignant transformation involves structural alterations of cancer-related genes. These genes have been divided into two categories based on whether the gene function is gained or lost. The first involves activated growth-promoting genes (oncogenes), and the second involves inactivated genes that are normally responsible for growth control in the cell (tumor suppressor genes) [40–42].

A large number of oncogenes have been identified, but those that play the most prominent role in cancers are the closely related H-, K-, and N-*RAS* genes [43, 44]. These genes encode for closely similar monomeric 21-kd guanosine nucleotide-binding proteins (RAS) with a weak intrinsic GTPase activity [45–50]. They are related to the G proteins that bind guanine nucleotides with high affinity and are located at the inner surface of the cell membrane, and they play an important role in signal transduction pathways

[47, 50]. The wild type KRAS, once activated by external stimuli, switches from an inactive guanosine diphosphate (GDP)-bound to an active guanosine triphosphate (GTP)-bound conformation. The *RAS* activation switch is catalyzed by the guanine nucleotide exchange factor SOS1 that displaces the GDP, allowing the protein to bind to a GTP. This GTP-bound RAS activates multiple downstream effectors, including those involved the RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK, and the phosphatidylinositol-3 kinase (PI3K)/−protein kinase B (Akt)/mTOR signaling cascades, and thus regulates cell growth/differentiation and apoptosis, respectively. To terminate the downstream signaling, the active RAS protein catalyzes the hydrolysis of the GTP to GDP through its intrinsic GTPase and returns to its inactive state. The catalytic reaction can be increased by the binding of the RAS protein to a GTPase-activating protein, P120GAP, enhancing the GTPase activity and, thereby, accelerating the conversion of GTP-bound to the GDP-bound ras conformation [49, 51–54].

Activation of the *RAS* genes occurs with specific point mutations at only a very few codons, including codon 12, 13, or 61. In wild type *RAS*, amino acids at codons 12, 13, and 61 are in direct contact with the phosphoryl group of GTP and are involved in the catalytic reaction of GTPase. A mutation occurring at any of these codons will induce structural changes within the *RAS* protein, resulting in a reduction or loss of GTPase activity and an activated mutant *RAS*. As a consequence, in cells with a missense mutation at codon 12, 13, or 61 of the *RAS* gene, the mutant *RAS* will remain in its active GTP-bounded state, and constitutively will activate its downstream signaling pathways, thereby increasing abnormal cell growth and differentiation and the risk of tumorigenesis [52, 53, 55]. However, this concept has been questioned because the type of mutation occurring at these codons may affect the ability of guanosine triphosphate to bind to mutant *RAS* [52, 53, 56].

The frequencies and types of mutated *RAS* genes have been found to vary among tumors, depending on the tissue of origin. For instance, KRAS gene was the most frequently mutated in lung, colon, and pancreas tumors [57, 58].

5.3 KRAS Mutations in Lung Tumors

There have been extensive studies of KRAS mutations in lung tumors and in other specimens from lung cancer patients, most of them were smokers, from the United States and other parts of the world. These studies showed that KRAS mutations were identified more frequently in the adenocarcinoma subtype (15–30%) and less so in other histological phenotypes, including squamous lung tumors (3–5%) [59–64]. The data also showed that gender did not affect the incidence of KRAS mutations in the lung adenocarcinomas from smokers. For comparison, nonsmoking lung cancer patients are mostly women, while their lung tumors are mostly of the adenocarcinoma subtype that less frequently harbored KRAS mutations (5–15%), compared with smokers. These data suggest that KRAS mutations are primarily associated with exposure to tobacco smoke. The reasons for these varied KRAS mutation frequencies in lung adenocarcinomas are unclear, although differences in sample size, methods used for DNA preparation and mutation detection, and geographical differences may play a role.

The mutations in lung tumors from smokers consisted predominantly of a G to T transversion (~60%), whereas a G to A transition accounted for ~30%. A transition is the conversion of purine (A, G) to another purine base or pyrimidine (C, T) to another pyrimidine base whereas transversion is the conversion of a purine into a pyrimidine or vice versa. The consistent predominance of a G to T transversion in the KRAS gene in lung tumors has been reported in several studies [60, 65, 66] and is characteristic of lung tumors, in contrast to other cancer types, such as colorectal carcinomas, in which the G to A transition predominated [67, 68]. The predominance of G to T transversion, along with the prevalence of the KRAS mutation in the smoking population, sug-

gests that carcinogens in cigarette smoke, in particular the polycyclic aromatic hydrocarbons, (PAHs) may cause these mutations. For instance, benzo(a)pyrene is a known carcinogen that forms adducts with deoxyguanine residues in DNA and to induce mostly G to T transversion in several in vitro systems [69, 70]. The varying incidence of G to A transitions found in the different studies reflects differing carcinogenic exposure, such as exposure to radon or nitrosamines, that can cause this type of mutation. In addition, nicotine can be activated to the nitrosamine 4-(methylnitrosamino)-1-(3- pyridyl)-l-butanone (NNK), which could contribute to G to A transitions observed in some smokers. In mice, NNK, one major tobacco smoke carcinogen, caused mutations in the KRAS gene of lung adenocarcinomas that were almost exclusively G to A transitions in codon 12 [71].

Compared with smokers, the KRAS mutations in lung tumors from nonsmokers consisted mainly of G to A transition [64, 72], suggesting different mutagen origins and/or mechanisms of tumorigenesis in nonsmokers who were mostly women. Nevertheless, most lung cancer is caused by exposure to smoke carcinogens from tobacco and/or from other sources. For instance, there was a high incidence of lung tumors among female nonsmokers in Xuan Wei County (XWC), Yunnan Province, China. These women were exposed to smoky coal emissions for generations, and their lung cancer rate was 5-fold and, in some communes, up to 24-fold greater than the Chinese national average. Investigation of KRAS mutations revealed that their lung tumors carried KRAS mutations, consisting highly of G to T transversion at codon 12 [66] (87–100%). Household fuel surveys indicate that lung cancer was highly correlated with the use of generations of "smoky coal" for domestic combustion [73, 74]. Smoky coal is a low-sulfur (0.2%) medium-volatile bituminous coal used for cooking and heating in XWC homes without chimneys. Characterization of the indoor air from homes using smoky coal showed that XWC residents were exposed to high concentrations of submicron particles that contain mostly organic matter, including large amounts of mutagenic/carcinogenic PAHs [75–77]. These results point to a strong etiologic link between exposure to smoky coal combustion and the high rate of lung cancer harboring KRAS mutations in women living in XWC.

5.4 KRAS Mutation Type and Status in the Prognosis of Lung Cancer

In spite of being studied for many years for their role in lung tumorigenesis, only recently have an increasing number of studies shown evidence of prognostic and predictive values of KRAS mutations in lung cancer, although the results were not consistent across studies. For instance, some studies showed that lung tumors with KRAS mutations were more likely to be resistant to therapies [78–82] and to engraft in immunodeficient mice and predict disease recurrence [83] than those without these mutations. Furthermore, it has been suggested that the different KRAS mutation types could lead to different oncogenic KRAS variants.

In lung tumors, KRAS mutations were found primarily at codon 12 (~93%), where cysteine, valine, and aspartate accounted for about 80% of the amino acid changes that substituted for the wild type glycine [60, 84]. These KRAS variants could possess distinct biologic manifestations, including their signaling pathways, transforming potential, and treatment outcomes. For instance, patients with tumors harboring a substitution of codon 12 of arginine, cysteine, aspartate, or valine had a poorer outcome than those whose tumors contained wild type or other amino acids. However, the results were not always consistent among studies, likely reflecting on the small numbers of the relevant amino acid substitutions and patient populations involved in these studies [60, 84].

On one hand, several other studies showed that patients with some types of KRAS mutations had significantly poorer survival, compared with patients with KRAS wild type. In particular, mutant cysteine substitution at codon 12 was associated with poor prognosis, compared with

other mutant KRAS or wild-type KRAS [79–82].

On the other hand, other studies showed there were no differences in prognostic value based on the type of KRAS amino acid substitution present, or the mutant KRAS variants versus wild type KRAS [85, 86]. These discrepancies across studies may reflect from a heterogeneity regarding the stages, the histology, and the treatment modalities of lung cancer. Furthermore, the different methods used for DNA preparation and mutation analysis could also explain the varying results from the different studies.

5.5 Mutant KRAS Signaling in Lung Tumorigenesis

Studies have shown that human lung tumors with activating KRAS mutation have higher levels of inflammation, compared with lung tumors without these mutations [87]. This indicates a link between activating KRAS and tumor-associated inflammation. In lung cancer, it has been suggested that KRAS mutation is important in the initiation but may not be sufficient for an effective and complete development of lung adenocarcinoma [88–94]. Lung tumorigenesis initiated by oncogenic KRAS may be further promoted or inhibited by genetic/epigenetic events that activate or suppress other signaling pathways. Factors capable of controlling such events and pathways may impact the development of an initiated cell into a malignant tumor and, therefore, lung tumor incidence.

Several mouse models have been developed to investigate the molecular pathways to lung tumors driven by mutant K-ras [87–94] (mouse homolog of human KRAS). One of such studies used a cohort of conditional mutant mice in which the aspartate substitution at codon 12 of allele of K-ras (K-ras^{G12D}) was expressed specifically in mouse CC10-positive bronchiolar epithelial cells [87]. The activation of oncogenic K-ras^{G12D} in these cells led to the development of lung adenocarcinoma. These cells produced inflammatory chemokines, characterized by the production of Macrophage Inflammatory Protein-2 (MIP-2), C-X-C motif chemokine 5 (CXCL5, LIX), and keratinocyte chemoattractant (KC) by cell lines established from the mouse lung tumors, and by the increase of these chemokines in the mouse bronchoalveolar lavage fluid (BALF). These chemicals attracted neutrophils and macrophages within the mouse lung, generating lung inflammation and a pro-tumorigenic environment within the lung.

Other studies showed that mutant KRAS cooperates with alterations of other genes, including the loss of tumor suppressor gene phosphatase and tensin homologue deleted from chromosome 10 (PTEN), one of the components regulating the PI3K/Akt pathway [80–82, 93, 95, 96]. For instance, human NSCLC cell lines that express no detectable PTEN frequently had KRAS mutations, suggesting that alteration in both genes confers a selective advantage in these cells [93]. Another study used mouse models of CCSP-driven expression of oncogenic *K-ras* (Pten$^{\Delta 5/\Delta 5}$; Kras$^{Lox/+}$; CCSP$^{Cre/+}$), in which conditional oncogenic K-ras and Pten null alleles can be targeted specifically in the CCSP-expressing bronchial epithelium. It was demonstrated that, by itself, Pten inactivation had no discernible effect, but it accelerated lung tumorigenesis initiated by oncogenic K-ras. The tumor microenvironment in these mice was enriched in endothelial cells and inflammatory cells and that the lungs expressed high levels of chemokines and growth factors [93]. It has been shown that the interaction between Pten loss and K-ras mutant alters PI3K pathway regulation, enhances the activation of the nuclear transcription factor NF-κB [95–97], up-regulation of downstream cytokines, and creates an inflammatory environment within the lung.

NF-κB plays an important role in the regulation of the expression of genes involved in inflammation, immune responses, cell cycle, apoptosis, and angiogenesis in a variety of cells, including epithelial cells, and deregulated NF-κB plays an important role in tumorigenesis [98–104]. Increased NF-κB activity correlates with expression of oncogenic KRAS and that the p65/RelA subunit of NF-κB is an important oncogenic KRAS effecter in lung cancer [105–107]. For

instance, in mouse studies, activation of the NF-κB pathway has been detected during KRAS oncogene-driven lung adenocarcinoma [105, 108]. Inhibition of NF-κB signaling in the airway epithelium significantly reduces the formation of lung tumors [100], while NF-κB activation in the lungs markedly increases tumor formation [109, 110]. This supports the concept that activation of NF-κB pathway plays an important role in lung carcinogenesis.

5.6 Extrinsic Inflammation Promotes Mutant KRAS-Initiated Lung Tumorigenesis

Inflammation is an essential process for host immune responses to prevent pathogen invasion and also involves in wound healing. However, persistent and uncontrolled inflammatory responses are associated with active recruitment of inflammatory cells and the production of mediators such as cytokines, chemokines, growth factors, and matrix-degrading enzymes leading to inflammatory microenvironment [111]. It has been reported that "smoldering" inflammation in the tumor microenvironment has many tumor-promoting effects such as tumor-cell migration, invasion, metastasis, epithelial-mesenchymal transition, and angiogenesis [112]. In addition, chronic inflammation also induces immunosuppressive mechanism associated with accumulation of suppressive cells like myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) as well as the increased immunosuppressive mediators such as Interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β), which help tumors escape from immune surveillance [113, 114]. Although the exact mechanisms of inflammation in promoting lung cancer remain unclear, two hypotheses proposed that an intrinsic pathway driven by genetic alternations leads to neoplasia and inflammation, and an extrinsic pathway driven by inflammatory conditions leads to increased cancer risk [112]. Several mouse models have been applied to explore the effects of extrinsic pulmonary inflammation induced by several agents on tobacco smoke carcinogen-mediated lung tumorigenesis.

Witschi et al. [115] evaluated the effects of the food additive phenolic antioxidant, butylated hydroxytoluene (BHT), on the development of lung tumor in male Swiss-Webster mice following exposure to the tobacco smoke carcinogen urethane. The development of lung tumors was enhanced by a single injection of urethane and chronic exposure to BHT. BHT acted as a promoting agent, as it effectively enhanced tumor formation in mice exposed to BHT after being injected with urethane, but not if they are treated with BHT before urethane injection. This suggested that this mouse treatment system provides an example of two-stage carcinogenesis consisting of initiation by a carcinogen and promotion by BHT [116].

Another study examined the effects of chronic BHT exposure following a single injection of 3-methylcholanthrene (MCA) into BALB/c mice. It was found that treatment with low doses of MCA in this strain does not induce lung tumors, unless BHT exposure follows MCA treatment. BHT administration promotes a 3-fold increase in urethane-induced lung tumor multiplicity [116, 117]. However, BHT administration promotes lung tumor formation only in BALB/c mice but not in CXB4 mice [118]. The MCA/BHT protocol in BALB/c mice thus offers an experimental model for determining the biochemical and cellular nature of how BHT stimulates the selective clonal expansion of initiated cells [117].

Administration of BHT to BALB/c and A/J mice at doses higher than 150 mg/kg caused infiltration of inflammatory cells into the alveoli [119, 120], followed by the reversible pneumotoxicity to mice. In addition, Bauer et al. [118] showed that BALB/c mice treated with BHT developed strong inflammatory responses characterized by transudation of proteins from the blood into the BALF, and an influx of macrophages and lymphocytes into the airspaces. There were also elevated pulmonary concentrations of cyclooxygenase-1 (COX-1) and COX-2 and increased prostaglandin synthesis [118]. For comparison, the CXB4 mice that did not show

any increase in lung tumor formation following treatment with the MCA/BHT protocol were found to be resistant to all of the BHT-mediated increases in inflammatory parameters that occurred in BALB/c mice [118].

Matzinger et al. [121] evaluated the two-stage model of lung tumorigenesis in A/J mice treated with the tobacco smoke carcinogen NNK. They demonstrated that BHT promotes an increased multiplicity of the mouse lung tumors, following NNK exposure. Furthermore, there were some differences in the K-ras mutation patterns identified in the lung tumors. While all mutations in the non-BHT-treated mice consisted of G to A transition occurring at the second base of K-ras gene codon 12, only about one-third of the mutations found in the BHT-treated mice were of this type. This suggests that the NNK-initiated lung tumorigenesis in these mice was altered by BHT-tumor promotion, from oncogenic K-ras-driven pathway to a non-K-ras mechanism.

Wang and Witschi [122] compared the promoting effects of BHT on lung tumorigenesis initiated by urethane or MCA in two mouse strains, male A/J and Swiss-Webster (SWR). MCA predominantly produces K-ras mutations in codons 12/13, whereas urethane affects codon 61 in these mice. Furthermore, in the A/J mice, unlike the findings using NNK/BHT by Matzinger et al. [121], both urethane and MCA induced K-ras mutations in lung tumors, and BHT treatment induced an increased frequency of K-ras mutations in both mouse strains. This result suggests that BHT promotes the activation of K-ras gene in lung tumors in A/J mice.

Other inflammation-inducing agents have also been investigated. Freire et al. [123] studied early molecular changes associated with lung tumorigenesis in a silica-induced chronic inflammatory microenvironment. Female BALB/c mice were treated by oropharyngeal aspiration with a single low dose of the tobacco smoke carcinogen *N*-nitrosodimethylamine (NDMA), silica, a combination of both, or saline [124]. They demonstrated that silica-induced strong inflammatory responses, characterized by increased expression of programmed cell death protein 1 (PD-1), TGF–β1, monocyte chemotactic protein 1 (MCP-1), lymphocyte-activation gene 3 (LAG3), forkhead box P3 (FOXP3), and the presence of regulatory T cells, compared with mice treated with NDMA alone. This created an immunosuppressive microenvironment favoring NMDA-induced development and progression of lung tumors in co-treated mice. There was also an increased incidence of lung tumors and multiplicity in mice treated with NDMA and silica, compared with those treated with NMDA alone. However, the mutational pattern was different between the NDMA-only and NDMA+silica–induced tumors. Specifically, the K-ras mutations in tumors from mice treated with NDMA+silica was primarily G to A transition in codon 12, while A to G transition in codon 61 was the most frequent alteration in mice treated with NDMA alone. Histopathologic analysis showed that tumors from mice treated with NDMA+silica accumulated more anergic and regulatory T cells, characterized by the expression of the PD-1 and Foxp3 markers, respectively, compared with tumors from mice treated with NDMA alone. The predicted reduction in tumoricidal T-cell activity associated with these changes is consistent with the escape of cancer cells from immune elimination. This led the authors to conclude that silica-induced chronic inflammation facilitates the development of preneoplastic lesions and subsequently lung cancer.

5.7 Bacteria-Induced Airway Inflammation and Lung Tumorigenesis

COPD is an independent risk factor for lung cancer [125–128], and the airways of COPD patients are commonly colonized by nontypeable *Haemophilus influenzae* (NTHi). Moghaddam et al. [129] showed that repeated exposure of mice to an aerosolized NTHi lysate causes lung inflammation with a profile of mediators and inflammatory cells similar to that observed in patients with COPD. In their follow-up study, they evaluated the effects of this NTHi-induced COPD-like inflammation on mouse models of lung cancer induced by K-ras mutant expression

in airway epithelial cells [130]. NTHi exposure results in leukocyte recruitment and increase in cytokines and chemokines in BAL. Furthermore, this NTHi-mediated, extrinsic COPD-like airway inflammation plays a role in the promotion of lung cancer in one of their mouse models, $CCSP^{Cre}$/LSL–K-ras^{G12D}, resulting in a 3.2-fold increase in lung surface tumor number. In addition, NTHi lysate challenge resulted in a shift from macrophage-predominant to neutrophilic airway inflammation in this mouse model, which is associated with significant tumor promotion.

The promoting effect of COPD-like inflammation on lung carcinogenesis was also assessed by using a mouse model with late-onset and low multiplicity lung tumor formation, combining exposure to NNK with NTHi exposure [131]. This mouse model is based on the knockout of the retinoic acid-inducible G protein-coupled receptor [132]. NTHi exposure is associated with activation of NF-κB, release of inflammatory mediators, recruitment of innate (neutrophil and macrophages) and adaptive inflammatory cells, and activation of Hypoxia-inducible factor 1-alpha (HIF-1α), HIF-1α-mediated angiogenesis. Mice exposed sequentially to NNK and NTHi showed a 3.5-fold increase in the multiplicity of surface lesions, compared with mice exposed to NNK alone [131]. Furthermore, a separate study showed that K-ras mutant-mediated lung tumorigenesis and its promotion by COPD-like airway inflammation is associated with significant tumor angiogenesis and activation of HIF-1α [133].

Our group evaluated the effects of inflammation induced by a bacterial component, the lipopolysaccharide (LPS) on lung tumorigenesis caused by exposure of FVB/N mice to NNK [71]. LPS is an endotoxin and a major cell wall component of gram-negative bacteria [134–136]. LPS also is an agonist for innate immune response through activation of the toll-like receptor 4 (TLR4) signaling cascade. Exposure to LPS has been shown to lead to a production of both pro- and anti-inflammatory mediators by myeloid lineage and other cell types including epithelial cells. It has been suggested that LPS is involved in bacterial infection-induced exacerbations of COPD and contributes to the progression of the disease [137]. The recurrent LPS instillation in our mouse model resulted in a promotion of neutrophil and macrophage-dominant chronic inflammation in both LPS + NNK- and LPS-treated mice that is similar to what has been observed in COPD patients.

Inflammatory cell counts in the BAL, including macrophages, neutrophils, and lymphocytes, were significantly increased in the LPS + NNK treatment group. The BAL fluid of chemokines/cytokines, as analyzed by Luminex assays, revealed higher levels of IL-17, CXCL10, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein 1 alpha (MIP-1α), and KC in LPS + NNK than in NNK treatment group [138, 139]. Flow cytometry analysis of the mouse lung tissue revealed that combined LPS and NNK exposure significantly increased $CD4^+$ T cells including Th1, Th17, Tregs, and MDSCs recruitment in the lung. T cell exhaustion related genes, including *Pdcd1, Ctla-4, Tim-3, Lag-3*, and *Foxp3*, and PD-L1 protein were significantly upregulated in the LPS + NNK treatment than NNK treatment. Our data suggest that chronic LPS exposure-promoted and NNK-induced lung tumorigenesis is associated with immunosuppressive tumor microenvironment. The changes include recruitment of Tregs and MDSCs, increased T cell exhaustion, and upregulated PD-1/PD-L1 pathway [138, 139].

We demonstrated that mice treated with LPS alone did not lead to any tumor formation, while mice treated with LPS + NNK developed an averaged 8-fold of synergistically increased incidence of lung tumors, compared with mice treated with NNK alone. There was also an increased rate of K-ras mutation in the tumors of LPS + NNK-treated mice, compared with mice treated with NNK alone (72% vs. 45%, respectively) using FVB/N mice. The mutations all involved the first G/C of the codon 12 of the K-ras gene and consisting of primarily G to A transition. These results suggest that LPS-induced inflammation enhanced the development and progression of K-ras mutant-mediated lung tumorigenesis in LPS + NNK-treated mice [71]. In our lung cancer

model where both LPS and NNK were administered simultaneously, it is likely that LPS-induced inflammation affects the promotion step of NNK-induced lung tumorigenesis. In a later study, Melkamu et al. similarly examined the effects of LPS on NNK-induced lung tumorigenesis in an A/J mouse model. The authors also showed that administration of LPS to NNK-pre-treated mice caused inflammatory responses and a significantly increased tumor multiplicity in the lungs, suggesting that LPS-induced inflammation acts in the promotion stage [139].

5.8 Persistent Inflammation Induces KRAS Mutation with Various Genotypes

It has been suggested alveolar macrophages play an important role in mediating the effects of LPS that enters the lungs. During the earliest event in LPS-induced inflammation, LPS is transferred to its cellular receptor complex formed between toll-like-receptor-4, pattern recognition receptor CD14, myeloid differentiation-2, and LPS-binding protein, leading to the signaling of the cellular interior and activation of the alveolar macrophages [140–143]. This leads to a pro-inflammatory cascade defined by the production of specific pro-inflammatory cytokines, such as tumor necrosis factor (TNF), followed by induction of IL-1α and IL-6 [144–146], recruitment of neutrophils to the wound, and a rapid neutrophil infiltration into the lung tissue and airspace [147–149]. In addition to macrophages and neutrophils, other studies suggested that airway epithelial cells, including Club cells and alveolar type II cells [150–153], are capable of producing a variety of pro-inflammatory cytokines that participate in the innate immune responses.

Therefore, extrinsic inflammation induced by various agents promotes lung tumorigenesis initiated by tobacco smoke carcinogens, characterized by a heightened inflammatory response and an increased lung tumor incidence, compared with mice treated with the carcinogen only. However, there were some differences in the K-ras mutational frequencies, patterns, or types in lung tumors without and with treatment with an inflammatory agent (e.g., LPS or BHT) that may indicate that extrinsic inflammation could alter the tumorigenic pathways initiated by a carcinogen. For instance, Matzinger et al. [121] found that the K-ras mutation rate was significantly lower in tumors produced by NNK + BHT than NNK alone. In our study, however, there was a significant increase in both the incidence of lung tumors and the mutation rate of K-ras-positive lung tumors developed in LPS + NNK-treated mice, compared with mice treated NNK only [71, 138, 139]. We also observed a slight change of K-ras mutation type in our study where a subset of G to A transition was identified in mice treated with LPS + NNK but was absent from mice treated with NNK alone [71, 139].

The reasons for the differences in K-ras mutation rates, types, and patterns in lung tumors following different inflammation-promoting agents' treatment are unclear. Mouse strains, carcinogens, inflammatory agents, and their associated inflammatory response patterns could all be a factor. For instance, all K-ras mutations identified in lung tumors from both A/J mice [121] and FVB/N mice [71] treated with NNK only were G to A transition at position 2 of codon 12, but only BHT-treatment altered the tumorigenic pathways from K-ras to a non-K-ras mechanism [121]. Nevertheless, the study by Wang and Witschi suggested that inflammatory agents may not be necessarily a critical factor [122]. Other underlying mechanisms may explain the differences observed. Inflammatory responses, especially induced by agents such as silica and LPS, produce reactive oxygen and nitrogen species that result in oxidative DNA damage, and also inhibition of DNA repair enzymes [154–157]. For instance, reactive oxygen species (ROS) can cause modified bases, apurinic/apyrimidinic sites, and strand breaks. It has been shown that oxygen free radicals and other oxidative agents cause activating K-ras mutations consisting mostly of G to T transversion [158, 159]. A fraction of the K-ras mutations found in tumors from mice treated with LPS + NNK in our study consisted of G to T transversion, compared with none in the NNK-treated group, suggesting that

some of the lung tumors may be initiated by this oxidative pathway following administration to inflammation-promoting agents.

5.9 Conclusion

KRAS is a potent oncogene and is mutated in about 25% of all lung cancers. Despite substantial progress made with regard to the cancer treatments, effective cures of the KRAS-associated cancers remain lacking and the KRAS mutation still indicates poor prognosis. Unlike EGFR mutations and ALK rearrangements that now have relatively effective therapies, KRAS mutations are still perceived as "undruggable." Oncogenic KRAS induces inflammation from tumor cells through intrinsic mechanisms, but extrinsic inflammation also results in increased KRAS mutations. A vicious cycle of chronic and persistent inflammation together with increased KRAS mutations creates an immunosuppressive microenvironment that potentiates lung tumorigenesis. Tolerogenic inflammatory cells including T cell exhaustion in KRAS-mutated tumor microenvironment further promote cancer progression. Since KRAS mutation-related lung cancers are strongly associated with inflammation, modulation of inflammatory response could be a target for therapeutic intervention including checkpoint blockade-based immunotherapy.

Acknowledgments This research is supported by NIH awards HL125128 and AI133351 (to YPD).

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MicroRNA Targets for Asthma Therapy

6

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Abstract

Asthma is a chronic inflammatory obstructive lung disease that is stratified into endotypes. Th2 high asthma is due to an imbalance of Th1/Th2 signaling leading to abnormally high levels of Th2 cytokines, IL-4, IL-5, and IL-13 and in some cases a reduction in type I interferons. Some asthmatics express Th2 low, Th1/Th17 high phenotypes with or without eosinophilia. Most asthmatics with Th2 high phenotype respond to beta-adrenergic agonists, muscarinic antagonists, and inhaled corticosteroids. However, 5–10% of asthmatics are not well controlled by these therapies despite significant advances in lung immunology and the pathogenesis of severe asthma. This problem is being addressed by developing novel classes of anti-inflammatory agents. Numerous studies have established efficacy of targeting pro-inflammatory microRNAs in mouse models of mild/moderate and severe asthma. Current approaches employ microRNA mimics and antagonists designed for use in vivo. Chemically modified oligonucleotides have enhanced stability in blood, increased cell permeability, and optimized target specificity. Delivery to lung tissue limits clinical applications, but it is a tractable problem. Future studies need to define the most effective microRNA targets and effective delivery systems. Successful oligonucleotide drug candidates must have adequate lung cell uptake, high target specificity, and efficacy with tolerable off-target effects.

Keywords

Antisense · Asthma · House dust mite · LNA · MicroRNA · Oligonucleotide · Ovalbumin · RNAi · Therapy

Abbreviations

AHR	airway hyperreactivity
APC	antigen presenting cell
ASM	airway smooth muscle
BALF	bronchoalveolar lavage fluid
CDG	cyclic diguanosine monophosphate, bis-(3′-5′)-cyclic dimeric GMP
HDM	house dust mite
IFN	interferon
IL	interleukin
LNA	locked nucleic acid

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_6

mAb	monoclonal antibody
miR	microRNA
mRNA	messenger RNA
OVA	ovalbumin
RISC	RNA-induced silencing complex
Th1	type 1 helper
Th17	type 17 helper
Th2	type 2 helper
Th2high	type 2 high
Th2low	type 2 low

6.1 Introduction

Asthma is a chronic obstructive disease defined by symptoms of wheezing, chest tightness, and shortness of breath. There is typically evidence of airway hyperreactivity and reversible airway obstruction. These hallmarks of asthma are thought to be due to inflammation of the airways and the vasculature, bronchoconstriction, and airway remodeling. Asthma was originally thought of as a single disease for which the efficacy of treatment was and is limited. The heterogeneity of asthma endotypes has recently been appreciated, and the resulting variability in phenotypes has undergone careful reclassification [1–3]. The concept of asthma endotypes that differ in severity, triggers, and treatment responses has revised the approach to developing novel, more targeted therapies. The standard of care for asthma is bronchodilators that temporarily reverse contraction of airway smooth muscle and inhaled corticosteroids to inhibit inflammation. Yet up to 10% of all asthmatics are steroid and/or beta adrenergic insensitive. This cohort makes up at least 50% of the health care cost associated with the treatment of asthma and can lead to fatal asthma [4, 5]. One important limitation of current therapy is that pathological airway remodeling is not reversed. To identify new targets to reverse obstructive airway remodeling, it may be useful to think beyond the current one drug-one target paradigm. It will be useful to develop drugs that target multiple aspects of remodeling including, mucosal metaplasia, smooth muscle hypertrophy and hyperplasia, interstitial fibrosis, and basement membrane thickening. To treat the multifactorial aspects of airways remodeling, it might be better to target molecules that regulate many pro-inflammatory molecules and biochemical processes. For this purpose, microRNAs are ideal molecular targets because they regulate expression of protein networks thus influencing multiple physiological pathways. The networks and pathways controlled by microRNAs contribute to both normal function and disease pathogenesis. In most diseases studied so far, there are sets of microRNAs dysregulated when compared to the healthy state. These dysregulated microRNAs can be either upregulated or downregulated as compared to a non-disease state. This is not to say microRNA dysregulation initiates disease progression but rather it may be an adaptive (or maladaptive) effect of the disease. Asthma is a disease where expression and function of many microRNAs are dysregulated (see Table 6.1). In this chapter, we will describe features of asthma pathology influenced by dysregulated microRNAs, the animal models used in asthma drug development, and the preclinical studies of microRNA-based drugs for asthma.

6.2 Lung Inflammation in Asthma

Asthma is associated with environmental triggers including cold air, air pollutants, molds, pollen, animal dander, and dust mite allergens. Many of these triggers elicit an allergic response in atopic asthmatics. Repeated allergen exposure causes type I hypersensitivity (immediate sensitivity). In atopic asthma, allergens activate an immune response by first being taken up and processed by antigen-presenting cells (APCs). The APCs then present the allergen peptide to Th0 cells which signal IL-2 and IL-4 release to differentiate into a Th2 cell. The Th2 cells stimulate B cells with the release of IL-4 and IL-13. The B cells then release IgE that binds to Fcε receptors on mast cells and structural cells in the lung and are "sensitized." Subsequent exposure of the same allergen cross-links bound IgE on mast cells. The cross-linking results in degranulation and secretion of inflammatory mediators including histamine, leukotrienes, and prostaglandins. The released

Table 6.1 MicroRNAs and validated targets in asthma and asthma models

microRNA	Targets	References
MiR-9 ↑	↓ Protein phosphatase 2A regulatory subunit B	[80]
miR-15a ↓	↑ VEGFA (vascular endothelial growth factor A)	[81]
miR-16 ↑	↓ ADRB2 (beta2 adrenergic receptor)	[82]
miR-19a ↑	↓ TGFβ2R (transforming growth factor beta2 receptor)	[83]
miR-21 ↑	↓PDCD4 (programmed cell death protein 4)	[84, 85]
	↓ PTEN (phosphatase and tensin homolog)	[86]
miR-23a ↑	↓ BCL2 and CXCL12	[87]
miR-34/449↓	↑ Notch, mucosal metaplasia	[88]
miR-106a↓	↑ IL-10	[89]
miR-126 ↑	↓ OBF.1(Oct binding factor 1) → ↓ GATA3	[90]
miR-133a ↓	↑ Rho A (Ras homolog gene family, member A)	[91]
miR-145 ↑	↓ KLF 4/5 (Kruppel-like factor 4/5)	[68, 92]
	↓ RUNX3 (runt-related transcription factor 3)	[93]
miR-146a ↓	↑ CCL20 in airway smooth muscle	[94]
miR-146b ↑	↑ TNFSF9 (tumor necrosis factor superfamily, member 9)	[65]
miR-155 ↑	↓ SOCS1 (suppressor of cytokine signaling 1), Pu.1, c-Maf	[95]
	↓ COX-2 (Cyclooxygenase-2)	[72, 96]
miR-181b ↓	↑ SPP1 (secreted phosphoprotein 1, osteopontin)	[97]
miR-221 ↑	↓ p21 and p27 (cyclin-dependent kinase inhibitors p21 and p27)	[71]
miR-221-3p	↑ CXCL17 (C-C-C motif chemokine ligand 17)	[98]
miR-218-5p↓	↑ CTNND2 (δ-catenin)	[99]
miR-223 ↑	↓ FOXO3 (Forkhead box O3)	[68]

↑ – indicates a validated upregulation
↓ – indicates a validated downregulation

inflammatory mediators trigger a cascade of events that includes bronchoconstriction, immune cell recruitment, and mucus secretion.

The general approach to treating asthma has been the same for more than 100 years: allergen avoidance, acute rescue treatment, and longer-term control treatment. Once physicians understood that there were environmental triggers, the standard of care was to avoid the triggers. Prior to 1850, identifying environmental triggers was done by taking a careful history, and sufferers were then instructed to avoid such triggers. Once skin tests of allergen sensitivity were developed for diagnosis of hay fever, the techniques were applied to allergens of asthma sufferers. While somewhat effective allergen avoidance is not always possible nor is it optimally effective. Bronchodilators and inhaled corticosteroids are now used routinely as rescue and prevention therapies, respectively. Additional asthma therapies, leukotrienes antagonists, short- and long-acting muscarinic antagonists, phosphodiesterase inhibitors, and several antibody-based therapies have been developed and optimized in the past 50 years. But there are still limitations due to the heterogeneity of asthma endotypes. Understanding the different asthma endotypes and careful phenotyping of asthma patients provides an opportunity to develop better drugs [3].

6.2.1 Asthma Endotypes

Asthma is not a homogeneous disease, but rather a heterogeneous syndrome divided into endotypes [6]. An endotype is a disease subtype defined by its pathophysiological or functional mechanism [1]. Asthma can be broadly divided into two endotypes, type 2 high ($Th2_{high}$) and type 2 low ($Th2_{low}$) [6]. $Th2_{high}$ is what is classically thought of as allergic asthma with an eosinophilic response, meaning that the asthmatic responds to an allergen that results in an increase in the number of eosinophils recruited to the lung [1, 6]. It is now clear that asthma is more than just $Th2_{high}$ asthma. $Th2_{low}$ asthma has less eosinophilia and more neutrophilia in bronchoalveolar lavage and lung tissue [1, 6, 7]. Another distinction between $Th2_{high}$ and $Th2_{low}$ endotypes is differences in cytokines released in response to allergen triggers. Individuals with $Th2_{high}$ asthma release type 2 cytokines -IL-4, IL-5, and IL-13, whereas

$Th2_{low}$ asthma individuals do not [4, 7]. In fact, $Th2_{low}$ asthma individuals appear to release more IL-17, IFN-gamma, and other type 1 immune cytokines [8, 9]. Each endotype can be further subdivided into more specifically defined endotypes such as type 17 high, early age onset, late age onset, obesity-associated, and others [1, 6]. Grouping of asthmatics into two endotypes is not entirely accurate because there is overlap between groups, and the endotypes are not yet fully defined. Some asthmatics may express a phenotype that includes features of more than one endotype [10].

6.2.2 Asthma Therapy

The pharmacological therapies available for the treatment of asthma fall into five general categories: (1) beta 2 adrenergic agonist bronchodilators, (2) anticholinergic bronchodilators, (3) corticosteroids, (4) PDE inhibitors, and (5) specifically targeted biologics [11, 12]. Inhaled corticosteroids (ICS) have become the standard care for the treatment of asthma because they reduce inflammation, and most patients respond well with tolerable adverse effects. Corticosteroids provide better control of the disease than bronchodilator therapy alone. Nonetheless, there are subsets of asthmatics who respond poorly to steroid therapy even at the higher doses. These patients are thus defined as steroid-resistant or steroid-refractory asthmatics [11–13]. It is the steroid-resistant asthmatic patients that are left without proper care and management of symptoms. Therefore, there is still a need for additional classes of drugs for a small, but important, group of asthmatics.

6.2.3 Biologics for Targeted Therapy of Lung Inflammation

As the understanding of asthma's complexity improved, much effort focused on targeting inflammatory mediators with protein-based drugs often termed "biologics" [14]. The first biologic therapy developed was humanized anti-IgE monoclonal antibodies (omalizumab) [15]. IgE is the dominant immunoglobulin that is produced in allergic asthma [16]. Busse et al. [17] determined that Omalizumab reduced IgE and asthma exacerbations, while improving asthma scores in patients with severe asthma. Omalizumab is now used as an "add-on" therapy for severe, uncontrolled asthma. Subsequently, several anti-interleukin therapies were developed using humanized antibodies. Anti-IL-5 antibodies are now used to treat asthmatics with high levels of IL-5 because it is the major interleukin responsible for differentiation of eosinophils [18, 19]. In 2000, a single infusion of a humanized monoclonal antibody to IL-5 (mepolizumab) was administered to patients with allergic asthma. The antibody reduced the level of eosinophils in the blood and sputum but did not protect against later exacerbations due to exposure to an allergen. These data suggested that eosinophils are not required for late asthmatic response [19]. However, additional studies of humans with a severe eosinophilic form of asthma demonstrated clear efficacy of anti-IL5 antibodies when administered to a carefully stratified cohort of asthmatics [20]. This was an important advance in asthma therapy that stimulated a more careful definition of asthma phenotypes and the underlying endotypes in order to optimize asthma therapy. It is an excellent example of using principles of "personalized medicine" that suggest further refinement of therapy using novel-targeted oligonucleotide drugs might be possible [21].

IL-13 is another Th2 cytokine associated with asthma that is considered a key driver of mucus secretion, airway remodeling [22, 23], and airway hyperreactivity (AHR) [24, 25]. De Boever et al. [26] determined that an anti-IL-13 mAb was tolerated in patients with severe asthma, but they did not demonstrate clinical improvements. Although these findings contrast with other reports, asthma patients with less severe asthma did respond [26]. A recent meta-analysis supports the further development and use of IL-13 mAb in uncontrolled asthma [27].

There are now FDA-approved monoclonal antibodies targeting IgE, IL-4, IL-5, and IL-13 as well as investigational monoclonal antibodies

targeting IL-25, IL-33, TSLP, and several T cell signaling proteins [14]. Although several biologics have some benefits and offer new therapeutic options, they are typically only effective in subpopulations of asthmatics. Another limitation is the potential for the development of resistance since these new biologics target a single molecule. To expand beyond the one drug-one target approach, drugs that have broad activity like corticosteroids but do not suffer from resistance would offer a new exciting approach to uncontrolled asthma. MicroRNAs are molecules that have broad activity in inflammation and tissue remodeling that could be novel drug targets for treating poorly controlled asthma. To expand the set of targeted asthma therapies, new agents must first be tested in preclinical animal models.

6.3 Experimental Asthma Models

Several animal models of lung inflammation in asthma have been used to investigate asthma pathology and to identify novel drug targets. Guinea-pigs and mice have been used extensively to induce asthma-like phenotypes using a variety of allergens to mimic atopic asthma. The allergens used included ovalbumin (OVA), house dust mite extracts (HDM) [28, 29], and combinations of allergens including HDM extract, ragweed, aspergillus [30], fungal proteases plus cockroach allergens (CRA) [31], animal dander [32], and mucosal adjuvants [33]. When identifying a suitable model for preclinical drug development, the following several criteria should be met: (1) the model must replicate the clinical condition; (2) the underlying biology should be as similar as possible to the human disease; (3) the model should be cost-effective and yield valuable information; and (4) the endpoints should be translatable to clinical trials [34]. Each model has its advantages and limitations. Since no single animal model can recapitulate all aspects of asthma, choosing a model that closely resembles particular features of human disease to test a particular question is reasonable. Choosing an allergen that is also a trigger in human disease and delivering that allergen by the most pertinent route is also important.

6.3.1 Ovalbumin Models

Ovalbumin (OVA) mouse models of asthma typically begin with inducing a response by intraperitoneal injection with the adjuvant aluminum hydroxide followed by a challenging phase when the ovalbumin is either aerosolized or administered intratracheally. After the challenge phase, responses to allergen exposure are measured. Responses include airway hyperreactivity, eosinophilia in bronchoalveolar lavage fluid (BALF) and lung tissue, Th2 cytokine levels in BALF, and mucosal metaplasia. The responses vary depending on the strain and gender of the mice used. BALB/c mice mount a Th2 immune response, whereas C57BL/6 mount a Th1 response [35]. Female mice elicit a greater inflammatory response than male mice [36, 37]. A major limitation to this model is the allergen itself. OVA is not an allergen in humans, so the lung inflammation in mice does not mimic atopic asthma at the molecular level even though the histological and immune cell responses are similar to human asthma. Another limitation of this model is the route of administration of the adjuvant which is not physiologically relevant. Humans do not sensitize in the lung through intraperitoneal injection of an adjuvant or inhalation of ovalbumin. Also, when mice are chronically exposed to OVA, the mice become tolerant and the lung inflammation resolves spontaneously [38, 39]. The inflammatory response to OVA effectively mimics acute and subchronic lung inflammation rather than persistent asthma. Although OVA models have important limitations, there is a large and influential body of knowledge relevant to lung immunobiology generated with these models and they are commonly used. An alternative approach is to use more physiologically relevant allergens and routes of administration for sensitization such as house dust mite (HDM) extract, fungi, and fungal proteases delivered via the airways.

6.3.2 House Dust Mite Models of Mild/Moderate Asthma

House dust mite (HDM) models of asthma are arguably more physiologically relevant because house dust mite allergens are common triggers for many atopic asthmatics. The Der1 and Der2 proteases in house dust mite cuticles are important allergenic components, along with lipopolysaccharide, which acts as an adjuvant [40]. In addition, the route of administration of the HDM is intranasal which is similar to the route of exposure in atopic humans [28]. When testing interventions to modify the responses to HDM allergens, the drug treatment may occur during allergen challenge in a prevention protocol or after allergen challenge in a rescue protocol. Short-duration exposure to HDM allergens elicits mostly a Th2 high phenotype with mild/moderate severity that is corticosteroid-responsive [41–43]. Like OVA models, HDM-sensitized mice can become tolerant after chronic exposure but that is determined by timing and concentration of HDM used in the protocol [29]. Short-term sensitization protocols lasting a few weeks have become popular models of mild/moderate, steroid-responsive asthma.

6.3.3 Mouse Models of Severe Asthma

One limitation of short-term HDM models is that they do not recapitulate severe or steroid-resistant asthma. This is an important limitation for drug development when the goal is to develop new therapy for individuals who do not respond well to current therapies. One approach is to use models of severe asthma that recapitulate the immunological profile and steroid resistance in humans with severe, poorly controlled asthma. This has proven to be a somewhat difficult task, but there is important recent progress. In order to overcome the development of tolerance, additional allergens have been added to OVA and HDM models. Goplen et al. [30] combined HDM, ragweed, and *Aspergillus* species to break tolerance and establish a chronic asthma model (DRA). This model shows increased airway resistance during methacholine challenge when compared to a single allergen or double allergen model. Treatment with anti-Th2 antibodies did not reverse the symptoms of the DRA chronic model unlike the OVA acute model [30]. Another severe asthma model is a triple allergen chronic (TAC) model composed of OVA, cockroach extract, and HDM extract [31]. Unlike the DRA chronic model, the TAC chronic model still used OVA as an allergen to achieve increased airway resistance. A major difference between the two models is that all Th2 cytokines increased in the TAC chronic model, which was not the case in the DRA chronic model. Duechs et al. [31] determined that immune cell infiltration and AHR were not suppressed with dexamethasone treatment whereas dexamethasone did suppress the same parameters in the OVA-chronic model. Inducing steroid resistance in a mouse model was a significant advance in translational relevance of animal models of asthma. A more recent model of severe asthma uses HDM plus the mucosal adjuvant bis-(3′-5′)-cyclic dimeric GMP (CDG) [33]. CDG is a second messenger produced by many bacterial species, some of which are associated with exacerbations of severe asthma (e.g. *H. influenza*, *Streptococcus sp*., *Moraxella catarrhalis*) [33, 44]. CDG activates the STING pathway in human cells resulting in significant amplification of the innate immune response to lung pathogens and allergens [33]. Raundhal et al. [33] found that dexamethasone treatment was significantly less effective in mice sensitized with CDG plus HDM. This is consistent with this model being a better model of steroid-resistant asthma than chronic OVA models. Increased IFNγ and decreased secretory leukocyte protease inhibitor (SLPI) were implicated as important molecular effectors of asthma severity and steroid resistance in both humans and the new mouse model. All multiple allergen and allergen plus adjuvant models have been important advances for comparing the molecular similarities and differences between mild/moderate asthma and severe, steroid-resistant asthma. The phenotypes of the severe models recapitulate key features of patients with Th1/Th17 high, Th2 low

endotypes. These models offer new opportunities to discover and test novel, disease-modifying treatments of severe, poorly controlled asthma.

6.4 MicroRNAs

Several epigenetic processes are emerging as targets for disease-modifying approaches in asthma therapy [45–47]. Epigenetic processes including DNA methylation/demethylation, histone post-translational modifications, chromatin remodeling pathways, and noncoding RNAs have been actively investigated [47–49]. MicroRNAs have high therapeutic potential because they typically repress (but sometimes enhance) gene expression by regulating translation, thus controlling protein abundance and cell phenotype. Most microRNA genes are transcribed by RNA polymerase II in the nucleus to form a primary miRNA, which is then processed by Drosha (Nuclear RNase III) to form precursor microRNA (Fig. 6.1). The precursor miRNAs are bound to proteins and are transported to the cytoplasm by exportin where Dicer further processes them to mature microRNA duplexes of 20 to 22 nucleotides. The duplex then complexes with Ago-1 and Ago-2 in the RNA-induced silencing complexes (RISC). RISC mediates post-transcriptional gene silencing by either degrading the mRNA or blocking translation as depicted in Fig. 6.1 [50, 51]. The 3′ untranslated regions of many mRNAs contain microRNA response elements (MREs) that are DNA sequences to which the microRNAs bind. Binding of the 5′ seed sequence of the microRNA to the MRE causes translational repression of the target mRNA by multiple biochemical mechanisms including destabilizing the target mRNA, a process termed RNA interference (RNAi). MicroRNAs are predicted to regulate abundance of most proteins coded in the human genome. Each microRNA can act synergistically with other microRNAs to silence the same target transcript, and each microRNA can regulate more than one target in networks of co-expressed protein genes. Sets of co-regulated proteins have been described that define the molecular phenotype of a cell. MicroRNA/protein networks have been described that provide insight into both normal and disease states. MicroRNAs are abnormally expressed in many diseases including asthma, which has stimulated a search for potential targets for new RNAi-based treatments [52–55].

6.4.1 MicroRNAs in Lung Diseases

Defining which microRNAs control which sets of proteins in normal lung development and function and in lung diseases is a very active area of research. Dysregulation of microRNA expression and function has been described in lung cancer [56], idiopathic pulmonary fibrosis [57, 58], cystic fibrosis [59, 60], pulmonary hypertension [61], and bronchopulmonary dysplasia [62, 63]. Expression of microRNAs becomes dysregulated (either up or down) causing abnormal expression of target genes. This disrupts normal expression patterns in protein networks, which can influence the progression and severity of the disease. Some microRNA dysregulation may also serve as diagnostic or prognostic markers of lung diseases [64]. Whether altered microRNA expression is cause or effect depends on the particular disease and is mostly undefined. However, it is clear dysregulated microRNA-mediated gene regulation contributes to pathology. Therefore, targeted approaches to restore normal microRNA levels might be effective therapies for those pathologies. To be effective therapeutic targets, microRNA expression in normal and disease states needs to be concordant in humans and animal models of the disease. The validated mRNA targets of a given microRNA need to be defined. Then preclinical efficacy and toxicity studies must establish both desired therapeutic effects and undesired off-target effects. These can be inferred initially from in silico pathway analysis of miRNA targets that are then validated with in vitro biochemical studies in cells and tissues. Efficacy and toxicity then must be assessed with in vivo studies of lung structure and function in animal models prior to initial safety trials in humans.

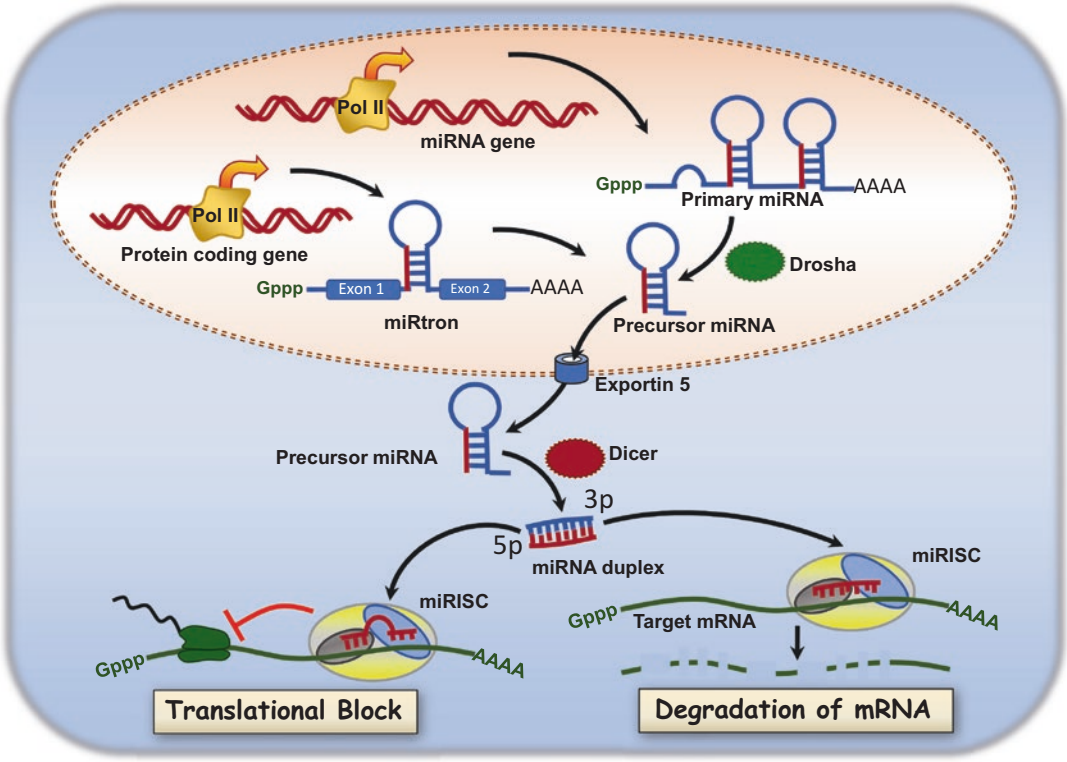

Fig. 6.1 MicroRNA biogenesis and mechanisms of gene silencing. The primary RNA transcript of miRNA genes and intronic primary miRNAs are transcribed by RNA polymerase II and processed by Drosha to pre-miRNA. Pre-miRNAs are transported from the nucleus via exportin-5 where they are further processed by Dicer. The mature miRNA is then bound to miRNA-associated RNA-induced silencing complexes (miRISC). Within miRISC, the miRNA can bind to the 3′-untranslated region (3′-UTR) of target mRNA to either repress translation or induce cleavage. This simplified schematic does not show other known interactions of miRNAs with 5′UTRs or with long noncoding RNAs, both of which are known to regulate expression of some proteins. (This image was published in Pharmacology and Therapeutics, vol. 147, Comer et al., Epigenetic targets for novel therapies of lung diseases, pp. 91–110, Copyright Elsevier, 2015)

6.4.2 MicroRNAs in Asthma

MicroRNAs contribute to asthma by regulating inflammation, cell migration, cell proliferation, and smooth muscle contraction in both animal models and isolated human cells [65–67]. In an early study of bronchial biopsies, Williams et al. [68] determined that global microRNA levels did not change when comparing healthy and mild asthmatics. However, once individual cell types were analyzed, there were significant differences between healthy and mild asthmatics [68]. Williams et al. [68] determined that miR-140 and miR-16 were expressed most predominantly in airway smooth muscle cells. Alveolar macrophages were found to possess the highest number of highly expressed microRNAs that included miR-223 and miR-146b, whereas fibroblasts expressed miR-125b and miR-214 [68]. Numerous subsequent surveys identified multiple microRNAs and microRNA host genes that are either upregulated or downregulated in mouse models of asthma [43, 65, 66, 69]. Table 6.1 summarizes microRNAs implicated in asthma. Many microRNAs altered in animal models are also altered in humans [70]. For example, Perry et al. [71] determined that miR-221 promotes increased proliferation of airway smooth muscle (ASM) isolated from severe asthmatics. Comer et al. [72] determined that miR-155 enhances cyclooxygen-

ase-2 expression in ASM cells from asthmatics. Additionally, Comer et al. [73] showed that miR-146a and miR-146b are induced by inflammation in human ASM cells. Both microRNAs negatively regulate cyclooxygenase-2 and IL-1β expression in human ASM cells. Recent network analyses of microRNAs in asthmatic human macrophages suggest that signaling pathways in both innate and adaptive immune responses are altered because microRNA expression is altered [74, 75]. The examples illustrated in Table 6.1 indicate the direction of change in expression and the putative mechanism by which microRNAs contribute to the pathogenesis of asthma. These initial descriptive studies have guided the design of translational studies summarized below. The reader is also directed to prior reviews of microRNAs in asthma for a more comprehensive view of the topic [75–79].

6.5 MicroRNA-Targeted Therapy for Asthma

Part of the appeal in targeting microRNAs for asthma is that microRNAs like glucocorticoids regulate expression of networks of inflammatory mediator proteins. One key question is which protein expression networks constitute the best targets for new anti-inflammatory and anti-remodeling drugs? If suitable targets are identified, then what constitutes an effective microRNA targeting agent? Ideally, microRNA-based drugs would be long-acting anti-inflammatory agents that reverse airway remodeling and avoid drug resistance seen in severe asthma treated with corticosteroids. Many studies seeking to alter microRNA-mediated gene expression have benefited from using microRNA mimics or antisense oligonucleotides as microRNA antagonists to exploit RNAi machinery [100]. Antisense oligonucleotides used for gene silencing are typically single- or double-stranded DNA molecules designed to reduce expression of a single protein by hybridizing and degrading the mRNA of the target protein. Native microRNAs use the same RNAi silencing machinery for gene silencing, but they typically bind to multiple target mRNAs, thus regulating networks of proteins rather than a single protein. Because single-stranded RNA molecules are unstable in vivo, chemical modifications were explored to increase efficacy, stability in vivo (i.e., half-life), and specificity for the target genes. One of the most commonly used strategies is to synthesize antisense oligonucleotides with chemical alterations to sugar residues at the 2′-O location to enhance cellular uptake [101]. Another useful modification is to use locked nucleic acids (LNA) and deoxyribonucleotides in an LNA/DNA mixmer. LNAs are nucleotide analogs that contain a methylene bridge on the sugar linking the 2′ oxygen with the 4′ carbon to "lock" the base into the optimal N-confirmation for high-affinity binding to the complementary mRNA [102]. The LNA modification increases specificity in vitro and improves resistance to endonuclease degradation in vivo. The incorporation of LNA bases increases the specificity dramatically for the target by removing the "wobble effect" of mismatched base pairs. In addition to altering the sugar structure, the phosphodiester bonds between nucleotides can also be modified to slow endonuclease degradation. Using phosphorothioate bonds or peptide bonds in place of the phosphodiester bonds reduces degradation significantly, increases cell uptake, and increases the half-life in vivo dramatically [103, 104]. This results in more effective and sustained RNAi with less frequent doses compared to unmodified oligonucleotides. Combinations of modified bases and modified backbones have been used successfully both in vitro in mechanistic studies of microRNAs and in vivo in preclinical translational studies listed in Table 6.2. One limiting factor in successful in vivo use is effective delivery to the target organ and effective uptake by target cells.

6.5.1 Lung Delivery of Oligonucleotide Drugs

First-generation antisense oligonucleotide (ASO) therapy for asthma targeted messenger RNAs of single proteins with delivery by inhalation. These initial approaches targeted proteins that regulate

Table 6.2 Preclinical trials of microRNA-based drugs in mouse models of asthma

microRNA	Mouse model	Drug formulation	Route	References
let-7	OVA, 4 wk	Anti-let-7, 2′-O-methyl	Inhalation	[127]
miR-9	OVA, 16 days	Anti-miR-21, 2′-O-methyl, phosphoramidites	Intranasal	[80]
miR-21	OVA, 4 wk	Anti-miR-21, 2′-O-methyl, phosphoramidites	Intranasal	[125]
miR-106a	OVA, 4 wk	mmu-miR-106a, uncomplexed	Inhalation	[89]
miR-126	OVA, 6 wk	Anti-miR-126, 2′-O-methyl, phosphoramidites	Intranasal	[69]
miR-133a	OVA, 8 wk	Adenovirus, miR-133a	Intravenous	[128]
miR-145-5p	OVA, 18 days	Anti-miR-145, 2′-O-methyl, phosphoramidites	Intranasal	[92]
	HDM, 18 days	Anti-miR-145, LNA/DNA mixmer, cationic pegylated lipid nanoparticles	Intravenous	[43]
miR-155-5p	OVA, 16 days	Anti-miR-155, 2′-O-methyl, phosphoramidites	Intranasal	[126]
	OVA, 24 days	Lentivirus, anti-miR155 shRNA	Intratracheal	[129]
	OVA, 25 days cockroach allergen, miR-155 −/− mice	Adeno-associated virus, anti-miR-155	Intravenous	[96]

Th2 inflammation including IL-5 [52], TNFα [105], STAT6 [106], p38 MAP kinases [107]. The ASOs were delivered by inhalation, and some had only modest efficacy. A more recent study targeting suppressor of cytokine signaling 3 (SOCS3) with a combination of antisense oligonucleotides delivered intranasally was more successful [108]. But the relatively low overall success rate of inhaled ASOs suggests additional approaches were needed. Targeting single genes might limit efficacy because lung inflammation in asthma is a complex syndrome depending on altered expression of many proteins.

One approach to overcome these limitations is to use antisense oligonucleotides targeting microRNAs rather than targeting the transcript of a single protein. In addition, it is important to optimize the delivery methods that enhance lung uptake of oligonucleotide drugs. Delivery methods have been tested that complex oligonucleotides to cationic liposomes [109, 110], anionic liposomes [111], and solid nucleic acid particles (SNALP) [112] and exosomes [113]. The use of polylactic acid-polyglycolic acid (PLGA) [114] and polyethyleneimine (PEI) polymeric nanoparticles for delivery of oligonucleotides to the lungs is well established. The reader is referred to prior reviews for details of chemistry, pharmacokinetics, and efficacy in using these approaches in lung cancer and other lung diseases (reviewed by [101, 115–117]).

6.5.2 Inhalational Toxicity

Nanoparticles can have significant respiratory toxicity when inhaled. Some classes of lipid nanoparticles are pro-inflammatory [118–120]. Therapeutic oligonucleotide can also modulate the innate immune response, acting as danger-associated molecular patterns (DAMPs) via Toll receptor activation, or as Toll receptor antagonists depending on the nucleotide chemistry (e.g., RNA, DNA, or LNA) [121]. Lipid nanoparticles are taken up by phagocytic cells as well as by pinocytosis and endocytosis into epithelial and endothelial cells [122, 123]. This explains effective lung delivery of oligonucleotides, but it also raises questions of off-target effects in other organs. Therefore, it is critical to test for hematopoietic toxicity and immune modulation by the delivery vehicle and the oligonucleotides. The liver and the kidney clear oligonucleotides and degrade them via nucleotide salvage pathways. Some modified oligonucleotide with LNA modifications, phosphorothioate backbones, and peptide nucleic acid modifications are very long-lived in vivo because they are highly resistant to nucleases [124]. This makes them effective in vivo, but also increases

the risk of chronic toxicity in the liver, kidney, spleen, and bone marrow. When using long-acting oligonucleotides and nanoparticle delivery systems, it is important to measure uptake in lung as well as uptake into kidney, liver, and spleen. Analysis of differential leukocyte counts, comprehensive metabolic panels, and blood-borne marker proteins is needed to detect hematopoietic, cardiac, renal, or hepatic toxicities.

6.5.3 Preclinical Efficacy Studies of MicroRNAs in Asthma Therapy

Translational research on microRNA targets has been guided by cell and animal studies (Table 6.1) and by surveys of microRNAs in humans with asthma. Informative changes in expression of microRNAs have been described using lung cell samples from asthmatics patients, some of which correlate with known mechanisms of asthma pathology. This rapidly expanding body of knowledge is the basis for numerous preclinical efficacy studies designed to reduce airway inflammation, reduce airway hyperreactivity, and reduce mucus hypersecretion in mouse models. Table 6.2 summarizes studies where a microRNA mimic or antagonist was delivered in vivo. In many cases, a positive therapeutic effect on lung inflammation was observed, but in several cases, the literature is contradictory (let-7, miR-21, miR-155). The positive outcomes were typically verified by adequate cell uptake of the drug and positive effects to reverse or rescue signs of allergic lung inflammation [86, 125]. The negative reports on the same microRNA could be due to issues with oligonucleotide chemistry, drug delivery, or inadequate dosing [92, 126]. Although a candidate drug might be taken up by lung tissue, it is important to define the cell types that take up the drug and to define the effects on target gene silencing by mimics or upregulation by antagonists (see [43]). Even with adequate drug distribution and cellular uptake, the microRNA mimics and antagonists may or may not adequately load the RISC complex to alter microRNA function. Careful transcriptomics, single-cell PCR for target mRNAs, and target protein detection are required to fully validate the molecular mechanisms of action of RNAi-based drugs. Variable efficacy when targeting microRNAs illustrates one of the inherent risks in translational studies of preclinical disease models. It also illustrates the need for multiple approaches to drug design, oligonucleotide chemistry, drug delivery, and disease models to arrive at a reliable conclusion about the value of a given microRNA target.

6.6 Future Directions

Despite some controversy in the literature, the weight of evidence supports continued research into microRNAs as valuable, druggable targets. There are important issues of drug delivery to be resolved as well as issues of potential acute and chronic adverse effects of oligonucleotide drugs. The problem of effective lung-directed delivery is tractable. Multiple studies in mice report that uncomplexed oligonucleotides administered intranasally or by inhalation are effective anti-inflammatory drugs (Table 6.2). In addition, several delivery vehicles are effective in delivering oligonucleotides to lung tissue including PGA, PEI, PEGylated cationic liposomes, adeno-associated virus, and lentivirus vectors. However, the use of nanoparticle carriers and viral vectors complicates regulatory approval for use in humans and adds to the expense of translation to a clinically useful drug. The delivery vehicles are themselves pharmacologically active even though they are often well tolerated in animal models – whether this is also true in human studies remains to be seen. Naked oligonucleotides delivered to the airways are viable alternatives that work well (Table 6.2). The agents must have adequate in vivo stability and cell permeability. They must have tolerable adverse effects in the lung and in other organs that take up and metabolize nucleotides such as kidney and liver. Future work in microRNA-based therapies must effectively address these issues. If successful, then there is potential for high impact on therapy of poorly controlled asthma.

Acknowledgments Preparation of this manuscript was supported by a grant to WTG from the National Institute of Allergy and Infectious Diseases (AI116985).

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7 Roles of Genetic Predisposition in the Sex Bias of Pulmonary Pathophysiology, as a Function of Estrogens

Sex Matters in the Prevalence of Lung Diseases

An Huang, Sharath Kandhi, and Dong Sun

Abstract

In addition to studies focused on estrogen mediation of sex-different regulation of systemic circulations, there is now increasing clinical relevance and research interests in the pulmonary circulation, in terms of sex differences in the morbidity and mortality of lung diseases such as inherent-, allergic- and inflammatory-based events. Thus, female predisposition to pulmonary artery hypertension (PAH) is an inevitable topic. To better understand the nature of sexual differentiation in the pulmonary circulation, and how heritable factors, in vivo- and/or in vitro-altered estrogen circumstances and changes in the live environment work in concert to discern the sex bias, this chapter reviews pulmonary events characterized by sex-different features, concomitant with exploration of how alterations of genetic expression and estrogen metabolisms trigger the female-predominant pathological signaling. We address the following: PAH (Sect.7.2) is characterized as an estrogenic promotion of its incidence (Sect. 7.2.2), as a function of specific germline mutations, and as an estrogen-elicited protection of its prognosis (Sect.7.2.1). More detail is provided to introduce a less recognized gene of *Ephx2* that encodes soluble epoxide hydrolase (sEH) to degrade epoxyeicosatrienic acids (EETs). As a susceptible target of estrogen, Ephx2/sEH expression is downregulated by an estrogen-dependent epigenetic mechanism. Increases in pulmonary EETs then evoke a potentiation of PAH generation, but mitigation of its progression, a phenomenon similar to the estrogen-paradox regulation of PAH. Additionally, the female susceptibility to chronic obstructive pulmonary diseases (Sect. 7.3) and asthma (Sect.7.4), but less preference to COVID-19 (Sect. 7.5), and roles of estrogen in their pathogeneses are briefly discussed.

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Keywords

Sex bias · Estrogens · Germline mutation · Pulmonary hypertension

Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_7

7.1 Introduction

The differential expression of certain genes that are either selective targets of, or capable of being regulated by sex hormones, is believed to be predisposed to, or protected from, cardiovascular and pulmonary diseases for either of the sexes. On the one hand, as one of the most important female hormones, estrogens have the property to regulate gene transcriptions during many cellular signal pathways via serving as ligands for nuclear hormone receptors (NHRs) such as estrogen receptors (ERs) or signal-regulated transcription factors (TFs), to ultimately determine expressive patterns of the gene [23]. On the other hand, the signaling responsible for estrogen actions and metabolisms is also controlled by specific gene(s) that guide a physiological balance between both aspects [26]. Thus, any pathological change(s) that interrupts the balance may yield the phenotype of female susceptibility to certain diseases, wherein pulmonary arterial hypertension (PAH) is a classical paradigm. The consensus that the female sex represents one of the most powerful risk factors for the development of PAH has been well accepted [92, 163]. The clinical relevance of estrogen signaling in PAH pathogenesis lies in the variations of generation, severity, and prognosis of PAH, correlated with the fluctuation of circulating estrogen levels during puberty, menstrual cycle, pregnancy, and menopause as well, although hormonal changes alone are not the primary contributors. To date, the issue that "estrogen matters" has long been a topic of interest, hence emerging valuable reviews summarizing studies of bench to bedside research. These articles, in particular, highlight the clinical importance of altered estrogen signaling in potentiating PAH, as a function of germline mutations. However, most of the studies separately discuss each single gene mutation, lacking a coordinated profile that reveals correlations of one gene mutation interfering with, or being interfered by, the other(s) to constitute an interactive component of altered estrogen signaling during the process of PAH development. To this end, PAH, characterized as a sexually dimorphic disease, is a major focus of this article. The interactions among the most recognized genetic candidates in response to pathological surroundings and the *Ephx2* gene, whose roles in the female susceptibility to PAH remain unaddressed yet, will be specifically explored, concomitant with brief discussions focused on pulmonary inflammatory disorders including chronic obstructive pulmonary disease (COPD) and asthma, since both possess female-predilection features as well.

7.2 PAH

PAH is a progressive disease, characterized by sustained pulmonary vasoconstriction that increases vascular resistance and pulmonary artery (PA) pressure (=>25 mmHg), and followed by elevation of right ventricular (RV) pressure and RV failure [93]. Classification of pulmonary hypertension was revised and defined as five groups [33], in which, the nomenclature of PAH as Group 1 lies primarily in its characteristics as idiopathic- and/or heritable/familial-based germline mutations, drug/toxin-induced alterations, and others associated with connective tissue diseases, schistosomiasis and portal hypertension (portopulmonary hypertension) etc., disorders that damage and eventually obstruct distal pulmonary arteries/arterioles; so dubbed as PAH [134, 156]. Current studies show a female to male ratio of 4.3:1 among the total PAH group [171] and 4.1:1 in the idiopathic pulmonary hypertension (IPAH) subcategory [8, 169]. Heritable PAH, most commonly caused by autosomal BMPR2 (bone morphogenetic protein receptor type II) mutation, has a female to male ratio of approximately 2.7:1, paralleled with an increased number of female carriers of the mutation [5, 108]. Other subcategories of PAH also present sex bias, as evidenced by female predominance in scleroderma-associated PAH [24, 38] and portopulmonary hypertension [79, 139]. This sexual dimorphism in PAH is believed to be mediated at least in part, by biologically relevant effects of female hormones/estrogen. This is indicated by an earlier onset and significant prevalence of PAH in women who have taken oral contraceptives [68, 83] or received hormone replacement therapy [159]. The sex differences in

hemodynamics disappeared in PAH patients older than 45 years [169]. Some clinical studies provide evidence that correlates the polymorphism of gene(s) involving estrogen metabolism with female predisposition to PAH [5, 7]. A cohort study that assesses roles of common single-nucleotide polymorphisms (SNP) in the development of the disease indicates that aromatase polymorphisms, a key enzyme responsible for tissue/local synthesis of estrogens, are more common in patients with portopulmonary hypertension. More importantly, these polymorphisms trigger higher estrogen levels in patients with portopulmonary hypertension than those with simple cirrhosis [138]. Also, a higher aromatase activity exists in both female PAH patients and PAH animal models induced by treatment with sugen5416 (an inhibitor of VEGF receptor)/hypoxia (SuHx-PH) [112, 138], as well as in male PAH patients associated with significantly higher plasma estrogen levels or a greater ratio of estrogen to testosterone [180]. Moreover, a currently completed clinical trial "Anastrozole in Pulmonary Arterial Hypertension (AIPH)" has demonstrated that pharmacological inhibition of aromatase with anastrozole for three months improves the 6-minute-walk distance (6MWD) in PAH patients, accompanied with significant decreases in circulating estradiol levels [78], although a direct causation between the reduced estrogen and improved walking capacity needs to be clarified. In contrast to the clinical studies that support pathogenic roles for estrogens in PAH, most animal studies, as well as some human data [64, 125], have shown that female sex or estrogen supplementation evokes protective effects against PAH [87, 89, 92] in an ovariectomy-reversible manner [2, 27, 167]. Thus, the concept of "estrogen paradox" emerged, defined as divergent actions (detrimental and beneficial) of estrogen in the incidence/generation and prognosis/survival of PAH [41, 169].

7.2.1 PAH Prognosis

In comparison with the incidence of PAH (see Sect. 7.2.2) that most likely occurs under physiological or periphysiological conditions, the progression of PAH involves a myriad of pathological changes, which together forms an integrated pathological network that elicits endothelial dysfunction and vascular remodeling, plexiform formation, and occlusive vessel lesions [134, 166], followed by RV hypertrophy and dysfunction [119, 166]. During this process, the severity of RV dysfunction serves as an indicator for evaluating PAH prognosis [111]. The RV dysfunction in PAH is not solely resulted from the pressure overload caused by increased pulmonary vascular resistance [13], but rather evoked by a multi-signaling engaged pathological complex that mediates the progressive process of PAH [19]. Thus, the PAH prognostic procedure is governed by pathological pathways that share similarities applicable to be challenged by estrogen [58, 59, 121]. In this context, the estrogen-driven improvement of PAH prognosis is not an etiologically specific response [112]. For instance, in SuHx-PH mice estrogen improves RV function via inotropic effects on the myocardium [100], restoration of PA compliance [101], and prevention of RV remodeling [43, 91]. In monocrotaline (MCT)-induced PH, estrogen prevents MCT-induced damage in antioxidant capability to preserve myocardial function [9, 105] and improves RV perfusion by increasing RV capillary density [167], leading to a restoration of RV structure and function and prevention of RV failure [113, 123, 167]. In the hypoxia-induced PH (HPH) model, estrogen protects against hypoxia-inducible factor (HIF)1α-induced PA remodeling in an ERβ-dependent manner [44]. In IPAH patients, women have a significantly higher survival rate attributed to a better maintenance of RV adaptive/compensative remodeling in response to an equal magnitude of elevated afterload compared to men [69]. Taken together, regardless of pathogeneses, different types of PAH may share similar pathological changes, yielding the non-specific nature of estrogen-evoked better prognosis, which is neither selectively driven by a specific PAH model nor triggered by particular target(s). This concept is important for spotlighting the relevancy of estrogenic protection in PAH prognosis. On the other hand, there is indeed an alternative interpretation for "estrogen paradox" based on divergent

outcomes derived from animal studies versus human observations. In this context, most parameters in animal studies seem to be more likely involved in RV function that represents PAH prognosis, while human data shown PAH registries denote a prevalence/incidence of the disease. Additionally, the "estrogen paradox" is also present in different animal models. For instance, PAH models of an upregulation of serotonin transporter gene (SERT+), overexpression of the calcium-binding protein S100A4/Mts1 that is a downstream effector of serotonin signaling, and treatment with dexfenfluramine (Dfen; a serotonergic agonist) are all characterized by female-predominant development of the disease [92], implicating estrogen as a responsible mediator. In contrast, female animals deficient in VIP, eNOS or ApoE and fed a high-fat diet, show a lesser prevalence of pulmonary hypertension than their male counterparts [53, 55, 144], an issue that will not be discussed in this review.

7.2.2 PAH Incidence

Regarding the incidence of PAH, certain genetic candidates from genetically engineered animal models are spotlighted as key players. Although these genetic models are criticized for failing to perfectly recapitulate the human PAH, they at the very least provide a platform to discern roles of intrinsic gene(s), as well as their interactions in potentiating females as a vulnerable population of PAH. For instance, the mutation of the *BMPR2* gene [6, 35, 94, 120], overactivation of SERT potentially instigated by Dfen-treatment [179] (a female-specific PAH model characterized as enhanced serotonin signaling for PASMC proliferation) [29], overexpression of S100A4/Mts1 (downstream effector of SERT) to initiate vascular formation of plexiform-like lesions in only females [30], and dysregulation of CYP1B1 (an enzyme for estrogen metabolism) [30, 177, 178] are all involved in the female-predilection for PAH. As such, signal correlations among estrogen, SERT, S100A4/Mts1, BMPR2, and CYP1B1 attract considerable attention. Intriguely, much fewer studies to date have paid attention to the *Ephx2* gene, whose expression is physiologically controlled by estrogen [184], an issue that will be addressed in Sect. 7.2.2.3.

7.2.2.1 BMPR2

Sex differences in the expression and activity of *BMPR2* (a gene encoding a TGF-beta receptor) exist even under basal conditions. In general, mutation in the *BMPR2* gene turns out to be one of the most prevalent genetic contributors for female susceptibility to heritable and idiopathic pulmonary arterial hypertension (HPAH and IPAH) [7, 109, 120, 179]. Approximately 3/4 of HPAH are carriers with the mutation of the *BMPR2* gene [107, 108], and females exhibit a two-fold greater PAH penetrance of the *BMPR2* mutation than males [124]. The BMPR2 mutation causes an irregular trafficking of ERα in pulmonary vasculature [37] forming a transcription complex of estrogen-ERα-estrogen responsive elements (ERE, located in the *BMPR2* gene promotor), which via recruiting co-repressor(s), downregulates the *BMPR2* gene [6, 7], subsequently suppressing downstream signaling of inhibitors of nuclear differentiation 1 & 3 (Id1/Id3), both inhibiting cell proliferation of pulmonary artery smooth muscle cells (PASMC) [33, 54]. As a result, PAH patients with the BMPR2 mutation display significant increases in PASMC proliferation and decreases in its apoptosis [32, 94]. Accordingly, an inhibition of estrogen restores suppressed BMPR2 signaling to mitigate PAH in BMPR2 mutant mice in an ER-dependent manner [22] and alleviates PAH in female SuHx-PH rats [112]. Using FK506 (tacrolimus; BMPR2 activator) causes a therapeutic reversion of IPAH via recruiting BMPR2 signaling in pulmonary vessels [157]. This study also demonstrates that conditional BMPR2 deletion in the endothelium exacerbates mouse PA responses to chronic hypoxia, an event that can be prevented by treating mice with FK506. In both MCT- and SuHx-PH models, normalization of BMPR2 signaling attenuates vascular remodeling [157]. On the one hand, a gene array also indicates that as a target of estrogen, ERα expression is upregulated in female BMPR2 mutation carriers with PAH compared with non-carriers [135], confirming further the importance of estrogen signaling in PAH. These studies have revealed a pathological

pathway that involves estrogen, ERs, BMPR2 signaling, and PAH, wherein CYP1B1 seems to be one of the crucially responsible intermediates (see Sect.7.2.2.2 and Fig. 7.1). On the other hand, BMPR2 mutation patients exhibit poorer RV adaptation and more severe RV dysfunction than PAH patients without the mutation [168]. This phenomenon, if indeed related to estrogen, is somehow contradictory to the concept of estrogenic protection of PAH prognosis. Nevertheless, it can serve as a paradigm for irregular estrogen signaling, that is otherwise protective of RV, but becomes maladaptive in the presence of a transcriptionally or epigenetically altered milieu.

7.2.2.2 CYP1B1

As an estrogen-metabolizing enzyme, CYP1B1 catalyzes the hydroxylation of estradiol to 2-OH-estradiol, ensued by the quick formation of 2-MeO-estradiol, an estrogenic metabolite that possesses anti-proliferative, -angiogenic, and -inflammatory effects [34, 162, 181], while metabolizing estrone into 16α-OH-estrone, a pro-inflammatory mediator that promotes cell proliferation [34, 127, 165]. This physiological balance between the two metabolites can be disrupted by dysregulation and/or genetic mutation of CYP1B1, therefore favoring a shift of estrogen metabolic singling away from the 2-MeO-estradiol pathway toward the 16α-OH-estrone axis. Indeed, there is a high ratio of 16α-OH-estrone to 2-MeO-estradiol in human HPAH patients. BMPR2 mutant mice receiving chronic 16α-OH-estrone express doubled PAH penetrance [37]. V*ice versa*, exogeneous administration of 2-MeO-estradiol to male and ovariectomized female MCT-PH rats significantly attenuates their vascular remodeling and inhibits inflammatory responses [162]. And while roles of irregular CYP1B1 intervention with PAH have been extensively investigated, there are some confounding outcomes. For instance, highly expressed CYP1B1 in pulmonary vasculatures in HPH and SuHx-PH models, as well as PAH patients, promotes their development of PH via at least in part, 16α-OH-estrone-mediated mitogenic effects on PASMC, responses that are reversible with blockage of CYP1B1 pathway [178]. Pharmacological inhibition of CYP1B1 activity or genetic deletion of the CYP1B1 gene reverses the development of PAH in SERT+ model that expresses upregulation of pulmonary CYP1B1 [72]. In line with these findings, a genetic deficiency of CYP1B1 fails to elicit Dfen-induced, estrogen-mediated increases in RV pressure and pulmonary vascular remodeling [29], suggesting an upregulation of CYP1B1 correlated to PAH development. Alternatively, a study using Affymetrix arrays to compare BMPR2 mutation carriers with (affected) and without (unaffected) PAH shows that CYP1B1 expression and activity were almost tenfold reduced in female BMPR2 mutation carriers with PAH [176]. Moreover, genotypes for CYP1B1 Asn453Ser (N453S) conducted on 140 BMPR2 mutation carriers indicate that wild-type CYP1B1 (N/N) is fourfold higher in affected patients than unaffected carriers who hold N/S or S/S genotypes. However, the former ironically displays a higher urinary ratio of 16α-OH-estrone to 2-MeO-estradiol [5]. These studies that reveal a discrepancy in CYP1B1 expression/activity raise not only questions as to whether an up- or down-regulation of CYP1B1 determines PAH pathogenesis, but also, more importantly, implicates CYP1B1 as a potential player in intervention with PAH in female BMPR2 mutation carriers. Thus, regardless of increased, decreased, or polymorphism of CYP1B1, dysregulation of this gene tips the estrogen metabolic axis away from the protective track toward the detrimental pathway during the process of PAH development.

Taken Sects. 7.2.2.1 and 7.2.2.2 together, aberrant estrogen signaling appears to be an essential predisposed factor to PAH, whereas contradictory results prevent clear conclusions from being drawn. As an illustration, Fig. 7.1 provides an integrated network that operates in concert with estrogen, BMPR2, and CYP1B1 to trigger PAH signal transduction, via a serotonin-mediated pathway that can be activated by the overexpression/overactivation of SERT and Dfen exposure, followed by stimulating downstream effector of S100A4/mts1. Thus, a positive feedback loop, centralized on CYP1B1-specific dysmetabolism of estrogen, characterized by an altered serotonin signaling forms [29, 72, 177], which contributes significantly to the penetrance

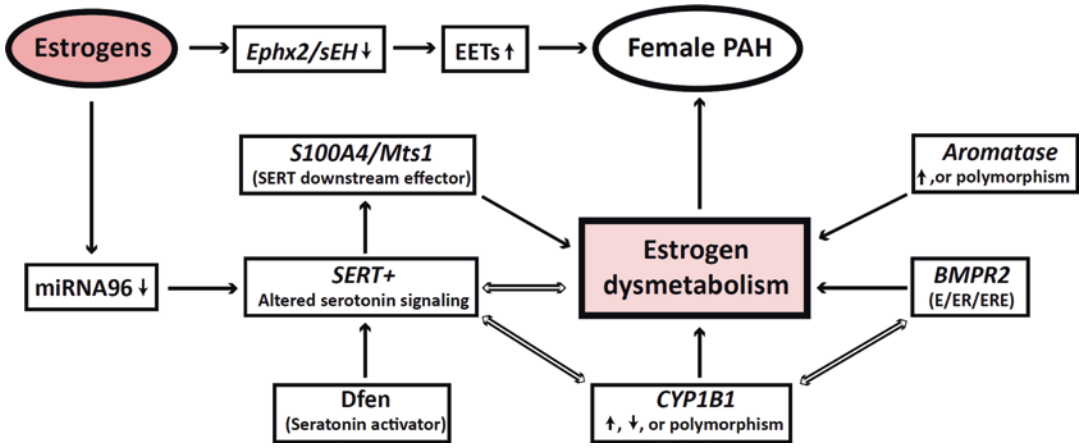

Fig. 7.1 A schematic of correlations among dysregulated genes/or proteins (*in Italic*) and their functional positions in processing the signaling. Estrogen downregulates the *Ephx2* gene to increase pulmonary vasoconstrictor EETs and suppresses miRNA-96 to upregulate serotonin transporter gene (SERT), followed by promoting serotonin pathway via activating a rate-limiting enzyme for serotonin synthesis. The overactivation of serotonin signaling stimulates its downstream effector of the calcium-binding protein S100A4/Mts1 to cause the estrogen dysregulation that, in turn, exacerbates SERT dysfunction. On the other hand, the altered serotonin signaling can also be elicited by exposure to serotonin agonists and/or in the presence of CYP1B1 dysfunction, the latter is either responsible for, or a consequence of the serotonin pathology. Furthermore, dysregulation of CYP1B1 not only directly instigates the estrogen dysmetabolism but also cooperates with BMPR2 mutation in a reciprocal causative manner, to worsen dysregulating estrogens, an event that can also be initiated by an augmented aromatase activity. Thus, the signal pathway mediating estrogen dysmetabolism/dysregulation becomes an ultimate approach to female-predominant PAH

of PAH in females with BMPR2 mutation [5, 37]. Hierarchically, as an upstream regulator of estrogen signaling, a downregulation of female-specific microRNA-96 is also recognized to intervene with PAH development via promoting its downstream-located 5-hydroxytryptamine 1b receptor (5-HT_{1b}R; a rate-limiting enzyme for serotonin synthesis) activity to stimulate serotonin-induced PASMC proliferation [172]. During this pathological cycle, each single gene mutation or factorial alteration may serve as a cause for, or consequence of, the other(s), and *vice versa*. Coordinated interactions among these genes, along with corresponding changes in their downstream pathways, constituting the main component of altered estrogen signaling, which yields a female-favorable phenotype of PAH, although it is not always possible to clearly separate the intrinsic (gene) versus sex steroid-induced differences. Of note, the RV protective actions of estrogen (better prognosis) seem not evident in these germline mutation-based PAH [92].

7.2.2.3 Soluble Epoxide Hydrolase (sEH)

Unlike other genetic candidates such as BMPR2, CYP1B1, SERT, and S100A4/Mts1, their roles in the female-specific development of PAH have already been extensively discussed [33, 90, 92, 133, 163, 172, 176, 177, 179]; studies implicating specific impacts of the *Ephx2* gene that encodes sEH, on the sexually dimorphic regulation of pulmonary/RV pathophysiology, are extremely limited, despite recognition that estrogen innately downregulates *Ephx2* expression via an epigenetic-based mechanism [59, 184]. To this end, ensuing discussions are particularly focused on this emerging research, along with the most updated findings regarding roles of estrogen and sEH in evoking PAH development. A broad spectrum that hierarchically addresses histological macro-/micro-fabrics (from the airway to vasculature) and biological cellular mediators contributing to the sex-different, or female-favorable occurrence of pulmonary disorders has been well illustrated

[163], suggesting the pulmonary vasculature as one of the major objectives targeted by sex hormones. In general, estrogen is known to stimulate endothelial production of vasodilator mediators, nitric oxide (NO) and prostacyclin, while inhibiting vasoconstrictor, endothelin (ET), in pulmonary vasculatures [28, 95, 118, 153]. These responses are ascribed to be responsible for estrogen-mediated protection [88, 89] but seem contradictory to the female-predisposition to PAH development. In current decades, endothelial mediators, called epoxyeicosatrienoic acids (EETs) that are metabolites from arachidonic acid through cytochrome P450 (CYP)/epoxygenase system, have come to the forefront of research inspired by their pathophysiological properties. EETs have been demonstrated to possess cardiovascular protection in the systemic circulation [66, 140]. The bioavailability of EETs is controlled by sEH that degrades EETs to their bio-inactive diols (DHETs) [59, 67]. As such, not only EET synthases but also sEH (EET hydrolase) can serve as therapeutic targets for cardiovascular diseases [67]. More importantly, their enzymatic activities are regulated by estrogen, leading to a sex discrepancy in the presentation of EET actions, in terms of a greater contribution of EETs in females than males [59]. In this context, EETs shed light on the nature of estrogen-paradoxical regulation of PAH rooted on two important features. First, the bioactivity of EETs acts in a female-favorable manner, as a function of estrogen-methylation of the *Ephx2* gene to downregulate protein expression of sEH [184], followed by increases in circulating/tissue EETs. Second, EETs possess divergent (detrimental and beneficial) actions in the pulmonary and systemic circulation, characterized as pulmonary vasoconstriction [75, 81, 129, 186] that acts as an initial trigger for the elevation of PA pressure [92, 160], and systemic vasodilation via hyperpolarizing vascular smooth muscle cells to decrease blood pressure [4, 39, 40, 60–62]. These two features match the nature of "estrogen paradox" precisely, since they articulately explain the increase in EETs and all the consequences arising therefrom, manifested by female susceptibility to PAH (incidence) via EET potentiation of pulmonary vasoconstriction, while favorable to RV function (prognosis) via EET mediation of systemic vasodilation, protection against inflammation [31, 104], apoptosis and oxidative stress [21, 146], and stimulation of angiogenesis as well [115, 117].

1. With respect to the incidence of PAH, increases in pulmonary EETs by either estrogen intrinsic downregulation of sEH (females), genetic deletion of the *Ephx2* gene (sEH-KO) or pharmacological inhibition of sEH activity (sEH inhibitors; sEHIs) significantly enhance hypoxia pulmonary vasoconstriction (HPV) and promote elevation of PA pressure in response to acute hypoxia [75–77, 80, 81]. Evidence for the female susceptibility to PAH in an EET-dependent manner has been provided by the finding that a thromboxane analog-induced pulmonary vasoconstriction and elevation of right ventricular systolic pressure (RVSP) are significantly greater in male sEH-KO (*Ephx2*$^{-/-}$) and female WT mice (estrogen downregulation of sEH), compared with male WT mice. Furthermore, female sEH-KO mice (intrinsic downregulation with additional disruption of *Ephx2*) exhibit the highest RVSP among all groups. These differences in the elevation of RVSP among these groups are eliminated by an EET antagonist, suggesting that EETs dose-dependently promote PH [76]. Specific mechanisms, by which EETs potentiate HPV and HPH, are not fully understood; however, roles of cyclooxygenases (COXs)-derived vasoconstrictor prostaglandins (PGs), Rho kinase, endothelin, and adenine nucleotides in processing the responses have emerged [85]. As reported, enhanced hypoxic responses can be reversed by inhibiting COXs and/or Rho kinase, and also by blocking EET activity [77, 80]. Hypoxia potentiates pulmonary production of EETs associated with a membrane translocation of a TRPC6-V5 fusion protein within PASMC, a response that is sensitive to a putative EET antagonist, and additionally, PH is failed to be elicited in mouse lungs deficient

in TRPC6 [80]. In chronic HPH, increases in pulmonary EETs, accompanied with intensified COXs/PGH2/thromboxane A2 (TXA2) signaling, great superoxide production, and impaired NO bioavailability, work reciprocally to provoke the development of PH, a response that is predominant in sEH-KO mice [74]. Moreover, HPV and pulmonary vascular remodeling to hypoxia are reversed by the inhibition of epoxygenase (EET synthase) and augmented by sEHIs [129]. Although multiple PAH and COPD models exhibit a downregulation of pulmonary sEH [128, 137, 149], the casualty between the disease pathology and reduced sEH remains elusive. To date, with respect to the correlation between EETs/sEH and pulmonary hypertension, most studies have focused on the HPH model because chronic hypoxia upregulates EET synthases [129] and downregulates EET hydrolase/sEH [74]. Our current studies using radiotelemetry, with implanted pressure probes in the PA of rats to dynamically monitor changes in PA pressure, have provided direct evidence indicating that being female, or estrogen, significantly promotes the generation of PAH in an EET-dependent manner, responses that are observed in both SuHx- and MCT-PH models [59]. It is worthy to note that physiological downregulation of the *Ephx2*/sEH by estrogen and ensuing increases in pulmonary EETs do not significantly affect basal PA pressure in normal conditions, as indicated by the comparable PA pressure and RVSP between male and female, and between sEH-KO and WT mice before their exposure to hypoxia and treatment with SuHx or MCT [59, 74, 76, 77]. This indicates that the physiological impact of increased EETs on PA pressure/RVSP is barely discerned, attributed to the *in vivo* presence of compensatory mechanisms, by which enhanced EET-induced pulmonary vasoconstriction can be compromised by estrogen-stimulated release of NO, and/or CYP-mediated release of 20-HETE to dilate PAs [160]. When the physiological balance is disrupted by any pathological stimulus as a "second hit," such as hypoxia, increased vasoconstrictor forces by ET, constrictor prostanoids and/or AngII, or altered estrogen signaling, EETs can potentially activate PAH signaling (Fig. 7.1).

2. Regardless of particular etiologies, the prognosis of PAH, evaluated by the severity of RV dysfunction, appears to be in an estrogen preventable manner [90, 92]. Searching literatures, estrogen-dependent protection against RV dysfunction leading to a better prognosis/survival and its mechanisms responsible for attenuation of pulmonary vasoconstriction and vascular remodeling, favorable maintenance of adaptive/compensated RV function, alleviation of inflammatory and toxic responses, and improvement of endothelial function via NO-dependent and/or -independent pathways have been well reviewed [7, 33, 41, 90, 92]. On the other hand, similar to the limited research on estrogen-dependent EET potentiation of PAH generation, studies of EET benefit to PAH prognosis also remain very rare, revealing a significant knowledge gap in the sexually dimorphic regulation of pulmonary/RV pathophysiology. Scattered studies have indicated that in MCT-PH rats, pharmacological inhibition of sEH significantly prevents vascular remodeling via EET-dependent anti-mitogenic effects to lower RV pressure and maintain RV function [137]. Interestingly, some clinical trials using sEHIs to treat COPD that exists with downregulation of pulmonary sEH show that beneficial effects of sEHIs may not be specifically mediated by the inhibition of sEH *per se* but rather by alternative pathways, such as blocking COX2- and p38β-mediated inflammatory signaling [65, 99], preventing vascular remodeling [137], and/or improving endothelial functions [96, 182]. Currently, our laboratories are studying specific roles of EETs in the estrogen prevention of RV dysfunction during PAH progression. Owing to limited studies that specifically explore actions of EETs in PAH prognosis, we, therefore, will introduce some of our preliminary studies/data, aiming to narrow the knowledge gap in the field. We identified a significant RV dysfunction in both

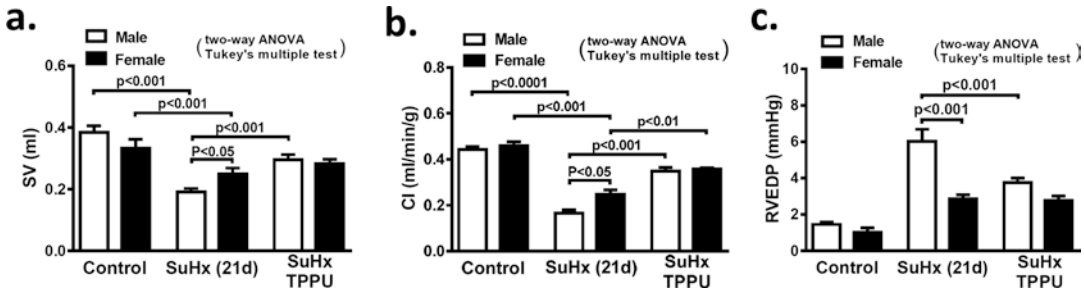

Fig. 7.2 RV stroke volume (SA; **a**), cardiac index (CI; **b**), and end-diastolic pressure (EDP; (**c**) in male and female controls, SuHx and SuHx with TPPU-treated rats ($n = 4$ for each group)

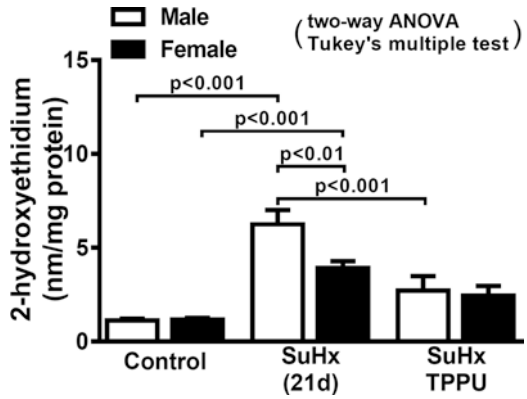

Fig. 7.3 RV superoxide levels in male and female controls, SuHx and SuHx with TPPU-treated rats ($n = 5$ for each group)

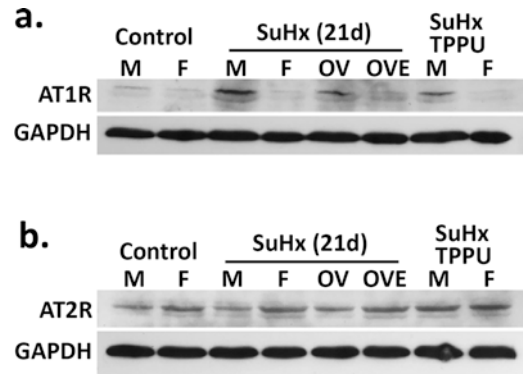

Fig. 7.4 Western blot analysis for RV protein expression of AT1R and AT2R in control male and female rats, SuHx-male, -female, -OV and -OVE rats, and SuHx rats treated with TPPU

sexes of rats treated with SuHx for 21 days (Fig. 7.2), as indicted by attenuated RV stroke volume (SV; a) and cardiac index (CI, b) associated with great increases in RV end-diastolic pressure (RVEDP; c). However, female SuHx rats as well as TPPU (an sEHI)-treated male SuHx rats displayed much lower reductions in both SV and CI, paralleled with a smaller magnitude of increase in RVEDP compared to TPPU-untreated male SuHx rats. These findings indicate a female-favorable maintenance of adaptive RV function in an EET-mediated manner, even though the RV afterload (pulmonary artery pressure) in the male and female SuHx-PH rats was comparable at that moment. In accordance, Fig. 7.3 shows the same responsive pattern of female/EET-inhibition of RV superoxide production in SuHx rats. Measurements for angiotensin-II receptor (ATR) expression in Fig. 7.4 indicate that TPPU partially but remarkably reversed SuHx-induced upregulation of AT1R in RV of male and ovariectomized (OV) female SuHx-PH rats, whereas intact female and OV with estrogen replacement (OVE) SuHx-PH rats maintained their downregulated AT1R expression in both normal and SuHx-PH conditions (a). Interestingly, an upregulation of RV AT2R existed in not only female phenotypic (intact female and OVE) SuHx-PH rats, but also in either sex treated with TPPU (b), confirming an EET-mediated response. These findings implicate that, in processing estrogen signaling, EETs serve as downstream mediators to recruit AT2R-protective pathway while compromising AT1R-detrimental actions, leading to better prognosis and survival in female PAH patients.

To date, the rapidly updating knowledge and overall progress in unraveling the estrogen puzzle warrant a new assessment for the profound impact of estrogen on PAH pathogenesis. In terms of pros and cons, increases in EETs may be a double-edged sword with disadvantages in the pulmonary circulation and advantages in systemic circulation, characterized as paradoxically promoting PAH incidence while benefiting its prognosis. Thus, incorporation of this concept to outline the estrogen-paradox phenomenon unravels EETs as indispensable mediators responsible for the regulation of PAH signaling in an estrogen-specific/paradoxical pattern.

7.3 COPD

Co-existing with PAH, COPD evokes a significantly surged mortality [3]. A recent study demonstrates that PA remodeling in COPD, characterized by distal vessel muscularization, medial hypertrophy and intimal reactions, fibrous vascular occlusion, and in severe cases with even plexiform lesions, progressively exacerbates PH [16]. There is a consensus that women inherently have a great susceptibility to cigarette smoke than men [17, 132]. However, while adult females display higher sensitivity to smoke, female fetuses exhibit lower sensitivity to maternal smoking in their prenatal environments [185], revealing a yet unknown pathophysiological significance for the phenomenon. In the stratification of smokers versus nonsmokers, women constitute a major component in a subgroup of COPD patients who are nonsmokers or have never smoked (excluding those with α-1 antitrypsin deficiency that evokes an early development of COPD with genetic matters). As reported, nearly 80% of cases with early-onset COPD is nonsmoking women and more than 85% of nonsmoking COPD cases are females [10, 154], who in most cases represent characteristics of early-onset and severe symptoms [155]. These findings negate the female susceptible to smoking as a major contributor. On the other hand, women develop symptoms of COPD at a younger age than men [45, 131, 163], concomitant with an increase in the number of women being diagnosed with COPD during puberty [163], indicating a higher predisposition to COPD that parallels with a surge of female hormones. Additionally, after quitting smoking, female COPD patients exhibit a greater improvement in lung function than males [25], revealing a female/estrogen-paradox in the generation and prognosis of the disease. By extending the context to lung cancer, which is highly correlated with cigarette smoke exposure, nonsmokers diagnosed with lung cancer are approximately three times more likely to be females [56, 73], yet still have a better prognosis within 5-years of survival than men in all stages and subtypes [36, 152]. Therefore, it is the female hormone, estrogen, that contributes crucially to the female susceptibility to COPD while benefiting its prognosis as well.

7.4 Asthma

Asthma is an inflammatory disease of the airways, characterized by progressive airway narrowing, leading to an expiratory airflow limitation, sometimes accompanied with dyspnea and wheezing [42, 70]. This pathological event originates from multifactorial-engaged pathways and involves both intrinsic and environmental alterations [42]. The National Health and Nutrition Examination Survey (NHANES) published in 2009 provided evidence indicating a significantly greater prevalence of asthma in US women than men [114]. As shown, male sex seems to be a risk factor for the development of asthma at early ages [122, 148] due perhaps, to inherent male-specific “dysanapsis.” This is a morphological phenomenon characterized by disproportionately fewer alveoli for the number of airways [126, 161], as a function of airway growth lagging behind lung growth [57]. Before age 5, the ratio of boys to girls with asthma is approximately 2:1, accompanied with an approximately fourfold increase in potential developing chronic asthma in boys before the onset of puberty (ages 10–14) [11, 15], after which the metric for boys and girls diagnosed

with asthma becomes equal [151, 170]. Ultimately, puberty switches the sex favorable distribution of asthma toward females, as evidenced by approximately two times more women diagnosed with asthma than men during their reproductive years [116, 136, 147]. Accordingly, women who undergo early menarche (before the age of 12) show a significant increase in the generation of asthma compared to those with delayed menarche [130, 145]. Furthermore, this puberty-driven female predominance in development of asthma persists until the onset of menopause, followed by a disappearance of the sex bias with aging [163]. In consistence, a prospective cohort study has reported a reduced risk of developing *de novo* asthma in postmenopausal women compared to age-matched premenopausal controls [164]. Additionally, postmenopausal women receiving hormone/estrogen replacement therapy (HRT) exhibit a dose-dependent increase in the prevalence of asthma with worsened symptoms [141, 164]. Taken together, human studies clearly reveal a correlation between female sex and the incidence and severity of asthma. The underlying multiplex mechanisms may be involved in the biological susceptibility, gestational maturation-related structural and functional changes, age-dependent alterations of hormonal milieu or receptors, and environmental exposures as well [163].

7.5 COVID-19

At the moment, there is no greater pressing, or more relevant topic regarding sex-specific susceptibility, than the coronavirus disease 2019 (COVID-19). The pandemic has brought forth a global crisis. Up until April 30, 2020, data from 35 countries using sex-disaggregated analysis show that the death rate of COVID-19 in both confirmed and suspected cases represents a significant sex bias, characterized as a remarkably lower mortality in female than male patients, whereas their morbidity is comparable (14) (https://doi.org/10.6084/m9.figshare.12240707.v1). Why? Once again, sex hormones play key roles via, at least in part, regulating the angiotensin-converting enzyme 2 (ACE2), a functional receptor for the viral entry into cells and upregulated by interferons (IFNs) [86, 97, 187], and the transmembrane serine protease 2 (TMPRSS2). Early in 2003, Nature [98] had published a study identifying the severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) spike (S)-protein-binding site on ACE2. This approaching pathway has been confirmed in the SARS-CoV-2 (coronavirus clade 2; COVID-19) infected airway, in which, when binding to ACE2, the virus S-proteins are activated by TMPRSS2 to facilitate the viral-cellular fusion and augment viral entry [187]. In addition to the respiratory system, both ACE2 and TMPRSS2 are highly expressed in multiple tissues such as gastrointestinal, renal, and cardiovascular systems, which may explain why the COVID-19 causes a wide spectrum of clinical symptoms with multi-organ dysfunction [110].

ACE2 The encoding gene for ACE2 is located on the X-chromosome [47], which raises a question as to whether the double X-chromosome in females protects against, or exacerbates, COVID-19 infection. Alternatively, ACE expression seems more likely to be regulated by sex hormones than X-chromosomes, since controversial results of either up- [14, 51] or downregulation of ACE2 by estrogens [46, 103] imply that the sex hormone-driven regulation of ACE belongs to a multi-mechanism-engaged response. Particularly, estrogen-modulation of ACE2 yields a feature of tissue-specificity, even in the presence of COVID-19 infection [86, 97]. Furthermore, studies also reported a SARS-CoV Spike protein-elicited downregulation of ACE2 [48, 84], and SARS-CoV-2-dependent, IFN-driven upregulation of ACE2 [1, 187]. Nevertheless, behind these confounding results, the pathophysiological significance in the infected hosts still remains unknown. To date, there is no consensus for changes in ACE2 expression being protective, or detrimental during pathological process of COVID-19 infection, nor evidence indicating the lower female mortality dependent of ACE2 responses. Thus, it is

worthwhile to note whether regulations of TMPRSS2 and interferons are also affected by sex hormones.

TMPRSS2 Indeed, androgen receptor (AR) activity has been considered as a requirement for the transcription of the TMPRSS2 gene (its promoter contains androgen-responsive elements; ARE) [106]. Currently, Wambier et al. demonstrated the severity of SARS-CoV-2 infection proportional to the androgenic actions. As reported, androgens (A) via the formation of transcription complex A-AR-ARE upregulates and activates TMPRSS2, an event required for SARS-CoV-2 infectivity [173–175]. Their findings provide mechanistically based explanations for the androgen-mediated SARS-CoV-2 vulnerability and higher mortality in males and also raise the therapeutic possibility for using anti-androgenic approaches in the clinical treatment of COVID-19 [49, 114].

IFNs It is known that IFNs, as key players in the immediate antiviral response, are crucial for blocking viral replication via type I IFN receptor (IFNAR) signaling. During the incubation phase, SARS-CoV-2 replicates in host cells without detectable IFNs, resulting in high viral loads [12]. In SARS-CoV-infected mice, local IFN responses in the lung have occurred later than peak viral replication, leading to the development of cytokine release syndrome (CRS; or the so-called cytokine storm), followed by lethal lung injury [20]. In consistence, the level of circulating IFNs appears to be inversely proportional to the severity of COVID-19 infection [52]. Although the dynamic changes in the systemic and local IFN responses in COVID-19 infection and their contributions to COVID-19 pathogenesis are poorly understood, IFNs seem important in compromising the "cytokine storm", composed of a great amount of NF-κB, TNF-α, IL-1β, IL-1Ra, sIL-2Rα, IL-6, IL-10, IL-17, IL-18, IFN-γ, MCP-3, M-CSF, MIP-1a, G-CSF, IP-10 and MCP-1 [63, 183], an event that is central to the pathophysiology of COVID-19 [1, 71]. As a result, the robust production of pro-inflammatory cytokines and chemokines, concomitant with a limited production of IFNs and impaired IFNAR signaling, work synergistically to promote the disease progression. To this end, it becomes imperative to clarify how SARS-CoV-2 antagonizes IFN induction and dysregulates IFNAR signaling, even though IFNα drives ACE2 expression while initiating protective immune responses. Regarding the sex-different regulation of IFNs, the estrogen promotion of IFN expression/activity and significant contributions of IFNs to high prevalence of autoimmune diseases in women have already been demonstrated [142, 143]. In particular, IFNα is a major subform of IFN family in driving COVID-19 pathogenesis [1, 187], as indicated by the evidence that in response to SARS-CoV-2, human plasmacytoid dendritic cells (pDCs: important sources of IFNs in RNA virus infections) [82] produce IFNα that, in turn, elicits virus clearance [18]. In this context, a sex bias in pDC-derived higher IFNα levels in women [50] has been demonstrated to be dependent of an ERα-mediated pathway [150]. Moreover, female upregulation of IFNα mRNA already exists in the basal condition, which is believed to be responsible for the female-favorable immunological production of IFNα [188].

Overall, given that androgen upregulates TMPRSS2 expression and activity to amplify COVID-19 progressive signaling, and that estrogen promotes IFNα production to potentiate the viral clearance and mitigates cytokine storm via impeding the release of inflammatory agents, including, but not limited to, NF-κB, TNFα, and IL-6 [102, 158], it is plausible to speculate a less preference of COVID-19 infection in females.

7.6 Perspectives and Conclusions

To date, the estrogen-paradox remains an elusive puzzle, which inspires further research in this realm. The genetic predisposition, including germline mutations and epigenetic mechanisms via DNA methylation or microRNA, appears to be a prerequisite for female susceptibility to the development of PAH. The sex-specific phenotype

depends primarily on differences in intrinsic (genes) and sex hormones, most likely with complex interactions between the two aspects. Particularly, the coordination of differential gene expression between sexes with the potential contributions of sex steroids, as well as a myriad of pathological factors, determines the sex-specific phenotype presented in the pulmonary circulation. This chapter aims to take a step closer to understand how the genetic predisposition interacting with sex hormones influences pulmonary pathophysiology. Looking forward, the responsible gene(s) and/or irregulating estrogen signaling could be explored in the near future, as therapeutic targets during clinical treatment of pulmonary diseases particularly in women who bear an oversensitivity to the diseases.

Acknowledgments This work was supported by NIH grants R01 HL129797, HL070653, and HL144528.

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8 Hypercapnic Respiratory Failure-Driven Skeletal Muscle Dysfunction: It Is Time for Animal Model-Based Mechanistic Research

Ariel Jaitovich

Abstract

Dysfunction of locomotor muscles is frequent in chronic pulmonary diseases and strongly associated with worse outcomes including higher mortality. Although these associations have been corroborated over the last decades, there is poor mechanistic understanding of the process, in part due to the lack of adequate animal models to investigate this process. Most of the mechanistic research has so far been accomplished using relevant individual stimuli such as low oxygen or high CO_2 delivered to otherwise healthy animals as surrogates of the phenomena occurring in the clinical setting. This review advocates for the development of a syndromic model in which skeletal muscle dysfunction is investigated as a comorbidity of a well-validated pulmonary disease model, which could potentially allow discovering meaningful mechanisms and pathways and lead to more substantial progress to treat this devastating condition.

Keywords

Myogenesis · AMPK · Protein anabolism · Protein catabolism · Respiratory failure · Muscle atrophy

8.1 Introduction

Locomotor (nonventilatory) skeletal muscle dysfunction is a common and devastating comorbidity in patients with chronic respiratory failure including chronic obstructive pulmonary disease (COPD) and others [32, 34, 64]. It is associated with worse clinical outcomes including disability, frequent hospitalizations, and mortality [46, 62, 65]. These associations persist even after multivariably correcting for the level of pulmonary disease, which suggests that muscle dysfunction could potentially be responsible for the poor outcomes [62, 65]. Although not every patient with COPD develops muscle dysfunction [40, 50, 51], it occurs more significantly in individuals with emphysema than in those with chronic bronchitis [75]. These clinical correlations, although highly corroborated over several decades, do not provide any insight on the specific mechanisms regulating the process of muscle dysfunction. A major limitation of previous research is that it is based on animal models that evaluate the effects of single stimuli to otherwise healthy animals [31, 72]; or on pulmonary inflam-

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_8

mation not characterized by the fundamental features of COPD, including chronic airways obstruction, increased lung volumes, and others [41, 42, 69]. Thus, the interaction between COPD as a complex disease and skeletal muscle dysfunction has never been evaluated using a comprehensive and well-validated animal model of pulmonary emphysema. Muscle dysfunction results from a complex and often nonlinear interaction between reduced muscle mass and altered fibers metabolism [5, 6], causing decreased force-generation capacity, either in the form of maximal or submaximal force [3, 4].

In this review, I argue that mechanistic research based on a validated animal model is urgently needed to produce meaningful information intended to develop innovative data and substantially advance the field. I start by outlying the basic principles of skeletal muscle dysfunction in chronic respiratory using COPD as an example. Then I present the problem caused by reductionist approaches to address this problem, followed by the description of current animal models of COPD. I then introduce data from my lab that has established new approaches with potentially translational value and propose future avenues that in my opinion should be pursued over the next few years to advance the field in an innovative way.

8.2 General Principles of Locomotor Skeletal Muscle Dysfunction in Pulmonary Diseases

Pulmonary disease-driven skeletal muscle dysfunction is a process that occurs in patients with acute [15, 26, 34, 59] and chronic pulmonary diseases [62, 64, 65], and that does not involve a primary muscle condition. Typically, it leads to decreased force generation capacity and involves, to some extent, a combination of muscle mass reduction or atrophy and metabolic disruption of muscle fibers [32]. Muscle atrophy causes a decreased maximal force-generation capacity; and metabolic disruption leads to higher fatigability, or reduced submaximal force, also known as endurance [3]. Clinical evidence indicates that both reduced muscle mass and force-generation capacity are associated with worse outcomes including mortality. Moreover, although the process of fiber metabolic disruption is complex, it is to some extent correlated with the isoform of myosin-heavy chain (MyHC) expressed by the muscle fiber [61]. Fibers that express MyHC type I are slow twitched, more fatigue resistant, relatively oxidative, and calcium sensitive, whereas MyHC type II fibers are fast twitched, more fatigable, less oxidative (also called glycolytic), and less calcium sensitive [61]. In chronic pulmonary diseases such as COPD, locomotor muscles repopulate the fiber composition toward a higher presence of type II fibers, a process known as fiber switch or transformation [11]. Therefore, even without necessarily losing muscle mass, these patients develop a more fatigable phenotype that impacts their force-generation capacity in the form of lower endurance. Moreover, fiber transformation is independently associated with higher mortality in COPD [53]. Patients with other chronic pulmonary diseases such as cystic fibrosis also develop muscle dysfunction, which indeed is associated with higher mortality [64]. We have also recently demonstrated that reduced muscle mass of patients with predominantly acute pulmonary diseases is strongly and independently associated with worse outcomes including higher mortality [34, 35]. At a mechanistic level, clinical observations suggest a role of distinct processes including muscle protein accelerated proteasomal degradation [54], reduced protein anabolism [54], dysregulated autophagy [23, 57, 70], reduced expression of oxidative enzymes [47, 48], and others [5, 6]. However, it is unclear the nature of these associations with muscle dysfunction. For example, clinical evidence indicates significant alteration of autophagy in COPD muscles [23]. While seminal research established that autophagy integrity supports muscle mass [49], controversy surrounds its relevance COPD [29]. Indeed, recent data suggest that inhibition of autophagy prevents COPD muscle fibers' atrophy [20]. Moreover, while some authors reported that muscle mRNA levels of BECLIN1 and LC3B are not

significantly different among COPD versus healthy controls [54], others have shown increased LC3B-II (marker of autophagosomes formation), BECLIN1 and SQSTM1 (p62) protein levels in muscle biopsies of COPD patients [23], suggesting that autophagy is significantly induced in this setting. Therefore, it remains undefined if *dys*regulated autophagy in COPD skeletal muscle represents global *down*regulation, *up*regulation, or deacceleration of autophagy flux with preservation of other regulatory components [44]. It is also unclear whether one or more autophagy specific steps such as nucleation [60] or lipidation [52] are affected, or whether there are different phenotypes variably expressed in the COPD population [37, 38]. COPD-driven autophagy dysregulation could be mediated by oxidative stress signals, which are ubiquitous in that population [57] and known to undermine critical autophagy steps including inhibition of Atg7 [18].

8.3 Reductionist Models to Investigate a Complex Comorbidity

The autophagy example presented few lines above illustrates how a reductionist system—autophagy loss at the muscle fiber level—fails to clearly explain a central clinical observation on these patients. Reductionist models indeed consist in exposing an otherwise "healthy" system such as a cell line of an animal strain to a stimulation that is known or presumed to be relevant in a certain disease; and drawing conclusion out of that setting assuming the observation reflects the disease setting. Reductionist systems are extremely popular and have predominantly served mechanistic research in muscle dysfunction associated with pulmonary diseases. We have investigated the role of elevated CO_2 exposure, or hypercapnia, in skeletal muscle turnover, both in vivo and in vitro [30, 31, 39]. Both mice and cultured primary myotubes demonstrate, under controlled high CO_2 conditions, decrease in fiber diameter that is associated with upregulation of muscle ring finger-1 (MuRF1) expression. Indeed, both silencing MuRF1 with siRNA and genetic knockout of that ligase in vivo abrogate the CO_2-induced muscle loss. These processes are regulated by the energy sensor AMP-activated protein kinase (AMPK), which targets the transcription factor FoxO3 at six specific serine residues and increases the binding of that factor with MuRF1 promoter in the DNA, leading to proteasomal degradation of myosin and muscle atrophy [21, 31]. This pathway is fully recapitulated with high CO_2 exposure of nontransformed C2C12 cells, which are cultured as myoblasts and can be differentiated into myotubes highly reminiscent of muscle fibers [66].

High CO_2 exposure also causes C2C12 cells' reduced expression of ribosomal gene expression [31, 39]. That observation led to the hypothesis that CO_2 could also cause depressed protein synthesis. We directly confirmed that process in skeletal muscle biopsies from patients with hypercapnic (elevated CO_2) pulmonary disease, which demonstrated significant downregulation of ribosomal gene expression as well [39]. Indeed, unbiased analysis of large-scale muscle proteome from mice's muscles exposed to a controlled high CO_2 environment (with normal oxygen) revealed reduced structural constituents of the ribosome as one of the most significantly downregulated terms, and critical components of the messenger RNA recruitment to the ribosome were also reduced [39]. Depressed ribosomal biogenesis associates with downregulation of protein synthesis, as shown by experiments performed with the synthetic amino acid puromycin both in vivo and in vitro [19]. Depressed ribosomal gene expression and protein synthesis in hypercapnia are controlled by AMPKα2 as revealed by the lack of CO_2 anabolic attenuation in cells with previous silencing of the AMPKα2 gene [39]. While previous research indicates that AMPK-driven depressed ribosomal gene expression is mediated by the transcription factor TIF1-A [28], we were not able to show that process in the context of hypercapnia, even after validation of the expression and silencing of TIF1-A gene with crispr-Cas9 technology [39].

However, attractive, reductionist approaches have the limitation of lacking a disease context

where the investigated signals operate. For example, most hypercapnic patients have also pulmonary disease, but hypercapnia research has been accomplished with CO_2 stimulation to otherwise healthy mice. Although we showed the relevance of AMPK signaling in hypercapnia, clinical studies have observed the upregulation of that signaling in nonhypercapnic patients [23]. Similar reductionist approaches are used with other common areas of interest such as cigarette smoking, hypoxia, and others [72, 73]. Moreover, some skeletal muscle research has been done with models that express a cytokine assumed as relevant in COPD but do not develop a phenotype reminiscent of a clinically relevant picture in the used models [41, 42, 69]. Therefore, complex interactions that occur in the clinical setting, such as the occurrence of hypercapnia in hypoxic individuals, cannot be analyzed with these reductionist models.

8.4 Animal Models of COPD to Investigate Muscle Dysfunction

Over the last decades, COPD has been investigated with different animal models that have provided incremental level of sophistication [9]. However, these animals have been mainly designed to investigate the pulmonary morbidity and no other comorbidities such as skeletal muscle dysfunction. I briefly describe below the currently available animal models to investigate COPD/pulmonary emphysema.

First-generation models: More than 50 years ago, the first reproducible rat model of emphysema was reported by instilling the proteinase papain intratracheally [22]. Further studies in other animals demonstrated that enzymes with the capacity to solubilize the extracellular matrix protein elastin can produce emphysematous lesions. These observations, coupled with the discovery of the enhanced risk for development of emphysema in individuals with α1-antitrypsin deficiency [16], led to the proteinase–antiproteinase hypothesis and to modern thinking about the pathogenesis of emphysema.

Second generation models: The ability to over express or delete mouse genes led to the establishment of genetic models, including the constitutive over expression of matrix metalloproteinase type 1 (MMP-1) (collagenase) [12], deletion of macrophage elastase (MMP-12) [24], or inducible overexpression of interleukin-13 (IL-13) in Club cells [78]. Genetic animals have the advantage of developing a robust and stereotyped phenotype, and in the inducible versions, being versatile enough to define the timing of pulmonary disease. Moreover, by exploiting the specific signaling leading to a phenotype, they can provide insight into that specific mechanism regulating pulmonary disease. For instance, some patients with COPD demonstrate elevation of IL-13 which is associated with the severity of airways obstruction [43], and thus the IL-13 animal has the advantage of not only reminiscing the phenotype but also the potential mechanism of emphysema.

Third generation models: Technology now allows targeted deletion of murine genes, followed by inserting (or "knocking-in") human genes under control of the murine promoters. The development of CRISPR/Cas9-mediated genome editing will likely facilitate the process. Recently, an animal model of α-1 antitrypsin (AAT) deficiency-associated pulmonary emphysema has been developed [8]. This animal has the advantage of developing a robust phenotype of a well-defined human disease caused specifically due to that gene alteration.

An ideal model of COPD-skeletal muscle dysfunction should fulfill the following conditions: (1) be inducible, in order to minimize temporal confounders such as muscle development and age-related sarcopenia [17, 36]; (2) be robust enough and slowly developing, to reminisce a level of disease severity and chronicity shown by the majority of COPD patients with muscle dysfunction [50]; (3) develop the muscle phenotype after, and not simultaneously with, the occurrence of pulmonary disease [27, 74]; (4) recapitulate features observed in humans including morphologic, metabolic, and functional aspects of muscle dysfunction [9]; and (5) develop muscle dysfunction in the context of COPD and

not due to a single stimulus to an otherwise healthy mouse. That animal model would represent a bottom line to understand complex interactions occurring in patients suffering from COPD-associated muscle dysfunction. Analysis of specific mechanisms driven by single stimuli can be performed independently, and eventually merged with a syndromic model such as the one we present, contributing clinically more meaningful data.

8.5 Current Platforms to Perform Mechanistic Research in Disease-Focused Animal Models

We have investigated skeletal muscle dysfunction in a previously established mouse model of pulmonary emphysema [78] based on the over expression of IL-13 in club cells. This mouse fulfills all the mentioned criteria in the previous section [2]: condition #1: doxycycline inducible, Club cell-targeted interleukin 13 overexpression (*IL13*^{TG}); condition #2: a very robust pulmonary phenotype (Fig. 8.1a) with significant hypoxemia (mean saturation ~77% at room air) but *not chronic CO_2 retention* (HCO_3^-23.6 ± 2.3 mEq/L); condition #3: a consistent trajectory of weight loss that occurs after emphysema development (Fig. 8.1b); condition #4: reduction of muscle mass, force-generation capacity; and metabolic dysfunction as shown by COPD patients (Fig. 8.1c–e). This model deliberately does not involve cigarette smoking as this exposure causes muscle toxicity independently of pulmonary disease [7, 10, 13] (contradicts condition #3), leads to minimal weight and muscle loss [73] (contradicts condition #4), and represent a single stimulus to an otherwise healthy animal (contradicts condition #5).

We have recently found that this animal skeletal muscle demonstrates elevation of three biomarkers identified with COPD [2, 33]: ceruloplasmin, haptoglobin, and hemopexin [76]. These oxidative stress-response proteins show high level of correlation in serum and muscle, both under steady-state conditions and after exercise; and the levels are highly correlated with muscle integrity measured by muscle mass, force generation capacity, and oxygen consumption [33]. Future human studies with more individuals could allow multivariable modeling and define whether these biomarkers have incremen-

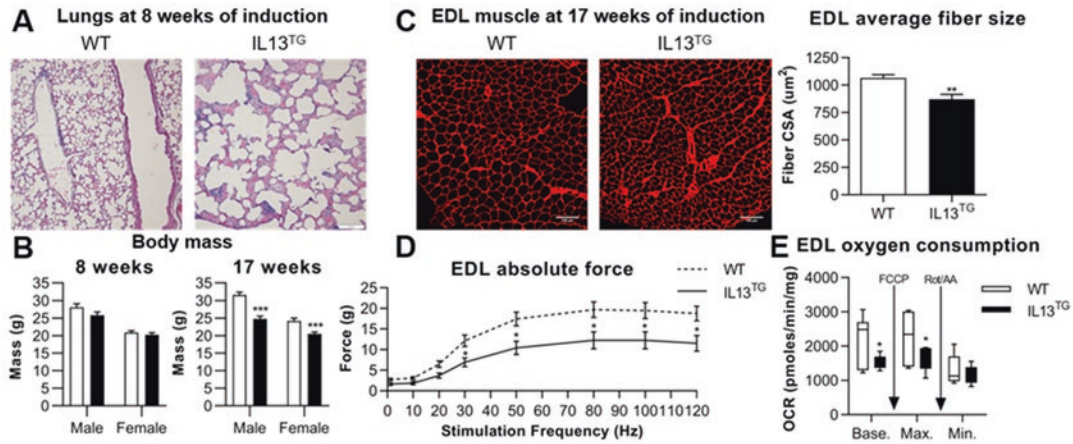

Fig. 8.1 Our established animal model of emphysema-induced skeletal muscle dysfunction [2] develops (**a**) pulmonary remodeling at 8 weeks post-induction; (**b**) body mass difference significant at 17 but not 8 weeks, indicating no muscle atrophy before lung remodeling. Extensor digitorum longus (EDL) muscle cross-sectional area (**c**), isolated muscle force-generation capacity (**d**), and Seahorse®-measured oxygen consumption (**e**), are all significantly reduced at 17 weeks post induction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $N = 8$ (4 males and 4 females)

tal effects given the persistent association of individual proteins with exercise-induced muscle recovery after adjusting for the other ones. That multivariable persistence could allow developing instruments to improve the biomarkers predicting capacity by combining determinations of haptoglobin, hemopexin, and ceruloplasmin to appreciate muscle dysfunction in COPD.

8.6 Future Avenues to Produce Innovative Research in the Field

Merging specific relevant stimuli with a platform of sophisticated "syndromic" animal models will contribute insight into complex interactions of multiple domains acting synergistically or antagonistically on specific phenotypes. Canonical processes such as proteasomal protein degradation, autophagy, or protein anabolism can be interrogated in a more comprehensive way. Other processes such as myogenesis—muscle turnover contributed by satellite (stem) cells could be investigated [77]. This process participates in muscle homeostasis in the context of organ development and injury-repair cycles [77]. Injurious events crucially occur in COPD in the setting of exacerbations and infections [58, 63], which lead to acute decompensations for limited periods of time after which patients typically fail to recover the baseline status they had before the acute event [1]. Indeed, frequency and severity of COPD exacerbations and infections powerfully associate with loss of muscle and lung integrity, and with higher mortality over time [1, 14, 68]. While strong evidence indicates that dysfunctional myogenesis contributes to muscle loss in different nonprimarily muscular conditions such as aging and cancer [25, 67], clinical observations suggest its possible role in COPD [25, 55, 56, 71]. Moreover, myogenesis has been recently demonstrated to alter the fiber-type population of skeletal muscle by contributing glycolytic phenotype at the expense of twist-2 myogenic progenitors [45]. Thus, research in the field of muscle injury/myogenesis could lead to better understanding of the process of fiber transformation. As COPD-induced muscle wasting leads to atrophy and disrupted metabolism, it is unclear if these two processes are obligatory associations. Rescue of metabolic properties via gain of function of oxidative enzymes could define if that intervention can reverse other nonmetabolic domains of muscle dysfunction such as maximal muscle force and others. The specific role of ongoing versus previous smoking on skeletal muscle phenotype can also be disaggregated with a syndromic model leading to the identification of specific interventions that could antagonize direct smoking toxicity.

8.7 Conclusion

Locomotor skeletal muscle dysfunction is a major comorbidity in chronic obstructive pulmonary disease and other pulmonary conditions, although available data were obtained either by clinical observations, or with models that use single stimuli to otherwise healthy animals. I advocate for a new phase of mechanistic research based on sophisticated animal models demonstrating a syndromic process in which muscle dysfunction occurs in the context of a well-established pulmonary emphysema. That approach will set up a foundation of innovative research where complex interactions between defined domains or noncanonical pathways such as myogenesis and metabolism are interrogated to allow novel treatment for this devastating condition.

Acknowledgments I thank Joseph Balnis for performing the experiments presented in this manuscript's figures.

Sources of Funding Part of the results reported herein have been funded by NHLBI of the National Institutes of Health under the award number K01-HL130704 and by the Collins Family Foundation Endowment.

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Role of Airway Smooth Muscle in Inflammation Related to Asthma and COPD

9

Hiroaki Kume

Abstract

Airway smooth muscle contributes to both contractility and inflammation in the pathophysiology of asthma and COPD. Airway smooth muscle cells can change the degree of a variety of functions, including contraction, proliferation, migration, and the secretion of inflammatory mediators (phenotype plasticity). Airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, and airway remodeling, which are fundamental characteristic features of these diseases, are caused by phenotype changes in airway smooth muscle cells. Alterations between contractile and hyper-contractile, synthetic/proliferative phenotypes result from Ca^{2+} dynamics and Ca^{2+} sensitization. Modulation of Ca^{2+} dynamics through the large-conductance Ca^{2+}-activated K^+ channel/L-type voltage-dependent Ca^{2+} channel linkage and of Ca^{2+} sensitization through the RhoA/Rho-kinase pathway contributes not only to alterations in the contractile phenotype involved in airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization but also to alteration of the synthetic/proliferative phenotype involved in airway remodeling. These Ca^{2+} signal pathways are also associated with synergistic effects due to allosteric modulation between β_2-adrenergic agonists and muscarinic antagonists. Therefore, airway smooth muscle may be a target tissue in the therapy for these diseases. Moreover, the phenotype changing in airway smooth muscle cells with focuses on Ca^{2+} signaling may provide novel strategies for research and development of effective remedies against both bronchoconstriction and inflammation.

Keywords

Large-conductance Ca^{2+}-activated K^+ channels · β_2-adrenergic receptors · Rho-kinase · Ca^{2+}signaling · Phenotype change · Allosteric effect

Abbreviation

ACh	acetylcholine
ADP	adenosine diphosphate
AF-DX116	11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	concentration of intracellular Ca^{2+}

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_9

CaM	calmodulin
cAMP	3′-5′-cyclic adenosine monophosphate
CCh	carbachol
cGMP	3′-5′-cyclic guanosine monophosphate
ChTX	charybdotoxin
COPD	chronic obstructive pulmonary disease
CPI-17	C-kinase potentiated protein phosphatase-1 inhibitor
CTX	cholera toxin
GPCRs	G protein-coupled receptors
CRAC	Ca^{2+} release-activated Ca^{2+} current
EETs	epoxyeicosatrienoic acids
G_s	a stimulatory trimeric G protein of adenylyl cyclase
G_i	an inhibitory trimeric G protein of adenylyl cyclase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HA-1077	fasudil hydrochloride
20-HETE	20-Hydroxyeicosatetraenoic acid
H_2O_2	hydrogen peroxide
IbTX	iberiotoxin
IP_3R	inositol-1,4,5-triphosphate receptor
K_{Ca} channel	large-conductance Ca^{2+}-activated K^+ channel
LABA	long-acting β_2-adrenergic receptor
LAMA	long-acting muscarinic receptor antagonist
Lyso-PC	lysophosphatidylcholine
MCh	methacholine
MLC	myosin light chain
MLCK	myosin light chain kinase
MP	myosin phosphatase
MYPT1	myosin phosphatase targeting subunit 1
nPo	open-state probability
NO	nitric oxide
$ONOO^-$	peroxynitrite
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PTX	pertussis toxin
RhoA	a monomeric G protein
ROC	receptor-operated Ca^{2+} entry
ROS	reactive oxygen species
RyR	ryanodine receptor
SOC	store-operated capacitative Ca^{2+} entry
S1P	sphingosine 1-phosphate
SR	sarcoplasmic reticulum
STOCs	spontaneous outward currents
TGF-β1	transforming growth factor beta 1
TRP	transient receptor potential channel
VDC channel	L-type voltage-dependent Ca^{2+} channel
Y-27632	(R)-4-(1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide dihydrochloride

9.1 Introduction

Airway smooth muscle contraction contributes to airflow limitation, which is implicated in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Airway smooth muscle tone is regulated by myosin light chain (MLC), which is phosphorylated by myosin light chain kinase (MLCK) and dephosphorylated by myosin phosphatase (MP). Activation of MLCK is mediated by an increase in concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via Ca^{2+} influx through various types of Ca^{2+} channels (Ca^{2+}-dependent mechanisms, Ca^{2+} dynamics). In contrast, inactivation of MP is mediated by an increase in the sensitivity to intracellular Ca^{2+} via Rho-kinase, which is a protein affected by RhoA, a monomeric G protein (Ca^{2+}-independent mechanisms, Ca^{2+} sensitization) [1].

Inhibition of both Ca^{2+} dynamics and Ca^{2+} sensitization is associated with the effects of β_2-adrenergis receptor agonists against muscarinic contraction [1–3]. Moreover, these agonists relax airway smooth muscle via 3′-5′-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (protein kinase A: PKA), leading to inactivation (phosphorylation) of MLCK. Large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels are markedly activated by PKA-induced phosphorylation [4–7] and G_s-induced membrane-delimited

action (G_s, a stimulatory trimeric G protein of adenylyl cyclase) (dual pathway) [5–8]. In contrast, K_{Ca} channels are suppressed by muscarinic receptor agonists via G_i, an inhibitory trimeric G protein of adenylyl cyclase (dual regulation by G_s and G_i) [8, 9]. The functional antagonism between β_2-adrenergic and muscarinic receptors (G protein-coupled receptors: GPCRs) may converge on these channels. Since K_{Ca} channels have a large conductance of outward currents and exist innumerably on the cell membrane in airway smooth muscle [10], the opening of these channels also regulates airway smooth muscle tone mediated by membrane potential-dependent Ca^{2+} influx (Ca^{2+} dynamics), such as L-type voltage-dependent Ca^{2+} (VDC) channels [11].

Airway smooth muscle cells play essential roles in the pathophysiology and therapy for asthma and COPD because these cells have the ability to change the degree of various functions, such as contractility, proliferation, migration, and synthesis of inflammatory mediators, referred to as phenotype plasticity [1, 12, 13]. The plasticity from a contractile phenotype to hyper-contractile and synthetic/proliferative phenotypes (proliferation, migration, or secretion of chemical mediators) may result in an increase in contractility and inflammation in the respiratory tracts, leading to airflow limitation, airway hyperresponsiveness, and airway remodeling (characteristic features of asthma and COPD). Therefore, these phenotype changes in airway smooth muscle cells may be associated with key characteristics of pathogenesis of these diseases.

Alterations of contractile phenotype, which is a characteristic feature of patients with asthma and COPD, may result from Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) and K_{Ca} channels in airway smooth muscle cells [1, 7, 14–17]. Alterations of synthetic/proliferative phenotype also results from Ca^{2+} dynamics [18, 19] and Ca^{2+} sensitization [1, 16, 17, 20–24]. Clinical trials have demonstrated that a VDC channel inhibitor reduces airway remodeling in patients with severe asthma [25], and that a novel African-specific coding polymorphism (the 818 T allele) in β_1 subunit of K_{Ca} channels is associated with severity and morbidity of asthma via inactivation of these channels [26]. In sensitized mice as asthma model, rottlerin, a K_{Ca} channel agonist, results in reducing both inflammation and hyperresponsiveness in the airways [27]. Ca^{2+} signaling and K_{Ca} channels may contribute not only to contraction but also to inflammation in the airways. Therefore, these processes may play key roles in research and development for remedy of asthma and COPD [28, 29].

In this chapter, the functional characteristics of airway smooth muscle involved in alterations of contractile and synthetic/proliferative ability (phenotype changes) are examined with a focus on Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) mediated by the G protein/K_{Ca} channel/VDC channel linkage and the RhoA/Rho-kinase processes. Moreover, data will be reviewed in detail from various fields (physiology—molecular biology) regarding phenotype changing in airway smooth muscle cells to seek a novel strategy for developing more effective agents for asthma and COPD that are beneficial both to contraction and to inflammation in the respiratory tracts.

9.2 Mechanical Characteristics of Airway Smooth Muscle

9.2.1 General

Contractile agonists acting on G protein-coupled receptors (GPCRs), such as methacholine (MCh), histamine, prostaglandins, leukotrienes, and endothelin, initially cause phasic contraction of airway smooth muscle, subsequent to tonic contraction with increasing concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) mediated by Ca^{2+} influx passing through various Ca^{2+} channels (Ca^{2+} dynamics) [30]. When these agents (ligands) are connected to the GPCRs, receptor-operated Ca^{2+} (ROC) entry is activated [31], and then Ca^{2+} is released from sarcoplasmic reticulum (SR) via the production of inositol-1,4,5-triphosphate receptor (IP_3R) and ryanodine receptors (RyR) in airway smooth muscle (Fig. 9.1) [32, 33]. This Ca^{2+} release from SR activates store-operated capacitative Ca^{2+} (SOC) entry, that is, Ca^{2+}

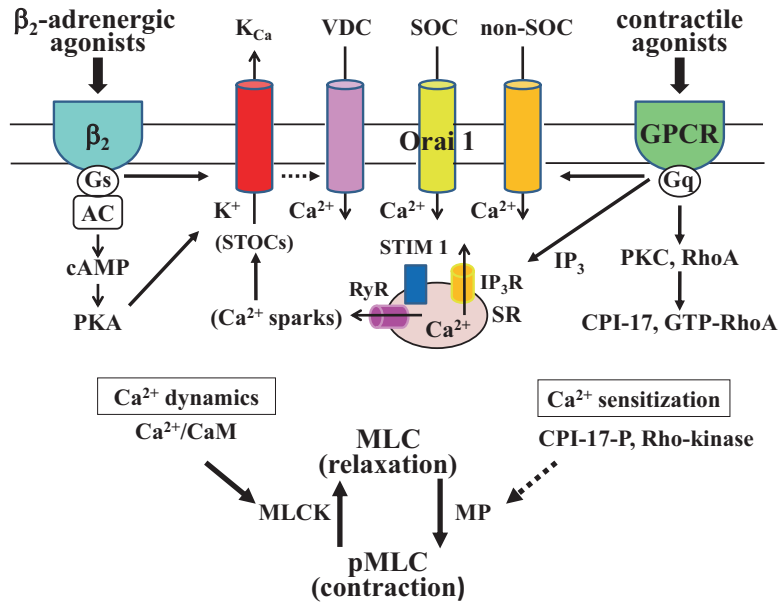

Fig. 9.1 Mechanisms of the regulation of airway smooth muscle tone. β_2-Adrenergic receptor agonists activate K_{Ca} channels via PKA and G_s, leading to inactivation of VDC channels. Contractile agonists that act on GPCRs activate SOC entry, non-SOC entry, and VDC channels. Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) contributes to the functional antagonism between β_2-adrenergic receptor agonists and these contractile agonists related to GPCRs. MLC phosphorylation (pMLC), which is regulated by the balance between MLCK and MP, is fundamental for controlling contraction in airway smooth muscle. GPCR-related agents cause Ca^{2+} influx and cause Ca^{2+} release from the SR by producing IP_3. The latter process induces Ca^{2+} influx via activation of SOC. An increase in concentration of intracellular Ca^{2+} mediated by these processes enhances the binding of Ca^{2+} to CaM. A Ca^{2+} – CaM complex (Ca^{2+}/CaM) augments MLCK activity, leading to MLC phosphorylation (Ca^{2+} dynamics: Ca^{2+}-dependent mechanisms). Contractile agonists acting on GPCRs activate PKC and RhoA. The PKC/CPI-17/CPI-17-P processes and the RhoA/GTP-RhoA/Rho-kinase processes phosphorylate (inactivate) MP, leading to MLC phosphorylation (Ca^{2+} sensitization: Ca^{2+}-independent mechanisms). Muscarinic receptor antagonists mainly suppress only Ca^{2+} dynamics; in contrast, β_2-adrenergic receptor agonists antagonize both Ca^{2+} dynamics and Ca^{2+} sensitization, ultimately inducing the inhibition of MLCK and muscle relaxation. ACh: acetylcholine, LTs: leukotrienes, PGs: prostaglandins, β_2: β_2-adrenergic receptors, GPCR: G protein-coupled receptor, AC: adenylyl cyclase, ROC: receptor-operated Ca^{2+} entry, SOC: store-operated Ca^{2+} entry, IP_3: inositol-1,4,5-triphosphate, IP_3R: IP_3 receptor, SR: sarcoplasmic reticulum, PKA: protein kinase A, PKC: protein kinase C, CPI-17: C-kinase potentiated protein phosphatase-1 inhibitor, CaM: calmodulin, MLCK: myosin light chain kinase, MLC: myosin light chain, MP: myosin phosphatase, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels, RyR: ryanodine receptor, STIM 1: Stromal interaction molecule 1, STOCs: spontaneous outward currents. Arrows: activation, dotted arrows: inhibition. Illustrated based on ref. [1–5, 11, 30, 32, 33, 37, 39–41, 44, 49–51, 53, 54]

release-activated Ca^{2+} (CRAC) currents [30]. Although transient receptor potential (TRP) channels may be involved in the conduction of SOC influx [34], it is recently considered that the pore-forming protein Orai 1 is an essential component of the CRAC currents [35, 36]. Stromal interaction molecule 1 (STIM 1), which is Ca^{2+} sensor for store depletion in the SR, activates SOC entry at the cell membrane formed by Orai 1. This STIM 1/Orai 1 process is associated with SOC entry in airway smooth muscle (Fig. 9.1) [37]. Moreover, ROC entry consists of not only SOC entry but also Ca^{2+} entry independent of the store-operated mechanisms (non-SOC entry) [30]. However, relationship between the pathway of Ca^{2+} release from SR (IP_3R or RyR) and the component of contraction (phasic or tonic) is not so clear in various smooth muscles. On the other

hand, Ca^{2+} entry passing through VDC channels are mainly activated by membrane depolarization under the condition of high K^+ at the extracellular side. VDC channels mainly contribute to high K^+-induced contraction. In contrast, VDC channels are partly involved in the GPCR-mediated Ca^{2+} entry [11]. β_2-Adrenergic receptor agonists activate K_{Ca} channels in airway smooth muscle cells [4, 5]. Since K_{Ca} channels may be involved in membrane potential, VDC channels are regulated by K_{Ca} channel activity [11, 38]. An increase in $[Ca^{2+}]_i$ enhances the binding of Ca^{2+} to calmodulin (CaM), a calcium-binding messenger protein. Myosin light chain kinase (MLCK) is activated by a Ca^{2+}-CaM complex (Ca^{2+}/CaM), and Myosin light chain (MLC) is phosphorylated (activated) by MLCK [1, 16, 29], leading to contraction of airway smooth muscle (Ca^{2+}-dependent contraction: Ca^{2+} dynamics) (Fig. 9.1) [1, 16, 29]. After activated MLC is dephosphorylated (inactivated) by myosin phosphatase (MP), contraction is reversed to relaxation. On the other hand, contractile agonists activate RhoA, a monomeric G protein, and protein kinase C (PKC) mediated by stimulating GPCRs. RhoA is activated by binding to GTP (RhoA-GTP: active form of RhoA). Rho-kinase, a serine/threonine kinase, is activated by RhoA-GTP, and MP is phosphorylated by Rho-kinase (MP inactivation) [40, 41]. MP is also phosphorylated by C-kinase potentiated protein phosphatase-1 Inhibitor (CPI-17), which is another potential mediator regulated by PKC [42, 43]. Since MLC activity is sustained, not suppressed, by loss of MLC dephosphorylation via inactivation of MP, airway smooth muscle tone is enhanced without increasing $[Ca^{2+}]_i$ (Ca^{2+}-independent contraction: Ca^{2+} sensitization) (Fig. 9.1) [39, 44]. Airway smooth muscle tone is regulated by the degree of MLC phosphorylation mediated by both MLCK and MP activity. Alterations of contractile phenotype, which are caused both by Ca^{2+} dynamics and by Ca^{2+} sensitization, have clinical relevance to airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization, which are implicated in the pathophysiology of asthma and COPD [1, 16, 17, 28, 29].

9.2.2 Ca^{2+} Dynamics

9.2.2.1 Membrane Potential–Independent Ca^{2+} Dynamics

When isometric tension and $[Ca^{2+}]_i$ are simultaneously recorded using fura-2-loaded tissues of tracheal smooth muscle, various contractile agonists (ACh, histamine, prostaglandins, leukotrienes, endothelin, etc.) including contractile agonists acting on GPCRs increase smooth muscle tension with elevated $[Ca^{2+}]_i$ in a concentration-dependent manner [39, 45, 46]. However, since these agents cause a modest depolarization of the cell membrane in a microelectrode experiment, airway smooth muscle contracts by Ca^{2+} entry via membrane potential–independent pathways. These Ca^{2+} dynamics with a modest depolarization are associated with Ca^{2+} entry through SOC and ROC entry [30, 31]. Depletion of the SR Ca^{2+} stores by thapsigargin, an inhibitor of the SR Ca^{2+}-ATPase, also leads to smooth muscle contraction with elevated $[Ca^{2+}]_i$ in the airways, demonstrating Ca^{2+} entry through SOC entry (Fig. 9.1) [30]. Since SOC entry is not inhibited by nifedipine, an inhibitor of VDC channels, VDC channels are not involved in SOC entry. GPCR-related agonists (MCh and histamine) cause further increases in $[Ca^{2+}]_i$ and tension under the condition that SOC entry is fully activated. These agonists activate not only SOC entry but also Ca^{2+} entry independent of SOC and VDC channels (non-SOC) [30]. The Ca^{2+} entry and contraction resulted from non-SOC is inhibited by Y-27632, an inhibitor of Rho-kinase. In contrast, Y-27632 did not affect SOC entry.

9.2.2.2 Membrane Potential–Dependent Ca^{2+} Dynamics

When concentrations of extracellular K^+ are increased more than 6 mM, smooth muscle tension is generated with elevated $[Ca^{2+}]_i$ in a concentration-dependent manner; high K^+ (40–60 mM)-induced contraction is approximately equivalent to MCh (1μM)-induced contraction in guinea pig tracheal smooth muscle. Since high concentrations of K^+ at the extracellular side causes membrane depolarization, high K^+-induced contraction results from the excitation-

contraction coupling, different from GPCR-related agonists; VDC channels are involved in this mechanism. Outward K^+ currents are suppressed under the condition of higher concentrations of extracellular K^+; K^+ channel closing generates smooth muscle tension. In contrast, K^+ channel opening leads to smooth muscle relaxation. VDC channel/K_{Ca} channel processes may be involved in the membrane potential–mediated Ca^{2+} dynamics. Activation of K_{Ca} channels serves as a brake on vasoconstriction in pulmonary vessels [47, 48]. Membrane hyperpolarization mediated by activation of K_{Ca} channels is proposed as the mechanism of bitter tastant-induced relaxation of airway smooth muscle [49], although an alternative pathway may also be an explanation. Since the membrane potential is elevated by inactivation of K_{Ca} channels, airway smooth muscle contraction may be caused by VDC channel activation via membrane depolarization [11].

In fura-2-loaded strips of tracheal smooth muscle, verapamil, an inhibitor of VDC channels, inhibits MCh-induced contraction with reduced $[Ca^{2+}]_i$; however, relaxant effects of verapamil are not so dramatic. VDC channels are partly involved in contraction mediated by GPCR-related agonists. Iberiotoxin (IbTX), an inhibitor of K_{Ca} channels, enhances muscarinic contraction with elevated $[Ca^{2+}]_i$ in airway smooth muscle. Since these effects of IbTX on tension and $[Ca^{2+}]_i$ are attenuated by verapamil [11, 38], K_{Ca} channel inactivation results in contraction with elevated $[Ca^{2+}]_i$ via opening VDC channels arisen from depolarization of cell membrane, whereas K_{Ca} channel activation results in relaxation with reduced $[Ca^{2+}]_i$ via VDC channel inactivation arisen from hyperpolarization of cell membrane.

When $[Ca^{2+}]_i$ is increased by Ca^{2+} entry resulted from various pathways explained before (Ca^{2+} dynamics), MLCK is activated by Ca^{2+}/CaM, leading to smooth muscle contraction via phosphorylation of MLC (Fig. 9.1). In airway smooth muscle, alteration of contractility regulated by Ca^{2+} dynamics is involved in the pathophysiology of asthma and COPD, such as airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization [1, 16, 17, 28, 29]. It is useful to suppress Ca^{2+} dynamics for improving these pathological conditions in the airways.

9.2.3 Ca^{2+} Sensitization

9.2.3.1 Characteristics of RhoA/Rho-Kinase

An increase in $[Ca^{2+}]_i$ results in airway smooth muscle contraction (Ca^{2+} dynamics, Ca^{2+}-dependent contraction) [11, 39]. However, it is generally considered that muscarinic receptor agonists and histamine increase tension without a marked increase in $[Ca^{2+}]_i$. This phenomenon is referred to as Ca^{2+} sensitization (Ca^{2+}-independent contraction) (Fig. 9.1) [50, 51] and is associated with G protein-coupled mechanisms. Rho is a monomeric G protein that belongs to the Ras superfamily. The Rho family makes up a major branch that contains Rho, Rac, and CdC42. Rho has isoforms of A-G; however, most of the function is described based on studies of RhoA. RhoA exhibits both GDP/GTP binding activity and GTPase activity, and it acts as a molecular switch between a GDP-bound inactive state (GDP-RhoA) and a GTP-bound active state (GTP-RhoA). When cells are stimulated with agonists related to GPCRs, GDP-RhoA is converted to GTP-RhoA. RhoA and Rho-kinase are widely distributed to many organs, including the respiratory system. Rho-kinase (160 kDa) is an effector molecule of RhoA [52, 53]. Rho-kinase activated by GTP-RhoA interacts with MP, and suppresses MP activity by phosphorylating threonine 696 and 853 of myosin phosphatase targeting subunit 1 (MYPT1), a myosin-binding subunit (Fig. 9.1) [54, 55]. Rho-kinase has effects on contraction resulted from Ca^{2+} sensitization, stress fiber formation due to actin (cytoskeletal) reorganization, cell migration, and cell proliferation [40, 56]. These phenomena are implicated in the major characteristics in the pathophysiological of asthma and COPD, such as airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, eosinophil recruitment, and airway remodeling [1, 16, 17, 28, 29].

9.2.3.2 Role of RhoA/Rho-Kinase on Tension

Y-27632, a pyridine derivative, was developed as a specific Rho-kinase inhibitor. Y-27632 relaxes vascular smooth muscle with reducing sensitivity to intracellular Ca^{2+} [41]. The effects of Y-27632 on MCh-induced contraction were analyzed by using strips of guinea pig airway smooth muscle treated with fura-2. In strips of guinea pig airway smooth muscle treated with fura-2, Y-27632 inhibits contraction induced by GPCR-related agonists, such as MCh, histamine, prostaglandins, and leukotrienes, in a concentration-dependent manner, but there is no significant decrease in $[Ca^{2+}]_i$ [39]. Y-27632 inhibits the phosphorylation of MYPT1, which is an effective protein for Rho-kinase action on MP in airway smooth muscle cells, in a concentration-dependent manner [55]. Fasudil hydrochloride (HA-1077), a specific inhibitor of Rho-kinase, is used clinically to suppress cerebral vasospasm following subarachnoid hemorrhage [57]. In allergen sensitized mice, HA-1077 suppresses MCh-induced lung resistance in a dose-dependent manner [58], indicating that Rho-kinase inhibition results in a decrease of bronchoconstriction. Alteration of contractility of airway smooth muscle regulated by Ca^{2+} sensitization is also involved in airflow limitation, airway hyperresponsiveness, and β_2-adrenegic desensitization [1, 16, 17, 28, 29].

9.2.4 Role of Ca^{2+} Signaling on β_2-Adrenergic Action

β_2-adrenergic receptor agonists (isoproterenol, procaterol, salbutamol) result in a concentration-dependent inhibition in both tension and F_{340}/F_{380} induced by MCh-induced contraction in the fura-2-loaded tissues of guinea pig tracheal smooth muscle [2, 3]. However, under the condition that these β_2-adrenergic receptor agonists cause roughly complete inhibition in tension, the values of F_{340}/F_{380} are still higher than that at the basal level [2, 3]. The concentration-inhibition curves for these β_2-adrenergic receptor agonists against MCh in tension are significantly dissociated from those curves in F_{340}/F_{380} [2, 3]. These results demonstrate that a reduction in tension is significantly greater than that in F_{340}/F_{380} in β_2-aderenergic action on airway smooth muscle. The tension–F_{340}/F_{380} curve for SKF-96365 (3–100μM), a non-selective inhibitor of Ca^{2+} influx, against MCh is on the lower side than those curves for these β_2-adrenergic receptor agonists. In contrast, the tension–F_{340}/F_{380} curve for Y-27632 (3–100μM), a specific inhibitor of Rho-kinase, is on the upper side than those curves for these β_2-adrebergic receptor agonists. The curves for these β_2-adrenergic receptor agonists exist between the curves for SKF-96365 and Y-27632 [2, 3]. These results demonstrate that a decrease not only in Ca^{2+} dynamics but also in Ca^{2+} sensitization contributes to β_2-adrenergic action on airway smooth muscle. On the other hand, glycopyrronium (a muscarinic receptor antagonist) causes a concentration-dependent inhibition of MCh-induced contraction with a marked reduction in $[Ca^{2+}]$ in fura-2-loaded tissues of tracheal smooth muscle [2], different from β_2-adrenergic receptor agonists. The concentration-inhibition curve for glycopyrronium against MCh in tension is not dissociated from those curves in F_{340}/F_{380} [2]. A decrease in Ca^{2+} sensitization may not be involved in the relaxant effect of a muscarinic receptor antagonist on muscarinic contraction. Involvement of Ca^{2+} signaling is not consistent between β_2-adrenergic receptor agonists and muscarinic receptor antagonists.

9.3 Large-Conductance Ca^{2+}-Activated K^+ Channels

9.3.1 General

Large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels are densely distributed on smooth muscle cell membrane in various organs including human airway smooth muscle [59–61]. K_{Ca} channels have a large conductance of about 250 pS in symmetrical 135–150 mM K^+ medium, as compared to other K^+ channels, and these channels are highly selective for K^+ despite their large conductance [62]. In freshly isolated human bron-

chial smooth muscle cells, single currents of K_{Ca} channels have been recorded in the electrophysiological technique such as cell-attached patches, inside-out patches, and outside-out patches [63, 64]. Typical action potentials are not observed in airway smooth muscle cells under physiological conditions (weak excitability). This lack of action potentials may result from a marked increase in outward K^+ conductance of the plasma membrane passing through K_{Ca} channels upon depolarization [65]. Augmented K^+ conductance of the membrane may lead to an inhibition in excitability in airway smooth muscle. Application of a K^+ channel opener results in a decrease in lung resistance (bronchodilation) [66]. Spontaneous phasic contractions can be generated along with electrical activities by applying K_{Ca} channel inhibitors, such as charybdotoxin (ChTX) and iberiotoxin (IbTX) [67]. Outward K^+ currents passing through K_{Ca} channels may be functioning in an important regulatory role in airway smooth muscle cells [68]. β_2-adrenergic receptor agonists increase K_{Ca} channel activity, and in contrast, muscarinic receptor agonists decrease this channel activity [4, 5, 8, 9]. Therefore, this channel may be target molecule in the functional antagonism between β_2-adreneric and muscarinic receptors [1, 16, 17, 28, 29, 69].

9.3.2 Structure

K_{Ca} channels are composed of a tetramer formed by pore-forming α-subunits along with accessory β-subunits, and these channels are activated by increased membrane potential and increased $[Ca^{2+}]_i$. The α-subunit is ubiquitously expressed by mammalian tissues and encoded by a single gene (Slo, KCNMA1) [70, 71]. The α-subunit transmembrane domains comprise seven membrane-spanning segments (S0-S6) with extracellular loops and share homology with all voltage-gated K^+ channels with six transmembrane domains (S1-S6) and a pore helix. S1-S4 are arranged in a bundle that forms the voltage-sensing component; S5-S6 and pore helices contribute to form the pore-forming component and the K^+ selective filter [72]. The C-terminal tail contributes to the Ca^{2+}-sensing ability of this channel with a pair of Ca^{2+}-sensing domains that regulate the conductance of K^+ (RCK), that is, RCK1 and RCK2 [73]. Although the Ca^{2+} sensor of K_{Ca} channels has high specificity for Ca^{2+}, other factors including divalent cations also influence the opening of these channels. Magnesium (Mg^{2+}) enhances activation of these channels via a distinct binding site in the voltage sensor and RCK1 domain [74]. On the other hand, intracellular protons (H^+) attenuate the opening of K_{Ca} channels [10, 75]. K_{Ca} channels are associated with modulatory β-subunits, which are expressed in a cell-specific manner and have unique regulatory actions on these channels. The β-subunits bring about diversity of K_{Ca} channels. There are four distinct β-subunits, β1–4, which are encoded by KCNMB1, KCNMB2, KCNMB3, and KCNMB4. These β-subunits in these channels consist of two transmembrane domains with intracellular N- and C-termini and a long extracellular loop [76].

9.3.3 Electrical Characteristics

The unitary amplitude of K_{Ca} channels is approximately 5 pA under the condition of approximately 6 mM K^+ at the cytosolic side and approximately 130 mM K^+ at the extracellular side held at 0 mV in tracheal smooth muscle cells [4]. Ca^{2+} sensitivity of K_{Ca} channels is increased by intracellular Mg^{2+}, as is the case in vascular muscle [77]; in contrast, Ca^{2+} sensitivity of this channel is decreased by intracellular H^+ in tracheal smooth muscle [10]. K_{Ca} channel activity is markedly inhibited by intracellular acidification by shortening the open state of the channel. On the other hand, intracellular alkalization has an opposite effect (increasing Ca^{2+} sensitivity and lengthening the open state of the channel). In the single-channel recording using outside-out patches of guinea pig and canine tracheal muscle cells, currents of K_{Ca} channels are reversibly blocked by external application of scorpion venom such as charybdotoxin (ChTX) or iberiotoxin (IbTX), selective antagonists of K_{Ca} chan-

nels. This effect is not a result of reduced current amplitude; rather, it is caused by reducing the open-state probability (nPo), the fraction of the time during which the channel is open [8, 78]. In contrast, tetraethylammonium (TEA, 1 mM) strongly reduces the unitary amplitude of single K_{Ca} channel current, different from the effects of ChTX (100 nM) on these channels without affecting current amplitude [60]. K_{Ca} channels are not affected by 4-aminopyridine (4-AP, 1 mM).

9.3.4 Effects on Ca^{2+} Signaling

In excitation-contraction coupling of airway smooth muscle cells [79], local increases in Ca^{2+} concentrations occur due to focal releases of Ca^{2+} through ryanodine receptors (RyR) from the sarcoplasmic reticulum (SR), termed Ca^{2+} sparks (Fig. 9.1). K_{Ca} channels are markedly opened by the Ca^{2+} sparks from SR close to the sarcolemma, resulting in spontaneous outward currents (STOCs) (Fig. 9.1). The coupling of ryanodine-mediated Ca^{2+} sparks to K_{Ca} channel-mediated STOCs, which is enhanced by β_1 subunit, causes hyperpolarization of smooth muscle cells, leading to smooth muscle relaxation via reduction of Ca^{2+} entry. In K_{Ca} channel β_1 subunit knockout mice, tracheal contraction induced by a muscarinic receptor agonist is enhanced as compared to wild-type mice, and not only the single channel activity of K_{Ca} channels in an inside-out patch but also STOCs in a whole cell configuration are markedly attenuated in tracheal smooth muscle cells of knockout mice as compared to wild-type mice [80]. IbTX (30 nM) enhances methacholine-induced contraction with elevating $[Ca^{2+}]_i$ in airway smooth muscle, and verapamil, an inhibitor of VDC channels, suppresses the effect of IbTX on both tension and $[Ca^{2+}]_i$, demonstrating that K_{Ca} channel inhibition augments contraction via a Ca^{2+} entry passing through VDC channels [11]. Therefore, K_{Ca} channel activity regulates the tone of airway smooth muscle; however, the Ca^{2+} sparks via ryanodine receptors may not be directly involved in this K_{Ca} channel-mediated bronchoconstriction and bronchodilation [81].

9.3.5 Effects on β_2-Adrenergic Action

β_2-Adrenergic receptor agonists cause relaxation of human and guinea pig tracheal smooth muscles with membrane hyperpolarization in the intracellular microelectrode technique [82, 83]. These agents also inhibit tracheal smooth muscle contraction with reducing $[Ca^{2+}]_i$ in a simultaneous recording isometric tension and F_{340}/F_{380} using fura-2-loaded tissues [2, 3]. The relaxant effects of cAMP-related agents, such as isoproterenol and forskolin, on muscarinic contraction are significantly reduced in the presence of ChTX, a selective inhibitor of K_{Ca} channels [84–86]. This phenomenon may result from Ca^{2+} dynamics based on K_{Ca} channel activation mediated by membrane hyperpolarization (Fig. 9.1).

9.3.5.1 Protein Kinase A

Application of PKA (10 units/mL) to the cytosolic side of inside-out membrane patches reversibly increases nP_o of K_{Ca} channels with no changes in the amplitude of single-channel currents in tracheal smooth muscle cells [4, 5], and the recovery from this activation is significantly delayed in the presence of okadaic acid, an inhibitor of protein phosphatases [4]. The open state of K_{Ca} channel may be enhanced by phosphorylation of this channel protein. External application of isoproterenol (0.2 μM), a β_2-adrenergic receptor agonist, and okadaic acid (10μM) also increases K_{Ca} channel activity in the cell-attached patch-clamp configuration, and the recovery from this activation was also significantly delayed by okadaic acid [4]. These findings demonstrate that PKA-mediated phosphorylation of K_{Ca} channel protein is involved in the β_2-adrenergic action on this channel (Fig. 9.1) [87]. Moreover, external application of forskolin (10μM), a direct activator of adenylyl cyclase, increases K_{Ca} channel activity in tracheal smooth muscle cells [84].

9.3.5.2 Stimulatory G Protein of Adenylyl Cyclase

External application of isoproterenol increases the open state of K_{Ca} channels without changes in the unitary amplitude in outside-out patches in

the presence of guanosine triphosphate (GTP, 100μM) at the cytosolic side of the patch [5, 8]. The recombinant α-subunit (α_s) of the stimulatory G protein of adenylyl cyclase (G_s) preincubated with GTP-γ-S (α_s*GTPγS, 100–1000 pM) similarly activates K_{Ca} channel in a concentration-dependent manner when applied to the cytosolic side of inside-out patches [8]. K_{Ca} channel activity is directly enhanced by G_s (membrane-delimited action), independent of cAMP-dependent protein phosphorylation (Fig. 9.1) [5, 8]. The effect of PKA on the gating kinetics of K_{Ca} channels is distinct from that of α_s, that is, PKA acts on the mean duration of the long openings; in contrast, α_s acts on the proportion of long open-time events [5]. K_{Ca} channels are activated by PKA (cAMP-dependent processes) and α_s (cAMP-independent processes); PKA and α_s affect these channels independently, that is, dual pathway [5] (Fig. 9.1).

β_2-Adrenergic receptor agonists cause membrane hyperpolarization in tracheal smooth muscle [82, 83]. This phenomenon may result from K_{Ca} channel activation by these agents. The relaxant effects of cAMP-related agents, such as isoproterenol and forskolin, on muscarinic contraction are reduced in the presence of a selective inhibitor of K_{Ca} channels [84–86]. Activation of K_{Ca} channels may be associated with β_2-adrenergic action on airway smooth muscle. After G_s activity is irreversibly enhanced by incubation with cholera toxin (2μg/mL) for 6 h, MCh-induced contraction is significantly attenuated, and this effect of cholera toxin is reversed in the presence of ChTX [7, 69]. Hence, the G_s protein/K_{Ca} channel stimulatory linkage may contribute to β-adrenergic relaxation in airway smooth muscle (Fig. 9.1).

9.3.6 Effects on Muscarinic Action

Methacholine (MCh)-induced contraction is significantly enhanced with elevating $[Ca^{2+}]_i$ in the presence of iberiotoxin, a selective inhibitor of K_{Ca} channels, in a simultaneous recording of isometric tension and F_{340}/F_{380} of fura-2-loaded tissues of guinea pig tracheal smooth muscle [11, 38]. Airway muscarinic contraction may result from Ca^{2+} dynamics mediated not only by ROC processes but also by K_{Ca} channel inactivation (VDC processes).

9.3.6.1 Inhibitory G Protein of Adenylyl Cyclase

External application of MCh causes a marked inhibition in K_{Ca} channel activity without changes in the amplitude of single-channel currents in outside-out patches of porcine or canine tracheal muscle cells [8, 9, 45]. This MCh-induced inhibition of K_{Ca} channels is potentiated by application of GTP in the cytosolic side, and in contrast, is abolished by incubation (4–6 h) with pertussis toxin (0.1–1.0μg/mL), which blocks signal transduction through ADP ribosylation of G_i, the inhibitory G protein of adenylyl cyclase [9]. The decreased nP_o of K_{Ca} channels results from a reduction in channel open times, probably reflecting a decrease in the Ca^{2+} sensitivity of the channel. The muscarinic inhibition of K_{Ca} channels may be partly responsible for the prolonged suppression by acetylcholine of STOCs following a transient increase [88, 89]. MCh-induced contraction of tracheal smooth muscle is significantly attenuated after incubation with pertussis toxin (1.0μg/mL for 6 h), and this effect of pertussis toxin is reversed in the presence of ChTX [69]. The G_i protein/K_{Ca} channel inhibitory linkage may be involved in the muscarinic-induced contraction in airway smooth muscle [1, 16, 17, 28, 29, 69].

9.3.6.2 Muscarinic M_2 Receptors

G_i protein couples with the M_2 subtype of muscarinic receptors, leading to an inhibition in cAMP. These muscarinic M_2 receptors exist on the surface of airway smooth muscle cells. A selective muscarinic M_2 receptor antagonist (AF-DX 116, a benzodiazepine derivative) suppresses MCh-induced contraction of tracheal smooth muscle in a concentration-dependent manner [69]. Muscarinic M_3 receptors, which are coupled with G_q, are the major muscarinic receptors that coupled to muscarinic receptor agonists. However, muscarinic M_2 receptors also contribute to airway smooth muscle contraction; K_{Ca}

channels regulate this M_2 muscarinic action [9, 69, 90].

9.3.7 Dual Regulation by G Proteins

K_{Ca} channel antagonists attenuate β_2-adrenergic relaxation [69, 85, 86], and in contrast, enhance muscarinic contraction in tracheal smooth muscle [11, 69]. K_{Ca} channel activity is markedly increased by β_2-adrenergic receptor agonists, and in contrast, this channel activity is markedly suppressed by muscarinic receptor agonists under the experimental condition that these two agents are sequentially applied to identical outside-out patches with GTP at the cytosolic side [8]. Moreover, internal application of GTP causes an activation of K_{Ca} channel in the presence of β_2-adrenergic receptor agonists at extracellular side in insideout patches, and in contrast, causes K_{Ca} channel suppression in the presence of muscarinic receptor agonists in the same condition [8]. The activation process is mediated by the stimulatory G protein, G_s; in contrast, the suppression process is mediated by the inhibitory G protein, G_i, that is, dual regulation by G proteins connected to β_2-adrenergic and muscarinic M_2 receptors [8]. The functional antagonism between β_2-adrenergic and muscarinic action converges on a single K_{Ca} channel current. Therefore, K_{Ca} channels may be key molecules in the regulation of airway smooth muscle tone [1, 16, 17, 28, 29, 69].

9.3.8 Regulation by Other Factors

9.3.8.1 NO, cGMP

Nitric oxide (NO), which is primarily generated by nitric oxide synthase (NOS) in the endothelium, results in smooth muscle relaxation on vessels via hyperpolarization of the cell membrane [91, 92]. NO also increases K_{Ca} channel activity in vascular smooth muscle; NO-induced vasodilation is attenuated by blockade of K_{Ca} channel activity [93]. The NO/3′-5′-cyclic guanosine monophosphate (cGMP) pathway plays an important role in smooth muscle relaxation in vessels and airways. K_{Ca} channel activity is markedly enhanced by cGMP-mediated processes, suggesting that cGMP-induced relaxation of smooth muscle results from activation of these channels [94, 95]. Vascular smooth contraction is enhanced in the K_{Ca} channel α-subunit null mice as compared to wild-type mice [96]. This phenomenon is caused by an impaired response to cGMP-dependent vasorelaxation, indicating that K_{Ca} channels are an important effector for cGMP-mediated action, similar to the cAMP/PKA processes (see 3.5.1.). Protein kinase G (PKG) increases K_{Ca} channel activity via the NO/cGMP pathway [97, 98]. Mechanisms of NO-induced K_{Ca} channel activation consists of dual pathway, that is, PKG-dependent phosphorylation [99] and NO direct action (PKG-independent) on channel protein [100]. PKG may also be cross-activated by cAMP to stimulate K_{Ca} channels [101]. Since the stimulatory effect of NO on K_{Ca} channels is abolished by knockdown of the β-subunit with antisense, the β-subunit acts as a mediator of NO [102].

9.3.8.2 Reactive Oxygen Species

Reactive oxygen species (ROS), which are synthesized during oxidative stress in endothelial and smooth muscle cells, exerts physiological and pathophysiological effects on smooth muscle via altering the intracellular reduction and/or oxidation (redox) status [103]. The redox state leads to the gating of K_{Ca} channels [104]. However, the effects of redox are complex. Preferential oxidation of methionine increases the activity of K_{Ca} channels, whereas oxidation of cysteines reduces the channel activity [102, 106]. K_{Ca} channel activity is enhanced by hydrogen peroxide (H_2O_2) in pulmonary arterial smooth muscle, resulting in vasodilation mediated by membrane hyperpolarization [107]. H_2O_2 may directly bind to K_{Ca} channels to regulate them, or it may increase this channel activity via the phospholipase A_2-arachidonic acid pathway and metabolites of lipoxygenase [108]. On the other hand, H_2O_2 causes contraction of tracheal smooth muscle with elevating $[Ca^{2+}]_i$ in a concentration-

dependent fashion [109]. Moreover, peroxynitrite ($ONOO^-$), an oxidant generated by the reaction of NO and superoxide, causes smooth muscle contraction in cerebral arterial resulted from inhibiting K_{Ca} channel activity [110].

9.3.8.3 Arachidonic Acid

Arachidonic acid and its metabolites such as 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) contribute to regulation of vascular smooth muscle tone. Arachidonic acid and EETs cause vasodilation as a result of an augmentation in K_{Ca} channel activity [111, 112]. 20-HETE also causes relaxation of airway smooth muscle with membrane hyperpolarization resulted from activation of K_{Ca} channels [113]. Acute hypoxia reduced the generation of 20-HETE, and subsequently the inhibitory action of 20-HETE on K_{Ca} channels results in relaxation of cerebral arterial smooth muscle [114]. On the other hand, 20-HETE acts as a vasoconstrictor via a decrease in K_{Ca} channel activity in renal arterial smooth muscle, and PKC is involved in this phenomenon [115].

9.4 Characteristic Action of Bronchodilators on Airway Smooth Muscle

9.4.1 General

GPCR-related agents such as β_2-adrenergic receptor agonists and muscarinic receptor antagonists are generally used as bronchodilators to improve symptoms and lung function for patients with asthma and COPD. The potency of these GPCR-related agents depends on its receptor affinity and intrinsic efficacy, which are influenced by pathophysiology of diseases and excessive administration. Therefore, alteration of affinity to its receptor and intrinsic efficacy may result in decreases/increases in the effects that these GPCR-related agents originally have. Although these issues are clinically important, little is known about clinical relevance of affinity to its receptor and intrinsic efficacy.

9.4.2 Intrinsic Efficacy

Intrinsic efficacy (intrinsic activity) refers to the ability of an agent to activate its receptors without regard for their concentration. Some agonists completely activate their receptors (full agonists), while other agonists activate their receptors only partially (partial agonists). The two subtypes of partial agonists are weak partial agonists, which have lower efficacy, and strong partial agonists, which have higher efficacy [16, 17, 28, 29, 116, 117]. Therefore, partial agonists need to occupy a large fraction of these receptors to produce an equivalent effect that full agonists achieve by occupying many fewer receptors. When the number of these receptors is decreased, and the function of these receptors is disordered, the ability of partial agonists to relax airway smooth muscle becomes less than their initial effect [16, 17, 28, 29, 116, 117]. On the other hand, full agonists resist reducing their responsiveness even under the conditions of reduced receptor number and disordered receptor function [16, 17, 28, 29, 116, 117]. Intrinsic efficacy would provide an important parameter for the rational clinical use of bronchodilators.

Intrinsic efficacy is commonly measured indirectly as a response to activation of the post-receptor signal transduction pathways; this response can be physiological (change in smooth muscle relaxation in vitro and airway resistance in vivo) [118]. Intrinsic efficacy depends markedly on variable factors in the target cells, such as the number of receptors and functional antagonism (activation of an opposing signal transduction process) [116]. In cells with a higher number of receptors (spare receptors), activation of a small fraction of receptors is sufficient to generate a full response [16, 17, 28, 29, 116, 117]. On the other hand, in cells with a lower number of receptors or functional antagonisms (desensitization or contraction of airway smooth muscle), even though a higher fraction of the receptors is activated, a full response may not be achieved [16, 17, 28, 29, 116, 117]. Many patients with COPD are older patients. Not only excessive exposure to bronchodilators, but also aging contributes to reduced receptor numbers and disor-

dered receptor functions. Hence, in the clinical use of bronchodilators, intrinsic efficacy may affect the expression of the effects of these agents on airway smooth muscle. The EC_{50} and the maximal effects in the concentration-inhibition curves for an agent against MCh express its potency and intrinsic efficacy, respectively [16, 17, 28, 29, 116]. When the functional antagonism was intensified by application of MCh (10μM, roughly 90% of the maximal contraction), isoproterenol causes complete relaxation against this contraction, indicating that isoproterenol behaves as a full agonist. In contrast, complete inhibition did not occur with other β_2-adrenergic receptor agonists [16, 17, 28, 29, 116, 117]. The maximal effects in the curves for these other agonists (excluding isoproterenol) are attenuated, indicating that these other agonists behave as partial agonists. Based on values of their intrinsic efficacy, they are classified into two types, that is, strong partial agonists (indacaterol, formoterol, procaterol, olodaterol vilanterol) and weak partial agonists (salbutamol, salmeterol, tulobuterol) [16, 17, 29]. Isoproterenol, a full agonist, causes β_2-aderergic desensitization greater than partial agonists, indicating that excessive application of a full agonist leads to reduced responsiveness to β_2-adrenergic receptor agonists in airway smooth muscle [11, 17, 28, 29, 38, 119]. In contrast, tulobuterol, which is the weakest partial agonist, causes a modest reduction in the relaxant effect, even in cases of repeatedly excessive exposure to tulobuterol [17, 28, 29, 120]. However, a loss of β_2-adrenergic action in partial agonists after exposure to these agonists is less potent in agonists with higher values of intrinsic efficacy (strong partial agonists) than in agonists with lower values of intrinsic efficacy (weak partial agonists) [17]. In a meta-analysis, indacaterol, which has a highest value of intrinsic efficacy in partial agonists, is most effective in improving lung function and clinical symptoms in patients with COPD [121].

On the other hand, in concentration-inhibition curves, muscarinic receptor antagonists (atropine, tiotropium, glycopyrronium, umeclidinium) cause complete inhibition against MCh (10 μM)-induced contraction of tracheal smooth muscle; values of EC_{50} are not significantly different in these muscarinic antagonists [38]. These antagonists behave as full antagonists. In a meta-analysis compared to placebo, these muscarinic receptor antagonists have no significant difference in increasing lung function for patients with COPD [122].

9.4.3 Allosteric Effects

Allosteric modulators connect to GPCRs (seven transmembrane receptors) at the allosteric site that is topographically distinct from the orthosteric site, and they result in an alteration in receptor conformation. Allosteric GPCR modulators impact on the orthosteric binding pocket and alter association or dissociation rates of an orthosteric ligand (affinity modulation). Allosteric effects also affect intracellular responses and alter the signaling capacity (intrinsic efficacy) of an orthosteric ligand (efficacy modulation) [123.124]. Antagonism of agonist response is caused by a reduction in affinity and/or efficacy resulted from allosteric effects. Isoproterenol (a full β_2-adrenergic agonist) competitively antagonizes MCh (10μM)-induced contraction, indicating that this agent acts on orthosteric sites, not on allosteric sites in β_2-adrenergic receptors [17, 28, 29, 38]. In contrast, partial β_2-adrenergic agonists, such as formoterol, procaterol, indacaterol, olodaterol, vilanterol, salmeterol, and salbutamol, noncompetitively antagonize MCh (10μM)-induced contraction (efficacy is attenuated by stimulating allosteric site), indicating that these agonists behave as allosteric modulators against β_2-adrenergic receptors (Fig. 9.2) [17, 28, 29, 38, 125]. Since allosteric modulators merely tune the signal in the receptors and have no effects on the receptors without endogenous ligands, partial agonists that act as allosteric modulators are less potent in causing tachyphylaxis (desensitization) after excessive exposure [17, 28, 29, 38, 125]. In concentration-inhibition curves for an agent, a reduction in maximal percent inhibition from complete inhibition indicates efficacy modulation with an agent (inhibition in response to orthosteric site involve-

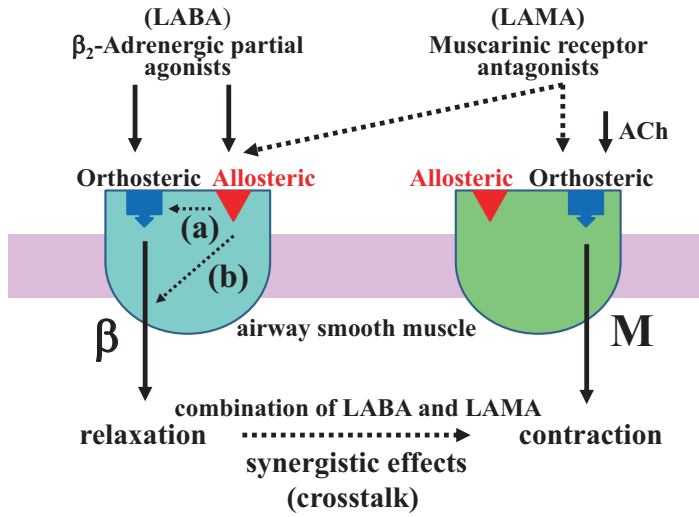

Fig. 9.2 Role of allosteric modulation in the synergistic effects between β_2-adrenergic receptor agonists and muscarinic receptor antagonists. Antagonism of the agonist response is observed via allosteric effects by a reduction in affinity and/or efficacy. Full β_2-adrenergic agonists act only on orthosteric sites, and do not act on allosteric sites in their receptors. On the other hand, partial β_2-adrenergic agonists act not only on orthosteric sites but also on allosteric sites in their receptors. Partial β_2-adrenergic agonists behave as allosteric modulators. By acting on allosteric sites in β_2-adrenergic receptors, this allosteric effect causes reduced responsiveness to agonists in orthosteric sites via affinity and/or efficacy modulation. Therefore, intrinsic efficacy is reduced with the use of partial β_2-adrenergic agonists. Muscarinic receptor antagonists act not only on orthosteric sites of muscarinic receptors but also on allosteric sites of β_2-adrenergic receptors, and this allosteric action increases affinity and intrinsic efficacy of partial β_2-adrenergic agonists. Therefore, muscarinic receptor antagonists markedly enhance effects of β_2-adrenergic receptor agonists. Allosteric GPCR modulation is involved in the synergistically relaxant effects of combination of these two agents via crosstalk of their receptors. β: β_2-adrenergic receptor, M: muscarinic receptor, ACh: acetylcholine, LABA: long-acting β_2-adrenergic receptor agonist, LAMA: long-acting muscarinic receptor antagonist, GPCR: G protein-coupled receptor. (a): affinity, (b): efficacy. Arrows: activation, dotted arrows: inhibition. Illustrated based on ref. [17, 29, 38, 123–125, 132]

ment). The ranking of alterations in efficacy modulation of partial β_2-adrenergic agonists is in reverse order to values of their intrinsic efficacy. In concentration-inhibition curves for an agent, value of EC_{50} of an agent is lower than that of isoproterenol, indicating that an agent causes an augmentation in affinity (association rate) to a ligand at an orthosteric site. The rank of augmentation in affinity modulation of partial β_2-adrenergic agonists is also in reverse order to values of their EC_{50}. On the other hand, all of muscarinic receptor antagonists cause complete inhibition against muscarinic contraction, and values of EC_{50} are not significantly different between these four antagonists [38]. Affinity and intrinsic efficacy of muscarinic receptor antagonists may not depend on each agent. Muscarinic receptor antagonists operate upon orthosteric sites, and do not act on allosteric sites on muscarinic receptors (Fig. 9.2) [38, 125].

9.4.4 Synergistic Effects of Bronchodilators

The COPD consensus report states that a combination of bronchodilators of different pharmacological classes may improve effectiveness and decrease the risk of adverse reactions compared to increasing the dose of a single bronchodilator. The two different types of bronchodilators, that is, long-acting β_2-adrenergic receptors (LABAs) and long-acting muscarinic receptor antagonists (LAMAs), are widely used as therapy for this disease, and a combination of these two agents has pharmacological rationale as a bronchodilator

therapy [16, 17, 28, 29, 38, 126–128]. Clinical trials have demonstrated that LABA/LAMA combination is beneficial to therapy for COPD (improving symptoms and lung function, and reducing exacerbations) [129–132].

Protein allosterism is the change in protein reactivity at one site arising from a molecule binding on the protein at another site. When one agent acts on its specific GPCRs, the effect of another agent on its specific GPCRs is altered. The effects of these two agents are mutually enhanced, leading to synergistic effects. Allosteric GPCR modulators lead to alteration in pharmacological properties such as affinity, efficacy, and agonism/inverse agonism [123, 124]. Since allosteric effects may be caused by the interaction mediated by ligands for GPCRs [123, 133], synergistic effects between β_2-adrenergic receptor agonists and muscarinic receptor antagonists against muscarinic contraction may result from allosteric GPCR modulation in airway smooth muscle [17, 28, 29, 38]. Treated with pertussis toxin and application of AF-DX 116 markedly shift the concentration-inhibition curves for isoproterenol against MCh to the left, and values of EC_{50} at each condition are markedly decreased. Muscarinic M_2 antagonism enhances affinity for β_2-adenergic receptor agonists via acting on allosteric sites on β_2-adenergic receptors (Fig. 9.2) [17, 28, 29]. In contrast, ChTX markedly shifts these curves for isoproterenol to the right, indicating that antagonists of K_{Ca} channels reduce affinity for β_2-adenergic receptor agonists by muscarinic M_2 receptor activation [17, 28, 29].

In concentration-inhibition curves, isoproterenol completely antagonizes muscarinic contraction [17, 28, 29, 38], and the complete inhibition is not attenuated at higher concentrations that produce the maximal relaxation. Isoproterenol operates orthosteric sites on β_2-adrenergic receptors, and does not operate on these receptors, demonstrating that isoproterenol acts as a full agonist [16, 17, 28, 29, 38, 117]. In contrast, since β_2-adrenergic receptor agonists except for isoproterenol and adrenaline incompletely antagonize muscarinic contraction, these agonists cause an inhibition in the signal capacity induced by efficacy modulation (reduced responsiveness to orthosteric sites via allosteric effects) (Fig. 9.2) [16, 17, 28, 29, 38, 117]. These β_2-adrenergic agonists cause a concentration-dependent contraction at higher concentrations that produce the maximal relaxation [38]. These agonists reduce intrinsic efficacy via operating allosteric sites on β_2-adrenergic receptors as allosteric modulators (partial agonists) (Fig. 9.2) [38]. In the concentration-inhibition curves for these partial β_2-adreneric receptor agonists with lower concentrations of these muscarinic receptor antagonists, values of EC_{50} for these curves are markedly decreased; the maximal effects of these partial β_2-adreneric receptor agonists are markedly augmented to complete inhibition at each experimental condition [38]. Moreover, these partial β_2-adreneric receptor agonists do not cause contraction in a concentration-dependent manner at higher concentrations that produce the maximal relaxation (complete inhibition), different from the curves without lower concentration of muscarinic receptor antagonists [38]. Muscarinic receptor antagonists may act not only upon orthosteric sites on muscarinic receptors but also upon allosteric sites on β_2-adrenergic receptors, and these antagonists enhance both affinity and efficacy to β_2-adrenergic receptor agonists; as a result, synergistic effects may be generated via crosstalk between these two GPCRs (Fig. 9.2) [38]. This synergism causes independent of the effects of muscarinic receptor antagonists on orthosteric sites on their receptors.

9.5 Role of Airway Smooth Muscle on Inflammation (Phenotype Plasticity)

9.5.1 General

Airway smooth muscle cells in culture have the ability to change the degree of various functions such as contractility, proliferation, migration, and the synthesis of inflammatory mediators (Fig. 9.3) [1, 12, 13, 29]. Contractile mediators result in shortening and contraction of airway smooth muscle, and airway smooth muscle has long been regarded as tissues that mainly contract passively

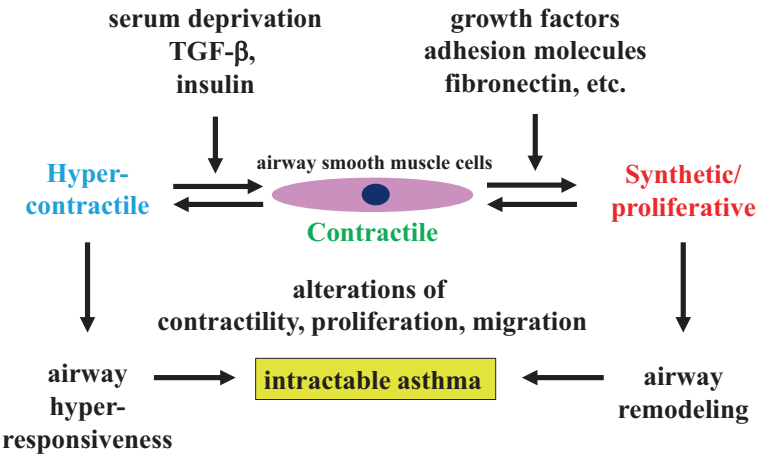

Fig. 9.3 Phenotype switching in airway smooth muscle cells. Important factors for phenotype switching are shown. Inflammatory processes alter phenotype of airway smooth muscle between the contractile phenotype and the synthetic/proliferative or hyper-contractile phenotype. These phenotype changes enhance contractility, migration, proliferation, and synthesis of inflammatory substances in airway smooth muscle cells, resulting in hyperresponsiveness and remodeling in the airways that cause an increase in the severity of asthma. Illustrated based on ref. [1, 12, 13, 29]

in response to various mediators for bronchoconstriction released from other cells. Increased contractile property of tracheal smooth muscle may be fundamental abnormality of asthma. Contractile response to muscarinic agonists and histamine in human bronchial smooth muscle from patients with asthma is greater than that from healthy subjects. This phenomenon is caused by increased proliferation of airway smooth muscle cells because an increase in thickening of airway wall, which is resulted from an increased airway smooth muscle mass, contributes to contractile hyperresponsiveness. Airways smooth muscle cells change to a proliferative phenotype in response to contractile agents, inflammatory mediators, and growth factors. In the presence of proliferating stimuli, airway smooth muscle cells change into a synthetic phenotype; these cells release several inflammatory mediators under various conditions of stimulation. Alteration of airway smooth muscle cells from a contractile to a synthetic or a proliferative phenotype is involved in the pathophysiology of asthma and COPD, such as in airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, and airway remodeling (Fig. 9.3).

9.5.2 Contractile Phenotype

In airway smooth muscle cell culture, phenotype plasticity is observed when cells grow to subconfluence in the presence of serum. A proliferative phenotype develops in airway smooth muscle cells under these conditions that is characterized by decreased expression of contractile proteins including smooth muscle–myosin heavy chain (sm-MHC), calponin, smooth muscle α action (sm-α actin), desmin, MLCK, and caldesmon [12, 29]. In contrast, airway smooth muscle cells with a contractile phenotype are characterized by augmented expression of contractile proteins and retain their ability to contract in response to various spasmogens. Trangestin (SM22), soothelin, metavinculin, and caveolin-1 are involved in modulation of airway smooth muscle cells toward a contractile phenotype [12, 29].

9.5.3 Synthetic and Proliferative Phenotypes

In addition to the effects of these endogenous factors, airway smooth muscle can change from

one phenotype to another after exposure to various exogenous stimuli including extracellular matrix (ECM, in particular collagen type 1 and fibronectin), PDGF, and TGF-β [13, 134]. Airway smooth muscle cells derived from healthy donors are less proliferative than those derived from asthmatic donors, who show alteration toward a more proliferative phenotype [135, 136]. After exposure to IL-13 and PDGF-BB, expression of the SR Ca^{2+} ATPase (a Ca^{2+} transporter) is attenuated, leading to recapitulation of a more secretory and proliferative phenotype [137]. A synthetic phenotype is characterized by an increase in synthetic organelles for protein and lipid synthesis such as the Golgi apparatus and numerous mitochondria, leading to an augmented proliferative capacity. Modulation toward proliferative and synthetic phenotypes is also associated with an increase in non-muscle MHC, l-caldesmon, vimentin, α/β-PKC, and CD44 homing cellular adhesion molecule [12]. Cells with this phenotype show increased proliferative capacity with a diminished abundance of contractile proteins, leading to attenuation of responses to contractile agents [12]. In airway smooth cell culture, 20–60% of the cells have a secretory phenotype; on the other hand, approximately 50% of the cells express proliferative capacity, indicating that cytokine production and proliferation may be overlapping and not independent functions [138].

9.5.4 Hyper-Contractile Phenotype

In contractile and proliferative states, intermediate or extreme phenotypes of each state may exist. Previous reports have demonstrated that prolonged starvation of canine airway smooth muscle causes a hyper-contractile phenotype (a third putative phenotype) [139, 140], which may contribute to hyperresponsiveness although this phenotype has not been replicated in human airway smooth muscle. Markers for this phenotype include a lack of smooth muscle myosin-B (SM-B; an isoform of MHC), and increases in expression of MLCK and muscarinic M_3 receptors. In human airway smooth muscle cells, prolonged serum starvation causes an increase in expression of muscarinic M_3 receptors on the surface of cells derived from healthy volunteers, but not on cells derived from patients with asthma. On the other hand, exposure to muscarinic receptor agonists for a longer period reduces expression of contractile proteins and responsiveness of airway smooth muscle cells [141].

9.5.5 Ca^{2+} Handling

The plasticity of cells that allows them to change from a contractile phenotype to other phenotypes (proliferation, migration, or secretion of chemical mediators) may be associated with Ca^{2+} dynamics [18, 19] and Ca^{2+} sensitization [20–24]. Phenotype plasticity in airway smooth muscle cells is associated with an alteration in the expression of ion channels such as voltage-gated sodium, inward rectifying K^+, and K_{Ca} channels [64]. K_{Ca} channels that are regulated by G proteins (G_s, G_i) contribute to Ca^{2+} dynamics, by regulating the passage of Ca^{2+} through VDC channels via membrane potential. In contrast, the phenotype plasticity in vascular smooth muscle cells is associated with various Ca^{2+} handling regulators such as SOC, ROC, transient receptor potential channel type C (TRCP), Orai 1 and Stromal interacting model 1 (STIM1) [142]. On the other hand, since RhoA/Rho-kinase acts on contractility and proliferation in airway smooth muscle, Ca^{2+} sensitization induced by this pathway may also contribute to phenotype change in this tissue. Exposure of airway smooth muscle to S1P results in airway hyperresponsiveness (hyper-contractile phenotype) that is mediated by Ca^{2+} sensitization via inactivation of myosin phosphatase, which links G_i and RhoA/Rho-kinase processes [55]. Inhibition of airway smooth muscle cell proliferation (proliferative phenotype) by simvastatin is due to prevention of geranylgeranylation of RhoA, which causes an increase in Ca^{2+} sensitization not by farnesylation of Ras, independent of reducing cholesterol synthesis. The inhibitory effect of simvastatin on cell proliferation is caused by Rho-kinase-induced Ca^{2+} sensitization [21].

9.5.6 Regulation of Phenotype Switching

Phenotype switching in airway smooth muscle is regulated by dynamic processes that are influenced by changes in the microenvironment of the cells. In vitro cell proliferation is increased by various factors such as peptide growth factors, agonists of $G_{q/i}$-involved GPCRs, inflammatory mediators and ECM proteins (collagen type I and fibronectin) [143–146]. Many of these factors are increased in the vicinity of the airway smooth muscle by structural cells of the airways, including by airway smooth muscle cells themselves in asthma [147–150]. In contrast, cell proliferation is inhibited by various factors such as glucocorticosteroids, agonists of G_s-involved GPCRs, NO, insulin, PGs, and ECM proteins (chondroitin sulfate, decorin, and laminins) [151–156]. Moreover, prolonged serum deprivation, insulin, and TGFβ induce a hypercontractile phenotype characterized by decreased proliferative response, increased contractive response, and enhanced expression of contractile proteins, such as sm-α-actin, sm-MHC, sm-MLCK, and calponin [157–159].

9.5.7 Modulation of Cell Phenotype by Cell Culture

Since phenotype change is markedly influenced by the surrounding conditions, this phenomenon may be due to the experimental environment used for analysis of the cell biology of airway smooth muscle in vitro. After single cells are isolated from airway smooth muscle bundles, these cells transiently enhance expression of contractile markers, and rapidly change to a synthetic/proliferative phenotype under the condition of exposure to serum-rich medium [13]. It is therefore possible that such phenotype change is an artifact of cell culture conditions in vitro. Little is known regarding whether this phenotype change occurs in vivo. This problem still remains to be solved. Although airway smooth muscle cell models using classical 2-dimensional cell type culture systems have provided a controlled environment suitable for assessing long-term control of cellar responses [160], there may be a limit as to what can be clarified using this method. Further research is required to increase the physiological relevance of these models [161].

9.5.8 Interaction Between Airway Smooth Muscle and Inflammatory Cells

Contractility of airway smooth muscle cells is altered by exposure to tryptase and S1P, which are released from mast cells, and Lyso-PC, which is synthesized in the membrane of various inflammatory cells (Fig. 9.4) [55, 162–164]. Ca^{2+} sensitization by RhoA/Rho-kinase processes contributes to this alteration of contractility. In sensitized mice by allergen challenges, eosinophil infiltration and responsiveness to MCh are markedly increased in the airways; pre-treatment with Rho-kinase inhibitors such as Y-27632 or fasudil hydrochloride (HA-1077) markedly suppresses increases in eosinophil recruitment and MCh-induced lung resistance in the respiratory tracts in a dose-dependent manner (Fig. 9.4) [58]. Thalidomide also inhibits both hyperresponsiveness and eosinophil inflammation in the respiratory tracts in sensitized mice by allergen challenges [165]. Pre-exposure of Lyso-PC and S1P to tracheal smooth muscle results in reduced responsiveness to β_2-aderenergic receptor agonists via the Rho-kinase-induced Ca^{2+} sensitization [163, 164], and administration of Lyso-PC to guinea pigs enhances eosinophil recruitment and resistance in the respiratory system (Fig. 9.4) [166]. S1P also increases mRNA and protein expression of vascular cell adhesion molecule (VCAM)-1 when S1P is applied to pulmonary endothelial cells, leading to eosinophil infiltration to the airways, and this upregulation of VCAM-1 is attenuated by C3 exoenzyme and Y-27632 [167]. Y-27632 reduces not only the number of eosinophils but also macrophages and neutrophils in an animal model of allergic asthma [22]. Hence, S1P causes eosinophil recruitment, hyperresponsiveness, and remodeling in the air-

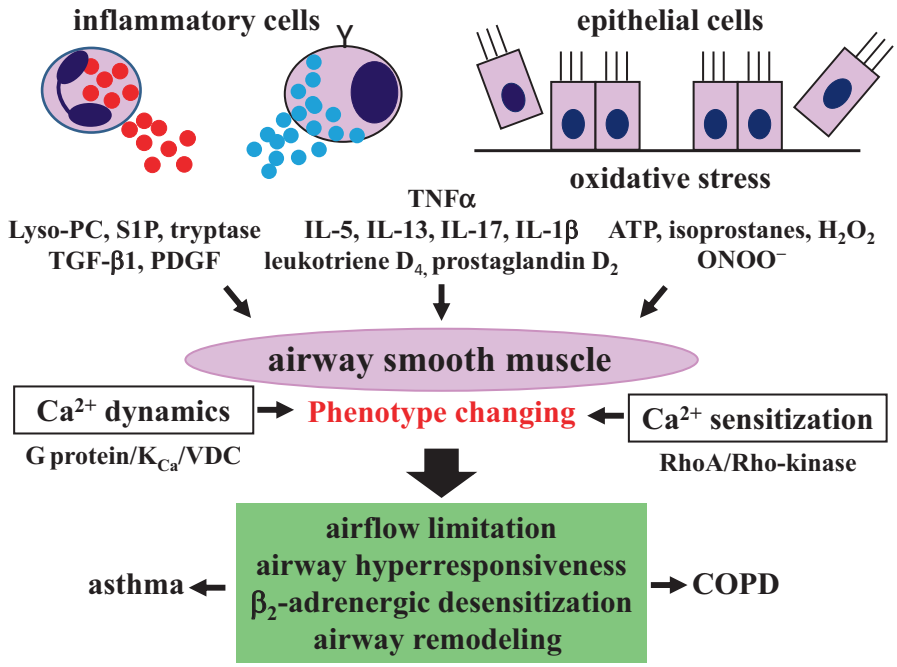

Fig. 9.4 Role of inflammatory cells on airway smooth muscle cells in the pathophysiology of asthma and COPD. In the respiratory tracts, inflammatory cells (eosinophils, mast cells) release interleukins, growth factors (PDGF, TGF-β_1), lipid mediators (Lyso-PC, S1P), and serine protease (tryptase). Oxidative stress generates isoprostanes, H_2O_2, and $ONOO^-$. Injured epithelium releases ATP and these growth factors. These substances contribute to alterations of airway smooth muscle functions by affecting Ca^{2+} dynamics due to the G protein/K_{Ca} channel/VDC channel linkage and by affecting Ca^{2+} sensitization due to the RhoA/Rho-kinase processes. These inflammatory processes cause not only alterations in contractility but also changes to the proliferative phenotype in airway smooth muscle, referred to as a phenotype change. Contractility change is involved in airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization; proliferative change is involved in airway remodeling due to cell proliferation and cell migration. Therefore, the G protein/K_{Ca} channel/VDC channel linkage and the RhoA/Rho-kinase processes are involved in almost all of the principal mechanisms of asthma and COPD. These pathways involved in Ca^{2+} dynamics and Ca^{2+} sensitization are molecular targets for therapy of these diseases. Lyso-PC: lysophosphatidylcholine, S1P: sphingosine 1-phosphate, PDGF: Platelet-derived growth factor, TGF-β_1: transforming growth factor beta 1, IL: interleukin, H_2O_2: hydrogen peroxide, $ONOO^-$: peroxynitrite, VDC: L-type voltage-dependent Ca^{2+} channels, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels. Illustrated based on ref. [1, 17, 20, 21, 24, 54, 55, 58, 109, 136, 144–147, 162–169, 171, 176–188, 195–197]

ways by RhoA/Rho-kinase processes [55, 167, 168].

After exposure to adenosine triphosphate (ATP), MCh-induced contraction is markedly enhanced without elevating $[Ca^{2+}]_i$ in fura-2-loaded tissues of guinea pig tracheal smooth muscle (Fig. 9.4) [169]. This phenomenon is inhibited by Y-27632, a selective inhibitor of Rho-kinase, and suramin, a non-selective P2 receptor inhibitor [169]. Pre-incubation with ATPγS, a non-hydrolysable analogue of ATP and α,β-meATP, a P2X agonist, also significantly increase methacholine-induce contraction [169]. In asthma, eosinophils are infiltrated to around the airway, leading to injury and detachment of airway epithelium. ATP released from these injured epithelial cells act on airway smooth muscle, resulting in airway hyperresponsiveness by RhoA/Rho-kinase-induced Ca^{2+} sensitization via the P2X receptors. Therefore, Ca^{2+} sensitization by RhoA/Rho-kinase processes contributes to the interaction between airway smooth muscle and inflammatory cells related to asthma [1, 16, 23, 24, 29, 170].

9.6 Role of Airway Smooth Muscle in the Pathophysiology of Asthma and COPD

9.6.1 General

An alteration of phenotype (contractile ~ synthetic/proliferative) in airway smooth muscle cells is caused by the inflammatory processes in the airways related to the pathophysiology of obstructive pulmonary diseases, such as asthma and COPD (Fig. 9.3). Ca^{2+} signaling by both Ca^{2+} dynamics and Ca^{2+} sensitization is involved in this phenotype change of airway smooth muscle cells resulted from interaction with airway constituent cells (inflammatory cells and epithelial cells), leading to airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, and airway remodeling associated with these diseases (Fig. 9.4).

9.6.2 Airflow Limitation (Bronchoconstriction)

Airway smooth muscle contraction caused by various spasmogens (ACh, histamine, prostaglandins, or leukotrienes) is associated with airflow limitation, which is a characteristic feature of asthma and COPD. These agonists generate force in airway smooth muscle with increasing $[Ca^{2+}]_i$ by Ca^{2+} dynamics via Ca^{2+} entry passing through SOC, non-SOC, and VDC (Fig. 9.1). Sphingosine 1-phosphate (S1P: a bioactive lysophospholipid) [55], tryptase (trypsin-like neutral serine-class protease) and SLIGKV (non-enzymatic activator of protease-activated receptor 2, PAR2) [162] released from mast cells cause airway smooth muscle contraction with increasing $[Ca^{2+}]_I$ (Fig. 9.4). Therefore, S1P and tryptase may be involved in the pathophysiology of asthma as novel mediators. ATP is released from injured airway epithelium during the inflammatory processes implicated in the pathophysiology of asthma. Extracellular ATP causes contraction of airway smooth muscle with increasing $[Ca^{2+}]_i$ (Fig. 9.4) [169]. Furthermore, oxidative stress and mechanical stress are related to the pathophysiology of not only COPD but also asthma. 8-iso-prostaglandin $F_{2\alpha}$, an isoprostane [171], and hydrogen peroxide (H_2O_2) [109] produced by oxidative stress cause contraction of airway smooth muscle by increasing $[Ca^{2+}]_I$ (Fig. 9.4). Therefore, ATP, H_2O_2, and 8-iso-prostaglandin $F_{2\alpha}$ may be involved in the pathophysiology of asthma as novel mediators.

Y-27632 suppresses smooth muscle contraction induced by spasmogens such as MCh, histamine, prostaglandins, and leukotrienes, which are involved in the pathophysiology of asthma and COPD, in a concentration-dependent manner, with no significant decrease in $[Ca^{2+}]_i$ in strips treated with fura-2 in guinea pig trachealis [30, 39]. Y-27632 also inhibits the following factors in a concentration-dependent manner with a modest effect on $[Ca^{2+}]_i$: 1) contraction due to S1P and tryptase released from mast cells [55, 162]; 2) contraction due to isoprostanes and hydrogen peroxide (H_2O_2) produced by oxidative stress [109, 171]; and 3) contraction due to ATP synthesized in injured airway epithelium [169]. These factors of contractility, which are implicated in the pathophysiology of asthma and COPD, cause force generation in airway smooth muscle via both Ca^{2+} dynamics and Ca^{2+} sensitization [172]. Force maintenance is caused by Ca^{2+} sensitization induced by Rho-kinase [173]. PKC, which is an intracellular signal transduction pathway for GPCR activation, also causes contraction of airway smooth muscle mediated by both Ca^{2+} dynamics and Ca^{2+} sensitization [42, 43].

9.6.3 Airway Hyperresponsiveness

Airway hyperresponsiveness is a hallmark of asthma, and any therapy cannot cure this characteristic feature of this disease. Airway hyperresponsiveness is also observed in some patients with COPD [174, 175]. This airway disorder is clinically defined as increased responsiveness to muscarinic receptor agonists (ACh and MCh) and histamine using provocation test. Airway hyperresponsiveness is due to various inflamma-

tory stimulations involved in the pathophysiology of asthma, such as antigens, chemical mediators, cytokines, and eicosanoids. In a postmortem study of airway smooth muscle strips of patients with asthma, the response to histamine and ACh is greater than in healthy individuals [176]. In human airway smooth muscle passively sensitized with human asthmatic serum, histamine-induced contraction is significantly elevated [177]. When airway smooth muscle is exposed for an extended period of time to interleukin (IL)-5, IL-13, IL-17, or tumor necrosis factor $(TNF)_{\alpha}$, which are released from inflammatory cells and epithelial cells in airways, contraction due to muscarinic receptor agonists and KCl is significantly increased (Fig. 9.4) [178–180]. This enhancement of contractility induced by TNF_{α} may be involved in Ca^{2+} sensitization via RhoA/Rho-kinase [181]. In the presence of a lower concentration of leukotriene C_4, KCl-induced contraction is markedly augmented, and this enhanced contraction due to high concentrations of K^+ is attenuated by Y-27632, a selective inhibitor of Rho-kinase [182]. After airway smooth muscle is exposed to S1P released from mast cells or ATP released from damaged epithelial cells, MCh-induced contraction is markedly increased without an increase in $[Ca^{2+}]_i$, and its augmented response to MCh is markedly suppressed by Y-27632 (Fig. 9.4) [55, 169, 183]. Furthermore, pre-treatment of 8-iso-prostaglandin E_2, an isoprostane, causes an increase in response to CCh in airway smooth muscle, and its augmented contractility is also suppressed by Y-27632 (Fig. 9.4) [184]. After cultured human bronchial smooth muscle cells were exposed for 15 min to serum-free medium in the absence (control) and presence of S1P (3μM), relative proportion of RhoA-GTP to total RhoA (RhoAGTP/total RhoA) is significantly increased compared with the control [21, 55]. S1P (0.3–3μM) causes a concentration-dependent increase in MYPT1 (Thr^{850}) phosphorylation in the cultured human bronchial smooth muscle cells. An increase in phosphorylation of MYPT1 (Thr^{850}) by 3μM S1P is significantly inhibited in the presence of Y-27632 (0.1–1.0μM) in a concentration-dependent manner [55]. These observations indicate that airway hyperresponsiveness is caused by direct interactions among inflammatory cells, airway epithelial cells, and airway smooth muscle cells. Ca^{2+} sensitization due to Rho-kinase-induced MYPT1 phosphorylation is involved in the airway hyperresponsiveness [54, 55]. Geranylgeranyltransferase activates RhoA activity [21]; airway hyperresponsiveness is attenuated by suppression of geranylgeranyltransferase in mice [185]. Alterations of sensitivity to Ca^{2+} in airway smooth muscle may play a key role in this phenomenon. Therefore, inflammatory processes involved in asthma directly affect the function of airway smooth muscle cells via the RhoA/Rho-kinase processes. In airway smooth muscle cells, this phenotypic change for contractility induced by not only Ca^{2+} sensitization but also cytoskeleton reorganization (cell stiffness), which are caused by Rho-kinase, may cause an augmented response to contractile agonists (airway hyperresponsiveness) [1, 186, 187]. Lung resistance in response to MCh is increased in mice sensitized by allergen challenges more than in control mice (airway hyperresponsiveness). Fasudil hydrochloride (HA-1077), an inhibitor of Rho-kinase, significantly inhibits the augmented response to MCh by allergen challenges [58]. On the other hand, Ca^{2+} dynamics also results in an alteration in the contractile phenotype of airway smooth muscle, leading to increased responsiveness to contractile agonists (airway hyperresponsiveness) [188]. Acidification of esophageal lumen increases the contractile response to ACh and KCl in guinea pig trachealis mediated by activation of VDC channels and Rho-kinase [189], indicating that both Ca^{2+} dynamics and Ca^{2+} sensitization play key roles in airway hyperresponsiveness (Fig. 9.4).

9.6.4 Desensitization of β_2-Adrenergic Receptors

An excessive activation of β_2-adrenergic receptors results in reduced responsiveness to an agonist. This phenomenon is referred to as desensitization of β_2-adrenergic receptors. The phosphorylation of β_2-adrenergic receptors leads

to desensitization to an agonist via uncoupling G_s from the receptors. This mechanism is involved in two protein kinases, that is, cAMP-dependent PKA and cAMP-independent protein kinases such as β_2-adrenergic receptor kinase (βARK) [190]. PKA-induced phosphorylation contributes to heterologous desensitization (a nonspecific reduced response to other agonists involving cAMP). This type of desensitization is caused by exposure to a low concentration of β_2-adrenergic receptor agonists [191]. On the other hand, βARK-induced phosphorylation contributes to homologous desensitization (a specific reduced response to β_2-adrenergic receptor agonists). This type of desensitization is caused by exposure to a high concentration of β_2-adrenergic receptor agonists [192]. These phenomena are also observed in tracheal smooth muscle, including human tissues [11, 119, 193, 194]. Reduced responsiveness to β_2-adrenergic receptor agonists (β_2-adrenergic desensitization) occurs subsequent to continuous [119, 195, 196] or repetitive administration [11, 119, 194] of an agonist, and to exposure to substances related to the inflammatory processes in asthma, including inflammatory cytokines such as IL-1β [195], growth factors such as transforming growth factor (TGF)-β1 [196] and platelet-derived growth factor (PDGF) [197], lipid mediators such as Lyso-PC, a lysophospholipid produced by phospholipase A_2 [163], and S1P [164], or PAR2 agonists such as tryptase and SLIGKV (Fig. 9.4) [162]. Therefore, β_2-adrenergic desensitization in airway smooth muscle is an extremely important phenomenon that occurs due to both the treatment and the pathophysiology of asthma.

In human tracheal smooth muscle, continuous exposure to isoproterenol for an extended period (45 min) causes a marked reduction in the relaxant action of isoproterenol (0.3μM) against MCh (1μM)-induced contraction [119]. However, pre-incubation of the tissue with cholera toxin (2μg/mL) for 6 h prevents this subsequent reduction in the inhibitory effects of isoproterenol after excessive exposure to the agonist [119]. As isoproterenol with MCh is repeatedly administered for 10 min every 30 min, the relaxant action of isoproterenol is gradually diminished in human and guinea pig tracheal smooth muscle [119, 194]. However, pre-incubation with cholera toxin also prevents this subsequent reduction in the effect of isoproterenol [17, 119, 196]. Since cholera toxin irreversibly activates G_s protein coupled to β_2-adrenergic receptors, β_2-adrenergic desensitization can be avoided by pre-activation of G_s protein [17, 192, 194, 198, 199]. In contrast, β_2-adrenergic desensitization is markedly enhanced in the presence of ChTX or IbTX, selective inhibitors of K_{Ca} channels [119, 194]. Therefore, inactivation of the G_s/K_{Ca} channel linkage plays an important role in β_2-adrenergic desensitization. As repeated exposure to forskolin and theophylline (agents not mediated by β_2-adrenergic receptors) with MCh in the same way, relaxant effects of these agents are not reduced, different from isoproterenol [11]. In single channel recording using tracheal smooth muscle cells, extracellular application of isoproterenol (1μM) markedly activates K_{Ca} channels in the cell-attached configuration; mean values of open probability (nP_o) increases to approximately 9.6-fold. However, after repeated exposure to isoproterenol for 5 min every 15 min, isoproterenol-induced K_{Ca} channel activation is gradually attenuated with no change in unitary amplitude of this channel. The values of fold stimulation of this channel by isoproterenol are deceased approximately 1.6-fold at the sixth application [11]. In contrast, application of 10 U/mL PKA to inside-out patches results in an augmentation of K_{Ca} channel activity [11]. Mean values of nP_o increase to approximately 5.2-fold, and activation of this channel is gradually enhanced with no change in unitary amplitude of this channel after repeated exposure to PKA. The values of fold stimulation of this channel by PKA at the sixth application are increased to approximately 9.6-fold [11]. These results demonstrate that β_2-adrenergic receptor/G_s protein processes are involved in reduced responsiveness to β_2-adrenergic receptor agonists after excessive exposure to airway smooth muscle, cAMP/PKA processes are not. β_2-aderenergic desensitization is suppressed by an augmentation of G_s and K_{Ca} channels in airway smooth muscle.

9.6.4.1 Effects of Ca^{2+} Dynamics

In fura-2-loaded tissues of guinea pig tracheal smooth muscle, the relaxant effect of isoproterenol is gradually attenuated with increasing $[Ca^{2+}]_i$ as isoproterenol with MCh is repeatedly applied for 10 min every 30 min [11, 119], and this reduced responsiveness to isoproterenol is prevented by pre-exposure to cholera toxin or the addition of verapamil with no change in $[Ca^{2+}]_i$ [11]. In contrast, as forskolin, db-cAMP and theophylline (agents not mediated by β_2-adrenergic receptors) are repeatedly applied with MCh, the relaxant effect of these cAMP-related agents is not diminished with no change in $[Ca^{2+}]_i$ (homologous desensitization) [11, 119]. Furthermore, pre-exposure to PDGF results in a marked reduction in the relaxant effect of isoproterenol against MCh-induced contraction with increasing $[Ca^{2+}]_i$, and this reduced responsiveness to isoproterenol is reversed by verapamil, an inhibitor of VDC channels [197]. The relaxant effects of not only β_2-adrenergic receptor agonists but also forskolin are markedly diminished with increasing $[Ca^{2+}]_i$ after exposure to growth factors, such as TGF-β_1 and PDGF (heterologous desensitization). In contrast, the relaxant effects of db-cAMP and theophylline are not attenuated after exposure to TGF-β_1 and PDGF. These results indicate that β_2-adrenergic desensitization is caused by dysfunction of the receptor/G_s/adenylyl cyclase processes in airway smooth muscle and that the cAMP-independent pathway contributes to this phenomenon [4, 5, 8, 16, 17]. Furthermore, Ca^{2+} entry through VDC channels is associated with β_2-adrenergic desensitization, and VDC channel activity may be enhanced by dysfunction of the G_s/K_{Ca} channel stimulatory linkage.

9.6.4.2 Effects of Ca^{2+} Sensitization

In fura-2-loaded tissues of guinea pig tracheal smooth muscle, pre-exposure to Lyso-PC results in a marked reduction in the relaxant effect of isoproterenol against MCh-induced contraction with no changes in $[Ca^{2+}]_i$ [163]. This phenomenon is reversed to the control response in the presence of Y-27632, a selective inhibitor of Rho-kinase, in a concentration-dependent manner. In contrast, the relaxant effect of cAMP-related agents (not mediated by β_2-adrenergic receptors) such as forskolin, theophylline, and db-cAMP, is not attenuated after exposure to Lyso-PC (homologous desensitization). Reduced responsiveness to isoproterenol with no changes in $[Ca^{2+}]_i$ is also caused after the exposure to tryptase and SLIGKV [162] and S1P [164]. The relaxant effects of forskolin are not reduced by pre-exposure to tryptase and SLIGKV; in contrast, these relaxant effects are markedly reduced by pre-exposure to S1P, indicating that the receptor/G_s/adenylyl cyclase process is also associated with the dysfunction of β_2-adrenergic receptors in airway smooth muscle; cAMP activity may be intact under this condition. Furthermore, in the presence of bisindolylmaleimide, a membrane-permeable inhibitor of PKC, reduced responsiveness to isoproterenol is not reversed after exposure to an agonist [119, 163, 194]. Therefore, tolerance to β_2-adrenergic receptor agonists caused by pre-exposure to lipid mediators and PAR2 agonists is involved in Ca^{2+} sensitization via the RhoA/Rho-kinase processes, not via PKC.

9.6.5 Airway Remodeling

In asthma, airway inflammation, which is mainly associated with mast cells and eosinophils, acts on the epithelium, subepithelium, and smooth muscle layers; bring about characteristic structural changes in the airways. Subepithelial fibrosis is resulted from the deposition of collagen fibers and proteoglycans under the basement membrane (thickening of the airway wall). This phenomenon leads to airway remodeling, which is thought to be related to asthma severity. In airway smooth muscle, mass formation is caused by cell proliferation and migration, resulting in airway remodeling [200, 201]. Increased proliferation of airway smooth muscle cell is not suppressed by glucocorticosteroids because of CCAAT/enhancer-binding protein (C/EBP)-α deficiency in airway smooth muscle cells of patients with asthma, different from them of normal subjects [202].

9.6.5.1 Cell Proliferation

Factors that cause proliferation of airway smooth muscle cells are divided into the following two groups: 1) polypeptide growth factors of tyrosine kinase receptors (RTKs), such as epidermal growth factor (EGF) and PDGF, and 2) contractile agents of GPCRs, such as leukotriene D_4, thromboxane A_2, and endothelin. When ligands bind to growth factor receptors, tyrosine kinase is activated, followed by Ras and extracellular regulated kinase (ERK)1/2, to transmit information to the nucleus [143]. Next, DNA synthesis and cell proliferation result from cyclin D1 activation [203]. In addition to this main pathway for smooth muscle proliferation, phosphatidylinositol 3-kinase (PI3K) activity is caused by gross factors via RTKs. Activation of the PI3-K/Akt signaling is also associated with the proliferation of airway smooth muscle cells [143]. Moreover, cross-talk between RTKs and GPCRs results from PI3K, p70S6 kinase, and glycogen synthase kinase-3 (GSK-3) [143, 204]. Involvement of the Rho family (RhoA, Rac, and Cdc42) is still unclear in the control mechanisms of airway smooth muscle cell proliferation. EGF- and PDGF-induced cell proliferation is not reduced by inactivation of RhoA/Rho-kinase signaling [186]; in contrast, activation of RhoA, not Rac or cdc42, results in the proliferation of human bronchial smooth muscle cells that are stimulated with serum. This proliferative reaction is reduced by Y-27632, C3 exoenzyme, and simvastatin, an HMG-CoA reductase inhibitor, which attenuate proliferation via the geranylgeranylation of RhoA (Fig. 9.4) [21]. Statins, inhibitors of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase, have pleiotropic effects, and results in an inhibition in cell growth in airway smooth muscle cells, independent of lowering the concentration of plasma cholesterol. The antiproliferative activity of statins may be caused by suppressing the synthesis of isoprenoids, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are associated with activation of small GTPases Ras and Rho families, respectively. Another factor, muscarinic M_2 receptor, causes the proliferation of airway smooth muscle cells [205, 206]. A clinical study has indicated that an antagonist of VDC channels suppresses airway remodeling in patients with severe asthma [25]. Ca^{2+} entry through VDC channels is enhanced since K_{Ca} channel activity is reduced by G_i coupled to muscarinic M_2 receptors when muscarinic receptor agonists are applied to airway smooth muscle [8, 9]. These results demonstrate that both Ca^{2+} dynamics (G_i/K_{Ca} channel/VDC channel linkage) and Ca^{2+} sensitization (RhoA/Rho-kinase pathway) is involved in the proliferation of airway smooth muscle cells (Fig. 9.4).

9.6.5.2 Cell Migration

Cell migration is a characteristic function of inflammatory cells, fibroblasts, and smooth muscle cells, and this function results in inflammatory cell recruitment and smooth muscle hyperplasia in the airways [207]. The extracellular matrix enhances the migration of airway smooth muscle cells [208]. Cell migration arises from contraction involving actin, myosin reactions, and actin reorganization. Since RhoA/Rho-kinase processes are an important factor in the regulation of the cytoskeleton of airway smooth muscle cells and other cells [209], these pathways may regulate the migration of airway smooth muscle cells due to changes in the cytoskeleton. Hence, RhoA/Rho-kinase may be associated with airway remodeling mediated not only by cell proliferation but also by cell migration (Fig. 9.4). Urokinase, PDGF, leukotrienes, and lysophosphatidic acid cause migration of human airway smooth muscle cells [20, 210–212]. Moreover, heat shock protein, PI3K, p38 mitogen-activated protein kinase, prostaglandin D_2, and IL-13 cause the airway smooth muscle migration [210, 213, 214]. Y-27632 significantly inhibits the increased migration of airway smooth muscle cells, due to PDGF or leukotrienes [143, 211]. Ca^{2+} sensitization related to the RhoA/Rho-kinase signaling is involved in regulation of cell migration. On the other hand, Ca^{2+} dynamics also regulate the migration of airway smooth muscle cells and inflammatory cells (Fig. 9.4). Ca^{2+} entry through SOC channels contributes to PDGF-induced migration of airway smooth muscle cells [19], and an increase in $[Ca^{2+}]_i$ due to other mech-

anisms also causes the substance P-induced cell migration of airway smooth muscle [18]. Since IL-13 causes Ca^{2+} oscillation in airway smooth muscle cells, cell migration induced by IL-13 may be associated with Ca^{2+} dynamics [215].

9.7 Bronchodilators on Airway Inflammation

Airway smooth muscle cells have the ability to alter the degree of various functions, such as contractility, proliferation, migration, and synthesis of inflammatory mediators [1, 12, 13, 29]. The plasticity from a contractile phenotype to other phenotypes (proliferation, migration, or secretion of chemical mediators) may enhance airway inflammation, leading to airway remodeling, which is also characteristic features of asthma and COPD (Fig. 9.3). Both Ca^{2+} dynamics [18, 19] and Ca^{2+} sensitization [20–24] may result in this phenotype change. Airway smooth muscle cells are associated with airway remodeling by mass formation due to proliferation and migration [25, 200]. Since isoproterenol inhibits muscarinic contraction mediated by a reduction in both Ca^{2+} concentrations and Ca^{2+} sensitization [2], β_2-adrenerguc receptor agonists may have effects on not only tension, but also inflammation in the airways. Formoterol, a long-acting β_2-adrenergic receptor agonist (LABA), suppresses infiltration of eosinophils and migration of myofibroblasts in the airway of patients with asthma (Fig. 9.5) [216]. Repeated contraction induced by muscarinic receptor agonists contraction independent of inflammation results in airway remodeling in patients with asthma (Fig. 9.5) [217]. These clinical trials have indicated that glucocorticosteroids have no effect on these phenomena, and that β_2-adrenergic receptor agonists may have prophylactic effects against airway remodeling. Moreover, since activation of muscarinic M_2 receptors causes the proliferation of airway smooth muscle cells [205, 206], muscarinic receptor antagonists may inhibit airway remodeling. β_2-Adrenergic action on airway smooth muscle competes with G_i/K_{Ca} channel inhibitory linkage connected to muscarinic M_2 receptors [8, 9, 17, 45, 69]. A clinical trial has demonstrated that an antagonist of VDC channels suppresses airway remodeling in patients with severe asthma [25]. Ca^{2+} entry through VDC channels is antagonized by activation of K_{Ca} channels, that is, the K_{Ca} channel/VDC channel inhibitory linkage [11]. β_2-adrenergic receptor agonists activate K_{Ca} channels via G_s and PKA [4, 5, 8, 69], indicating that these agonists may be useful for preventing airway remodeling resulted from a reduction in VDC channel activity [218].

Since atropine, a muscarinic receptor antagonist, suppresses G_i/K_{Ca} channel inhibitory linkage induced by muscarinic M_2 receptors [8, 9], these antagonists may have effects not only against tension, but also against inflammation in the airways, similar to β_2-adrenergic receptor agonists. Acetylcholine production in the airways is not restricted to the parasympathetic nervous system; acetylcholine is also released from non-neuronal origins, such as the bronchial epithelium and inflammatory cells [219, 220]. Furthermore, acetylcholine (either neuronal or non-neuronal) may cause inflammation and remodeling in the airways in asthma and COPD; muscarinic M_3 receptors may be associated with the pathophysiology of these diseases [221–224]. Long-acting muscarinic receptor antagonists (LAMAs) may suppress airway inflammation related to these diseases. However, it still remains to be determined clinically whether muscarinic receptor antagonists are useful for the inflammation and the remodeling in these diseases. Even though long-acting β_2-adrenergic receptor agonists (LABAs) are partial agonists, these agents can antagonize muscarinic action [17, 28, 29]. There is no signal transduction pathway that is unresponsive to β_2-adrenergic receptor agonists in muscarinic action. Both Ca^{2+} dynamics and Ca^{2+} sensitization are involved in the inhibitory effect of LABAs; in contrast, Ca^{2+} sensitization is not involved in that of LAMAs [2, 3, 29]. Therefore, combination of LABA and LAMA may be beneficial to improving contraction and inflammation in the airways [17, 28, 29, 38, 126–128]. LABAs have effects on an imbibition of migration of myofibroblasts, which are associated with airway remodeling [24]; in contrast, glucocorticoste-

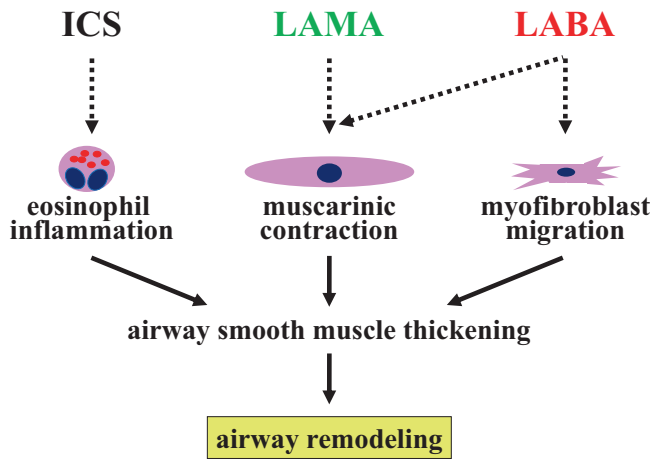

Fig. 9.5 Airway remodeling related to asthma independent of eosinophil inflammation in the respiratory tracts. Eosinophil inflammation causes remodeling in the respiratory tracts; inhaled glucocorticosteroids (ICS) is beneficial to this inflammatory disorder. However, repeated muscarinic contraction and myofibroblast migration causes thickening of airway smooth muscle, resulting in airway remodeling independent of eosinophil inflammation. Long-acting muscarinic receptor antagonists (LAMAs) and long-acting β_2-adrenergic receptor agonists (LABAs) are effective for these phenomena, respectively; in contrast, ICS is not. Arrows: activation, dotted arrows: inhibition. Illustrated based on ref. [216, 217]

roids do not (Fig. 9.5). Therefore, LABAs may be useful for preventing airway remodeling related to asthma. Moreover, LABAs may have an effect on an increase in glucocorticosteroid action in airway smooth muscle [225]. Since effects of LABAs are synergistically increased in the presence of LAMAs [17, 28, 29, 38, 125–128], the effect of inhaled glucocorticsteroid may be further enhanced by combination of LABA and LAMA [226]. Arrows: activation, dotted arrows: inhibition.

9.8 Conclusions

Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) is associated with alterations of contractility, proliferation, and migration in airway smooth muscle cells, resulting in airway disorders (airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, and airway remodeling), which are characteristic features of asthma and COPD (Figs. 9.1 and 9.4). This phenomenon is caused by induction of a change from contractile to synthetic/proliferative and hypercontractility phenotypes (Fig. 9.3). These phenotype changes based on Ca^{2+} dynamics and Ca^{2+} sensitization are due to the intracellular signal transduction pathways, such as G protein/K_{Ca} channel/VDC channel and RhoA/Rho-kinase pathways (Figs. 9.1 and 9.4).

Allosteric effect, which is a pharmacological characteristic in GPCRs, has not been taken into consideration so far in the use of β_2-adrenergic receptor agonists and muscarinic receptor antagonists for asthma and COPD. Allosteric GPCR modulation, which is caused by the G protein/K_{Ca} channel/VDC channel pathway, is associated not only with β_2-adrenergic intrinsic efficacy but also with synergistic effects between β_2-adrenergic receptor agonists and muscarinic receptor antagonists (Fig. 9.2). These two types of bronchodilators may be useful for preventing airway remodeling in asthma via inhibitions of muscarinic contraction and myofibroblast migration because glucocorticosteroids are not effective for these phenomena (Fig. 9.5).

Therefore, Ca^{2+} dynamics modulated by the G protein/K_{Ca} channel/VDC channel pathway and Ca^{2+} sensitization regulated by RhoA/Rho-kinase processes may be therapeutic targets for asthma and COPD, and research in these areas (pheno-

type changes and allosteric effects) may provide novel strategies in the development of agents for these diseases that will be effective for both bronchoconstriction and airway inflammation.

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Systemic Sclerosis and Pulmonary Disease

10

Khoa Ngo

Abstract

Systemic sclerosis is a complex, often progressive, multisystem autoimmune disease. It is commonly categorized into limited cutaneous or diffuse cutaneous systemic sclerosis. There is near universal involvement of skin fibrosis and gastrointestinal dysfunction, but lung disease is not only common but also a most serious complication. Severe lung disease is the top cause of mortality, displacing scleroderma renal crisis as the leading cause of death. Whether there is limited cutaneous or diffuse cutaneous manifestations can be predictive of what type of lung disease that can present in the patient. Limited cutaneous systemic sclerosis patients tend to have pulmonary hypertension whereas diffuse cutaneous systemic sclerosis patients tend to have interstitial lung disease. There are more rare phenotypes associated with antibodies Th/To and U3RNP that can have both pulmonary hypertension and interstitial lung disease concomitantly. There are inherent challenges in the management for both pulmonary hypertension and interstitial lung disease but with the focus on early diagnosis for each of these lung complications, treatment may have a higher chance of efficacy.

Keywords

Limited cutaneous systemic sclerosis · Diffuse cutaneous systemic sclerosis · Fibrosis · Vasculopathy · Raynaud's phenomenon · European League Against Rheumatism · American College of Rheumatology · Pulmonary arterial hypertension · Interstitial lung disease · Scleroderma Lung Study · Cyclophosphamide · Mycophenolate mofetil · Autologous hematopoietic stem cell transplantation

Abbreviations

ACA	anticentromere antibody
ACEI	angiotensin converting enzyme inhibitor
ACR	American College of Rheumatology
ANA	antinuclear antibody
dcSSc	diffuse cutaneous systemic sclerosis
ECG	electrocardiogram
EULAR	European League Against Rheumatism

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_10

EUSTAR	EULAR Scleroderma Trials and Research group
GERD	gastrointestinal reflux
GIT	gastrointestinal tract
ILD	interstitial lung disease
lcSSc	limited cutaneous systemic sclerosis
MAHA	microangiopathic hemolytic anemia
MRI	magnetic resonance imaging
mRSS	modified Rodnan skin score
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
RNP	ribonucleoprotein
SIBO	small intestinal bacterial overgrowth
SMR	standardized mortality ratios
SRC	scleroderma renal crisis
SSC	scleroderma
ssSSc	systemic sclerosis sine scleroderma
Scl70	Scleroderma 70
RNAP III	ribonuclear antigen polymerase III
Pm-Scl	polymyositis scleroderma
AA	African American
PFT	pulmonary function test
DLco	diffusion capacity of the lungs for carbon monoxide
MCTD	mixed connective tissue disease
SLE	systemic lupus erythematosus
FVC	forced vital capacity
WHO	World Health Organization
NT-proBNP	N-terminal probrain natriuretic peptide
TTE	transthoracic echo
RHC	right heart catheterization
mPAP	mean pulmonary artery pressure
TR	tricuspid regurgitation
PF	pulmonary fibrosis
RLD	restrictive lung disease
6MWT	6-min walk test
SLS	Scleroderma Lung Study
MMF	mycophenolate mofetil
AHCT	autologous hematopoietic stem cell transplant

10.1 Introduction

Systemic sclerosis (SSc) is a chronic progressive connective tissue disease that has a complex, heterogeneous pathogenesis. Its hallmark features include vasculopathy, autoimmunity, and finally fibrosis [1]. The early disease course has varying degrees of microvascular dysfunction and autoimmunity with subsequent tissue injury and inflammation with then eventual collagen deposition and fibrosis. There is evidence that similar to other autoimmune conditions, susceptibility to SSc is due to both genetic and environmental factors [2]. A recent paradigm of the disease focuses on a dysfunctional repair response to an initial injury which then leads to fibrosis [1]. It is a rare disease with an incidence of 8–20 cases per million per year in the United States [3]. Globally, around 1 in 10,000 people is estimated to be affected by systemic sclerosis [4] Female-to-male ratio is 3:1 [5] and it disproportionately affects women in their 30s to 50s [6]. It is arguably the most challenging rheumatologic disease to manage as there is no disease modifying agents but rather palliative treatments and interventions [7] It also has the highest mortality among rheumatic diseases despite improvements in survival in the past 10 years, adding to the complicated nature of caring for these patients [1]. A meta-analysis of 40 years of publications with a compilation of nearly 2700 SSc patients provided standardized mortality ratios (SMRs), which is the ratio of deaths compared to expected deaths in the general population matched for age, sex, and for the disease. Analysis of these data revealed that the pooled SMR for SSc was 3.5 with minimal change over the past four decades despite some improvements in treatment [8]. Making an early diagnosis is paramount as there is evidence that early intervention can be associated with better outcomes [9].

The 2013 European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) classification criteria, though is primarily for research purposes, can be utilized as a guide in making a diagnosis [10]. The differential diagnosis can also be challenging as there are multiple diseases that can also

result in skin tightening of different parts of the body. This can be due to sequelae of uncontrolled metabolic diseases, paraproteinemia, infectious diseases, or medications. Of the latter group, certain chemotherapy drugs like taxanes and gemcitabine, as well as radiotherapy can trigger systemic sclerosis [11].

10.2 Multisystem Disease

Raynaud's phenomenon is typically the first physical exam sign that is almost universal in its presence. It is a vasospastic response to cold exposure, typically in the hands but can also occur in the feet, and color changes can include white and blue that indicate transient hypoperfusion then red upon reperfusion. Nailfold capillaroscopy can be a useful physical exam exercise and abnormal findings in the capillary nail bed would point away from primary Raynaud's phenomenon and instead toward a diagnosis of secondary Raynaud's phenomenon. A connective tissue disease would then be high on the differential with SSc being at the top [12].

The heterogeneity of SSc is apparent when viewing the disease from several methods of subcategorization. Most often it is by the pattern of skin sclerosis, but it can also be subdivided by positive serology, namely, the dominant antibody, and more recently there has been efforts to uniquely characterize patients by gene expression profiles of their skin [13]. The two primary subsets of SSc by skin involvement are limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc). There is also a third subset called systemic sclerosis sine scleroderma (ssSSc), comprising of <5% of the patient population (though this may be underrecognized), which has no skin involvement at all but rather the fibrosis is exclusively internal [14]. The skin sclerosis in lcSSc and dcSSc starts distally typically in the hands and feet. lcSSc can then extend proximally up to the elbow and knee and also involve the face and neck. By contrast, dcSSc can extend proximally past the elbows and knees and tends to involve the face, neck, and trunk. Time to peak sclerosis for dcSSc is 12–18 months, though each patient can be different [15]. Notably, worsening skin fibrosis can correlate with worsening internal fibrosis. Fibrosis can also occur internally, involving the lungs, gastrointestinal tract (GIT), and heart. Similar to Raynaud's phenomenon, GIT involvement is almost universally seen in all subsets of SSc. It can be extensive with wide variation in segment involved and severity [16]. Certain findings like gastrointestinal reflux (GERD) and esophageal hypomotility can portend lung involvement [17, 18]. Other findings include esophageal dysmotility, lower esophageal sphincter incompetence, gastroparesis, small intestinal bacterial overgrowth (SIBO), large-mouthed diverticuli, and anal sphincter incompetence. As a consequence, patients can experience myriad of symptoms corresponding to the above pathology, including: dysphagia, sensation of food being stuck mid-esophagus, early satiety, weight loss, alternating diarrhea and constipation, abdominal pain, hematochezia, and stool incontinence. Cardiac involvement is one of the more rare organ manifestations; however, it may be underreported [19]. Cardiac fibrosis can result in left ventricular dysfunction, conduction abnormalities, arrhythmias, or heart failure. In one major patient database, EULAR Scleroderma Trials and Research group (EUSTAR), prevalence of left ventricular dysfunction was found to be about 5.4%. Arrhythmias was the primary cause of death (6%) and combined ventricular failure accounted for 5% of deaths [20]. Of note, patients can have coronary involvement and present with atypical symptoms or it can be silent [21]. Pulmonary disease is the leading cause of mortality for SSc patients [7] and an extensive word on this is warranted later. The two primary lung pathologies are interstitial lung disease (ILD) and pulmonary hypertension (PH). Knowing whether a patient has either lcSSc or dcSSc can have anticipatory value as to what pulmonary complication that may manifest. lcSSc patients are at higher risk for PH whereas dcSSc patients are at greater risk of developing ILD. There are certain subsets that are at higher risk of developing both, depending on the antibody that returns positive. Given the significant morbidity and mortality,

there is great emphasis on early detection of any pulmonary involvement.

Renal disease, namely, scleroderma renal crisis (SRC), used to be the most feared complication as historically it was the #1 cause of death [22, 23]. Pathogenesis starts with severe vasculopathy involving the renal vasculature, leading to first accelerated-phase hypertension, then microangiopathic hemolytic anemia (MAHA) and progressive renal failure. MAHA can result in pancytopenia. Some patients lack the first phase of hypertensive emergency, and they tend to have worse outcomes. There is suspicion that this presentation of SRC is possibly due to concomitant left ventricular dysfunction. With the advent of angiotensin converting enzyme inhibitors (ACEI) treatment, the natural history of the disease has improved dramatically with less deaths and permanent renal replacement [24]. Patients are advised to conduct home blood pressure monitoring daily in order to have continual surveillance for SRC.

Vasculopathy is one of the primary features of SSc and can be extensive, leading to significant morbidity and at worst the aforementioned mortality in SRC. Multiorgan involvement is the hallmark of vasculopathy and can be severe in Raynaud's phenomenon, leading to digital ulcers, gangrene, and at worst amputation. Telangiectasias can be seen on physical exam and they are more likely to appear on lcSSc patients. They have a high correlation with development of PH, particularly if there are eruptions of new clusters on exam [25]. Relatedly, vasculopathy at the level of pulmonary vasculature can result in pulmonary arterial hypertension (PAH). Telangiectasias can also involve the GIT (gastric antral vascular ectasia; also called "watermelon stomach"), and this can lead to gastrointestinal bleeds. Other GIT involvement can lead to local ischemia and then fibrosis which is manifested as hypomotility or sphincter incompetence. Other musculoskeletal findings include calcinosis, arthralgias or arthritis, acroosteolysis, and myalgias or myopathy. Fatigue is a prominent symptom which can be due to the above complications or exist independently.

Screening for nonpulmonary complications involves serially extensive review of systems and physical exam. An assessment of skin involvement can be conducted with scoring using a validated tool like the modified Rodnan skin score (mRSS). A complete metabolic panel and urine studies can help monitor for SRC. If there is suspicion for significant cardiac involvement, namely, fibrosis, then a cardiac magnetic resonance imaging (MRI) with gadolinium can be useful to detect late gadolinium enhancement, which can be indicative of fibrotic change of the cardiac parenchyma [26]. Electrocardiograms (ECGs) can assess for arrhythmias.

10.3 Associated Antibodies

It is important to note that most organ involvement occurs early in the disease and thus the major challenge is to properly risk stratify patients for anticipated organ complications with the goal of providing early therapeutic intervention [27, 28]. What may be helpful in this intensive endeavor is knowing the positive SSc related labs of the patient. While there are multiple commercially available antibodies associated with SSc, only a few have been extensively studied. They are grouped into ones associated with lcSSc and dcSSc and some have certain associated phenotypic manifestations. The most common lcSSc associated antibody is anticentromere antibody (ACA). It can be reported as "anticentromere" or as a pattern in a positive antinuclear antibody (ANA). These patients tend to develop PAH, telangiectasias, and calcinosis. There is less likelihood for cardiac involvement and it is considered "protective" against ILD development [27]. Ribonucleoprotein (RNP) antibody is another lcSSc antibody and has similar associations as ACA but has a higher risk for PAH and not considered protective against ILD. It is also called U1RNP. Anti-Th/To is rare but is significant for twin higher risks of both PAH and ILD concomitantly. As for dcSSc, the two most common antibodies are scleroderma 70 (Scl70), which is also called topoisomerase I, as well as ribonuclear antigen polymerase III (RNAP III). The former

has the highest risk of ILD, can have extensive GI involvement, and has a noted risk for cardiac involvement. There is a smaller but notable risk for SRC. There is an independent risk factor for mortality [29]. The RNAP III phenotype is similar to Scl70 but has a comparatively lower risk of ILD and cardiac involvement and confers the highest risk of SRC (25% of patients have this complication). They have the worst skin disease, where it is the most rapid in its progression and peaks in the shortest duration of time. There is also a malignancy association. Other dcSSc antibodies include U3RNP and polymyositis-scleroderma (Pm-Scl). With the latter, it is an overlap disease with polymyositis, which is an idiopathic inflammatory myopathy. Like Th/To, U3RNP patients have a twin risk of both ILD and PAH development. There is also another ANA pattern called "nucleolar" which can have either lcSSc or dcSSc phenotypes. There is generally less incidence of telangiectasias and calcinosis in dcSSc patients. Importantly, dcSSc patients have a higher associated mortality, which is primarily due to earlier major organ involvement, as the mortality rate coincides between the two subsets after >5 years of disease duration [30]. This is likely due to complications with ILD and SRC.

In recent years, there has been work on developing yet another method to better categorize patients by way of gene expression profiles from skin biopsies. SSc skin can be differentiated from normal skin with the presence of CXCL4 and interferon-associated cytokines. The former chemokine in particular has been shown to predict the risk and progression of SSc [29]. Significant work has been done on developing subsets within dcSSc by this method as well and there are plans to do the same with lcSSc. What is notable is the gene signatures themselves are independent of actual skin sclerosis involvement [13].

10.4 Ethnicity Impact

The impact of race is yet another notable factor in disease complications and prognosis. Central to this point is that African Americans (AA) tend to have a higher mortality compared to Caucasians. They tend to have a higher incidence of dcSSc, early and severe ILD, higher rates of and progressive PH, and higher incidence of SRC. The incidence of dcSSc among AA is approximately 20 cases per million per year with a peak age onset between 35 and 44 years. Alternatively, incidence of dcSSc among Caucasians is about 8 cases per million per year. Peak age of onset is between 45 and 54 years of age. Diffuse disease occurs in approximately 70% of AA women whereas it is only in 30% of Caucasian women. These are all in socioeconomically matched groups [3]. AA also had a higher proportion positive for RNP and U3RNP [31]. Other nonwhite groups that have a worse prognosis due to early lung involvement are Japanese and Choctaw Indians [32, 33]. Hispanic patients were intermediate between white and AA patients in terms of rates of ILD and PH [17, 18]. Compared to AA, Hispanic SSc patients had similar pulmonary function test (PFT) values except the latter group had a higher mean percent predicted diffusion capacity of the lungs for carbon monoxide (DLco). This was independent of smoking history [31]. Asian cohorts also have more pulmonary complications compared to those from Europe and North America [34].

10.5 Pulmonary Disease

Many studies have reported a 17–24% reduction in survival rate if pulmonary involvement exists in SSc [35]. Overall, Ssc mortality rate is 250–280% greater than expected when compared to the general population. By comparison, rheumatoid arthritis has a 20–70% greater than expected mortality rate [36]. Since pulmonary disease is the top cause of mortality in SSc patients, any pulmonary involvement warrants closer monitoring. The challenges of both management of ILD and PH are similar: both require early diagnosis in order for early intervention to have the maximal therapeutic efficacy.

10.5.1 Pulmonary Arterial Hypertension

PAH affects approximately 15% of SSc patients. Incidence rates are approximately 1–2% per year [27]. In contrast, a French study noted that the incidence of PAH developing within 5 years for both lcScc and dcSSc was 50% [37]. Three-year survival for SSc patients with PAH has been 56% compared to 94% for those without [25]. A study focusing on lcSSc patients, the subset more likely to develop PAH, found that those who would go on to have this disease had progressive decline of DLco for >10 years [6]. Among the connective tissue diseases, SSc patients do not have the highest prevalence of PAH (10%); mixed connective tissue disease (MCTD) (20–29%) and systemic lupus erythematosus (SLE) (14%) have higher prevalence [20]. And yet among the same group of diseases, SSc has the worst outcomes, followed by patients with MCTD and then SLE [38]. Compared to idiopathic PAH, it has a worse prognosis and lower therapeutic response [19]. Risk factors include decreased DLco, increased forced vital capacity (FVC) to DLco ratio, ACA positivity and lcSSc disease, increased disease duration, and advanced age [27, 39]. The major causes of precapillary PH in SSc are obstructive proliferative vasculopathy in small- and medium-sized pulmonary arterial vessels and chronic hypoxia from advanced lung disease [40]. PH can develop in SSc from any of the first three World Health Organization (WHO) Groups. PAH (WHO Group I) is the most likely, followed by WHO Groups III and II. There are inherent challenges in making a distinction between the three groups by symptoms and signs alone and typically it is only with invasive imaging can a definitive diagnosis be made. A serologic screening tool can be N-terminal probrain natriuretic peptide (NT-proBNP) and uric acid, where both would be high [41]. Current professional consensus in assessment for PAH involves monitoring for associated symptoms and signs (such as dyspnea or signs of right heart failure like jugular venous distention or lower extremity edema on exam) as well as serial transthoracic echo (TTE) studies. The gold standard for diagnosis of PAH remains right heart catheterization (RHC) [39]. A mean pulmonary artery pressure (mPAP) of ≥25 mm Hg and pulmonary capillary wedge pressure ≤ 15 mm Hg meets the diagnosis of PAH [25]. RHC can also make a distinction between precapillary PH, from WHO Groups I and III, and postcapillary PH, from WHO Group II. Studies have found that TR > 40 mm Hg have a positive predictive value of having PH of 92% with a sensitivity of 58% and specificity of 87% [3]. Tricuspid regurgitation (TR) on TTE is the most widely used as an estimation of mPAP on RHC. However, use of this estimation can miss 30% of cases of PAH as 15–20% of patients can have an absent TR [42]. Additionally, studies have found patients with PH on RHC due to left heart disease despite a prior TTE that revealed normal left ventricular function [43]. In the DETECT study, a more robust algorithm involving a two-step process with assigned risk scores for each step performed better than consensus guidelines. The first step included assessment of the following parameters since it was identified they conferred the highest risk for PAH development: telangiectasias, ACA+, NTproBNP, FVC/DLco, right axis deviation on ECG, and high uric acid level. The second step was TTE based with examination of right atrial enlargement and TR velocity. This scoring system-based referral for RHCs resulted in only 4% of missed cases of PAH compared to the usual 30% and earlier diagnoses of PAH despite lack of symptoms for a substantial number of participants [25]. Treatment of pulmonary hypertension in SSc is built on evolving evidence-based studies and follows consensus recommendations. Options include prostacyclins, endothelin-1 antagonists, and phosphodiesterase 5 inhibitors [44].

10.5.2 Interstitial Lung Disease

In SSc patients, nonspecific interstitial pneumonia is more common than usual interstitial pneumonia [1]. Predictors for clinically significant pulmonary fibrosis (PF) development were: dcSSc, advanced age at onset, lower FVC and DLco, and +Scl70. The presence of ACA+ is con-

sidered protective [27]. In one study, it was found that ILD is very common, with findings of some degree of interstitial fibrosis in up to 90% of SSc patients, resulting in restrictive lung disease (RLD) in 30–50%, and progression to significant lung destruction in approximately 15% [31]. Another study corroborated this with findings of 80% of SSc patients having lung fibrosis and 25–30% developing progressive disease [45]. Yet another study found 50% of patients had developed ILD after 3 years and 75% had signs of the disease at 5 years. The overall survival estimate for those who have ILD is 57% in lcSSc and 50% in dcSSc [27]. Most studies suggest that early treatment is more effective as it can capture the disease during its inflammatory state before the fibrotic state has begun [31]. As the above data illustrate, most patients develop severe RLD in the first 5 years after onset of SSc-related symptoms. However, it can also stabilize during the first 4–6 years. Again, this highlights the need to intervene early before irreversibility settles in. Screening should be conducted with both PFTs and an HRCT at baseline. Subsequent assessments can include repeat PFTs and 6-min walk tests (6MWT), for the first 3–5 years while the disease can be active [46, 47]. In a systematic review of 20 publications involving a total of approximately 1500 patients, DLco was the most consistent predictor of mortality in SSc lung disease, whereas extent of ILD on HRCT was an independent predictor of overall mortality and ILD progression [48]. Thus, screening for disease then upon recognition of disease development, treatment initiation is paramount. But the decision of when to treat is not streamlined. Accepted indications for treatment include the following: pulmonary fibrosis >20% on HRCT, FVC <80% predicted, decline of >10% in FVC, or decline of >15% in DLco [49]. Given the wide variance of when to treat but also of treatment toxicity, a simplified algorithm would be of great benefit. Such a prognostic algorithm was put forth by Goh et al., and was validated against mortality. It featured two simple steps: (1) disease severity stratification by HRCT as either minimal (<10% involvement), severe (>30% involvement), or indeterminate (10–30%) and (2) indeterminate cases were then added either to the minimal or to the severe disease group depending on FVC % performance. If >70%, the patient was added to the minimal disease group; if <70%, they were added to the severe disease group. Of note, on linear regression models a cutoff of FVC 70% correlated with roughly 20% ILD involvement on HRCT, which in turn correlates with significant disease [50]. While there are multiple treatment options, there is no set algorithm. There have been three Scleroderma Lung Study (SLS) trials. In the first SLS study, 1-year treatment of cyclophosphamide (CYC) was compared with placebo and was able to demonstrate modest preservation of lung function, with FVC % predicted improvement of 2.5% [51]. In the second SLS trial, mycophenolate mofetil (MMF) treatment for 2 years was compared to CYC. MMF demonstrated similar efficacy to CYC but with less toxicity. MMF also demonstrated approximately 2% FVC % predicted improvement and had less leukopenia and thrombocytopenia [52]. A third SLS trial is underway with an antifibrotic medication, pirfenidone, added in combination treatment with MMF compared to MMF alone. It has already demonstrated safety in SSc patients [53]. In the SENSCIS trial, another antifibrotic medication, nintedanib, became the first FDA approved treatment for SSc ILD patients. It was compared to placebo and demonstrated an improvement of 41 mL in FVC after 1 year [54]. The choice of immunosuppressant and/or antifibrotic medications may be dependent on the inflammatory state of the disease process.

Autologous hematopoietic stem cell transplant (AHCT) has also been studied with positive results. The three trials are ASSIST [55], ASTIS [56], and SCOT trials [57]. AHCT had a significantly higher likelihood of improving skin disease and activity as well as preserving lung function [58], when compared to standard of care, which was intravenous CYC. The lung parameters that specifically improved were FVC and total lung capacity [1]. Comparatively, the CYC arms had worsening pulmonary function over time. Of note, both ASTIS and SCOT trials used CD34-selected grafts, which should be the preference if available, as it has a higher success

rate [1]. AHCT can be considered for SSc patients with moderate and progressive ILD. 85% of ASTIS patients had ILD, and 93% had it in the SCOT study [1]. It is suspected that the success from AHCT is due to a "re-setting" of the immune system, to where it was prior to the autoimmune antigenic triggers. Restoration of human T regulatory cells has also been observed with treatment [59]. The major caveat is the risk of death (5–10%) during the treatment process, which is primarily due to opportunistic infections or cardiac injury which is suspected to be from conditioning doses of CYC. There is a proposed inclusion and exclusion criteria for treatment which highlights those patients who are still early in their disease process but already have significant and progressive ILD. Inclusion criteria: dcSSc disease with internal organ involvement, age < 65 years, disease duration <5 years, MRSS > 15, early pulmonary involvement by HRCT, or PFTs revealing FVC or DLco between 45% and 80%, a decline in FVC of >10% or DLco decline >15%. Exclusion criteria: FVC or DLco <45%, PAH, cardiac insufficiency, renal insufficiency, or prior CYC treatment for >6 -month duration [1].

An emerging possible option is interleukin 6 (IL-6) inhibitors. IL-6 appears to play a role in SSc pathogenesis and is expressed in endothelial cells and skin fibroblasts, especially for dcSSc patients. It has been implicated as a potential biomarker for poor outcomes in lung fibrosis. Tocilizumab may be the first targeted therapy to show benefit in improvement of skin sclerosis and prevention of pulmonary decline [60, 61]. Rituximab has also been used as rescue therapy [62].

10.6 Summary

SSc is a challenging disease for the patient but also for the practitioner. This is due to the heterogeneity of the disease, early onset of multiple severe organ involvement, and high mortality. The disease can be subdivided into pattern of skin sclerosis involvement or by the representative positive antibody involved. By demographics, there is a female predominance, roughly 3:1. AA are disproportionately affected, where they have more serious lung and skin disease and have a higher risk for scleroderma renal crisis. Many consider it the most challenging rheumatic disease to manage. The focus for ILD and PAH is to make an early diagnosis in order to have better outcomes. There are recently proposed algorithms with that aim. Though the options for PAH treatment has not changed, there are emerging therapies for ILD. The hope is that with early intervention and optimization of current and new therapeutics, the mortality rate for SSc will finally improve after 40 years of marginal change.

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Innate Lymphoid Cells in Airway Inflammation

11

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Abstract

Airways are constantly exposed to antigens and various pathogens. Immune cells in the airways act as first line defense system against these pathogens, involving both innate and acquired immunity. There is accumulating evidence that innate lymphoid cells, newly identified lymphoid lineage cells, play a critical role in regulating tissue homeostasis and in the pathogenesis of inflammation. Cytokines produced by other cells activate innate lymphoid cells, which in turn produce large amount of cytokines that result in inflammation. Type 2 innate lymphoid cells (ILC2s) are recognized as key component of T helper type 2 (Th2) inflammation, and are known to be elevated in type 2 (T2) human airway diseases (asthma). Th2 cytokines produced by ILC2s amplify inflammation via activation of eosinophils, B cells, mast cell, and macrophages. "T2 high asthma" has an increased Th2 response triggered by elevation of IL-4, IL-5 and IL-13 and other inflammatory mediators, leading to increased eosinophilic inflammation. The growing evidence of ILC2 contribution in the induction and maintenance of allergic inflammation suggests that targeting upstream mediators may affect both the innate and adaptive immune responses and all disease phenotypes. Blocking ILC2 activators, activation of inhibitory pathways of ILC2, or suppression of ILC2-mediated pathways may be therapeutic strategies for the type 2 airway diseases.

Keywords

Type 2 innate lymphoid cells · Th2 cytokines · IL-5 · IL-13 · IL-33 · TSLP · T2 high asthma · T2 low asthma · Type 2 inflammation · Eosinophilic inflammation · Airway hyperresponsiveness

Abbreviations

AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
BMT	Bone marrow transplant
CysLT1R	Cysteinyl leukotriene receptor
FeNO	Fractional exhaled nitric oxide
FEV-1	Forced expiratory volume in one second
GMCSF	Granulocyte macrophage colony stimulating factor

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_11

HDM	House dust mite
IFNγ	Interferon gamma
ILCs	Innate lymphoid cells
LTD_4	Leukotriene D_4
ROR γt	Retinoic acid receptor–related orphan receptor
T2	Type 2
T-bet	T-box transcription factor
TCF-1	T-cell factor 1
TSLP	Thymic stromal lymphopoietin
VEGFA	Vascular endothelial growth factor

11.1 Introduction

The discovery of innate lymphoid cells (ILCs), in the past decade, has greatly expanded our knowledge of lymphocyte biology in health and disease. ILCs are a unique subset of lymphocytes that do not express antigen receptors or lymphocyte surface markers, but transcriptionally and functionally mirror T-helper cells [1]. Based on their different cytokine production profile and expression of transcription factors, ILCs have been categorized into three different groups: ILC1s, ILC2s, and ILC3s. ILC1s, which include natural killer cells, express T-box transcription factor (T-bet). These cells resemble Th1 cells and produce interferon gamma (IFNγ), and are involved in anti-viral immunity. ILC3s express retinoic acid receptor–related orphan receptor (ROR γt) and produce cytokines such as IL-17A, IL-22, and granulocyte macrophage colony stimulating factor (GMCSF), and are considered to be involved in antibacterial immunity and autoimmune diseases [1].

ILC2 can induce a type 2 inflammatory response in allergic diseases, and share many similarities with Th2 lymphocytes as they also produce type 2 cytokines, IL-4, IL-5, IL-13, as well as other growth effector molecules including vascular endothelial growth factor (VEGFA) [2–10]. ILC2s also respond to nonspecific cell-derived factors, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) [3–5, 7, 11–14]. Both human and mouse ILC2s produce significant amounts of IL-4, IL-5 and IL-13, cytokines which promote airway inflammation and airway hyperresponsiveness (AHR).

Asthma is characterized by airway inflammation, AHR, and reversible airflow obstruction [15]. Asthma is known to involve mostly Th2 cells, mast cells, and eosinophils indicative of primarily an adaptive immune response [16]. Allergic inflammation in asthmatics driven by type 2 cytokines, IL-4, IL-5, and IL-13, was thought to be produced only by CD4+ Th2 cells. However, numerous studies in the past decade have shown that ILC2s are another major source of Th2 cytokines.

This chapter will focus on the role of ILC2 in the pathogenesis of airway disease, particularly asthma. We will review the findings from murine models and data obtained from human studies, and potential strategies targeting ILCs for the treatment of asthma.

11.2 Biology and Development of Innate Lymphoid Cells

Like other lymphocytes, innate lymphoid cells (ILCs) derive from bone marrow lymphoid progenitors [17]. Despite a lack of clonally distributed antigen receptors, ILCs share striking molecular and functional similarities with T cells. T-cell factor 1 (TCF-1) is a transcriptional factor that drives early T-cell development and is also required for the proper development in all ILC subsets, particularly ILC2s [17]. Nevertheless, unlike T cells that develop in the thymus, the development of ILCs does not require the thymus [17]. In addition, early ILC development relies on ID2, a transcriptional inhibitor whose overexpression may inhibit early T-cell development [17]. The intriguing developmental and molecular similarities and differences between T cells and ILCs remain an area of intense investigation.

11.3 Activation and Cytokines Production by ILC2s

Although Th2 (T-helper-2) cells are a major source of type 2 cytokines, including IL-4, IL-5, and IL-13, which promote allergic inflammation. Increasing evidence suggests that ILC2s are also

implicated in the production of these cytokines. The initial studies, which established the role of ILC2s in allergic inflammation, were performed in murine models [4, 5, 7]. Subsequent studies indicate that ILC2s play an important role in production of these cytokines in the human respiratory tract [18–20].

One of the earlier state-of-the-art studies in a mouse model showed high level of IL-5, IL-6, and IL-13 production by ILC2s in response to IL-25 and IL-33 [4]. The amounts of Th2 cytokines produced by ILC2 (FALC c-Kit$^+$Sca-1$^+$) cells were substantially higher than those produced by CD4$^+$ T cells. IL33 and a combination of IL2 and IL25 induced extremely high levels of IL5 and IL13 (microgram amounts from 5000 cells) but little IFNγ. IL5 and IL6 were detected even in the culture with IL7 alone, and IL2 increased the production of these cytokines. IL-5 is important for eosinophil survival, activation, and migration; IL-13 promotes AHR, recruitment and activation of immune cells, and airway remodeling with increased mucous production and fibrosis. Later studies in mice showed significant production of IL-4 by ILC2s, in response to leukotriene D_4 (LTD_4) [21]. This study shows that Cysteinyl leukotriene receptor (CysLT1R) is expressed on lung and bone marrow ILC2s in unchallenged mice and remains expressed on lung ILC2s after allergen challenges independent of B and T cells. Stimulation of purified ILC2s with leukotriene D4 results in robust IL-4, IL-5, and IL-13 production dependent on CysLT1R. This study also demonstrated that LTD4 administered to mouse airways regulates ILC2 proliferation and IL-5 production, and potentiates allergen-induced airway eosinophilia independent of adaptive immunity. The results of this study have significant implications for type 2 diseases including allergic asthma where CysLTs are increased and available to potentially activate lung ILC2 (Fig. 11.1).

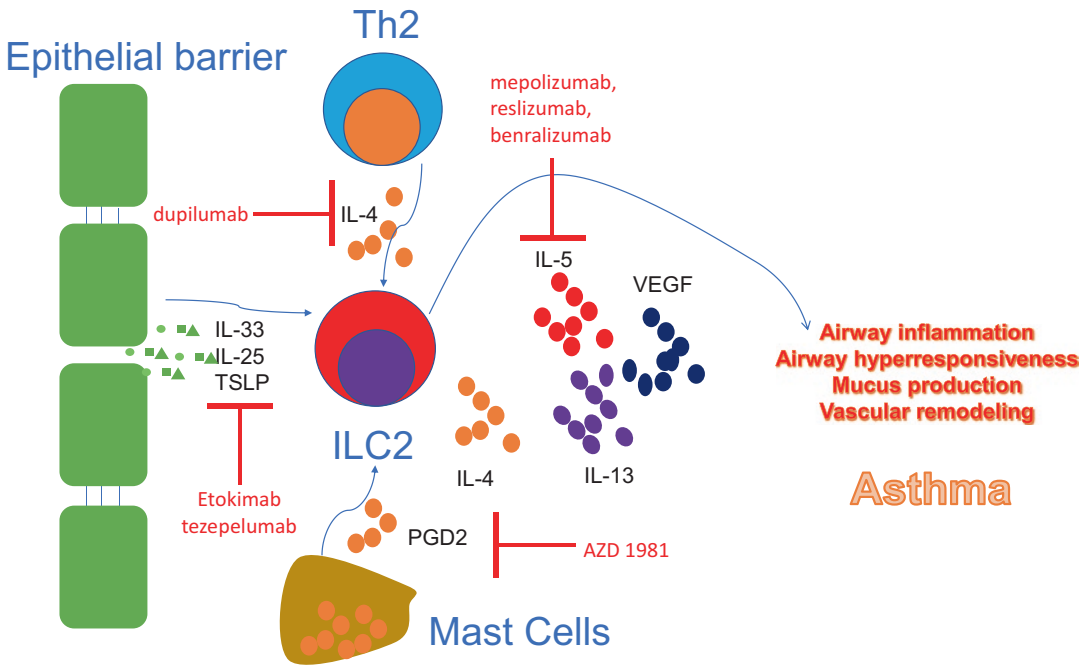

Fig. 11.1 Function and regulation of group-2 innate lymphoid cells in asthma. Group-2 innate lymphoid cells (ILC2) are activated by epithelium-derived IL-33, IL-25 and TSLP. The T helper type 2 cytokine IL-4, and some lipid mediators such as PGD2, further enhance ILC2 function. Activated ILC2 produce IL-5, IL-13 and VEGF that promote airway inflammation, airway hyperresponsiveness, mucus production and vascular remodeling. Biological therapies recently used in asthma may alleviate ILC2 function, by targeting key ILC2 regulators or effector molecules

11.4 Cellular Interactions and Upregulation of ILC2

A number of mediators that regulate the function of ILC2s have been identified. Studies in a murine model have shown that both IL-25 and IL-33 can induce ILC2 proliferation and production of type 2 cytokines as well as other effector molecules [4, 5, 7, 22]. Eosinophils, macrophages, and T cells have been shown to produce IL-25 that activates and further promotes type 2 cytokines production. Their expression in bronchial epithelial cells is also shown to be increased in Th2 asthma phenotype [23]. IL-33 is thought to be potent activator of ILC2s that induce Th_2 cytokines production and allergic inflammation. IL-33 is one of the "alarmins" expressed in the cells at mucosal barrier and released in response to tissue damage. However, some studies suggest that IL-33 may be actively secreted from bronchial epithelial cells even without injury [24]. Epithelial damage caused by cigarette smoke or diesel exhaust particles exposure stimulates expression and release of IL-33, leading to increased allergic inflammation. In a mouse model of airway inflammation induced by fungus *Alternaria*, IL-33 has shown to produce a type 2 response [25].

Thymic stromal lymphopoietin (TSLP), produced by epithelial cells, dendritic cells, mast cells, and basophils, is another activator of ILC2s. TSLP is produced mainly by airway epithelial cells in response to viruses and fungi [26]. Some human studies have shown that TSLP may be associated with steroid-resistant asthma. These studies show an increased number of Th2 cytokine producing ILC2s in the sputum, compared with those with mild asthma [27]. Anti-TSLP antibody treatment in moderate to severe steroid-resistant asthmatics has been shown to reduce asthma exacerbations and inflammatory biomarkers including both peripheral eosinophils and fractional exhaled nitric oxide (FeNO) [28]. These findings suggest that the TSPL/ILC2 axis may play a role in a subgroup of severe steroid-resistant asthmatics. Targeting the TSPL/ILC2 axis may be therapeutic strategy in these patients.

The ***lipid mediators***, Cysteinyl leukotrienes (CysLTs) as well as prostaglandin (PGD2) are products of arachidonic acid and known to be major pro-inflammatory mediators of allergic disorders. Eicosanoids, which help to promote or inhibit Th2 inflammatory response, have been found to be important in ILC2 responses [4]. ILC2 expresses PGD2 receptor (CRTH2), and PGD2 influences their migration and production of IL-13 [3, 29, 30]. CysLTs produced by mast cells, eosinophils, and basophils act directly on ILC2s to enhance their ability to produce Th2 cytokines [30]. Lipid molecules, PGI2, PGE2 and lipoxin A2 suppress ILC2s activation and cytokines production [30].

Our recent work has further indicated that IL-4 may play an important role in promoting human ILC2 responses in asthma patients [31]. Human ILC2 expresses both IL-4 and IL-4Ra. Inhibition of IL-4Ra by anti-IL4Ra repressed the numbers of ILC2 as well as their cytokine production capability in asthma patients, IL-4, but not IL-13, enhanced ILC2 activity in vitro. Results with mouse models have also indicated that IL-4 signaling promotes ILC2 function [31].

11.5 Asthma Heterogeneity and Phenotypes

Asthma is a chronic disease characterized by reversible airflow obstruction precipitated by bronchospasm and airway inflammation. It is a complex and heterogeneous disorder with several distinct phenotypes [32]. Type 2 (T2) inflammation plays a key role in the pathogenesis of asthma. IL-4, IL-5, and IL-13, along with other inflammatory mediators, lead to increased eosinophilic inflammation. Based on pathologic mechanism, asthma patients exhibiting an increase in airway T2 inflammation are now classified as having "T2- high" asthma whereas the remaining patients have "T2- low" asthma. Given the role of IL-5 and IL-13 in T2 inflammation and their effect on eosinophils, eosinophilia is a predominant feature of airway inflammation in T2-high asthma [33]. Potential mechanisms of T2 low asthma include the following: (1) non-T2 inflammation within the lung (airway neutrophilia, type 1 [T1] [IFN-mediated] or type 3 [IL-17–medi-

ated] immune pathways); (2) systemic inflammation associated with IL-6, obesity, and metabolic dysfunction; and (3) non-inflammatory (pauci-granulocytic) mechanisms. In addition to explaining T2 low asthma, these mechanisms may also co-occur with T2 inflammation [34].

A number of clinical features of T2 high asthma have been described. There is evidence to suggest that T2 high asthma is associated with a greater degree of airway responsiveness, reduced forced expiratory volume in one second (FEV-1), and increased asthma severity [33, 35–38]. T2 high asthma is also associated with increased health care utilization, resulting from poor asthma control causing exacerbation, increased systemic steroid use and emergency room visits [36, 38, 39].

Multiple pathways have been implicated in the clinical heterogeneity seen among asthmatics and asthma phenotypes. It is well established that the Th2 response involves both the innate and adaptive immune response. T cells (CD4+) contribute to the adaptive response, whereas type 2 innate lymphoid cells (ILC2s) and natural killer cells play an important role in the innate response [40].

Asthma is mediated by Th2 cells which play a critical role in this disease, by producing cytokines, IL-4, IL-5, and IL-13. IL-25, IL-33, and TSLP are also called epithelial alarmins, activating T cells and ILC2s [41]. IL-33 acts through its transmembrane receptor, leading to production of inflammatory cytokines and Th2 response [42]. CD4 T cells differentiate into Th2 cells by the action of IL-25 and IL33, whereas TSLP activates dendritic cells to induce Th2 responses [43]. T2 cytokines are also produced by resident memory T cells with the help of alarmins [44]. IL-4 is an important cytokine which promotes isotypes class switching and IgE production [45]. IgE binds to affinity IgE receptors on the mast cells and basophils causing activation and release of inflammatory mediators, such as histamine, leukotrienes, tryptase, and prostaglandins [46, 47]. These mediators cause airway smooth muscle contraction and mucous hypersecretion.

There is emerging evidence that asthma may not be simply Th2, IgE-mediated allergic inflammatory disease, but that it also involves an innate pathway in which ILC2s provide a cellular source of IL-5 and IL-13, important for adaptive type 2 immune responses [48–50]. Both IL-5 and IL-13 play a role in increasing airway eosinophils, mucous production, airway hyperresponsiveness, and airway remodeling [45]. This shows that both adaptive and innate immune responses contribute to Th2 inflammatory response.

11.6 Role of ILC2 in Type 2 Inflammation

11.6.1 Allergen and ILC2 Interaction (Murine Model)

Most initial studies that associated ILC2s with asthma have been performed in murine models. A number of allergens, including house dust mite (HDM), *Alternaria*, and Ovalbumin, activate ILC2. These allergens are known to possess protease activities by which they induce airway inflammation through ILC2 activation by IL-33 [51]. Airway challenge of naïve mice to *Alternaria*, a fungal allergen which is related to severe asthma exacerbations, caused increased IL-13 levels in bronchoalveolar lavage (BAL). This was followed by IL-5 and IL-13 production and airway eosinophilia without T or B cells' involvement (adaptive immunity). In IL-33R-deficient mice, this innate response to *Alternaria* was abolished [48]. HDM allergy commonly contributes to airway inflammation in asthma and ILC2s play a critical role in this response. When mice deficient in IL-33, IL-25, or TSLP signaling were exposed to intranasal HDM allergen, HDM-induced allergic asthma required IL-33 but not IL-25 or TSLP signaling. This may be due to IL-33 ability to upregulate ILC2 frequency in vivo [52].

There have been a number of attempts to develop a murine model deficient in the adaptive immune response but with an intact innate immune response. The nuclear receptor RAR-related orphan receptor α (RORα) is a transcription factor specifically important for ILC2

development [53, 54]. RORα bone marrow transplant (BMT) has been used to study ILC2 responses to investigate the adaptive responses to allergens [53, 55, 56]. RORα-deficient mice, when challenged with HDM allergen and papain, failed to produce an adaptive response despite the presence of the adaptive immune system. These observations indicate that ILC2s not only stimulate Th2 cytokines production, but also play an important role in adaptive immune responses.

11.6.2 Role of ILC2 in Human Airway Disease (Asthma)

ILC2 numbers are increased in the tissue of patients with various allergic disorders: allergic rhinitis, asthma, atopic dermatitis, and eosinophilic esophagitis [13, 30, 57–59]. Work from several groups has indicated critical roles for ILC2s, a type of lung-resident innate effector cells, in asthma pathogenesis [10, 57, 60].

ILC2s have been proposed to play a role in eosinophilic inflammation and have been identified in peripheral blood from asthmatics. There has been increased interest in utilizing ILC2 as a non-invasive marker predicting eosinophilic airway inflammation in asthmatics. A study compared the relationships of ILC2s and other biomarkers, blood eosinophils, FeNO, and IgE with sputum eosinophils [61]. This study revealed that the percentage of ILC2, blood eosinophil count, and FeNO were correlated with sputum eosinophil count, with ILC2 percentage most highly correlated with eosinophilic airway inflammation. This study also demonstrated that ILC2s were increased in eosinophilic asthmatic patients compared with non-eosinophilic asthmatics. Another study looked at potential role of ILC2s in driving chronic airway eosinophilia in severe corticosteroid-dependent asthmatics [20]. This study compared induced sputum-activated ILC2 with the measurements of CD4+ lymphocytes, which are another source of Th2 proinflammatory cytokines. Blood and sputum ILC2s and levels of intracellular IL-5 and IL-13, in patients with severe asthma, were compared to patient with steroid-naïve mild atopic asthma and nonatopic controls. The numbers of IL-5+ ILC2s were significantly increased in the sputum of severe asthmatics compared with mild asthmatics. In addition, patients with severe asthma had significantly increased levels of sputum IL-5+ and IL-13+ ILC2s, with sputum eosinophilia, despite normal peripheral eosinophil levels. These findings may suggest that a subgroup of steroid-resistant asthmatics has airway ILC2s which produce localized IL-5 and IL-13 promoting airway eosinophilia leading to severe corticosteroid- dependent asthma.

Another study examined the role of ILC2s in the maintenance of existing airway hyperreactivity, in addition to their involvement in asthmatics airways. Besides establishing the role of IL-13 through a positive feedback mechanism in a mouse model of airway hyperreactivity, this study also examined the clinical relevance of ILC2s and IL-33 in human asthma [19]. BAL fluid from asthmatic patients showed significantly elevated numbers of ILC2s and increased levels of IL-33 compared with a control group. Levels of IL-13 in BAL fluid negatively correlated with airway flow volume (FEV-1) and asthma control test scores are an indirect measure of asthma severity. Additionally, increased IL-33 levels predicted increased ILC2 number in the airways. Another study examined the presence of ILC2s in the airways of children with steroid-resistant asthma [58] compared with a control group with recurrent lower respiratory tract infections, without atopy or asthma [58]. The comparison was made between bronchoscopy, BAL, blood test, and induced sputum. Higher ILC2s were identified in BAL, induced sputum than in peripheral blood of pediatric patients with steroid-resistant asthma. Patients with steroid-resistant asthma were found to have significantly higher proportion of ILC2 compared with patients without asthma. Moreover, the presence of ILC2s in BAL of pediatric patients with steroid-resistant asthma compared with patients with lower respiratory tract infection shows that these cells may play a role in allergic airway disease.

11.7 Therapeutic Target

As ILC2s appear to play an important role in type 2 airway inflammation, it is intriguing that inhibition of ILC2 could be a therapeutic option in a subgroup of patients. Recently, a number of biologics have been approved by the FDA, primarily targeting Th2 inflammation. These include anti-IL-5 antibody (mepolizumab, reslizumab, and benralizumab) and anti-IL4Ra (dupilumab) [62]. Others, including anti-IL-13 antibody, lebrikizumab, and tralokinumab, are still under development [63]. Therapeutic agents targeting ILC2s activation pathways, including anti-TSLP antibodies (tezepelumab), anti-IL-33 antibody (etokimab) IL-33R, and CRTH2 (AZD 1981) antagonists, represent a promising new class of therapeutic agents under clinical development for the treatment of asthma. Humanized antibodies directed against epithelial alarmins, such as IL-33 and TSLP target upstream mediators of the airway inflammation pathway, may affect both innate and adaptive immune responses and as a result of this unique action may be effective for all asthma phenotypes. In a randomized, double blind, placebo controlled phase 2 trial of subcutaneous tezepelumab in patients with moderate to severe asthma treated with long-acting beta-agonists and medium-to-high doses of inhaled corticosteroids, there was a reduction in the annualized exacerbation rate compared with placebo group [28]. This effect was independent of baseline eosinophil count or other Th2 biomarkers. Tezepelumab also reduced blood biomarkers, blood eosinophil count, FeNO, and total serum IgE levels, indicating its important effects on IL-4, IL-5, and IL-13 pathways. These changes support the concept that inhibition of an upstream cytokine such as TSLP may have broader physiological effects than the targeting of individual downstream pathway, Th2 cytokines. These therapies will likely benefit not only patient with "T2 high asthma" phenotype but those with "T2 low asthma" as well, given that the innate lymphoid cells respond directly to release of alarmins. Other therapeutic options include activation of inhibitory pathways, which include cytokines (IL-10, IL-27, interferons) and lipid mediators [64].

11.8 Conclusion

There is accumulating evidence that ILC2s are present in the airways and play an important role in airway inflammation, particularly in asthma. ILC2s produce Th2 cytokines IL-5 and IL-13, which are important in the pathogenesis of asthma. Due to the Th2 blocking activity, new biologics such as anti-IL5, anti-IL4/IL13 antibodies act on both Th2 and ILC2s. Other therapeutic agents, primarily targeting Th2 responses contributed by ILC2, deserve further investigation. Anti-TSLP antibodies, anti-IL-25, and anti-IL-33 neutralizing antibodies are under investigation for the treatment of asthma. As there is significant evidence now that ILC2s play an important role in Th2 airway inflammation, therapies targeting interference with activation or stimulating inhibitory pathways represent promising tool for the treatment of airway inflammation in asthma.

Acknowledgments We thank Dr. Russell Hopp and Dr. Paul Feustel for their critical reading of the manuscript.

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Sjogren's Syndrome and Pulmonary Disease

12

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Abstract

Sjogren's syndrome is an autoimmune connective tissue disease targeting the exocrine glands and frequently affecting the respiratory system. The pulmonary disease is the most important extra-glandular manifestation as it carries most of the morbidity and mortality. Typically, it affects the small airways ranging from mild to severe respiratory symptoms. The upper airways are also commonly involved, predisposing sinusitis to occur more frequently than in the normal population. Lymphocytic interstitial pneumonia was initially thought to be the prevailing parenchymal disease; however, multiple cohorts report non-interstitial pneumonia to be the most frequent subtype of interstitial lung disease. In the review of high-resolution computed tomography scans, cystic lesions are commonly found and associate with both the small airways and parenchymal disease. Under their presence, amyloidosis or lymphomas should be considered in the differential. Overall, Sjogren's syndrome has a higher risk for lymphoma, and in lungs this condition should be thought of, especially when the images reveal pulmonary nodularity, lymphocytic interstitial pneumonia and lymphadenopathy. Although, pulmonary artery hypertension was traditionally and exceptionally linked with Sjogren's syndrome, together with systemic lupus erythematosus, they are now acknowledged to be the most common pulmonary vascular disease in east Asian populations, even over patients with systemic sclerosis. Although there are no controlled prospective trials to treat pulmonary disease in Sjogren's syndrome, the mainstay treatment modality still falls on glucocorticoid therapy (systemic and inhaled), combined with immune modulators or alone. Most of the evidence sustains successful outcomes based on reported cases or case series.

Keywords

Sjogren's syndrome · Airway disease · Interstitial lung disease

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12.1 Introduction

Sjogren's syndrome (SS) is a connective tissue disease (CTD) characterized by the lymphocytic infiltration of exocrine glands, leading to their

Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_12

dysfunction and the eventual destruction. The subsequent dryness of the mucosae, mainly in eyes and mouth, becomes clinically evident years after the pathogenic process has started [1]. It is an autoimmune disease characterized with an array of antibody production, with the anti-SSA/Ro and anti-SSB/La antibodies as the most representative and significant ones. In addition to targeting the exocrine glands, the disease extends beyond the extra-glandular tissues, involving a vast number of tissues and organs. Approximately, a third of patients will develop one or more extra-glandular manifestations, ascribing for most of the morbidity and mortality. In addition, a fraction of them will evolve into glandular lymphoma, also known as the mucosal-associated lymphoid tissue (MALT) lymphoma, or in advanced cases non-Hodgkin's, (usually large B-cell) lymphoma. Most of patients may complain of fatigue, an elusive manifestation of a presumed inflammatory immune state, causing a strong impact on patients' quality of life [2, 3]. Sjogren's syndrome may present alone and is labeled as primary SS (pSS), while presenting in association with another CTD it is known as secondary SS (sSS).

The extra-glandular tissues seem to follow a similar pathogenic mechanism as compared to the salivary glandular tissue. Pathology reveals invasion of lymphocytic and other mononuclear inflammatory cells, grouping adjacent to the glandular ducts and intact glands in the salivary glands and next to epithelial cells in other tissues. The concept of "epithelitis" labels this mechanism in which the epithelial cells of ducts, and glands in different tissues are recognized as antigens, eliciting a cellular and humoral immune response with inflammatory cell invasion [4]. Antigen driven T-cell mediated antibodies contribute to the tissue damage and organ dysfunction [5].

Patients with extra-glandular involvement, may present with a variety of manifestations, depending on the tissue/organ affected [1]. The most frequently affected are the pulmonary, neurologic, musculoskeletal, hematologic, renal, cardiac, reticuloendothelial, endocrine, cutaneous, and gastrointestinal systems. Due to the protean manifestations, atypical presentations may confuse the most experienced clinicians, delaying the diagnosis and the subsequent management. Consequently, and in average, it takes 4–5 years to make the diagnosis. Historically, the incidence and prevalence has been difficult to define, mainly due to the recurrent changes in the definition criteria and the methodological variances (age groups, gender, population selection). It affects more women than men with an average of 9:1, with a peak prevalence in the fourth to fifth decade of life [6]. The incidence varies from 0.4 to 3.9 million adults and a prevalence from 0.09% to as high as 1.4% [7]. Sjogren's syndrome is considered perhaps the most common, but still unrecognized, CTD affecting between 1% and 3% of the general population [8].

Criteria to define SS have changed several times throughout the last four decades, an indicative of the difficulties met to understand of the underlying pathogenic mechanisms. Newly uncovered findings and research experience, however, have allowed defining SS with an optimal sensitivity and specific, and designed for research purposes. Currently, the EULAR-ACR Classification Criteria Consensus Group defines SS as follows (see Table 12.1) [9].

The pulmonary manifestations account for most of the morbidity, if not mortality in SS patients [10], being one of the most common findings.

12.2 Pulmonary Manifestations

Sjogren's syndrome may affect different segments within the respiratory system, such as the airways, lung parenchyma, pleura, and other structures, either in the upper and lower airways, or presenting with a combination of them. The small airway disease seems to be the most common presentation [11, 12], and possibly linked with a variance of pathogenic mechanisms, ranging from xerotrachea to follicular bronchiolitis (FB). Remarkably, the respiratory involvement may impair not only the quality of life but also is a predictor of poor survival [10], and is associated with a fourfold increase in mortality risk after 10 years of the disease [13].

Table 12.1 SS classification criteria proposed to the ACR-EULAR [9]

The classification of SS applies to any individual who has a score of ≥4 when summing the weights from the following items. The evaluation should meet (1) the inclusion criteria, and (2) do not have any condition listed as exclusion criteria	
Inclusion criteria*	
At least one symptom of ocular or oral dryness (based on the AECG questions): (1) Have you had daily, persistent, troublesome dry eyes for more than 3 months? (2) Do you have a recurrent sensation of sand or gravel in the eyes? (3) Do you use tear substitutes more than 3 times a day? (4) Have you had a daily feeling of dry mouth for more than 3 months? (5) Do you frequently drink liquids to aid in swallowing dry food? Or suspicion of SS from the ESSDAI questionnaire (at least one domain with positive item)	
Exclusion criteria**	
Patients taking anti-cholinergic drugs should be evaluated for OSS, Schirmer's and UWS flow after a sufficient interval off these medications. Prior diagnosis of any of the following conditions would exclude participation in Sjögren's syndrome studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: • history of head and neck radiation treatment • hepatitis C infection • acquired immunodeficiency syndrome • sarcoidosis • amyloidosis • graft versus host disease • IgG4-related disease.	
Item	**Score**
FS ≥ 1	3
SSA/Ro (+)	3
OSS ≥ 5	1
Schirmer ≤5 mm/5 min	1
UWS ≤ 0.1 mL/min	1
Total	9

pSS primary Sjogren's syndrome
FS focus score. The histopathologic examination should be performed by a pathologist with expertise in the diagnosis of focal lymphocytic sialadenitis, and focus score count (based on number of foci per 4 mm^2) following a protocol described in Daniels et al. 2011 [104]
OSS ocular staining score, described in Whticher et al. [105] and van Bijsterveld score [106]
UWS unstimulated whole salivary flow, described in Navazesh and Kumar [107]
ESSDAI EULAR Sjogren's syndrome disease activity index
ACR American College of Rheumatology
EULAR European League Against Rheumatism
*reference: Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: A revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002; 61: 554–558.
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The prevalence of pulmonary manifestations in Sjogren's syndrome will depend on the criteria used to define abnormalities within the respiratory system (e.g., clinical, subclinical, imaging, or functional test). Studies defining lung involvement, and integrating the respiratory symptoms, pulmonary function tests (PFTs) or imaging on high-resolution chest tomography (HRCT), show a prevalence range from 9% to 24% [13–15]. Compared to these frequencies, in large studies that include PFTs and HRCT, the frequency of pulmonary involvement raises up to 75% [16].

The lung involvement implies a higher systemic inflammatory state. Subsequently, the laboratory findings will show an active inflammatory profile and specific serology. The most common findings are hypergammaglobulinemia, presence of high acute phase reactants, lymphopenia, and a higher focus scoring in the minor salivary gland histopathology [17]. The positive serology tests are: ANA, anti-SSA/Ro and anti-SSB/La antibodies [14, 15, 18–20]. Additional serology in SS patients linked with pulmonary features are the rheumatoid factor [17], anti-CCP [21], anti-Ku

[22], and anti-RNP antibodies [23], being these later antibodies associated with other CTDs and important to differentiate them from a possible coexistence. It is worth to mention that serology is not always present as described in a large cohort [24]. Another important feature of pulmonary involvement is its presence in SS patients with longer disease duration and older age at disease onset [13, 24–26]. In a large-population study, the cumulative incidence of interstitial lung disease (ILD) in individuals with primary SS was 10% (±3%) at 1 year of the pSS diagnosis and increased up to 20% (±4%) by 5 years after the pSS onset [10].

Suspected patients with SS and possible early pulmonary compromise may present subtle symptoms and signs associated with bronchial mucosal dryness and expressed as dry cough.

12.2.1 Nose, Mouth, and Upper Airway Disease

Sicca mucosae contribute to an inflammatory environment in the mouth, ears, sinuses, and other areas in the upper respiratory tract. The resultant xerostomia, and periodontal disease, is a common finding in SS patients with gum retraction and occasional caries at the neck of teeth. In addition, candida colonization presents with a variety of findings in the tongue, and gums, along with burning mouth syndrome [27]. Sjogren's syndrome is an independent risk factor for chronic rhinosinusitis, with a hazard ratio of 2.51 (95%CI2.22–2.84;$p < 0.001$) [28], in addition to presenting with other features like nasal crusting and epistaxis in 18.5% and 31.8%, respectively [29]. Hoarseness presenting in around a third of individuals [30], might reflect either sicca trachea-larynx alone or as a consequence of reflux due to esophageal motility dysfunction and inability for saliva to buffer the gastric fluid [31]. As in other CTDs, inflammation of vocal cords with a *bamboo node* appearance has been reported in SS and accounting for hoarseness [30]. Xerotrachea associates with cough and as described, it may carry most of the complications, and lasting for a prolonged time. In the ears, Sjogren's syndrome individuals may report present with pruritus [32].

12.2.2 Clinical Evaluation of Airway Disease

Cough is the most common symptom of airway disease, with a prevalence range from 41% to 61% [32]. It associates with poor quality of life while the severity correlates with the inflammatory degree [32]. Reports showed correlations of the dysfunction in the tracheobronchial mucociliary clearance with the inflammatory lymphocytic infiltrates [33] and additional submucosal gland atrophy [30]. Infiltration of CD4-(+) lymphocytes prevails in the infiltrated tissue [26, 34]. Moreover, gastroesophageal reflux, commonly present in SS, contributes to the inflammatory process [32]. The inflamed mucosae impair secretion clearance secretions. In turn, this will perpetuate the inflammation provoking bronchial hyperreactivity, and potentially triggering further complications in the distal airways. The inflamed trachea, also known as xerotrachea, may coexist with inflamed bronchi or xerobronchitis. Distally, the bronchioles may be affected also.

12.2.2.1 Lower Airway Disease

Bronchial hyperreactivity has been reported in 42–60% of cases and usually presents with dry cough [32]. Patients with SS report more sensitivity to environmental exposures (smoke, dust, etc.). In the pathogenesis, the inflammatory infiltrates invading the mucosal are composed of lymphocytes predominantly, but also mast cells and neutrophils. Contrary to the expected cellular population seen in bronchial asthma, eosinophils are not present in SS [34]. Another feature of bronchial involvement is the greater response to the methacholine challenge test, but not to adenosine monophosphate challenge, cold, or hyperventilation tests [35]. Both of these features, the cellular profile and the methacholine challenge test, suggest a unique inflammatory pathway causing hyperreactivity [36]. Furthermore, the dysfunctional capabilities to clear the secretions may play a main role in hyperreactivity rather than the submucosal gland atrophy [33]. Conventional therapy with inhaled glucocorticoids may not be as effective as in other causes to treat hyperreactive airways and tracheobronchitis, which adds to the elusive knowledge of airway disease in SS [37].

Bronchiolitis

This is the most frequent presentation of airway disease (small airways) in SS [11]. Bronchiolitis refers to the inflammation of the small airways. In SS, it has distinctive histopathologic features of what is known as follicular bronchiolitis. FB consists of hyperplastic lymphoid follicles, containing germinal centers, distributed along the bronchovascular bundles [38, 39]. Other histopathology patterns have been described showing various forms of severity/chronicity including chronic bronchiolitis, obliterative bronchiolitis, lymphocytic bronchiolitis, constrictive-destructive bronchiolitis, and panbronchiolitis [32]. Bronchiolitis may present alone or with additional tissue invasion into the parenchyma known as interstitial pneumonitis [38, 40]. Peribronchiolar hypertrophic lymphocytic aggregation causing a mechanic valve-effect may play a role in the bullae and cystic formation [41], a frequent finding seen on the HRCT (Fig. 12.1). Combined, the anatomical and radiographical criteria identifying bronchiolitis, it is present in 24% of cases, a higher number than previous reports [40]. The symptoms are dry cough, wheezing, dyspnea, and infections. They may last for months and recur, but overall bronchiolitis has a benign prognosis [42]. Evidence supporting specific therapies is weak, but inhaled glucocorticoids, rituximab, and/or chronic macrolide therapy may improve the symptoms [32].

Bronchiectasis

The permanent enlargement and/or dilatation of the airways defines bronchiectasis, and, in most SS cases, the cylindrical pattern on the HRCT is the predominant feature [32] (Fig. 12.2). The presenting frequency varies from 7% to 54%, depending on the studies [32]. The most frequent symptoms are cough, dyspnea, hemoptysis, and recurrent infections. Patients with SS and bronchiectasis are more likely to be females, have concomitant chronic sinusitis, are older at the time of diagnosis, have associated hiatal hernia and a higher frequency of anti-smooth muscle antibody, and a lower frequency of anti-SSA/Ro antibodies as compared to those without bronchiectasis [43, 44].

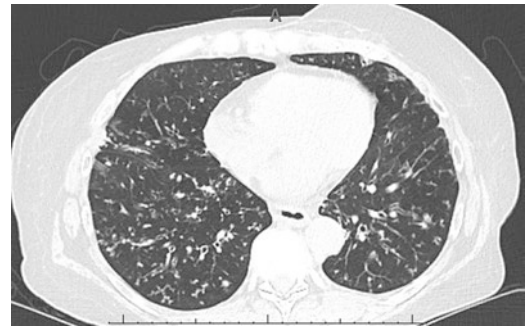

Fig. 12.1 Follicular bronchiolitis—HRCT demonstrates a few centrilobular ground-glass nodules as well as perilymphatic nodules. In addition, there is bronchial thickening and mucus impaction

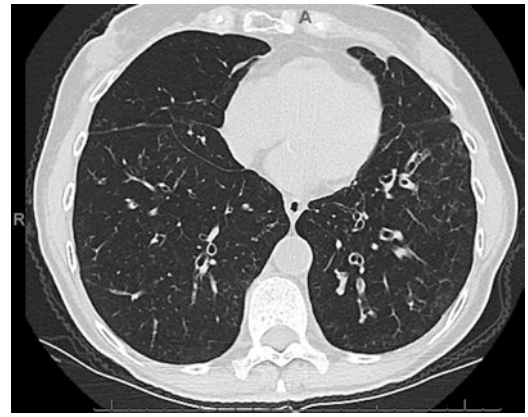

Fig. 12.2 HRCT imaging reveals dilated airways with bronchial wall thickening

12.2.3 Parenchymal Lung Disease

Interstitial lung disease (ILD) is the most serious form of lung involvement because of the association with most of the morbidity and early mortality [10], with a cumulative 5-year mortality rate up to 16% [10]. The prognosis of ILD is in most cases benign with stable PFTs in most of SS patients [19] contrasting with idiopathic ILD whose carriers may have a worse outcome [40]. However, a subset of patients will suffer from complications of respiratory dysfunction/failure [45]. Factors associated with poor outcome (mortality) were found to be lower levels of FVC percentage and higher levels of serum Krebs von den Lunden (KL-6) (with a cutoff >800 U/mL) [46].

The overall 5-year survival rate is of 89.8% and the 10-year survival rate is of 79% [46].

Prevalence of ILD has been reported to be between 3% and 60% [42, 47], depending on the methodology used to detect ILD. The incidence fluctuates between 8% and 17% [48, 49]. The EULAR-Task Force reported indirect evidence of parenchymal disease in 163 patients showing a restrictive pattern in 64%. The HRCT findings of ground glass-opacities/interstitial changes were identified in 49% of 526 patients, revealing a high prevalence of parenchymal lung involvement [50]. The HRCT is the most sensitive test, describing lung infiltrates in SS patients in up to 90% [32]. Sjogren's syndrome can present with many different patterns interstitial lung disease (ILD) in SS, including non-specific interstitial pneumonia (NSIP), organizing pneumonia (OP), usual interstitial pneumonia (UIP), lymphocytic interstitial pneumonia (LIP), and diffuse interstitial amyloidosis. In initial studies, Sjogren's syndrome was linked with a form of ILD, LIP, and primary pulmonary lymphoma. Larger cohorts, however, reclassified the frequency presentations. NSIP is the most frequent presentation of ILD. In a large database analysis of 146 SS cases with lung biopsies, the different patterns of ILD were 45% for NSIP, 16% for UIP, 15% for LIP, 7% for OP, 6% for amyloidosis, and 11% for other pathologies [50]. This distribution has been reported in other recent large studies [47]. Clinical features in SS patients with ILD show an older age at disease onset, higher median disease duration, presence of fever, xerostomia, xerophthalmia, and neuropathy [51]. Interstitial lung disease can be the initial presentation of SS in one-third to half of the patients [52], making the diagnosis difficult if serology is absent. A lip biopsy or newly described specific features on major salivary glands may help achieving the diagnosis.

The main findings are dry cough and dyspnea in the history, and rarely clubbing and crackles on the physical exam [32, 42, 53]. An inflammatory pattern is present in the laboratory profile, with hypergammaglobulinemia, lymphopenia, high acute phase reactants, and lactate dehydrogenase [42]. Also, positive serology, anti-SSA/Ro antibodies, and a positive rheumatoid factor have been found to associate with ILD [32, 51]. Pulmonary function test demonstrated a restrictive pattern with additional low DLco that prevails in most of patients (64%); additionally, an obstructive pattern was present in 21% and a mixed pattern in 25% [52]. The chest radiography, although of great utility showing linear and reticular patterns in up to 30% of suspected cases [32], may overlook early and subtle cases of ILD. Compensating this drawback, the HRCT is the modality of choice due to better resolution demonstrating different patterns: a reticular form, ground-glass attenuation, interlobular septal thickening, and cystic formations [32, 47]. Associated bronchovascular bundle thickening is reported, representing the mixture pattern of airway disease over the insterstitial component. Many other features may be found, including pleural effusion, airspace consolidation, nodules, fibrotic streak, honeycombing, subpleural curvilinear shadows, lung bullae, and mosaic perfusion. Other common characteristics in the HRCT are the bilateral lung infiltrates, localized in the lower lobes and subpleural spaces, with few lesions in the hilum [47]. Bronchoalveolar lavage will reveal a predominance of T-cell infiltrates of lymphocytic alveolitis [32].

12.2.3.1 Nonspecific Interstitial Pneumonia (NSIP)

As described, this is the most frequent presentation of ILD in SS. NSIP is characterized by varying degrees of alveolar inflammatory infiltrates with an admixture of fibrosis. Depending on the degree of inflammatory pattern versus fibrosis, NSIP will be subdivided in the cellular (cNSIP) (Fig. 12.3) or fibrotic form (fNSIP) (Fig. 12.4) [17]. The later variant is the most common NSIP form in SS, with bilateral basal reticular infiltrates and traction bronchiectasis, peribronchovascular extension, ground-glass attenuation and pulmonary consolidation, and classically sub-pleural sparing [54]. Features of honeycombing are exceptionally rare helping distinguishing them from the UIP pattern. The reticular pattern, with or without traction bron-

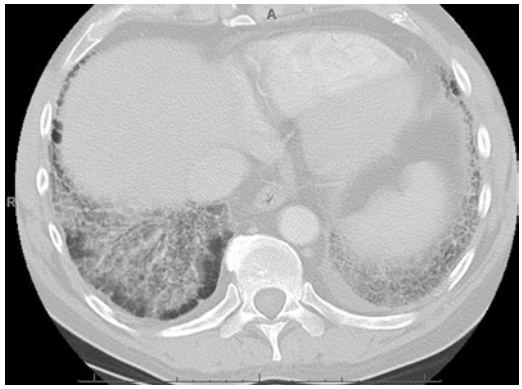

Fig. 12.3 Non-specific interstitial pneumonia—HRCT shows NSIP with a basilar and peripheral predominance. There is diffuse ground glass with subpleural sparing on the right and intralobular thickening

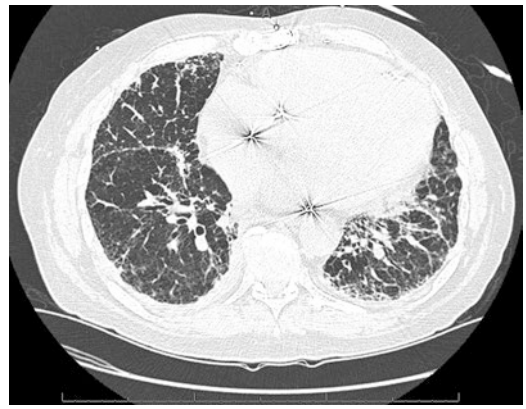

Fig. 12.5 Usual Interstitial Pneumonia—HRCT reveals a UIP pattern. Subpleural and basilar predominant distribution of honeycombing, reticulation, and traction bronchiectasis

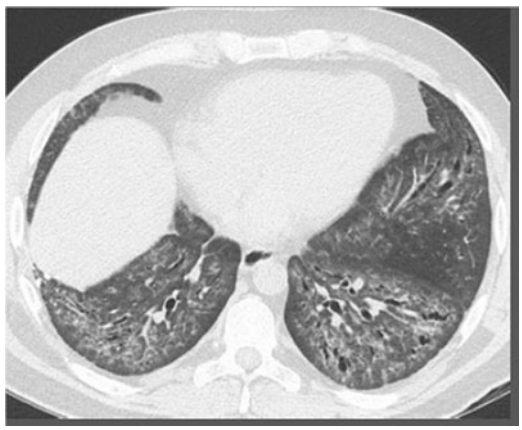

Fig. 12.4 Fibrotic NSIP—HRCT demonstrates fibrotic non-specific interstitial pneumonia (fNSIP). Imaging shows basilar and subpleural predominant ground-glass opacities with traction bronchiectasis (fibrotic) and classic subpleural sparing

chiectasis, may be correlated with the amount of fibrosis seen in the histopathology. More relevant, the subpleural sparing and tracking of opacities along the lower zone bronchovascular bundles correlate with the histopathological diagnosis of NSIP [55]. The outcome will depend on the fibrotic pattern with better survival in those with the cellular pattern [56]. Overall, the 5-year survival rate in NSIP is of 83% [40]. Treatment options are many, but there are not prospective controlled studies available exploring efficacy of a pharmacologic therapy. The mainstay of therapy relies on glucocorticoids, at 0.5–1 mg/kg/daily, and tapered while simultaneously the patient may be on an additional drug, mainly cyclophosphamide or azathioprine, with variable results [53, 57, 58]. Little data is available for mycophenolate mofetil, alone or with a combination with glucocorticoids; however, safety has shown promising outcomes [59]. Rituximab is efficacious for some cases [60], and perhaps in the future the combination of mycophenolate mofetil and rituximab may be the ideal therapy. Our experience reveals improvements on this later combination.

12.2.3.2 Usual Interstitial Pneumonia (UIP)

Main differences of UIP versus NSIP are in the histopathology. The relevant UIP features are patchy areas of fibrosis alternating with well-delineated normal lung; most of the worse findings are under the pleura and in the periphery of lobules; there is minimal interstitial inflammation; and the fibrotic tissue shows scattered fibroblastic foci [32]; honeycombing is the representative lesion [61] (Fig. 12.5). Although uncommon in Sjogren's syndrome, UIP may carry most of the morbidity, and poorer prognosis. Its prevalence is between 11 and 17% [47, 50, 53]. UIP pattern in SS patients was shown more in males, with an older age at disease onset and

had negative anti-SSA/Ro antibodies [51]. Despite the poorer prognosis than NSIP, UIP pattern in SS may have a better outcome than in patients with idiopathic pulmonary fibrosis [62].

12.2.3.3 Lymphocytic Interstitial Pneumonia (LIP)

LIP seems to be a continuum of follicular bronchiolitis, meaning the extension of small airway inflammation into the pulmonary parenchyma. Histopathology findings show a diffuse interstitial infiltration of T and B lymphocytes and plasma cells in the alveolar septa as and the small airways [17]. Initially thought to be unique for pSS, it may present in other CTDs. Its frequency ranges between 3.9 and 15%, depending on the methodology used [47, 50], with an estimated 1% of patients with pSS to endure LIP during their lifetime [63]. Patients may present with dyspnea and cough and inspiratory crackles during the exam [64] and occasionally pleuritic chest pain [65]. In the chest HRCT scan, relevant patterns are the thickened bronchovascular bundles (representing follicular bronchiolitis), centrilobular and subpleural nodules, and ground-glass opacities and thickening of the interlobular septa [42] (Fig. 12.6). Cysts are not uncommon with variable size with thin walls, and important to differentiate them from amyloidosis and lymphoma [32]. The biopsy will help determine the later [17]. Response to glucocorticoids will help controlling the disease in most of patients. However, LIP may worsen the honeycombing, cyst formations, and fibrotic changes all of which may carry a worse prognosis. Other immune suppressor therapies are of benefit, like cyclophosphamide, chlorambucil, azathioprine. Rituximab is a promising therapy [32].

12.2.3.4 Organizing Pneumonia (OP)

OP is characterized by the histopathologic hallmark of intraluminal inflammatory debris of fibroblastic and myofibroblast infiltrates and decomposing cells in the alveolar ducts and contiguous airspaces with inflammatory extension to the surrounding alveoli [30]. This is a rare entity reported in SS, but more frequently present in rheumatoid arthritis. In addition to dyspnea, patients may endure fever and malaise. The HRCT scan will reveal peripheral patchy areas of parenchymal consolidation, with subpleural or peribronchovascular distribution with air bronchograms, centrilobular nodules, and variable associated ground-glass opacities [30, 32] (Fig. 12.7). The frequency ranges between 3.9 and 11% [40, 50–53]. The main known drug therapy is glucocorticoids [40]. Other immune suppressors have been used also, including azathioprine, cyclosporine, infliximab, rituximab [66], and even tocilizumab [67].

12.2.3.5 Pulmonary Lymphoma

Sjogren's syndrome is the most susceptible CTD to develop lymphoma (Fig. 12.8). The lymphoproliferative predisposition seems to be selective for SS as compared with other CTDs or vasculi-

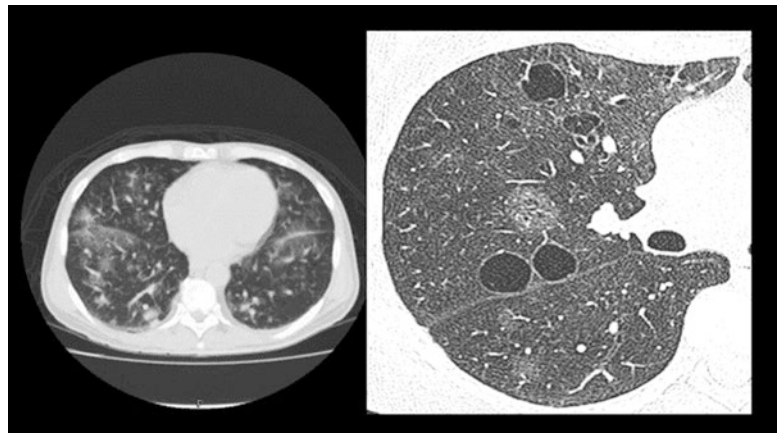

Fig. 12.6 Lymphoid Interstitial Pneumonia **Left: HRCT demonstrates patchy ground-glass opacity and a few scattered, subcentimeter, solid nodules Right: HRCT demonstrates several thin-walled cysts of varying size and scattered ground-glass opacities**

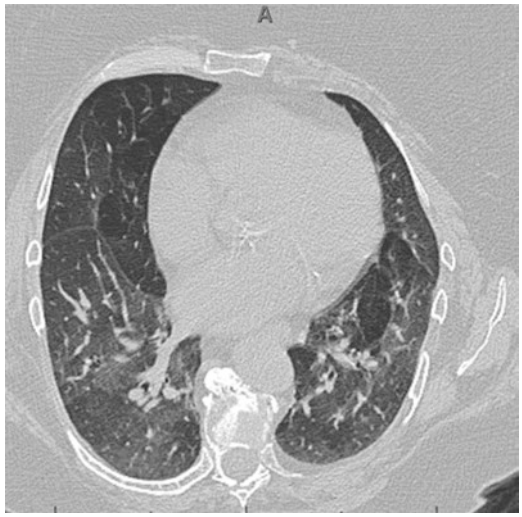

Fig. 12.7 Bronchiolitis obliterans—HRCT imaging shows mosaic attenuation with areas of increased attenuation and area of low attenuation which indicates air trapping

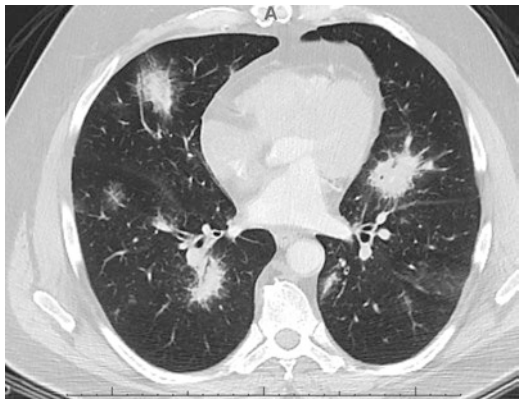

Fig. 12.8 Pulmonary lymphoma—HRCT demonstrate pulmonary lymphoma peribronchovascular nodules and masses in relation to airways. In addition, there are air bronchograms within some masses

tis. Subsequently, primary Sjogren's syndrome has a 10–44-fold greater risk for lymphoma as compared with healthy controls, and even higher than in systemic lupus erythematosus (sevenfold) and rheumatoid arthritis (fourfold) [68]. In a large cohort, men had a higher (20-fold) risk as women (10-fold) did [69]. Most of the lymphoma types are of B-cell lineage, more often associated with MALT (Mucosal-Associated Lymphoid Tissue), but also with other types of B-cell lymphomas. In the proposed lymphomagenesis, the continuous inflammation of MALT stimulation, B-cells in the exocrine glands (mainly parotids) may predispose activation of pro-oncogenic genes by the autoimmune process. This environment may promote the abnormal B-cell survival, with subsequent clonal transformation [70, 71]. Uncovered initially in 1973, MALT changes in bronchi were described as peribronchiolar lymphoid aggregates similar to Peyer's patches [72]. They are labeled as pseudolymphomas, which is a benign lesion of mature polyclonal lymphocytes and plasma cells [17]. MALT lymphomas originate from the marginal zone B-cells that surround the mantle zone and germinal centers, and the precise nomenclature is as a non-Hodgkin's lymphoma, arising from the extra-nodal marginal zone B-cell lymphoma (MZL). Usually they are low-grade, but occasionally will transform into a high-grade type. Other types of lymphoma in SS are the diffuse large B-cell lymphomas and behave much more aggressively. Clinical predictors for lymphoma are parotid gland swelling, palpable purpura, lymphadenopathy, splenomegaly, higher ESSDAI-score (European League Against Rheumatism, Disease Activity Index), peripheral neuropathy, skin ulcers, younger onset of SS, disease duration, and Raynaud's phenomenon. Laboratory features indicative of lymphoma are the low C4 and/or C3, cryoglobulins, low CD4, leukopenia, rheumatoid factor, monoclonal gammopathy, especially IgM kappa, anti-SSA/Ro, anti-SSB/La, anti-centromere antibodies, anemia, and high Beta-2 microglobulin [69, 73, 74]. Predictors for pulmonary lymphoma are not well defined, as it affects 1% of all the lung cancers, being lymphoma one of the fewest cancer types. In SS, the frequency is of 1–2% [75]. Few patients may have representative pulmonary symptoms with dry cough and slowly progressive dyspnea, otherwise, in most cases the disease runs unnoticed [76]. Even so, a small fraction may have B-symptoms [77]. In a series of 13 patients, seven (54%) had a diagnosis of SS, with female predominance as seen in other series [77–79]. The laboratories showed positive

ANA, anti-SSA/Ro antibodies, rheumatoid Factor, high ESR and lymphopenia, but no cryoglobulinemia and only a minority exhibited monoclonal gammopathy [77]. The HRCT scan findings are more informative. They may reveal airway involvement with bronchial wall thickening and bronchiectasis present bilaterally and preferably in lower lobes. The lung parenchyma surrounding the abnormal airways may associate with confluent alveolar opacifications or ground-glass changes. Multiple nodular densities are described, pseudocavitation, peripheral, wedge-shape infiltrates, cystic structures, and mediastinal lymphadenopathy [77]. Remarkably, most of the lymphomas show lymphoepithelial lesions of the bronchial and bronchiolar epithelium, and positive CD20 cells, with abnormal Kappa/Lambda ratio and clonality [77]. The average 5-year survival rate varies and fluctuates between 65% and 90% [32].

12.2.3.6 Pulmonary Amyloidosis

Amyloidosis associated with SS is uncommon and occurs primarily in the skin, lungs, tongue, lacrimal glands, and mammary glands [80–82] and is more often in women [42, 82, 83]. The combination of amyloidosis and SS suggests a reflection of the polymorphic spectrum of lymphoproliferative diseases related to SS. As described, it is often of Amyloidosis Light chain (AL) type, whereas Amyloidoidosis related to serum Amyloid protein (AA) was uncommon [80]. Pulmonary amyloidosis in association with Sjogren's syndrome is rare [84]. Pulmonary amyloidosis may cause dyspnea, cough, pleuritic chest pain, wheezing, post-obstructive pneumonia, and hemoptysis [80, 81]. Tracheobronchial amyloidosis may be a source of bleeding and pseudo-asthma, and endoscopic interventions with/without laser therapy are options to treat the obstruction and control the bleeding source [81, 85]. Nodularity is the most frequent presentation [82, 83], and among the several presentations (generalized, diffuse, pleural and localized, the later is the most frequent presentation in SS [30]. The biopsy is crucial to differentiate it from lymphoma, as it may associate with LIP and with cystic lesions. Other underlying findings in lungs may associate with amyloidosis, as is NSIP [86]. On HRCT, nodules with and without calcification are the most common findings. Local therapy palliates the symptoms and no clear systemic therapy is ideal, but glucocorticoids are of great help [32, 86] (Fig. 12.9).

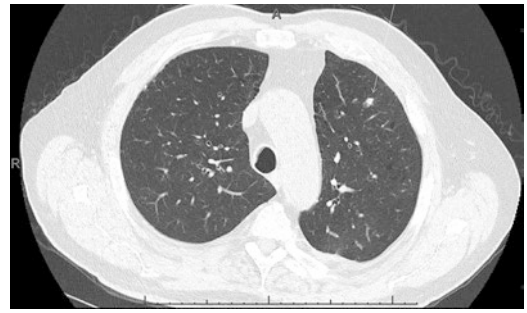

Fig. 12.9 Pulmonary Amyloid—HRCT shows a discrete nodule with paraseptal emphysema. Other levels demonstrate nodules

12.2.4 Pulmonary Hypertension

Pulmonary hypertension in Sjogren's syndrome is infrequent [30], but the true prevalence remains unclear. The difficulties to obtain this information rely on the disease's nature, as it may present with subtle clinical features, and often the serology is negative. In addition, in several cohorts, Pulmonary Arterial Hypertension (PAH) often precedes SS, and may remain as idiopathic, while SS runs undetected. In a review of patients with PAH and pSS, 12 (41.4%) out of 29 patients presented initially with PAH [87]. Female predominance and at ages between 30 and 40 are almost all the cases reported [41]. Several cohorts, mostly from Asian countries, suggested that the prevalence of PAH is higher in SS and systemic lupus erythematosus (SLE) than other CTDs, including systemic sclerosis [88]. Consistent with these findings, in a report of PAH of CTDs, out of 129 consecutive adult Chinese patients with PAH, 49% had SLE or pSS. In contrast, patients with systemic sclerosis were only 6% [89]. Moreover, when the systematic review in

patients with PAH considered as idiopathic was applied, some of them had pSS. In a prospective study of 40 patients without obvious etiology of PAH, five were diagnosed with pSS during their course of the disease [90], emphasizing a repeated evaluation for CTDs in follow up assessments. The systematic approach comprehends a compelling clinical review, laboratory and ancillary tests. In the directed interview, exploring sicca symptoms and performing the in-office Schirmer's test and saliva collection are of great help. Further information relies on the key findings of pSS associated with PAH: chest pain, Raynaud's phenomenon, cutaneous vasculitis and ILD with associated dyspnea, hepatic injury, and pericardial effusion [87, 91]. Serology is crucial to suggest if pSS or if any other CTD may be present. The most frequently reported positive tests were ANA, rheumatoid factor [87], anti-SSB/La [92], anti-SSA/Ro, and anti-RNP antibodies [17, 93]. The newly available ELISA-kit tests to detect ANA are more sensitive as they have the anti-SSA/Ro as antigen [94]. Hypergammaglobulinemia is present in most of patients representing active disease (ILD) [95], despite that the ESSDAI is low in most of patients with PAH in other cohorts [52, 90]. Other clinical features will help identifying a patient with pSS if serology is negative, like an exhaustive nailfold capillaroscopic exam with features suggestive of pSS [96], and the lip biopsy of minor salivary glands [97]. In the assessment to evaluate pSS, the utility of ultrasound of major salivary glands has become a firm tool, supporting the notion that the images of glands in patients with SS have unique features [98, 99]. The only inconvenience is that this later procedure is operator-dependent and not all institutions may offer this resourceful technique. Prognosis of PAH-associated pSS will depend on the delay of PAH onset and the SS diagnosis, a lower cardiac index [93]. The overall 1-, 3-, and 5-year survival rates were 80.2%, 74.8% and 67.4%, respectively [93]. In this same study, intensive immune suppressant therapy was predictive of better survival. In a cohort from Chinese patients with SS, the predictors of worse hemodynamic measurers were those with positive anti-SSB/La antibodies [92]. Recent studies have shown promising outcomes in selected patients with PAH and SS who underwent aggressive immune suppressive therapy [90], in addition to the conventional therapy for PAH. However, this notion needs further prospective larger studies.

12.2.5 Thromboembolic Disease

A subgroup of patients with pSS may have class 4 PAH, when pulmonary artery obstructions are identified and mostly in concurrence of antiphospholipid antibodies. As in many CTDs, antiphospholipid antibody syndrome (APS) may also be part of the spectrum of the autoimmune process. The prevalence of pSS and anti-phospholipid antibodies have been found in 14–16% [100, 101], with a low number of APS (3–5%). From another perspective, PAH is most frequently associated with lupus, and primary APS may present in 1.8–3.5% depending on the series [102]. We do not know the exact prevalence of PAH-associated pSS to be linked with APS, but case reports revealed this link [103]. Anticoagulation in this setting is recommended.

12.3 Conclusions

Respiratory manifestations in SS associate frequently as part of the SS extra-glandular features. It is important to highlight the alertness of presence of respiratory symptoms as they are many. The more frequent symptoms are those affecting the small airways disease, but also any parenchymal findings are common. Given the higher risk to develop malignancies in SS overall as compared with other CTDs, it is important to identify any possible features suggestive for their presence. Predictors for lymphoma related with lung malignancy are lymphadenopathy, exocrine gland enlargement or cryoglobulins. On the CT images, cystic lesions, nodules, organizing pneumonia, lymphocytic interstitial pneumonia may be part of this differential.

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13 Redox Regulation, Oxidative Stress, and Inflammation in Group 3 Pulmonary Hypertension

Olena Rudyk and Philip I Aaronson

Abstract

Group 3 pulmonary hypertension (PH), which occurs secondary to hypoxia lung diseases, is one of the most common causes of PH worldwide and has a high unmet clinical need. A deeper understanding of the integrative pathological and adaptive molecular mechanisms within this group is required to inform the development of novel drug targets and effective treatments. The production of oxidants is increased in PH Group 3, and their pleiotropic roles include contributing to disease progression by promoting prolonged hypoxic pulmonary vasoconstriction and pathological pulmonary vascular remodeling, but also stimulating adaptation to pathological stress that limits the severity of this disease. Inflammation, which is increasingly being viewed as a key pathological feature of Group 3 PH, is subject to complex regulation by redox mechanisms and is exacerbated by, but also augments oxidative stress. In this review, we investigate aspects of this complex cross-talk between inflammation and oxidative stress in Group 3 PH, focusing on the redox-regulated transcription factor NF-κB and its upstream regulators toll-like receptor 4 and high mobility group box protein 1. Ultimately, we propose that the development of specific therapeutic interventions targeting redox-regulated signaling pathways related to inflammation could be explored as novel treatments for Group 3 PH.

Keywords

Group 3 Pulmonary Hypertension · Hypoxia · Inflammation · Redox signaling · Oxidative stress · NF-κB

Abbreviations

(GSH/GSSG)	Glutathione
AECOPD	Acute exacerbation of COPD
Akt	Protein kinase B
ALK1	Activin receptor-like kinase 1
BMP9	Bone morphogenic protein 9
BMPR2	Bone morphogenic protein receptor II
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_13

CSE	Cystathionine gamma lyase
CXC4	C-X-C chemokine receptor type 4
CXCL12/SDF-1	C-X-C motif chemokine 12/ stromal cell-derived factor 1
DAMP	Damage-associated molecular pattern
EC	Endothelial cell(s)
EGFR	Epidermal growth factor receptor
EndoMT	Endothelial-to-mesenchymal transdifferentiation
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extra-cellular signal-regulated kinases 1 and 2
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
H_2S_n	Hydrogen polysulfides
HAPE	High-altitude pulmonary oedema
HIF-1	Hypoxia-inducible factor – 1
HIMF	Hypoxia-induced mitogenic factor
HMGB1	High mobility group protein box 1
HOCl	Hypochlorous acid
HPV	Hypoxic pulmonary vasoconstriction
ICAM-1	Inter-cellular adhesion molecule *1*
IKK	I-κB kinase or trimeric nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IPF	Idiopathic pulmonary fibrosis
IRAK	IL-1 receptor-associated kinase
MCP-1	Monocyte chemoattractant protein
MCT	Monocrotaline
MCTP	Monocrotaline pyrrole
MD2	Myeloid differentiation factor-2
MEK1/2	Mitogen-activated protein kinase kinases 1 and 2
MEKK1	Mitogen-activated protein kinase kinase 1
MMP-9	Matrix metalloproteinase
MPO	Myeloperoxidase
MYD88	Myeloid differentiation primary response 88
Ndufs2	NADH Dehydrogenase [ubiquinone] iron-sulfur protein 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
Nox	NADPH (nicotinamide adenine dinucleotide phosphate) oxidase
PA EC	Pulmonary artery endothelial cell (s)
PA	Pulmonary artery or pulmonary arterial
PAH	Pulmonary arterial hypertension
PAMP	Pathogen-associated molecular pattern
PH	Pulmonary hypertension
PKA RIα	Type I regulatory-RIα subunit of protein kinase A
PKG	Cyclic guanosine monophosphate (cGMP)-dependent protein kinase G
PPHN	Persistent pulmonary hypertension of the new-born
P-SNO	Nitrozated protein
$P\text{-}SO_2H$	Protein sulfinic acid
$P\text{-}SO_3H$	Protein sulfonic acid
P-SOH	Protein sulfenic acid
P-SSG	Glutathionylated protein
P-SSH	Persulfidated protein
Pyk2	Proline-rich tyrosine kinase 2
RAGE	Receptor for advanced glycation end products
RANTES	Regulated upon activation normal T cell expressed and presumably secreted
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RSS	Reactive sulfur species
SMC	Smooth muscle cell(s)
SOD	Superoxide dismutase(s)
TAK1	TGFβ-activated kinase 1
TGF-β	Transforming growth factor-β
TIRAP	Toll/interleukin-1 receptor domain-containing adapter protein
TLR	Toll like receptor
TNF-1α	Tumor necrosis factor 1 alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
Trx/TrxR	Thioredoxin/thioredoxin reductase
VEGF	Vascular endothelial growth factor
XDH	Xanthine dehydrogenase
XO	Xanthine oxidoreductase
α-SMA	Alpha-smooth muscle actin

13.1 Introduction

Pulmonary hypertension (PH) is the common name for a pathological condition in which the mean pulmonary arterial (PA) pressure is greater than 25 mmHg at rest [1, 2]. Patients with PH develop shortness of breath and a reduced ability to exercise, and often lead a sedentary lifestyle [3]. If left untreated, PH leads to a progressive decline in cardiac output and right-sided heart failure, with a mean survival time of only a few years after diagnosis [1]. The prevalence of PH is estimated at ~1–2% of the global population, which sometimes promotes the misleading assumption that PH is a rare condition. However, this increases up to 10% in patients ≥65 years old [2]. Although PH often occurs secondary to other disorders such as left heart disease (Group 2) or hypoxic lung disease (Group 3), historically the majority of fundamental, preclinical and clinical studies have focused on Group 1 PAH [2, 4]. Ultimately, current therapeutic approaches help to slow the progression of the disease but do not offer a cure [4].

PH is classified into five groups, based on their pathological and clinical features. Common pathophysiological features in all PH groups involve pulmonary vasoconstriction, remodeling, thrombosis, and inflammation [5]. Reactive oxygen species (ROS) production is yet another integral part of the PH pathology, particularly for Group 3 [6]. Elevated ROS may contribute to disease progression; at the same time, they can act as intra-cellular mediators of redox signaling in health, and promote adaptation to pathological stress that limits disease [7–9]. Antioxidant supplements have been commended as a panacea for many disorders, PH included [10, 11]; however, in general, these have failed to improve health in clinical trials and even have proven harmful when administered to humans with diseases [12–14]. One reason could be that antioxidants may prevent intrinsic protective cellular responses, for example, by neutralizing ROS that may initiate adaptive signaling. Therefore, it is important to consider the possible cross-talk between hypoxia, oxidative stress, and redox signaling for a better understanding of Group 3 PH, and future development of novel treatment options.

The subject of this chapter will be Group 3 PH, with a particular focus on redox-signaling pathways and mechanisms related to inflammation. The current clinical picture and PA pathological changes observed in hypoxic PH will be briefly overviewed. We will then describe the production of oxidants, their deleterious and protective roles in hypoxic PH, redox signaling and inflammation, starting with observations made in commonly used animal models and human PH Group 3. We will go on to describe transcription factor signaling pathways that are involved in the initiation and propagation of pulmonary vascular inflammation proceeding remodeling processes. In particular, we will focus on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a major "rapid-acting" regulator of inflammation which is known to be activated by cytokines, oxidants, and extra-cellular factors, in turn causing the upregulation of the target genes, that is,

pro-inflammatory cytokines, chemokines, and adhesion molecules. We will also highlight the role of toll-like receptor 4 (TLR4) and high-mobility group box protein 1 (HMGB1), as selective mediators of NF-κB signaling. We will conclude by providing specific evidence to illustrate a role for oxidants and oxidative post-translational modifications of NF-κB, TLR4, and HMGB1, and their cellular effects that may be involved in inflammation in Group 3 PH.

13.2 Clinical Picture for Group 3 PH

PH secondary to left heart failure (Group 2) and lung diseases (Group 3) are the most common causes of PH worldwide [2, 15]. For example, Group 3 alone or in combination with other comorbidities accounts for about half of all PH cases in Canada [16]. These data suggest that Group 3 may occur more often within the population than previously assumed. The World Health Organization defines Group 3 as PH caused by lung diseases and/or hypoxia, which encompass chronic obstructive pulmonary disease (COPD), interstitial lung disease (e.g., idiopathic pulmonary fibrosis, IPF), sleep apnea, and high-altitude exposure [17, 18]. Of note, hypoxia is also thought to be present in Group 1 PH (termed pulmonary arterial hypertension, or PAH), as a secondary event to falling cardiac output [19], such that hypoxia-induced gene expression is apparent in the remodeled PAs of Group 1 PAH patients [20]. With regard to pharmacological interventions, Group 1 PAH current treatment options provide only temporary, symptomatic relief in Group 3 PH, rather than long-term benefit, and disappointingly do not reverse or ameliorate remodeling processes [4]. Since most therapeutics cause acute vasodilation, they may even worsen the disease scenario in hypoxia-induced PH by affecting the ventilation–perfusion ratio [15, 18]. It is therefore not surprising that these limited treatment options have not shown benefit in controlled trials for Group 3 PH [4, 15]. As such, Group 3 PH remains a lethal condition with an unmet clinical need and a significant collective impact on global population health and healthcare. Hence, a deeper understanding of the pathological and adaptive molecular mechanisms within this Group is required to develop effective treatments.

13.3 Cellular Pathological Changes in PH Due to Hypoxia

Brief episodes of alveolar hypoxia, ranging from seconds to minutes, initiate acute hypoxic pulmonary vasoconstriction (HPV), a local adaptive, rapid, and reversible physiological response in affected lung segments that aids blood oxygenation by maintaining gas exchange [20–23]. This response is specific to the pulmonary circulation and is distinguished from the hypoxic vasodilatory response observed in systemic circulation [24]. HPV in humans is characterized by at least two constrictor phases: an initial response that reaches a plateau within minutes (although in animals the first phase is often transient), and a second, slower increase in tone which develops after 0.5–2 h [23]. The pivotal mechanism of the rapid HPV phase is elicited by hypoxia-induced reduction of potassium channel activity, membrane depolarization, and cytosolic calcium elevation in smooth muscle cells (SMCs) [25–28]. Endothelin-1 released by endothelium has been shown to sensitize SMCs to a hypoxia-induced cytosolic calcium increase, and contribute to the second, prolonged response to acute hypoxia [29, 30]. Upon long-term hypoxia, that is, ranging from hours to days, persistent HPV associates with sustained constriction of the entire pulmonary circulation and a pathological increase in PA pressure and pulmonary vascular resistance [31]. In addition, prolonged hypoxic exposure prompts phenotypical, biochemical, and functional changes in each of the PA cell types, leading to what is commonly known as PA remodeling [24, 31–33].

Hypoxia-induced structural PA changes first appear as muscularization of previously nonmuscularized distal arteries, with a further increase in muscularization of more proximal partially or

fully muscularized ones [32, 34]. Notably, hypoxia-induced hypertrophy, hyperproliferation, and migration of SMCs in the PA medial layer account for PA remodeling [32, 34, 35], while increased deposition of extra-cellular matrix proteins by SMCs, recruitment of nonresident smooth muscle-like cells [32], and endothelial-to-mesenchymal transdifferentiation (EndoMT) [36] can also contribute. In animal models, PA muscularization initiates at around 2–4 days of hypoxic exposure [37, 38], and usually reaches its plateau after 3 weeks [34, 39]. The magnitude of muscularization depends on the species, gender, and the developmental stage of the animal, which is used in chronic hypoxia model [32, 35].

Several rodent models have been employed to aid the mechanistic understanding of Group 3 PH. These include exposure to chronic hypoxia (either as a single physical stimulus or in combination with chemical stimuli) and models of interstitial lung disease (e.g., pulmonary fibrosis induced by bleomycin). While there is no perfect animal model that fully recapitulates human PH, these approaches have been valuable for mechanistic studies into signaling pathways, including inflammatory component, underlying Group 3 PH. The widely used chronic hypoxia model requires exposing animals to low oxygen air (usually 10% inspired O_2) at normal pressure or normoxic air at hypobaric pressure [5]. This produces a strong and pronounced HPV which is sustained as long as the hypoxic stimulus is present, and with time leads to medial vascular hypertrophy which to some extent mimics that observed in human Group 3 PH [34].

At least in animal models, chronic hypoxia alone over several weeks does not cause RV failure. For this reason, mixed models emerged, that are still related to Group 3 PH, but combine chronic hypoxia stimulus with other insults, and typically result in a more severe PH phenotype, heart failure and often death within weeks. The most widely used is a model that combines chronic hypoxia with vascular endothelial growth factor (VEGF) pathway inhibition, and was established in 2001 in rats [39] and a decade later in mice [40]. Rodents treated with chronic hypoxia and SU-5416, a selective pharmacological inhibitor of the VEGF receptor 2 tyrosine kinase activity, develop severe PH and pulmonary vascular remodeling characterized by SMC and precapillary arterial endothelial cell (EC) proliferation, leading to the development of heart failure [40, 41].

Other components of the PA vascular wall, including resident fibroblasts and ECs, are also involved in hypoxic PA remodeling [31]. The former contribute to adventitial thickening by proliferating, migrating, and secreting extra-cellular matrix proteins (e.g., collagens, elastin, fibronectin)—all of which have been reported in chronic hypoxia [42]. Hypertrophy and hyperplasia of the latter contribute to intimal thickening in human Group 3 PH [32]. ECs may also account for PA differential responses to chronic hypoxia between various strains and species [32]. In vitro, ECs exposed to 1% O_2 proliferate faster than normoxic controls [43, 44]; however, while in vivo intimal thickening has been observed in hypoxic animals, it is (with the exception of cows [20, 35]) usually minimal.

ECs undergo structural and phenotypic changes due to hypoxia that unequivocally promote functional changes and interactions with SMCs and pro-inflammatory circulating cells which collectively participate in the PH response. The nitric oxide (NO) level is generally elevated in hypoxia [45], and eNOS knock-out mice develop less PA wall thickening in response to hypoxia than do wild-type controls [38], indicating that NO produced by endothelium contributes to muscularization of small PAs. Hypoxia also causes changes in the membrane properties of ECs. Pulmonary ECs isolated from rabbits, exposed to hypoxia for 3–5 h, are characterized by decreased membrane fluidity due to changes in fatty acid composition, higher cell surface to volume ratio, and potentiated caveolar density, all of which compromise endothelial barrier function [46].

EndoMT is a phenotypical shift of ECs toward a mesenchymal-like cellular state [47], which typically occurs during embryonic development, wound healing, or inflammation [36, 47], and is thought to play a crucial role in the progression

of Group 1 PAH [36, 48, 49]. In addition, EndoMT has been reported in PAs of rats subjected to 4 weeks of hypoxia [50] and mice after 3 weeks of SU-5416/chronic hypoxia exposure [51]. Induction of EndoMT involves activation of transcription factors Twist, Snail, and Slug which work together to repress or activate various endothelial and mesenchymal genes. As a result, there is a visible loss of EC marker protein expression, including Von Willebrand Factor, cluster of differentiation 31, and a gain of expression of mesenchymal proteins such as α-smooth muscle actin (α-SMA), transgelin, fibronectin, and vimentin, shifting the EC shape from cobblestone to spindle-like and prompting their migratory and hyper-proliferative characteristics [36, 48, 49].

All in all, hypoxia-induced PA contraction and remodeling, together with increased blood viscosity [31], contribute to decreased PA lumen diameter and elevation of pulmonary vascular pressure and resistance, resulting in RV hypertrophy and chronic PH, as observed in animal models [5, 31, 34]. In high-altitude dwelling individuals [52], or those with pulmonary hypoxemia due to chronic lung disease (i.e., COPD or IPF) [53], a progressively increased RV afterload and hypertrophy that cannot be tolerated eventually lead to heart failure, a primary cause of death in patients in this group. Inflammation often occurs during systemic responses to hypoxia, subsequently affecting all vascular cell types involved in PA remodeling [54]. In addition, inflammation has been linked to production of oxidants [55], which can mediate the adaptive processes as well as further exacerbate PA remodeling.

13.4 Oxidant Production and Redox Signaling in PH

ROS, reactive nitrogen species (RNS), and reactive sulfur species (RSS) comprise a broad range of molecular oxidants that can oxidize biological molecules, although the reactivity varies between different species [56–59]. Oxidants can be produced constitutively, or in response to stimuli (e.g., exposure to chemicals, pollutants, and radiation), by chemical reactions (e.g., the Fenton reaction), as by-products of other reactions (e.g., electron leakage from electron transport chains, endothelial nitric oxide synthase (eNOS) uncoupling, drug metabolism), by a single enzyme, or by a clustered group of enzymes [8, 57]. One example of stimuli-induced oxidant production is by macrophages that utilize it in host defense during inflammation [55]. The balance between oxidant generation and utilization (the reduction-oxidation or redox state) may differ within and between intra- and extra-cellular compartments under a specific physiological or pathophysiological scenario [56].

Oxidants may react with deprotonated protein cysteine residues (or thiols). This results in a range of reversible or irreversible oxidative post-translational modifications [60] which can change the three-dimensional structure, localization or, sometimes, the function of a target protein or regulate its trafficking [7, 8]. Figure 13.1 shows a schematic of various oxidative post-translational modifications as a result of reaction of ROS, RNS, or RSS with the thiols (Fig. 13.1). Protein disulfide bonds can be formed between vicinal cysteine residues (i.e., intra-protein disulfides) or between cysteine thiols on two protein subunits, resulting in either homo- or hetero-dimers (inter-protein disulfides) [60]. Protein S-nitrozation (also known as S-nitrozylation), or the formation of a nitroso protein thiol (P-SNO), is the oxidative post-translational modification that occurs as a result of the reaction between the thiol and NO, or nitrozating variants of NO [60, 61]. Although S-nitrozation is thought to be a widespread stable oxidative post-translational modification that provides regulation of protein functions, this idea has been recently challenged by Wolthuter et al. who provided evidence that S-nitrozated proteins react rapidly with abundant thiols to form protein disulfides, thus implying that S-nitrozation is an intermediate redox state [62]. RSS, including hydrogen sulfide (H_2S) and hydrogen polysulfides (H_2S_n), have been recently proposed to mediate protein persulfidation (also sometimes described as S-sulfuration or S-sulfhydration), a modification where H_2S or H_2S_n reacts with the thiol to form persulfides (i.e., P-SSH or P-S_n-SH) to convey signaling by these

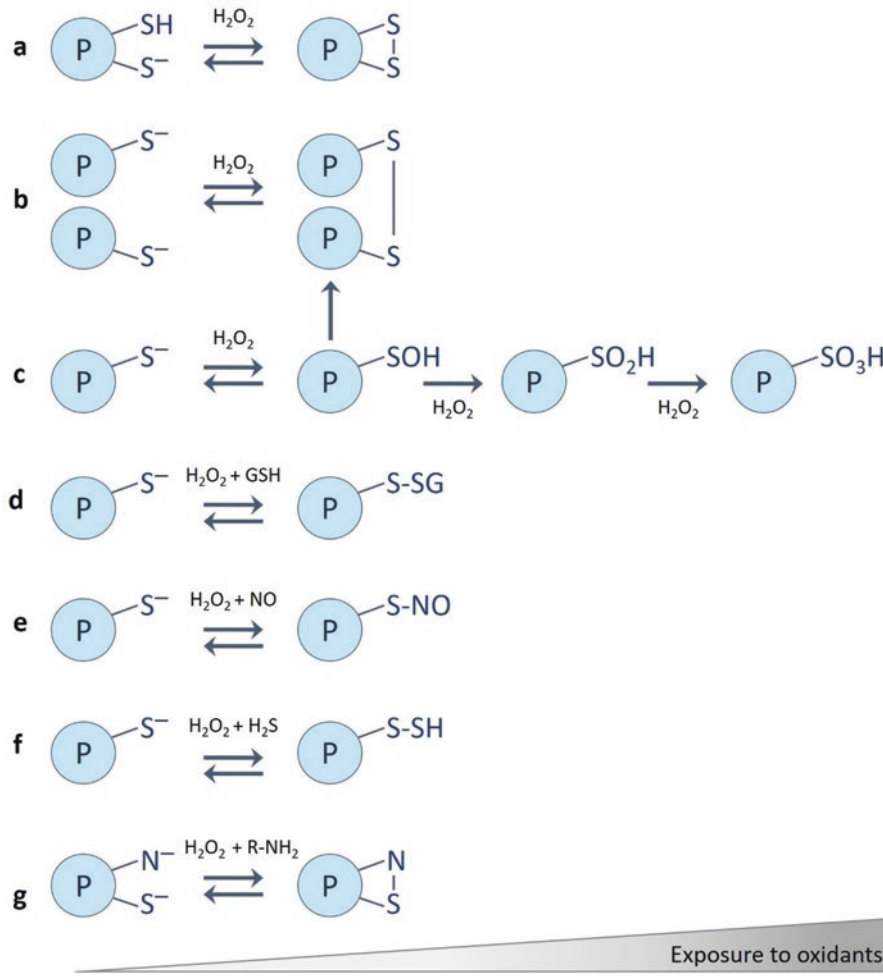

Fig. 13.1 Schematic of various oxidative post-translational modifications formed in protein thiols as a result of reaction with oxidants. Deprotonated protein thiols can react with reactive oxygen species (i.e., H_2O_2) to allow formation of reversible intra-molecular disulfide bond (**a**), inter-molecular disulfide bond (**b**), or sulfenic acid (P-SOH) (**c**), which under prolonged exposure to oxidants can further (mostly irreversibly) oxidize to sulfinic acid (P-SO_2H) and then sulfonic acid (P-SO_3H). Protein thiols can react with glutathione to yield S- glutathionylated protein (P-SSG) (**d**). Some forms of reactive nitrogen species can induce a reversible modification termed protein nitrozation (P-SNO) (**e**), while some reactive sulfur species or sulfenamide can cause protein persulfidation (P-SSH) (**f**) or sulfenamidation (**g**) respectively. Of note, some protein oxidative post-translational modifications, that is, nitrozation, persulfidation, sulfenic acid, or sulfenamide formation can be intermediate transition states to disulfide bonds [7, 60, 61, 63]. Abbreviations: *H_2O_2* hydrogen peroxide, GSH glutathione, *NO* nitric oxide, *H_2S* hydrogen sulfide

RSS [60, 63]. Since H_2S is unlikely to directly react with cysteine thiols, due to its low oxidation state, H_2S_n that may form as a result of H_2S oxidation are more likely to mediate the effects of RSS signaling [64].

The reactivity of an individual protein thiol toward oxidation is determined by the negative log of its acid dissociation constant, or p*K*a, which is influenced by proximity to proton accepting amino acids (histidine, lysine, arginine), a higher pH, the proximity of two thiols (in case of a disulfide bond formation), and its propinquity to oxidase enzymes [60]. The formation of oxidants, and therefore the accumulation of

various oxidative post-translational modifications, is tightly regulated by cell antioxidant systems. These include: (i) oxidant scavengers (e.g., glutathione, vitamins C and E, urate, polyphenols); (ii) enzymes that eliminate oxidants or their precursors (e.g., superoxide dismutases (SOD), catalases, glutathione peroxidase, peroxiredoxins, thioredoxin/thioredoxin reductase (Trx/TrxR)); (iii) damage repair systems (e.g., disulfide isomerases, glutaredoxins, sulfiredoxins, proteasomes, phospholipases, DNA repair enzymes) [57].

Growing evidence demonstrates that protein cysteine modifications play an important role in cell function, analogous to that of the phosphorylation of serine, threonine, or tyrosine [7]. RNS can also nitrate serine, threonine, and tyrosine residues in a reversible or irreversible manner [65]. 3-nitrotyrosine formation, as a result of the nonenzymatic nitration of tyrosine by peroxynitrite (a product of the reaction of superoxide with NO), is one example [19]. It is thought that tyrosine nitration is likely to cause modification of specific proteins rather than widespread, nonspecific protein dysfunction, as will be mentioned below. Overall, oxidants can serve intra-cellular messengers that mediate redox signaling (also termed oxidative "eustress" [56]), and so they are essential for healthy tissue functions at rest or during exercise, and in adaptation to pathological stress that limits disease progression [7, 8]. However, elevated levels of oxidants may override cell antioxidants' regulatory mechanisms and cause oxidative stress, thereby disrupting physiological or adaptive (protective) redox signaling by irreversible protein oxidation and damage to DNA and lipids. Indeed, imbalance between oxidants production and their utilization have been reported for various cardiovascular diseases [66], including hypoxia-induced PH [6, 67–71].

Patients with Group 1 PAH and Group 4 PH have increased levels of markers of oxidative stress [72], which supposedly play a role in pathogenesis of this disease both in patients and in experimental models [19, 73]. Generally speaking, it is assumed that elevated ROS and RNS levels are causatively involved in potentiated responses to vasoconstrictor and proproliferative stimuli; however, their role is likely to be specie-defined and therefore more complex [19, 74]. Oxidant production in hypoxia-induced PH involves the mitochondrial electron transport chain and the membrane NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Nox) family (comprising Nox 1–5 and Duox 1 and 2) [9, 65, 69, 75]. Nox is a cytoplasmic oxidoreductase enzyme which transfers electrons from cytosolic NADPH to molecular oxygen, generating superoxide. The latter is highly reactive but unstable, and so is rapidly transmuted to the more stable hydrogen peroxide (H_2O_2) by SOD [75]. Superoxide can also react with NO producing the RNS peroxynitrite; while H_2O_2 can be reduced to water by catalase or converted to hydroxyl radical via the Fenton reaction. Below we will describe in more detail how oxidants are involved in redox regulation of pulmonary vasotone during acute hypoxia, and both deleterious and adaptive PA responses to chronic hypoxia.

13.4.1 Regulation of Pulmonary Vasotone by Oxidants during Acute Hypoxia

Oxidants regulate pulmonary vascular tone in response to physiological agonists and cellular stressors, including acute hypoxia. For example, the initial rapid phase of acute HPV is mediated by immediate changes in oxidant production in SMCs, which in turn causes intra-cellular calcium elevation [25, 26]. There is an ongoing debate regarding the direction of this change, and the nature of oxygen sensor and effector [23, 30, 76]. One source of oxidants in pulmonary vasculature is mitochondria, which, as first proposed by the 'redox mode', also serves as an oxygen sensor [29]. Recently, the chemical reduction of cysteines in mitochondrial subunit NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (Ndufs2) has been suggested as a requirement for oxygen-sensing and HPV [77]. The 'redox model' of HPV is based on the concept of normoxic vasodilation of PAs, which reside in a relatively high oxygen tension milieu compared to

systemic blood vessels [78]. During acute hypoxia, the redox state of pulmonary SMCs becomes pro-reducing due to decreased availability of molecular oxygen in the electron transport chain of mitochondria and a rapid accumulation of reducing equivalents [79]. This is indicated by studies monitoring NADH/NAD, NADPH/NADP, and glutathione (GSH/GSSG) redox couples [79–82], and direct measurements of reduced H_2O_2 levels in acute hypoxia using compartment-specific redox probes [77]. When pulmonary vessels become hypoxic, a marked change in cellular redox state is predicted to affect effector protein oxidative post-translational modifications that have occurred basally, that is, during normoxia. Indeed, cysteine-rich voltage-gated potassium channels Kv1.5 close when reduced by hypoxia. In turn, this leads to membrane depolarization, opening of voltage-dependent L-type calcium channels and calcium influx, followed by activation of myosin light chain kinase and contraction of SMCs which initiates HPV [30].

The reduced state in acute hypoxia can affect the disulfide level of cyclic guanosine monophosphate (cGMP)-dependent protein kinase G (PKG). In its 'classical' activation mode, cGMP released due to activation of NO-soluble guanylate cyclase pathway, binds and activates PKG to induce phosphorylation-dependent signaling. The α isoform of PKGI (PKGIα) is also susceptible to oxidation by forming an inter-protein disulfide homo-dimer at Cys42 which is linked to kinase targeting and activation [7]. The serine/threonine kinase PKGIα plays an essential role in vasodilation and SMC proliferation, and its post-translational regulation has recently been a topic of interest in systemic blood pressure as well as PH [9, 83]. In normoxia, PKGIα oxidation by disulfide dimerization mediates vascular relaxation and contributes to normal pulmonary pressure. This is illustrated by the observation that PAs from 'redox-null' Cys42Ser PKGIα knock-in mice, which cannot form PKGIα disulfide, demonstrate deficient H_2O_2-induced relaxation [9]. Unsurprisingly, during acute pulmonary hypoxia, lower levels of disulfide-PKGIα and the anticipated loss of kinase activity have been reported [84]. However, a direct comparison of PA responses to acute hypoxia in wild-type and 'redox-null' Cys42Ser PKGIα knock-in mice ex vivo and in vivo is required to confirm the effector role of this kinase in HPV. Consistent with these findings, oxidants have been shown to block HPV in vivo [80]. More recently, chronic hypoxia was suggested to attenuate acute HPV by the increase in extra-cellular H_2O_2 and elevated level of disulfide-PKGIα [85]. At the same time, reducing agents (e.g., dithiothreitol) mimicked the HPV response in PA SMCs [77].

Although it has been studied less than acute initial phase HPV, there is evidence that the prolonged (sustained) phase is also redox dependent, and, as opposed to the rapid phase, is mediated by increased ROS production [27, 28]. This is interesting in relation to hypoxia-induced PH, as the sustained phase of HPV is thought to eventually develop into the chronic disease state, as evidenced by the finding that Rho kinase inhibitors, which effectively inhibit the second phase of HPV, can immediately reverse the elevated PA pressure in a rat model of chronic hypoxia-induced PH [86]. Although it is generally assumed that chronic hypoxic PH is an extension of the sustained phase of HPV, some high-altitude dwellers are more resistant to elevation of PA pressure and develop less pulmonary vascular remodeling due to genetic adaptations [20]. In addition, studies in both rats and humans show that exposure to chronic hypoxia in perinatal period may predispose some individuals to an increased pulmonary vascular reactivity in response to acute hypoxia in adult life [87, 88]. Taken together, these data support a crucial role for oxidants, in particular H_2O_2, in the regulation of pulmonary vascular function.

13.4.2 Deleterious Role of Oxidants in PH

Upregulation of Nox4 during PH has been frequently observed in PAs and lungs of mice subjected to chronic hypoxia, and in lungs of patients with Group 1 PAH [9, 69, 89]. The Nox4 level in lungs of mice subjected to hypoxia for 3 and

14 days was positively correlated with the level of H_2O_2, a primary product of this enzyme [9]. Knock-down of Nox4 prevented hypoxia-induced hyper-proliferation of cultured human PA SMCs [69], or fibroblasts [90]. In another study, pharmacological inhibition of Nox4 decreased PA SMC proliferation in vitro and RV hypertrophy and vascular remodeling in vivo [91], consistent with a causative role for Nox4 in PH. Of note, the specificity and isoform selectivity for many of Nox inhibitors has been questioned [92], and so their protective effect may result from a widespread inhibition of superoxide-producing Nox isoforms, rather than Nox4 alone. Surprisingly, although a large body of literature is consistent with the detrimental role of Nox4 produced H_2O_2 in PH, Nox4 constitutive or conditional knockout mice develop a similar PH phenotype to the wild-type mice subjected to chronic hypoxia [93]. Therefore, it could be argued that Nox4 upregulation and subsequent oxidative protein modifications may play a dual—detrimental as well as a protective—role (as will be discussed in the next section), and this can explain the somewhat contradictory results.

Nox2, the primary function of which is to produce superoxide, has also been implicated in the development of PH. Chronic hypoxia increased Nox2 protein expression in mice, and Nox2 knock-out mice demonstrate a less severe PH phenotype associated with decreased superoxide levels [94]. Further evidence of the deleterious role of superoxide in PH comes from studies in Fawn-hooded rats which are characterized by spontaneous PH. These exhibit attenuated H_2O_2 but increased superoxide levels, together with lower than control SOD2 activity [95]. Treatment of Fawn-hooded rats with a SOD2 mimetic, which is anticipated to enhance H_2O_2 derived from superoxide, abrogated RV pressure and vascular remodeling [95]. Consistent with the adaptive role of SOD enzymes to limit PH, increased SOD3 expression was shown to be required to protect the lungs from hypoxia-induced PH [96], and SOD1 knock-out mice develop spontaneous PH under normoxic conditions [97]. These reports imply that any significant alterations causing elevated superoxide and decreased H_2O_2 levels may initiate PH-like phenotypes [95, 97]. Taken together, oxidants may contribute to hypoxic PH, although the roles of specific ROS (or RNS) in evoking constriction remain unclear. Below we will provide a few examples of how increased oxidant production can cause deleterious effects in PH.

Protein kinases are essential components of the majority of redox-regulated signaling pathways [8, 98]. Oxidants can alter localization or function (substrate phosphorylation or targeting) of several members of this enzyme group. For example, an impairment of PKGI activity due to tyrosine nitration has been reported in pulmonary vessels after 4 h of hypoxia [99] and was associated with worse outcomes in caveolin 1-deficient mice during PH [100]. Subsequently, tyrosine nitration was identified in human PAH Group 1 by two different groups [100, 101], with one study showing that nitration of Tyr247 causes attenuation of cGMP binding and hence, a decreased "classical" PKGIα activity [101]. These studies are consistent with a deleterious effect of RNS in PH, as reviewed elsewhere [102, 103]. Tyrosine nitration of multiple proteins was evident in skin fibroblasts from patients with familial Group 1 PAH, that is, due to bone morphogenic protein receptor II (BMPR2) mutation, as well as in pulmonary microvascular endothelial cells from BMPR2-mutant mice [19], implying more than one target of this modification in PH.

Cysteine oxidation stimulates tyrosine kinase activity, and increased oxidant production is associated with a rise in total cellular tyrosine phosphorylation in pulmonary vessels [98]. One example is Src-family kinases, the largest subfamily of nine closely related nonreceptor tyrosine kinase members, of which c-Src is highly expressed in SMCs [65]. Src-family kinases play a role in distributing pro-mitogenic signaling coming from receptor tyrosine kinases, and if deregulated, contribute to PA remodeling in Group 1 PAH [104]. In addition, Src-family kinases can be activated by oxidants due to an inter-molecular disulfide bond formation between cysteines in the SH2 domain [65], consistent with

increased oxidative stress being deleterious and causative to PH progression.

Another example is the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which mediate numerous cellular processes including growth, proliferation, and differentiation. In addition to ligand-dependent activation and concomitant tyrosine phosphorylation, EGFR stimulation is coupled to H_2O_2 generation by Nox. In turn, H_2O_2 functions as a secondary messenger to promote EGFR oxidation at active site redox-regulated Cys797 to sulfenic acid leading to enhancement of its tyrosine kinase activity [105]. This EGFR sulfenylation is thought to be a putative signaling mechanism that may regulate other receptor tyrosine kinases, and irreversible inhibitors that target Cys797 are being developed [106]. To the best of our knowledge, the role of EGFR Cys797 oxidation in hypoxia-induced PH or Group 1 PAH has been overlooked. However, it has been shown that oxidation of EGFR by an alternative mechanism, that is, H_2O_2-induced ligand-independent activation via tyrosine dimerization, occurs in experimental PH and in patients with Group 1 PAH [107]. As described in Section 13.6, there is evidence that inflammatory stimuli can cause the incorporation of the cellular Src and the EGFR into signaling complexes which mediate the activation of the transcription factor NF-κB, thus promoting the synthesis of cytokines and adhesion molecules.

Bone morphogenic protein 9 (BMP9) is a member of the transforming growth factor β (TGFβ) superfamily and the main ligand of activin receptor-like kinase 1 (ALK1). ALK1 is expressed in ECs, where it plays an essential role in maintaining vascular quiescence [108]. The liver constitutively releases BMP9 into the circulation, and the mechanisms maintaining BMP9 bioavailability are crucial for optimal ALK1 signaling. Interestingly, Cys73 of BMP9 is shown to be responsible for the formation of an intermolecular disulfide bond between the two BMP9 monomers [109]. The disulfide bond is not required for BMP9 signaling activity per se; however, it makes the oxidized, dimer form of BMP9 less susceptible for proteolytic cleavage, whereas the reduced monomeric form of BMP9 can be effectively regulated by redox-dependent proteolysis [109]. At present, there is no in vivo evidence for BMP9 disulfide dimer formation, the detection of which is a challenging although not entirely impossible task. Redox-regulation of BMP9 by disulfide may contribute to accurate maintenance of low concentrations of BMP9 necessary for ALK1 specific signaling [109]. However, hypothetically, increased oxidant levels can facilitate the formation of proteolysis-resistant BMP9 dimer, leading to its accumulation and potential contribution to the development of PH. Consistent with this suggestion, a recent study reported that the loss of BMP9 by deletion or inhibition protected mice against the development of hypoxia-induced PH [110]. Ultimately, further studies are needed for a better understanding of redox-regulated BMP9 signaling pathway in PAH. Potentially, the generation and assessment of a Cys73Ser 'redox-null' BMP9 transgenic mouse model would help to address this question.

13.4.3 Protective Role of Oxidants in PH

Interestingly, oxidants may also play an adaptive role in PH. A paradoxical increase in PKGI total protein expression [9, 111], or its disulfide-dimerized form [9, 85] was observed in rodents subjected to chronic hypoxia, or in lung tissues of patients with PAH Group 1 [9]. Even though PKGI activity in chronic hypoxia can be compromised by tyrosine nitration, as discussed above, it is plausible that such PKGI upregulation mediate a protective mechanism which may have evolved to counter the damaging effect of oxidants in cells in a range of situations where oxidative stress may arise. Indeed, consistent with upregulation of the kinase being adaptive, PKGI knock-out mice develop spontaneous PH even without any additional stimulus [112]. Rudyk et al. have recently reported elevated levels of disulfide-PKGIα in PAs and lungs from mice subjected to chronic hypoxia for 3, 14, and 28 days. This PKGIα oxidation was likely

caused by H_2O_2 that was elevated in hypoxic tissues together with increased expression of oxidant-producing enzymes Nox4 and SOD3. Interestingly, the production of low molecular weight hydropersulfide species in lung tissues was decreased after 3 and 14 days of hypoxia [9]; a similar observation has been made recently in the lungs of patients with COPD [113]. Low molecular weight hydropersulfides can act as superior reductants that determine the cellular redox state by rapid neutralizing reactions with oxidants, and reduction of disulfide-containing molecules [59]. Notably, potassium polysulfides induced PKGIα oxidation in human PA SMCs and alleviated the PH phenotype when administered to mice exposed to hypoxia for 14 days. In addition, Cys42Ser PKGIα knock-in mice developed more severe hypoxia-induced PH, compared with wild-type mice, and appeared to be resistant to a therapeutic effect of polysulfides [9]. The underlying mechanisms involve vasodilatation, consistent with the pressure-lowering role of disulfide-PKGIα, and, likely, disulfide-PKGIα mediated prevention of EndoMT, which otherwise contributes to PA muscularization. This study provided cause and effect evidence that disulfide-PKGIα which accumulates in hypoxia-induced PH serves an endogenous, adaptive redox mechanism that limits PH and the associated adverse PA remodeling. Conceivably, pharmacological agents that induce disulfide-PKGIα may have therapeutic potential in Group 3 PH [9].

Further evidence for a protective role of oxidants comes from an elegant study showing that resveratrol induces disulfide-PKGIα, at least in the vasculature, and that this mediates the therapeutic effect of this antioxidant. This concept ensued from the observation that Cys42Ser PKGIα knock-in mice appear to be resistant toward the protective antihypertensive effect of resveratrol which occurs in wild-type mice [114]. Such effect may seem counterintuitive, as the ability of this nonflavonoid polyphenolic compound to promote health and prevent disease has been attributed primarily to its antioxidant activity, and to a lesser extent, to direct interaction with target molecules. Nevertheless, it is plausible that some antioxidants (e.g., resveratrol) can induce cysteine oxidation, and by doing so, upregulate protective redox signaling [114, 115]. Hence, oxidation of PKGIα may contribute, at least partially, to the protective mechanism of resveratrol in two PH models [116, 117].

The type I regulatory-RIα subunit of protein kinase A (PKA RIα) is another cyclic nucleotide-dependent kinase that, like PKGIα, is also regulated by inter-protein disulfide bond formation [69]. PKA activity contributes to prostacyclin-triggered vasodilator pathways to maintain healthy pulmonary vascular function [118]. Previous work has addressed the role of PKA RIα disulfide in cardiac contractility [7], migration, and growth of endothelial cells during angiogenesis [119] and vasodilation induced by CysNO [120]; however, not much is currently known about the role of this disulfide-PKA RIα in PH. When 'redox-null' Cys17Ser PKA RIα knock-in mice were subjected to chronic hypoxia for 28 days, there were no differences in RV hypertrophy, compared with the wild-type mice [9]. Although this is consistent with a specific adaptive role for pulmonary disulfide-PKGIα in hypoxia-induced PH [9], more work needs to be done to address the possible role of disulfide-PKA RIα in the pulmonary system.

Receptor-tyrosine kinase protein BMPR2 is the key heritable risk factor for the development of Group 1 PH [121]. BMPR2 binds BMPs followed by the initiation of paracrine, protective antiproliferative signaling. It is of interest that that BMPR2 possesses 10 cysteine residues in the ligand-binding domain, which can form five disulfide bonds [122]. Group 1 PAH is strongly associated with mutations of some of these cysteines, which are functionally linked to retention of mutant BMPR2 protein in the endoplasmic reticulum, its reduced trafficking and significant disruption of normal BMPR2 signaling [121]. It appears that the formation of these BMPR2 disulfide bonds may indeed play an intrinsic protective role in health, as their mutation to other residues during disease prevents this oxidative post-translational modification from occurring [121]. In line with this,

Frank et al. demonstrated that mutant mice carrying heterozygous BMPR2 mutations develop more severe PH induced by chronic hypoxia, due to endothelial dysfunction in the pulmonary vasculature, however, without an associated increase in pulmonary vascular remodeling as compared with the wild-type mice [123].

13.5 Inflammation in Animal Models and Patients with Group 3 PH

Inflammation is often referred to as a complex interplay between cells and humoral factors in response to injury or insults. Inflammatory responses are controlled by innate and adaptive immunity systems that act together to defend against these insults. Innate immunity is usually a rapid response, which is nonspecific to an insult and occurs within hours. In contrast, an adaptive immune response commences four to seven days after the initial insult to mobilize inflammatory cells and target the specific antigen [124]. Cells that are involved in the innate immune response, such as macrophages, mast cells, neutrophils and dendritic cells, produce cytokines or chemokines (i.e., chemotactic cytokines that regulate migration) to potentiate the adaptive immune response through autocrine and paracrine mechanisms and an "amplification cascade." The latter system includes monocytes, macrophages, neutrophils, basophils, dendritic cell types, mast cells, T-cells, and B-cells [125]. The inflammatory response is reinforced by various signaling pathways, including the ubiquitously expressed NF-κB, activator protein-1, histone modifications, and others that coordinate and regulate expression of pro-inflammatory mediator genes in tissues. Transcriptional pathways, such as nuclear factor erythroid 2-related factor 2 can also provide feedback regulation of both innate and adaptive immune responses, and their dysregulation can contribute to the pathogenesis of inflammatory diseases [126]. Although inflammation is crucial for the immune response, if uncontrolled or prolonged, it can lead to development of inflammatory or autoimmune diseases.

Over the past few decades, it has become increasingly apparent that inflammation (either acute or as an underlying low-level condition) is a risk factor for systemic vascular disorders [66]. Physical trauma from balloon angioplasty, transplantation, coronary bypass surgery, or other vascular injuries such as ischemia/reperfusion, elevated levels of oxidized low-density lipoprotein, diabetes or cigarette smoking have all been established as vascular inflammatory insults. These insults can cause direct or indirect damage to the vascular endothelium and trigger an inflammatory cascade followed by the release of mediators, which in turn affect blood vessel composition, function, and integrity [127]. Growing evidence demonstrates the significant contribution of inflammation to PA remodeling processes in patients with PAH Group 1, and possible triggers include viruses, parasites, infectious and toxic factors (e.g., some oxidants), or transforming growth factor-β (TGF-β)/BMPR2 signaling cascade dysfunction [54]. Increased levels of circulating cytokines and chemokines are reported on the systemic level [54], together with evidence for a local perivascular invasion of inflammatory cells in lung tissue from patients with familiar Group 1 PAH [128]. These cytokines include interleukin 1 (IL-1), interleukin 6 (IL-6), and interleukin 8 (IL-8); monocyte chemoattractant protein 1 (MCP-1), regulated upon activation, normal T cell expressed and presumably secreted (RANTES), tumor necrosis factor 1 alpha (TNF-1α), and C-reactive protein (CRP). A link between circulatory levels of various pro-inflammatory cytokines and survival in Group 1 PAH was suggested [129], implying a relationship between inflammation and progression of the disease. Furthermore, a recent analysis of the proteomic profile of cytokines, chemokines, and growth factors in the blood of patients highlighted remarkable heterogeneity in Group 1 PAH [130]. With the help of unsupervised machine learning, it extracted patient subsets with distinct blood proteomic profile patterns of inflammation (i.e., immune phenotypes) that are independent of Group 1 PAH subtypes, demographic features, comorbid conditions, or background therapies, and associated with differing

clinical disease severity and outcomes [130]. Numerous animal and human studies have focused on either systemic, pulmonary or perivascular inflammation in Group 3 PH, which will be discussed in a greater detail in this section.

13.5.1 Cytokine Production in Group 3 PH

Hypoxia induces an early and persistent PA-specific vascular inflammatory response in rodents [20]. For example, hypoxic mice demonstrated early recruitment of macrophages and monocytes to the lungs [131]. Exposure of rats to hypoxia for 1–8 h caused an upregulation of alveolar macrophages and a related increase of inflammatory mediators and chemokines which was associated with increased pulmonary vascular permeability [132]. In another study, rats subjected to more prolonged bouts of hypoxia (7 and 28 days) demonstrated progressive accumulation of monocytes and dendritic cells in the PA vessel wall as well as increased expression of numerous pro-inflammatory chemokines, including C-X-C motif chemokine 12/stromal cell-derived factor 1 (CXCL12/SDF-1), MCP-1, IL-6, complement component 5 and their respective receptors [133]. Similarly, chronic hypoxia in mice for up to 2 weeks resulted in a marked accumulation of pro-inflammatory macrophages, and neutrophils in lung sections and broncho-alveolar lavage fluid together with their chemokines including MCP-1, macrophage inflammatory protein (MIP)-2, IL-1 and IL-6 that peaked after 2 days of hypoxia and had entirely subsided after 2 weeks [134]. Chronic hypobaric hypoxia in rats (4 weeks) and calves (2 weeks) resulted in pronounced perivascular inflammation, caused by the infiltration of "fibrocytes," that is, mononuclear cells of a monocyte/macrophage lineage, derived from circulatory mesenchymal precursors, that can produce collagen and express α-SMA [135].

Interestingly, the combined SU-5416/chronic hypoxia PH rat model was initially seen to demonstrate a minor or indeed nonexistent lung perivascular inflammatory response [39]. However, macrophage recruitment to the lung was subsequently seen in mice subjected to hypoxia alone, and this was reportedly potentiated by combined hypoxia and SU-5416 treatment [40]. Cytokine biomarker profile in response to SU-5416/chronic hypoxia in mice correlated with that of patients with idiopathic PAH [40], although this was contradicted by another study [136]. More recently, a progressive temporal increase of perivascular macrophages, together with the contemporaneous increase in the proportion of macrophage-positive intima was observed in rats treated with SU-5416/chronic hypoxia for 3 weeks and then allowed to recover for 10 weeks [137]. Gene expression of IL-6, MCP-1, matrix metalloproteinase 9 (MMP-9), cathepsin-S, and RANTES was progressively up-regulated in the lungs of rats after three and 5 weeks of SU-5416/chronic hypoxia treatment. These inflammatory changes correlated with phenotypical changes in SMCs (i.e., prevalence of phenotypically modulated immature, highly-proliferative α-SMA and vimentin-positive SMCs in intimal and plexiform lesions) that resemble those during experimental obstructive pulmonary disease [137]. In summary, exposure to chronic hypoxia or SU-5416/chronic hypoxia in rodents and calves lead to a marked induction of pro-inflammatory cytokines and chemokines, consistent with pulmonary vascular inflammation that precedes the development of PH. Therefore, hypoxia-induced PA remodeling is likely to be causatively dependent, at least in part, on active inflammatory and progenitor cell recruitment.

When it comes to human Group 3 PH, the potential contribution of inflammatory responses in PA remodeling has been studied in COPD with PH (which is typically characterized by poorer prognosis compared to "PH-free" COPD). Higher levels of circulating TNF-α and C-reactive protein (CRP), a robust marker of systemic inflammation, correlated with PA pressure in COPD patients with PH [138]. IL-6 and CRP were later confirmed as independent risk factors for PH in COPD patients [139]. A systematic review of possible biomarkers to diagnose acute exacerbation of COPD (AECOPD) concluded that only CRP was consistently elevated com-

pared to control patients, while IL-6 and TNF-α showed variable results [140]. However, the ratios of neutrophils to lymphocytes, platelets to lymphocytes, and the systemic-immune-inflammation index were elevated to a greater extent in patients with PH secondary to AECOPD, compared to AECOPD patients without PH [141]. In addition, COPD patients demonstrate evidence of perivascular inflammation, with infiltration of leukocytes in small PA; this becomes more apparent as the disease progresses, and is associated with increased wall stiffness [142].

Interestingly, there is little evidence for the role of local or systemic inflammations in the development of early noncardiogenic high-altitude pulmonary oedema (HAPE) in humans [143, 144]. HAPE usually occurs upon a rapid ascent to high altitude, due to increased permeability of the alveolar-capillary barrier as a result of severe, sustained and uneven HPV and high capillary pressure, which may be lethal in susceptible individuals [31]. Inflammatory activation is likely to occur in later stages, mediated by spontaneous cytokine release by pulmonary artery endothelial cells (PA ECs) and leukocytes, leading to increased capillary permeability and neutrophil recruitment to the lung if HAPE is untreated for several days [31]. In line with this, it is speculated that individuals may be predisposed to HAPE if they have an underlying infection or inflammatory condition [31, 145].

Inflammation is also an essential feature of the PH model induced by monocrotaline (MCT) [146], a toxic pyrrolizidine alkaloid compound which is activated to the reactive pyrrole metabolite of dehydromonocrotaline (MCTP) by cytochrome *P*-450 in the liver [147]. When administered, MCT leads to acute PA injury followed by progressive pulmonary vasculopathy and pulmonary oedema leading to PH and death within weeks [148]. The original seminal paper by Kay et al. demonstrated a pronounced increase of RV pressure and PA medial thickness in MCT-treated rats [149]. Inflammatory cells, mainly neutrophils, macrophages, dendritic cells, and lymphocytes infiltrate the lung, mostly in perivascular areas in response to MCT in rats [148], even though the blood cytokine biomarker profile does not appear to correlate well with that of patients with Group 1 PAH [136]. Recently, a rat model of severe PH was developed by Coste et al., which combined a single administration of MCT with 4 weeks' exposure to chronic hypoxia [150]. This model displays pronounced RV failure, PA remodeling with thrombotic, neointimal and plexiform-like lesions, and inflammation which are similar to those observed in severe PAH Group 1 [150].

Thus, inflammation has a multifaceted role in PH and involves the pleiotropic release of cytokines from various cellular sources driven by various signaling pathways, complex cell–cell interactions, and impaired regulatory immune responses, which affects all vascular cell types. More comprehensive and rigorous studies in patient populations, like the one identifying distinct immune phenotypes [130] would be of interest for further hypothesis-generating and mechanistic studies in Group 3 PH, which may help to inform about future therapies targeting immune response in this particular group.

13.5.2 Evidence for Upregulation of pro-Inflammatory Pathways in Group 3 PH

As we discussed above, the lungs (which usually reside in a high oxygen condition) need oxidants for their normal pulmonary vascular function. However, increased oxidative as well as cytokine stress, as seen in Group 1 PAH and Group 3 PH, have been implicated in the initiation of local lung inflammation via activation of various transcriptional signaling pathways. In particular, many studies have examined the role in PH of NF-κB, an archetypical oxidant-regulated immunological pathway which is known to regulate the synthesis of many of the cytokines shown to be elevated in PH. Although oxidants act directly on some key regulators of NF-κB signaling, such as the inhibitor of κB (I-κB) and NF-κB itself, they can also affect NF-κB indirectly by activating its upstream regulators HMGB1 and TLRs, which are both redox-regulated, and which recent work has highlighted as being involved in PH. Thus,

oxidants can play a causative role in triggering the inflammatory response by activating NF-κB, TLR4, and HMGB1, which in turn can be involved in pathogenesis of Group 3 PH. We will focus on these pathways in this and the following section.

13.5.2.1 Overview of NF-κB

There has been an increasing interest in NF-κB signaling pathway in PH. The NF-κB family of inducible transcription factors is important in orchestrating cellular immune and stress responses when activated by variegated stimuli including inflammatory cytokines, engagement of antigen receptors, and the binding of microbial products by TLR. Once NF-κB is activated, for example, by oxidative stress, it can mediate an inflammatory response by inducing gene transcription of cytokines such as IL-1, IL-6, TNF-α, and inter-cellular adhesion molecule 1 ICAM-1 [151]. In most types of unstimulated cells, NF-κB exists as inactive homo- or hetero-dimers formed from a family of five proteins (p65 (RelA), RelB, c-Rel, p105/p50, and p100/52) containing a conserved amino-terminal Rel homology domain. These are localized to the cytoplasm by virtue of being bound to members of the I-κB protein family and can be activated via either canonical or noncanonical signaling pathways [126]. In canonical activation, inflammatory signals from immune receptors activate TGFβ-activated kinase 1 (TAK1) or other kinases and subsequently, the trimeric nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, I-κB kinase (IKK) complex [126]. The latter, which is composed of catalytic IKKα or IKKβ and regulatory IKKγ subunits, then phosphorylates IκBα or other IκB family members. The regulation of NF-κB by I-κB varies depending on which particular family members of both proteins are involved, but for the canonical forms p50/p65 and I-κB, activation and translocation to the nucleus of NF-κB occur when I-κB is phosphorylated by IKK, causing its ubiquitination, dissociation from NF-κB, and proteasomal degradation [152]. It is established that IKKβ and IKKγ are essential for phosphorylation-dependent IκBα degradation and canonical NF-κB nuclear translocation, whereas IKKα plays only a supporting role in the canonical pathway. The noncanonical NF-κB pathway can be induced by TNF receptors and is conveyed by a slow but continuous activation of NF-κB-inducing kinase, which in concert with IKKα mediates the processing of p100 into p52. p52 lacks the auto-inhibitory domain on p100 which blocks its nuclear localization sequence, this allowing it to translocate into the nucleus in complex with RelB [126].

13.5.2.2 Role of NF-κB in PH

Evidence for NF-κB activation in Group 3 PH comes mostly from rodent and cell studies, as emphasized below. In particular, rats exposed to chronic hypoxia for 2 weeks demonstrated increased canonical NF-κB activity, assessed by nuclear translocation of p50 and p65 proteins and inflammatory gene expression in the lungs along with the development of RV hypertrophy and dysfunction [153]. This was ameliorated by twice-daily injection of a nonselective NF-κB inhibitor, the thiol compound pyrrolidine dithiocarbamate [153], suggesting that canonical NF-κB activation may contribute to Group 3 PH. In a different study, DNA binding activity of both canonical and noncanonical NF-κB family members (e.g., NF-κB1, NF-κB2, p65, and RelB) were increased in lung homogenates of mice exposed to chronic normobaric hypoxia for 3 weeks, and in ECs subjected in vitro to 1% O_2 for 24 h [154]. A selective knock-down of specific NF-κB subunits with siRNA in hypoxic ECs demonstrated that only canonical, p65, and no other NF-κB subunits, can mediate NF-κB-dependent regulation of endothelin-1 expression and subsequent PA SMC proliferation [154]. NF-κB was also activated in rats with MCT-induced PH, and its blockade ameliorated the PH phenotype [155, 156].

In relation to human studies, increased nuclear expression of p65 protein was observed in the bronchial biopsies of smokers with COPD and correlated with the degree of airflow limitation, as compared with control nonsmokers [157]. In this study, at least one-third of both CD4+ and CD8+ T-cells expressed p65, providing additional evidence for the involvement of these cells

in the cytokine production induced by NF-κB activation in subjects with COPD [157]. In patients with Group 1 PAH, increased canonical NF-κB activation, as assessed by expression of p65 protein, was demonstrated in alveolar macrophages obtained from bronchoalveolar lavage [158] and in lung sections, lung tissue macrophages, lymphocytes, PA ECs, and SMCs [159]. In the latter study, enhanced expression of p65 (which enabled to overcome the inhibitory effects of NF-κB by I-κB), led to the increased mRNA level for the NF-κB-regulated genes endothelin-1 and RANTES in the whole lungs of Group 1 PAH patients compared with the control patents [159]. On the cellular level, proliferation of PA SMCs from patients with Group 1 PAH was reduced after knocking down NF-κB with small interfering RNA [160]. In the same study, the compound celastramycin significantly reduced the nuclear level of p65 as well as *RelA* and *TLR4* gene expression in PA SMCs from Group 1 PAH patients, compared with vehicle-treated controls, implying that the therapeutic mechanism of this drug involves inhibition of TLR4-NF-κB-dependent inflammatory signaling [160]. It is conceivable that specific inhibition of the canonical Nf-κB pathway could be a novel therapeutic approach for Group 3 PH.

13.5.2.3 Overview of TLR4 and HMGB1

TLRs are transmembrane proteins containing an extra-cellular leucine-rich repeat domain which, in concert with the protein myeloid differentiation factor-2 (MD2), recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs) such as HMGB1. The ectodomain is linked through a transmembrane domain to an intracellular toll - IL-1 receptor (TIR) domain which, upon activation by PAMPs or DAMPs and dimerization of the TLR-MD2 complex, recruits a variety of adaptor proteins, thereby initiating specific intra-cellular signaling pathways [161, 162]. TLR4 signals to NF-κB through two such pathways, involving the adaptors toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), myeloid differentiation primary response 88 (MyD88), TRIF (TIR-domain-containing adapter-inducing interferon-β), and TRAM (TRIF-related adapter molecule). In a pathway causing rapid activation of NF-κB, TIRAP binds to the TIR, and recruits MyD88, which then recruits IL-1 receptor-associated kinase (IRAK) 1, IRAK2, IRAK4, and tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 then binds to and activates a signaling complex containing TRAK1 which acts through IKK to phosphorylate IKβs, thus causing translocation of NF-κB to the nucleus. TRAF6 also stimulates mitogen activated protein (MAP) kinases, causing production of inflammatory cytokines [161]. TLR4 is subsequently endocytosed and becomes localized in endosomes. Here, it binds to TRAM, which recruits TRIF, initiating a pathway which causes a slower and more sustained activation of NF-κB. In this case, the recruitment of TRAF6 and other proteins results, not only in the activation of the TRAK1 complex but also in the production of interferons and TNF-α resulting in further activation of NF-κB [162]. As described in Section 13.6, several components of these TLR4 signaling pathways are influenced by cell redox state. Moreover, there is evidence that TLR4 can stimulate the production of ROS by Nox, and that in some types of cells, this process is important in mediating TLR4 dependent activation of NF-κB.

The role of TLR4 has been closely studied in relation to its ligand HMGB1, which is a ubiquitously expressed multifunctional protein that is involved in DNA repair, replication, recombination, and transcription. Under nonstressed conditions, HMGB1 is localized to the nucleus where it binds to chromatin and is involved in transcriptional regulation. Upon cellular injury/necrosis, however, HMGB1 can translocate to the cytoplasm and leak out into the extra-cellular space, where it can bind to numerous cellular receptors, the most important of which are thought to be TLR4, TLR2, receptor for advanced glycation end products (RAGE), and C-X-C chemokine receptor type 4 (CXCR4), thereby causing various pro-inflammatory actions [163]. HMGB1 is therefore categorized as a DAMP molecule, a term which typically refers to intra-cellular pro-

teins or other biomolecules that play defined roles in normal cellular function but upon cell damage are released to the extra-cellular space where instead they promote immune responses. In addition, HMGB1 is actively secreted from some types of cells, for example, macrophages, dendritic cells, and natural killer cells [163]. HMGB1 is also actively secreted during, and contributes to, cardiac, hepatic, and renal ischemia/reperfusion injury [164].

13.5.2.4 Role of TLR4 and HMGB1 in PH

As TLR4 is a key downstream receptor for HMGB1 [163] and mediates its pro-inflammatory effect via binding to TLR4/MD2 receptor complex on macrophages and stimulating TNF-α release [165], its role in PH is closely related to HMGB1. Tsung et al. first suggested an interaction between HMGB1 and TLR4, leading to an activation of the inflammatory cascade in a model of hepatic ischemia and reperfusion injury [166]. More recent studies confirmed that HMGB1 is involved in PH, indeed through the activation of TLR4 [167]. Consistent with the direct effect of HMGB1 on TLR4 receptors, when TLR4 knock-out mice were compared with RAGE knock-outs and corresponding wild-type mice, a major contribution of TLR4 in the development of PH phenotype was evident [167], even though the role of RAGE in PH could not be ruled out, as described later. Treatment with exogenous HMGB1 resulted in more severe increase of RV pressure and hypertrophy, PA muscularization and vessel wall thickening as well as exacerbation of endothelial dysfunction and inflammation in wild-type but not TLR4 knock-out mice subjected to chronic hypoxia [167]. In further support of a causative role of TRL4 and HMGB1 in PA muscularization and remodeling, Wang et al. provided evidence of exogenous HMGB1 stimulating cell cycle progression in PA SMCs, which was prevented by TLR4 inhibitors [168].

Consistent with the above, either global TLR4-knock-out mice generated on a pure C57BL/6 J background [167] or TRL4-deficient mice expressing mutant protein (C3H/HEJ mice) [169] are resistant to hypoxia-induced PH. Along with this, Ma et al. demonstrated that while TLR4 deficient mice were resistant to PH pathological changes induced by chronic hypoxia, they developed a mild spontaneous PH, with some variability, as assessed by RV pressure, hypertrophy, and PA wall thickness [170]. Although it is a notable observation, differences in genetic background, supplier, age and sex of animals, and variability between animals within experimental groups could all contribute to this seeming discrepancy. Importantly, TLR4-deficient mice (C3H/HEJ) demonstrated a decreased pulmonary expression of inflammatory cytokines (i.e., TNF-α and IL-1β) and decreased the activity of MMP-9 in response to chronic hypoxia [169], consistent with reduced inflammation as a result of TLR4 deficiency. Follow up work by Bauer et al. demonstrated that TLR4 expressed in platelets contributes to the pathogenesis of PH, as genetic deletion of platelet TLR4 attenuated hypoxia-induced PH [171].

Interestingly, HMGB1 could be detected in serum, broncho-alveolar lavage, and lung tissue of mice as early as 8–16 h after their exposure to hypoxia. In addition, translocation of HMGB1 from the nucleus, necessary for it to be exported from cells, which is required for its action, was evident within 48 h after hypoxia in lungs [167]. Treatment of mice subjected to chronic hypoxia with HMGB1 neutralizing antibody every other day prevented RV pressure increase and hypertrophy, as well muscularization of arterioles, as compared with IgG-treated controls [167]. Furthermore, rats treated with SU-5416/chronic hypoxia and HMGB1 neutralizing antibody also developed lower RV pressure and less PA remodeling than control-treated animals [172].

HMGB1 treatment stimulated migration and proliferation of PA SMCs as well as down regulation of BMPR2 signaling pathway (i.e., reduced phosphorylation of Smad1/5/8) which was abrogated by HMGB1 inhibitors saquinavir and glycyrrhizin [168]. Exposure to hypoxia also caused PA SMC proliferation, and the possible underlying mechanism may include HMGB1 causing the release of IL-6 and CXCL8 through advanced glycation end products [173]. HMGB1 can also induce endothelin 1 secretion

in PA ECs, either in a TLR4-dependent or -independent manner [167].

Lin et al. have recently proposed that the interaction of HMGB1 and its receptor RAGE plays a pivotal role in PH [174]. This laboratory had previously discovered that a protein of the resistin-like molecule family with mitogenic, pro-contractile, and chemokine properties, which they christened hypoxia-induced mitogenic factor (HIMF), is up-regulated in the lungs of rats subjected to chronic hypoxia [175]. The PH and associated pulmonary and cardiac pathologies induced by hypoxia were diminished by knocking down HIMF in vivo using shRNA, and HMIF gene transfer into the lungs caused PH pathological changes similar to that caused by exposure to chronic hypoxia [176]. Lin et al. reported that the effect of HIMF, which their work suggests may play a crucial role in causing PA remodeling in PH due to chronic hypoxia, is mediated largely via HMGB1 released from pulmonary microvascular endothelial cells (PMVEC) acting on RAGE in the PA SMCs. They observed that hypoxia increased the protein expression of HMGB1 and RAGE in PMVECs from rats subjected to chronic hypoxia, and that treating cultured human PMVEC with HIMF increased HMGB1 and RAGE release. Conditioned medium from these cells promoted the proliferation of human PA SMCs more strongly than did HIMF alone, and preincubation with antagonists of HMGB1 or RAGE almost completely blocked the enhanced growth of PA SMCs induced by the conditioned medium. In further experiments, they showed that HIMF was acting through HMGB1 and RAGE to inhibit autophagy of PA SMCs and also to reduce the expression in these cells of BMPR2, both effects leading to their proliferation [174].

Although several publications have mechanistically addressed the contribution of TLR4 to Group 3 PH in animal models and cell experiments, less is presently known about its role in relation to human pulmonary vascular disease, apart from the work showing increased expression of TLR4 in the lungs of patients with Group 1 PAH [167, 172]. However, a recent study in COPD patients with PH demonstrated increased HMGB1 serum levels, compared with the control patients [177]. Along with this, lungs from COPD patients with PH contained HMGB1-positive cells in remodeled PAs [177]. Of interest, the serum levels of HMGB1, TNF-α, and IL-6 were elevated in newborns with persistent PH (PPHN, a type of Group 1 PAH) at the onset and PPHN alleviation, while the serum levels of TNF-α and IL-6 were positively correlated with HMGB1 levels both at PPHN onset and after remission [178]. In addition, an increased circulating level of HMGB1 [177], and the positive correlation of circulating level of HMGB1 with PA pressure [167, 179] were observed in Group 1 PAH patients. Meanwhile, HMGB1 mRNA and protein levels were increased in the perivascular adventitia and intima [172], and a diffuse extranuclear staining pattern of HMGB1 protein around concentric and plexiform vascular lesions [167] in lungs of patients with Group 1 PAH.

Thus, both animal and human data support the role of TLR4 and HMGB1 as important mediators of PH across many species. TLR4 receptors can be activated during chronic alveolar hypoxia (e.g., directly by HMGB1), and this can have downstream signaling effect leading to release of inflammatory mediators which potentiate deleterious PA remodeling and contribute to the pathogenesis of PH.

13.6 Oxidative Post-translational Protein Modifications Within Inflammatory Pathways

Not surprisingly, pulmonary inflammation has been linked to ROS production [55], with PA ECs, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells being major sites of oxidant generation [180]. Oxidant concentrations in PH are enhanced by their release from macrophages and neutrophils, which localize to areas of inflammation [181]. Although several enzymes are recognized to produce oxidants in inflammatory cells, superoxide derived from membrane Nox appears to be the most important in polymorphonuclear cells, leukocytes, and macrophages [180]. However, Nox is

undoubtedly of more general importance in the inflammatory response, as it seems to be activated ubiquitously as pulmonary cells respond to inflammatory stimuli, and functions to provide ROS which drive multiple inflammatory cascades. However, other sources of ROS have also been linked to Group 3 PH. Xanthine oxidoreductase (XO), an enzyme that catalyzes the oxidation of hypoxanthine to xanthine and uric acid, exists as xanthine dehydrogenase (XDH) and XO [181]. The activity of XDH/XO, which can be induced by inflammation, was also upregulated in a hypoxia-induced PH scenario. In the same study, administration of the XO inhibitor allopurinol prevented pulmonary vascular remodeling, which highlights a role for XO-derived oxidants in hypoxia-induced PH [181]. Another ROS-producing hem enzyme in inflammatory cells is myeloperoxidase (MPO), which is expressed in neutrophils and some tissue macrophages at sites of inflammation. MPO can form hypochlorous acid (HOCl) and hypothiocyanous acid by catalyzing the reaction of H_2O_2 with chloride and thiocyanate ions [55]. HOCl is a very potent oxidant that reacts rapidly with many biological molecules and causes extensive oxidative damage in different cell types [55]. MPO has been implicated in many diseases, including PH. Its plasma levels are elevated in patients with PAH Group 1 and positively correlate with the adverse outcome; while MPO knock-out mice appeared to be protected from hypoxia-induced PH [182].

Thus, the activation of inflammatory pathways in pulmonary ECs and vascular SMCs caused by cell damage and cytokines associated with the local perivascular invasion of inflammatory cells is further exacerbated by a concomitant increase in ambient ROS concentrations. Having focused our discussion of inflammation in Group 3 PH on the interconnected NF-κB, TLR-4, and HGMB1 pathways, in part because their regulation by ROS, RNS, and RSS appears to be particularly salient [183, 184], below we describe some of the current evidence attesting to redox regulation of these pathways, including wherever possible examples relevant to Group 3 PH.

13.6.1 Redox Regulation of NF-κB

As described in Section 13.5, activation of NF-κB, particularly via the canonical pathway, contributes to the pathogenesis of Group 3 PH. Although antioxidants have been shown to generally attenuate NF-κB activation in many types of cells, the effects of cell redox state on NF-κB activation are remarkably complex, in that NF-κB, IKK, and multiple upstream regulators such as TLR4 and its partner proteins all contain thiol switches. Specific oxidative modifications of the different components of the system can either inhibit or activate NF-κB [185, 186].

For example, Lin et al. reported that 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2, an electrophilic prostaglandin derived from prostaglandin D_2, induced cysteine modifications at positions Cys38 (see also below), Cys160, and Cys216 in the Rel homology domain of p65, thereby inhibiting NF-κB translocation to the nucleus [187]. IKKβ is another important target of the NF-κB pathway for the redox regulation by endogenous or exogenous oxidants, which appears to confer an anti-inflammatory action [188, 189]. Initially, redox reactive cysteine residue Cys179 which is located directly between IKKβ phosphorylation sites Ser177 and Ser181 was shown to be a direct target for S-nitrozation. This was associated with inhibition of IKKβ enzymatic activity and NO-related anti-inflammatory signaling in lung epithelial cells [188]. A similar inhibitory effect of S-glutathionylation at Cys179 was subsequently described [190]. IKKβ phosphorylation can also be attenuated indirectly by the inhibitory S-glutathionylation of its upstream regulator mitogen-activated protein kinase kinase kinase 1 (MEKK1) [191].

Recently, Zhang et al. used a modified biotin switch assay [189] to show that exogenous treatment with the H_2S donor NaHS can cause persulfidation of Cys179 in IKKβ, leading to an inhibition of NF-κB in human PA ECs. This inhibitory effect was lost when IKKβ Cys179 was mutated to Ser179. The Cys179Ser mutation of IKKβ also reduced inflammatory responses in PA ECs treated with MCTP, the active metabolite

of MCT [189]. Furthermore, consistent with the protective role of RSS in PH [9, 192], H_2S donor administration to MCT-treated rats suppressed PH, PA remodeling and plasma release of inflammatory markers ICAM-1, IL-6, and TNF-α. However, this protection due to IKKβ persulfidation at Cys179 was lost in rats injected with adenovirus containing 'redox-null' Cys179Ser IKKβ [189]. This study is particularly interesting in that pulmonary levels of persulfide species are reportedly depressed in several animal models of PH, as is the expression of cystathionine γ lyase (CSE), the enzyme thought to be the main source of cardiovascular H_2S (see [192] for a review). However, CSE protein expression was also reportedly up-regulated in lungs of mice subjected to chronic hypoxia [9] and patients with COPD [113].

Zhang et al. also found that in lung tissue and PA ECs, respectively, MCT and its active metabolite MCTP decreased the persulfidation of the NF-κB component p65 on Cys38, and at the same time increased p65 phosphorylation on Ser468 and also ICAM-1 expression. Treatment with NaHS reversed both effects, and it appeared that these events were causally linked, since in PA ECs transfected with p65 in which Cys38 was mutated to a serine, the effects of both MCTP and NaHS were abolished [189]. This is consistent with a previous report that Ser468 phosphorylation increases NF-κB transcriptional activity in other types of cells by promoting its translocation to the nucleus [193]. Thus, it appears that a MCT-induced suppression of CSE expression and H_2S synthesis results in a fall in basal persulfidation of both IKKβ and p65, and that as a consequence NF-κB activity is promoted, causing pro-inflammatory effects which contribute to the development of PH. It is worth noting, however, that Sen et al. [194] reported the contradictory observation that Ser 38 persulfidation activates NF-κB in liver cells, perhaps reflecting the apparent context-dependency of ROS-induced effects of this transcription factor.

Although MEKK1 is inhibited by S-glutathionylation, other upstream activators of IKKβ phosphorylation can be stimulated by ROS. For example, IKK in HeLa cells was shown to be activated by oxidants through a pathway in which c-Src signals through Abl, another nonreceptor tyrosine kinase, and also through PKCσ, to phosphorylate protein kinase D on Tyr463 and then Ser738 and Ser742. Protein kinase D then phosphorylates IKKβ resulting in dissociation of IKβ from NF-κB [195, 196]. c-Src is targeted by oxidants, which activate it both directly (as mentioned in Section 13.4.2) and indirectly by inhibiting a c-Src specific tyrosine phosphatase [65]. Inflammatory stimuli typically act through Nox to generate the H_2O_2 which induce c-Src activation [183]. In rat aortic SMCs, there is evidence that upon stimulation by thrombin, c-Src can cause the activation of NF-κB by transactivating the EGFR. This leads to the sequential stimulation of mitogen-activated protein kinase kinase (MEK1/2) and extra-cellular signal-regulated kinase (ERK1/2) [197], the latter of which can phosphorylate IKKβ in these cells [198]. Interestingly, proline-rich tyrosine kinase 2 (Pyk2), another nonreceptor tyrosine kinase which has been shown to promote chronic hypoxia-induced PA remodeling by upregulating hypoxia-inducible factor-1 (HIF-1) [199], was also reported to mediate thrombin-induced activation of NF-κB via ERK1/2, in this case associated with an IKK-dependent phosphorylation of NF-κB on Ser538 [200, 201]. Furthermore, this group provided evidence for feed-forward mechanism in which activated NF-κB then increased the expression of Nox4, causing further stimulation of NF-κB through H_2O_2-dependent activation of ERK1/2 and inhibition of peroxisome proliferator activated receptor gamma [202]. Although they did not study the role of c-Src in these responses, it is known that Pyk2 is activated by c-Src directly and also indirectly via protein kinase C and phospholipase Cσ [75].

Of note, toll-like receptors, and particularly TLR4, assemble signaling complexes which play an important role in activating NF-kB in response to inflammatory stimuli. As described in the next section, these complexes contain multiple redox active thiols, conferring additional indirect redox-dependent mechanisms by which NF-kB is regulated.

13.6.2 Redox Regulation of TLR4

The TLR4-induced activation of NF-κB occurs through the formation of MyD88-dependent and MyD88-independent pathways. Stottmeier & Dick demonstrated that low concentrations of H_2O_2 cause MyD88 to form several different disulfide-linked conjugates, and that this process is inhibited by redox-nulling mutations of the seven cysteines present within the TIR domain on MyD88. Overexpression of MyD88 activated NF-κB activity, and this effect was increased by mutation of the seven TIR domain cysteines, implying that they are involved in inhibiting MyD88 function. Conversely, mutation of Cys113, which is in the region of MyD88 thought to be important for its interaction with IRAK4, greatly decreased NF-κB activity, suggesting that it promotes TLR4 signaling. Intriguingly, nucleoredoxin, a member of the Trx family of oxoreductases, engaged in disulfide exchange with the oxidized cysteines which were involved in conjugate formation, suggesting that it may function to reduce them, thereby modulating signaling through TLR4/MyD88 [203].

TRAF6, which operates within both the MyD88-dependent and independent pathways to activate the TAK1/TAK1-binding protein (TAB) 2/TAB3 complex to phosphorylate IKKβ, is also redox sensitive. TRAF6 is an E3 ubiquitin ligase, which when activated undergoes auto-polyubiquitination, allowing it to bind TAB2 and TAB3 and thereby activate TAK1 to phosphorylate IKKβ. Using HEK293 and HeLa cells, Chantzoura et al. demonstrated that under basal conditions, the E3 ubiquitin ligase activity of TRAF6 is suppressed by S-glutathionylation within its really interesting new gene (RING) finger motif, which is required for E3 ubiquitin ligase activity [204]. Moreover, activation of TRAF6 was associated with its deglutathionylation by glutaredoxin-1, the primary cytoplasmic member of the Trx family, indicating that glutaredoxin-1 is involved in regulating TRAF6 function. Notably, glutaredoxin-1 has been reported to exert analogous regulatory effects on signaling through NF-kB in lung epithelial cells by deglutathionylating cysteines on p65, IKKβ, and IKKα [205, 206].

An additional interaction between TLR4 and redox signaling emerged from the observations that stimulation of TLR4, for example, by lipopolysaccharide (LPS), the archetypical PAMP, recruits Nox4 to the TIR domain in HEK293T and U937 monocyte cells, and that this interaction results in ROS production which promotes the activation of NF-κB [207]. In a subsequent paper, this group showed that the C-terminal region of Nox4 binds in the region of amino acids 739–763 within the TIR domain of TLR-4. They then demonstrated that transfection of HEK293K cells overexpressing TLR4 with the C-terminal region of Nox4 blocked LPS-induced degradation of IκBα and activation of NF-κB, presumably by antagonizing the binding of endogenous cell Nox4. Additional studies in human aortic endothelial cells showed that knocking down NOX4 strongly inhibited LPS-induced degradation of IκBα and NF-κB activation [208]. Along the same lines Cho et al. found that in human pulmonary alveolar epithelial cells, the activation of NF-κB by LPS required the formation of a complex containing TLR4, MyD88, TRAF6, c-Src, p47phox, and Rac1 [209]. Using both pharmacological inhibitors and siRNA knock-down of various proteins, they presented evidence that this led to c-Src- and Nox-dependent oxidant production which then trans-activated platelet derived growth factor and epithelial growth factor receptors, resulting in phosphoinositide 3-kinases/protein kinase B (Akt) and P42/44 MAP kinase-dependent stimulation of NF-κB activity. It is tempting to speculate that formation of this type of complex may also be involved in the effects of inflammatory stimuli on PA remodeling in human Group 3 PH, although evidence for this is currently lacking.

13.6.3 Redox Regulation of HMGB1

Both the release and functional effects of HMGB1 are modulated by redox reactions [210]. Tang et al. showed that low (noncytotoxic) concentrations of H_2O_2 induced translocation to the cytoplasm and release of HMGB1 from human peripheral blood mononuclear cells and cultured

macrophage-like RAW 264.7 cells [211]. This may have been mediated by its binding to the nuclear export factor exportin 1, as they found that H_2O_2 increased the co-immunoprecipitation of these proteins. They also showed that H_2O_2-induced HMGB1 release was blocked by MEK and c-Jun N-terminal kinase inhibitors, with the latter also attenuating HMGB1 translocation to the cytoplasm. Furthermore, Tsung et al. reported that hypoxia-induced release of HMGB1 from hepatocytes was mimicked by H_2O_2, blocked by the antioxidant N-acetylcysteine, and occurred in the absence of cell damage, implying the involvement of oxidant-dependent active secretion [164]. The hypoxia-induced increase in oxidants (detected using dichlorofluorescein) was greatly diminished in cells from TLR4 knock-out mice, as was HMGB1 release, consistent with the concept discussed above that ROS production is integral to TLR4 signaling. Additional in vivo experiments revealed that hepatic ischemia-reperfusion injury in mice was also ameliorated by N -acetylcysteine and TLR4 knock-out and was associated with the release of TNF and IL-6, suggesting the involvement of the disulfide bridge containing-form of HMGB1 [166].

Indeed, HMGB1 contains three redox active cysteine residues, Cys23, Cys45, and Cys106, which are involved in oxidative post-translational modifications either via disulfide bond formation or hyperoxidation by sulfonic acid formation. These cysteines define the pro-inflammatory activity of HMGB1 as well as its inactivation [212]. In particular, the redox state of the cysteines dictates which receptor HMGB1 interacts with, and therefore defines the nature of its inflammatory actions. The fully reduced form of HMGB1, in which all three cysteines exist as protonated thiols, forms a complex with CXCL12, potentiating the affinity of this chemokine for CXCR4, its receptor, and in doing so potentiating leukocyte recruitment. At a moderate level of oxidative stress, Cys23 and Cys45 form a disulfide bridge while C106 remains a thiol. This form of HMGB1 binds to the TLR4/MD-2 complex, leading to a sustained NF-κβ-mediated release of cytokines such as TNF, IL-1, IL-6, IL-8, and macrophage inflammatory protein (MIP)-1. During prolonged and more severe oxidative stress, further (irreversible) oxidation of HMGB1 occurs, leading to all three cysteines forming a sulfonic acid state which renders it inactive [212]. The disulfide form of HMGB1, which does not act via CXCR4, also has the highest affinity for binding to RAGE [213]. Importantly, HMGB1 forms complexes with a large variety of other pro-inflammatory species, including cytokines, nucleic acids, histones, and lipopolysaccharide, and there is evidence that the interaction of HMGB1 with RAGE results in endocytosis of these complexes into the endolysosomal compartment. HMGB1 then acts to permeabilize lysosomes, allowing its partner molecules to enter the cytoplasm and bind to their receptors, causing, for example, autophagy or stimulating NF-κβ via TLR4 [214].

Dai et al. found that protein expression of total HMGB1 as well as its oxidation-induced tetrameric form (which was abrogated by chemical reduction with dithiothreitol or 2-mercaptoethanol) was increased nearly twofold in the lung of rats subjected to chronic hypoxia for 4 weeks [215]. HMGB1 monomer and tetramer were also increased in the lungs of rats treated with MCT for 3 weeks. Whether this tetramer is formed due to a disulfide bond between two dimer forms is yet to be established, however, it is likely that it included an active form of this protein [215].

It was mentioned earlier that rats treated with SU-5416/chronic hypoxia and HMGB1 neutralizing antibody developed lower RV pressure and less PA remodeling than control–treated animals [172]. However, owing to the fact that HMGB1 exists in three different redox states, it may be not possible to achieve a therapeutic effect with conventional inhibitors. P5779 compound has been proposed for precise targeting of extra-cellular HMGB1 in its disulfide form, disrupting its interaction with the TLR4 adaptor MD-2, and thus specifically and accurately disrupting HMGB1/TLR4 signaling [216]. Indeed, P5779 treatment resulted in lower RV pressure and less PA remodeling than scrambled peptide in MCT-induced PH in rats. Accordingly, when given in a delayed fashion (from day 21) to rats treated with

SU-5416/chronic hypoxia, P5779 alleviated RV pressure and hypertrophy, PA remodeling, restored RV function, and reduced PH-related mortality [172]. In a cell model, P5779 prevented HMGB1-induced migration of PA SMCs and their hypertrophy [172].

Taking together, these studies demonstrate a fundamental role for HMGB1 in PH pathogenesis and suggest it as a redox therapeutic target for this disease. Peptide that specifically blocks the interaction between disulfide HMGB1 and the TLR4 adaptor MD-2 improved disease severity and mortality, even when given to animals with established disease. Testing such approach in human Group 3 PH would be a logical next step.

13.7 Concluding Remarks

Systemic, pulmonary parenchymal and perivascular inflammation, together with oxidative stress are common in Group 3 PH (as evidenced by both animal models and human studies), and it is becoming increasingly apparent that there is an interplay between oxidants, inflammation, and Group 3 PH which involves oxidative modification of some key "rapid-acting" players and regulators of inflammatory responses. As it is a relatively novel area of research, it is likely that a similar interplay will be uncovered in other PH groups, in particular, Group 1 PAH, as earlier in this chapter we have provided evidence for increased oxidants production in this group. At this point, however, it is not entirely clear to what extent chronic hypoxia causes an elevation in levels of reactive species that cause inflammatory cascades by upregulating oxidant-sensitive signaling pathways, or whether hypoxia induces a "cytokine storm," thereby activating ROS-producing enzymes and producing reactive species which in turn modify proteins and affect redox signaling. The complexity of this interplay is further entangled by various pulmonary cell types which all may respond to oxidative stress in a heterogeneous way. Hence, a deeper understanding of how these pathways are redox regulated, by dissecting multiple redox cysteine modifications and addressing their functional outcomes in different pulmonary vascular cell types would lead to a better understanding of the PH pathology and novel translational opportunities. Furthermore, this would allow the development of specific redox-related therapeutic interventions for this detrimental clinical condition. With the ongoing development of novel drugs in the PH field, redox-based therapies should also be considered.

There are several approaches to target oxidation therapeutically. As discussed in Section 13.4, protein oxidation as well as protein oxidative post-translational modifications are limited by endogenous defense systems, including enzymes that remove oxidants or their precursors [57]. For example, disulfide bond accumulation is achievable by preventing its reduction by inhibition of their reducing systems (e.g., Trx/TrxR inhibition with gold compounds) [217]. Trx/TrxR inhibitors will likely induce oxidation of many proteins, and the selective role for the protein of interest can be addressed by employing 'redox-null' transgenic cells or animals and comparing them with wild-types. Ionizing radiation induces protein oxidation [218], and there are a few emerging reports on low-dose lung irradiation causing a beneficial effect in PH patients [219] and animals [220], possibly by eliminating bone marrow progenitor cells which are involved in inflammation and PH pathology. Although it is plausible that radiation-induced oxidation of proteins would mediate the reversal of PA remodeling, this field is only emerging, and meticulous 'cause and effect'studies need to be done to explore this further. In addition, some chemicals, usually emerging from high-throughput screens of electrophiles libraries, can directly induce protein oxidation, for example, of PKGIα [221]. Such compounds could bind to the oxidized form only, for example, HMGB1 [172, 212], or could prevent oxidation by covalent binding to redox-active cysteine, for example, EGFR [106]. Selected candidate compounds can be further screened in vitro for their ability to induce oxidation [221], potentially leading to a unique drug class, stimulating or blocking an endogenous mechanism responsible for in vivo oxidation of a protein of interest, depending on the desired functional outcome. From the other side, the use of

antioxidants in PH may need to be revisited, as it is clear that oxidants may have both deleterious and protective effects, at least in Group 3 PH. In addition, some substances that have been thought as putative antioxidants clearly can have oxidant activity (e.g., resveratrol), and so perhaps it may not be even entirely accurate to use the term "antioxidants" as an umbrella term. In any case, it seems apparent that interest in the exploitation of specific redox-based approaches to treat inflammation in PH is likely to increase in the coming years.

Acknowledgments Dr. Olena Rudyk is supported by the British Heart Foundation Intermediate Basic Science Research Fellowship (Sponsor reference FS/14/57/31138) and the British Heart Foundation Centre of Research Excellence Award (King's College London) (Sponsor reference RE/18/2/34213).

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Sex-Steroid Signaling in Lung Diseases and Inflammation

14

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Abstract

Sex/gender difference exists in the physiology of multiple organs. Recent epidemiological reports suggest the influence of sex-steroids in modulating a wide variety of disease conditions. Sex-based discrepancies have been reported in pulmonary physiology and various chronic inflammatory responses associated with lung diseases like asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and rare lung diseases. Notably, emerging clinical evidence suggests that several respiratory diseases affect women to a greater degree, with increased severity and prevalence than men. Although sex-specific differences in various lung diseases are evident, such differences are inherent to sex-steroids, which are major biological variables in men and women who play a central role to control these differences. The focus of this chapter is to comprehend the sex-steroid biology in inflammatory lung diseases and to understand the mechanistic role of sex-steroids signaling in regulating these diseases. Exploring the roles of sex-steroid signaling in the regulation of lung diseases and inflammation is crucial for the development of novel and effective therapy. Overall, we will illustrate the importance of differential sex-steroid signaling in lung diseases and their possible clinical implications for the development of complementary and alternative medicine to treat lung diseases.

Keywords

Estrogen · Testosterone · Progesterone · Asthma · COPD · Sex difference

14.1 Introduction

Sex is considered the greatest invention of all time: it not only is important in sexual reproduction, but facilitates the evolution of higher life forms and also had a profound impact on human culture, history, and society. In the current health care system, along with social sectors, "sex" (biological basis between females and males) and "gender" (roles in society and behaviors) variables have been considered important parameters for research and action [54, 126]. As a difference in biology among the sexes decides various diseases specific to males and females, however, the role of sex/gender in disease pathophysiology is not yet fully explored. Overall, the knowledge gap is high in many areas like (1) differences in

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_14

disease prevalence in men and women, (2) reasons behind that difference, (3) is there any difference in signaling mechanisms, and (4) how to design preventive and therapeutic treatment approaches concerning the change in disease prevalence. This situation has been reformed continuously with recent breakthrough research work and curiosity in determining and reacting to sex and gender differentials in disease conditions [7, 126]. However, to date, there is no clear evidence about the role of major sex-steroids (sex/gender determining factors) and their signaling in the pathophysiology of respiratory diseases. The goal of this chapter is to delineate the influence of sex-steroids (estrogens, progesterone, and testosterone) and their signaling in lung diseases.

Sex differences in health and disease conditions have been gaining considerable interest and widely explored in cardiovascular structure/function [15, 17, 194, 260], neurological research [117, 152, 160, 161, 210, 284, 324], and metabolism [30, 38, 127, 338]. Furthermore, the role of sex difference in clinical pharmacology is well evident and provides details about the basic mechanisms to understand their role in drug pharmacokinetics and pharmacodynamics for therapeutic optimization of the frequency of dose or their effects along with possible adverse effects [30, 38, 111, 211, 305]. There is growing evidence that reports the effect of sex-steroids in different lung components, and how it contributes to various diseases like pulmonary fibrosis, cancer, chronic obstructive pulmonary disease (COPD), asthma, and even pulmonary hypertension [10, 11, 13, 36, 37, 58, 71, 169, 171, 204, 215, 216, 294, 295], but still need more thorough investigations. Intrinsic sex differences in the growth of lungs and function are present even before birth *in utero* and are visible during the different stages of human life from childhood to old age [140, 257]. Changes in lung physiology and their functions due to sex differences during the various stages of the lifespan, such as puberty, pregnancy, menopause, and during aging propose additional modulatory roles of sex-steroids and/or their metabolites [45, 214, 215]. Multiple recent *in vitro* and *in vivo* studies established the crucial role of sex-steroids in modulating lung pathophysiology [8, 10, 13, 36, 37, 168–170, 204, 258]. For example, the prevalence of asthma is more common in boys, which is more than double the risk of developing asthma in girls [39, 60, 62, 163, 229], and as age increases interestingly this trend reverses. Adult women, after puberty, tend to show higher chances of asthma occurrence with greater severity compared to men [39, 60, 62, 163, 229]. Also, few female patients with existing asthma experience exacerbated asthma symptoms in their premenstrual or menstrual phases [2, 220]. Higher chances of asthma or poor prognosis in women suggest the importance to explore the role of inherent sex difference versus sex-steroids signaling especially female sex-steroid, estrogen *per se* in the pathophysiology of lung diseases. Furthermore, studying the comparative effects of sex-steroids and their locally produced metabolites will further improve our understanding of disease pathophysiology [214, 320].

Epidemiological studies demonstrate a critical role of sex-steroid signaling to control the mechanisms associated with the inflammatory response in the lungs [5, 49, 52, 55]. Multiple reports suggest a higher susceptibility in women to an inflammatory response with worse complications of lung disease associated with inflammation compared to men [19, 55, 67, 216, 307]. Besides, earlier *in vivo* studies showed sex-steroids differentially regulate lung immune responses in the mouse model of asthma [55, 109]. Notably, data from clinical studies have shown that circulating sex-steroid levels may contribute significantly in regulating innate immune responses and affects inflammation and airway tone during the menstrual cycle in female asthmatic patients; however, the exact signaling mechanism of sex-steroids and the associated mechanisms are multifaceted and not fully explored [96, 208, 234, 252, 282]. This chapter explores our attempts to comprehend the influence of sex-steroids signaling in lung diseases and inflammatory response in the lungs. Accordingly, exploring the mechanisms of sex-

steroids signaling becomes important in both ways of appreciating sex differences in normal lung physiology, and disease development and its ultimate therapeutic approach. The major goal of this chapter is to highlight the growth in research relating to the differential role of sex-steroids and the lung physiology.

14.2 Sex-Steroids and Their Biology

Sex is the major characteristic by which all individuals are differentiated with respect to reproductive organs and functions as female or male, whereas gender is described as a way of social conduct, manhood, and femaleness [23, 111, 294, 295, 342]. In 2001, the Institute of Medicine (IOM) of the National Academy of Sciences (NAS) demonstrated exclusive evidence showing that "sex matters"; especially "being male or female is an important variable in human that must be contemplated as a biological variable in disease pathophysiology as well, while designing and analyzing studies at all health research spectrum" [56]. As per IOM, biological variations correlates with sex-based differences among males and females, while the cultural and social distinction is characterized as gender [305].

Sex-based specific effects in the context of disease pathophysiology and occurrence are often connected to the changed sex-steroid milieu in males and females [341]. Sex-steroids, comprising estrogens, progesterone, and testosterone have been traditionally defined by their association in the normal functioning of reproductive system function. However, multiple earlier studies demonstrated the importance of sex-steroids in the field of cardiovascular [187, 194, 205, 260], metabolic [38, 127, 338], and neurological research [152, 161, 210, 225, 284]. Also, emerging clinical and epidemiological data have proven the crucial role of sex-steroids in the modulation of various lung diseases [8, 13, 36, 169–171, 278, 294, 324, 337]. Among all sex-steroids, estrogens and progesterone are reflected as key sex-steroids secreted by the ovary, while testosterone by the testes. Moreover, in addition to steroidogenic organs, sex-steroids are also produced by local peripheral tissues [215, 294]. After production, these hormones may act in a paracrine manner or circulate in the bloodstream to act at target tissues in an endocrine fashion [231, 341]. Typically, sex-steroids are produced in the gonads, adrenal glands, and the fetoplacental unit from common precursor cholesterol by well-known biosynthetic pathways (Fig. 14.1). In the ovary, the onset of the steroidogenic pathway is theca cells, where first cholesterol converted to pregnenolone using the cytochrome P450 (CYP) side-chain cleavage enzyme (CYP11A1) in the mitochondrial membrane. Whereas, a similar biochemical process happens in the Leydig cells of the testes. Pregnenolone further forms progesterone (via 3β-hydroxy-steroid dehydrogenase, 3β-HSD) or dehydro-epiandrosterone (DHEA) via CYP17A1 and then diverges toward the formation of androstenedione (via 3β-HSD) and sub-sequent testosterone (via 17β-HSD). Furthermore, testosterone converts to estrogens by formation of an aromatic ring by utilizing aromatase enzyme (CYP19A1) [63, 232, 277, 294].

14.2.1 Estrogen

Estrogens are major female sex-steroids that are important for sexual and reproductive development, especially in women. In women, there are three main steroidal estrogens; estrone (E_1), estradiol (17β-estradiol; E_2), and estriol (E_3) which are produced by steroidogenic cells (e.g., ovaries, placenta, and adipose tissue) as well as extrahepatic tissues [84, 189, 192, 206, 294, 295, 324]. Another form of estrogen called estetrol (E_4) is produced only during pregnancy [320, 321]. Estrogens are also produced by ovarian androgens, specifically testosterone and androstenedione via the steroidogenesis pathway in the presence of aromatase enzyme [294]. Endogenously, estrogens are metabolized by CYP enzymes CYP1A1 and CYP1B1 into two catechol estrogens, 4-hydroxyestradiol (4-OHE_2) and 2-hydroxyestraidol (2-OHE_2), while

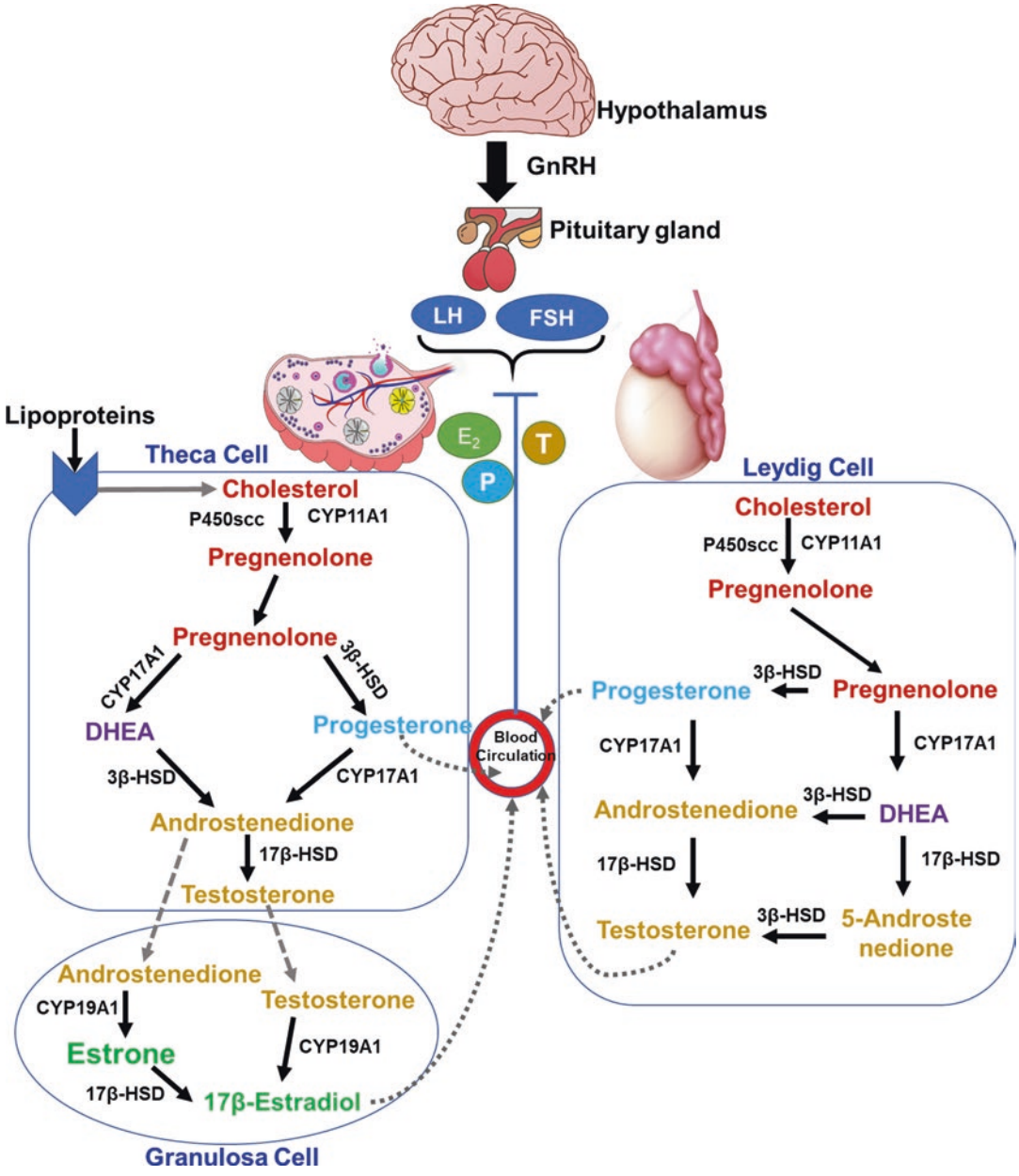

Fig. 14.1 Sex-steroid hormone biosynthesis from cholesterol. In steroidogenesis pathway cholesterol converts to pregnenolone which further cleaved via two different pathways and leads to the production of testosterone and subsequent estrogen. P450scc, P450 side chain cleavage; CYP11A1, cytochrome P-450, family 11, subfamily A, polypeptide 1 gene; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP17A1, cytochrome P-450, family 17, subfamily A, polypeptide 1 gene; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19A1, cytochrome *P*-450, family 19, subfamily A, polypeptide 1 gene

CYP3A4 converts estrogen into 16α-hydroxyestradiol (16α-OHE_2) [95, 190, 294, 295, 320, 324]. Furthermore, 4-OHE_2 and 2-OHE_2 are metabolized to methoxyestrogens via catechol-O-methyltransferase (COMT) and forms 4-methoxyestradiol (4-ME) and 2-methoxyestradiol (2-ME), respectively [22, 95, 190, 199, 202, 207, 288]. Recent reports from our groups and others have described the potential capability of the lung tissue to locally metabolize thereby inactivating sex-steroids and modulating the actions of sex-steroids at a cellular level [8, 108, 324]. Among all estrogens, E_2 possesses the highest estrogenic activity and is found to be more abundant in blood circulation, particularly during reproductive ages [294]. However, the level of estrogens fluctuates in women throughout their lifetime based on the menstrual period, pregnancy, menopausal periods, ages, and some other factors. In premenopausal conditions, normal estradiol levels, which range from 40 to 400 pg/mL drops considerably to almost 10 to 20 pg/mL after menopause [151, 308, 316]. During the menstrual cycle, estradiol increases in the follicular phase (from days 0 to 14) up to the range of 40 to 100 pg/mL, which can reach up to the highest level of 100 to 400 pg/mL on day 14. During the luteal phase, the levels of estradiol drop up to 40–250 pg/mL and return to lower levels before starting the next menstrual cycle (Table 14.1) [22, 151, 316]. Men also produce estrogen ranges from 15 to 50 pg/mL in normal, healthy condition [324], albeit comparatively lower than women. In men, testes form estradiol by converting testosterone by aromatase in Leydig cells and germ cells [151, 316]. Notably, the estradiol levels in elderly males are comparatively high (20–30 pg/mL) than menopausal females (10–20 pg/mL), which accord with earlier reports suggesting increased aromatase activity with age converts testosterone to estrogens in males [332]. Values provided in Table 14.1 for estradiol and progesterone in different life stages of humans are derived from multiple established reports.

14.2.2 Progesterone

Progesterone is considered as a second major female sex-steroid hormone that regulates important cellular pathways of the inner lining of the uterus to maintain the pregnancy [317]. Progesterone helps the transition of endometrial from a proliferative to the secretory stage successively, which forms blastocyst nesting, and provides essential support during the preservation of pregnancy [77, 263, 327]. Ovaries, placenta, and adrenal glands are the main gonadal organs that produce progesterone, additionally, brain and adipose tissue also produce a small amount of progesterone in both males and females [306, 317]. Progesterone also is a part of various metabolic and physiological modifications in different stages of life which includes puberty [40, 242], menstrual cycle [287, 336], and embryogenesis [133, 248, 313]. In addition to the reproductive system, progesterone also has a critical role in other tissue systems, such as the mammary gland in preparation for breastfeeding [51, 156], the cardiovascular system [314, 319], neurodevelopment process in CNS [124, 132, 158], and bones [274]. In the bloodstream, progesterone circulates bound form by attaching to cortisol-binding globulin and serum albumin. Circulatory progesterone in bound form possesses very short half-

Table 14.1 Circulating levels of sex-steroid in males and females during the various stages of life

		Estradiol (pg/mL)		Progesterone (pg/mL)	
Stages		Female	Male	Female	Male
Normal		40–400	15–50	<890	0–480
During menstrual cycle	Follicular phase (day 0 to 14)	40–100	N/A	200–1200	N/A
	Ovulation (day 14)	100–400		200–2000	
	Luteal phase (day 15 to 28)	40–250		2000–10,000	
After menopause/elderly men		10–20	20–30	<400	145

life (t1/2) of about only 5 min. After metabolism mainly in the liver, progesterone gets converted into sulfates and glucuronides and eliminated from the body through urine [317].

Progesterone levels are comparatively low in women during the follicular phase of preovulation of the menstrual cycle, which increases significantly after the ovulation phase and is elevated further in the luteal phase of menstruation [296]. In females, the progesterone levels in preovulation were observed <890 ng/mL [300] which alters during ovulation stages. During the ovulation period the levels of progesterone changes are episodic, prior to ovulation, it tends to decrease to about 200–1200 pg/mL which increases up to 2000–10,000 pg/mL after ovulation. However, in normal, healthy males the level of progesterone is 0–480 pg/mL [296], which declines with age up to 145 pg/mL (Table 14.1) [32]. During pregnancy, the levels of progesterone are maintained steadily by human chorionic gonadotropin (HCG) dependent corpus luteum. After 7 weeks of pregnancy, the placental membrane starts producing progesterone instead of a corpus luteum, and this phase is called the luteal-placental shift. After this phase, the levels of progesterone further increase high and are maintained throughout the pregnancy [83, 131, 324].

14.2.3 Testosterone

Testosterone is the main male circulating hormone produced by the Leydig cells of the testes. Testosterone regulates various sex-associated functions such as fabricating male sex characters, sex differentiation, production of mature spermatozoa, and fertility [115, 294]. Traditionally testosterone was considered as a male hormone, albeit it is also produced in theca cells of the ovaries and by the adrenal gland in minuscule amounts [173, 235]. The initial effect of testosterone in human life is observed as early as in the uterine fetus during the second trimester of pregnancy when the reproductive organs are identical in the fetus [78]. In addition to the major reproductive role, testosterone also employs a wide range of physiological effects in regulating the structure and function of nonreproductive organs, including the heart [78, 283], lungs [195], bone [159], liver [3, 250, 251, 299], intestine [255], kidney [105, 318], adipocytes [221], anabolism [75, 186], metabolism [115, 268, 310, 333], and cognition [25, 162] both in men and women.

Recent studies had been reported reduced serum testosterone levels in the elderly men, while the mechanistic approach is disputed among researchers, has this naturally occurred process or secondary comorbidities and associated factors play any role [331]. Moreover, comprehensive prospective trials, such as the Massachusetts Male Aging Study, evidently demonstrated a decrease in circulating testosterone is associated with aging [125]. This was further confirmed with some other longitudinal studies showing continuous drops in serum testosterone levels independent of any associated comorbidity and risk factors [247]. The gradual increase in age has shown to decline in free and total serum testosterone levels, with rises in gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Although the levels of LH increase with age, it does not correlate with testosterone secretion, which points to change in feedback mechanisms of these hormones with the unknown reason [287]. Furthermore, a decrease in free testosterone levels is greater than total testosterone, which eventually leads to reducing the biological activity of testosterone [247, 329]. The "normal" or healthy serum total testosterone levels vary widely among individuals over time, contingent to the normal functioning of the thyroid gland, serum protein levels, and other factors. But in general, men tend to have a higher testosterone level than women. In both males and females the peak level of total serum testosterone reaches around the age of 18 or 19 up to 3000–12,000 pg/mL and 80–330 pg/mL, respectively, then it gets declining throughout the remaining age (Table 14.2; values derived from earlier published reports) [41, 178, 290]. Interestingly, earlier reports also suggest that testosterone levels decrease with age as the aromatase activity increases, which converts testosterone to estrogen [332]. As per the American Urological Association's (AUA) recent

Table 14.2 Average total testosterone levels in male and females through various ages of life

Age	Male	Female
18 years or below	50–9750 pg/mL	80–330 pg/mL
18 to 19 years	3000–12,000 pg/mL	200–750 pg/mL
19 years and above	2400–9500 pg/mL	40–480 pg/mL

guidelines, the serum testosterone levels of equal to 3000 pg/mL are considered to be normal in males. While for women at a young age (19 years and up), the serum testosterone from 80 to 330 pg/mL are considered to be normal [41]. Other androgens, including the three pro-androgens: dehydro-epiandrosterone-sulfate (DHEAS), dehydro-epiandrosterone (DHEA), and androstenedione (A) are followed by conversion to active sex-steroids; testosterone and dihydrotestosterone (5α-DHT/DHT) [53]. Here, estrogen or DHT can be formed upon irreversible aromatization of testosterone [66, 113].

14.2.4 Crosstalk Between Sex-Steroids

Sex-steroid hormones include estrogens, androgens, and progesterone, overall which are synthesized from cholesterol via steroidogenic pathways [311] and are present in both sexes (males and females) from the time of birth, while the levels in circulation vary greatly [93, 104, 340]. It is always believed that crosstalk among sex-steroids may occur only in women due to predominant levels of estrogens and progesterone [295]. However, multiple clinical and epidemiological studies reported the presence of all three hormones in both males and females at a variable concentration [90], which provides the possibility for local metabolism and interaction between these hormones in both sexes.

Sex-steroids control cellular mechanisms and functions through the membrane, intracellular, and/or nuclear receptors, which further interact with discrete nucleotide sequences to alter gene expression [294]. Sex-steroid receptors such as estrogen receptors (ERs; ERα and ERβ), progesterone receptors (PRs; PR-A and PR-B), and androgen receptor (AR) are typically considered as nuclear receptors [294] and studies revealed that sex-steroid receptors generally have some common structural domains: C-terminus ligand binding domain (LBD), variable N-terminus with transcription activation function (AF-1) domain, mid-region DNA-binding domain (DBD), and hinge region. The LBD region of C-terminus carries another AF-2 domain which acts as a second region for transcriptional activity and various functions depend upon the interacting ligands [28, 227]. These similarities between the sex-steroid receptors improve the chances of interaction/crosstalk at multiple signaling levels in the target cells. Whereas specific ERβ activation shows opposite effects to ERα in cell proliferation [9, 11–13, 144], extracellular-matrix (ECM) modulation, and calcium handling. In the nucleus, ERα and ERβ can act as heterodimers or homodimers. Notably, emerging reports also propose the change in transcriptional activity due to heterodimer formation between ERα and AR [34, 294]. Unfortunately, limited data are available suggesting the interaction between sex-steroids, whereas minimal data showing the effects in the lung [294]. It would be very interesting to study the interaction and signaling mechanisms of combined sex-steroids at a different time of lifespan in both males and females. Additionally, to understand the contribution of individual sex-steroids signaling at the cellular and molecular level with their site of action will provide future prospective to better understand the pathophysiology of the disease.

14.3 Role of Sex Steroid Signaling in Lung Diseases

Sex-steroid signaling and their effects are very complex, and cell-, tissue-, and context-dependent. Although sex-steroids are primarily gonadally derived, they are also locally produced in many tissues, and mediate their cellular actions via genomic and nongenomic receptor activation, which further complicates the overall interpreta-

tion. The nongenomic and genomic effects of sex-steroids have been extensively reviewed in various tissues including lung tissues [8, 13, 36, 168, 293–295, 323–326]. Conventional thinking was steroid receptors are generally located in the cytoplasm of target cells, which upon activation translocate into the nucleus to change the gene expression, which needs at least 30–60 min. Conversely, additional signaling regulatory actions of sex-steroids are displayed in seconds to a few minutes. This time is too rapid to produce genomic changes due to which they are typically termed nongenomic or rapid actions, which distinguish them, from classical sex-steroid dependent genomic actions of regulating gene expression [215, 294, 295]. Nongenomic effects of sex-steroids typically are diverse, from activation of adenylyl cyclase (AC), protein kinase C and A (PKC, PKA), mitogen-activated protein kinases (MAPKs), and heterotrimeric guanosine triphosphate-binding proteins (G proteins) [341]. These nongenomic effects of sex-steroids sometimes are mediated via the classical steroid receptors, which can act as ligand-activated transcription factors, while on other occasion's classical sex-steroid receptors do not involve in these rapid effects [129, 341, 346]. Emerging indication suggests that the classical sex-steroid receptors can be present at the plasma membrane, which upon activation may trigger a chain of reactions in the cytoplasm [43, 196, 298]. Identification of interaction domains on the classical sex-steroid receptors responsible for the nongenomic effects or functions, and separation of this function from the genomic effects, should pave the way to a better understanding of the receptor-specific action of sex-steroids for therapeutic management of lung diseases.

14.3.1 Asthma

Asthma is a respiratory disorder associated with chronic inflammation causing significant morbidity and mortality worldwide [13, 20, 89, 191, 269–271, 273, 279, 292]. It is an intricate disorder that involves diverse pathophysiology affecting the airway structure and function in the lungs [270, 272, 280, 324]. The most substantial characteristic of asthma is a chronic inflammation of conducting airways, which is often associated with airway hyperresponsiveness (AHR) and remodeling [89, 191, 269–271, 292]. Although multiple genetic and environmental factors play a crucial role in the prevalence of asthma, sex/gender difference also acts as a notable driving force [62, 63, 294, 295]. It is challenging to determine the role of sex-steroids and their signaling mechanisms involved in asthma considering the wide array of factors affecting asthma pathophysiology. Here, intrinsic sex differences play an important role in lung physiology [294, 324], which concurs to apparent epidemiological evidence suggesting the impact of sex differences in asthma incidence, prevalence, and severity [39, 63, 293, 294, 303]. Clinical evidence suggests the prevalence of asthma is more common in boys, which is more than double the risk of developing asthma in girls [39, 60, 62, 163, 229], and as age increases this trend reverses. Adult women, after puberty, show greater incidence, frequency, and severity of asthma compared to men [39, 60, 62, 163, 229]. Similarly, few female patients with asthma experienced an exacerbation of asthmatic symptoms during premenstrual or menstrual phases of their cycle, suggesting a potentially crucial role of sex-steroids, especially estrogen [2, 42, 60, 62, 86, 179, 220, 293, 294]. Sex differences in the development of fetal lungs, as well as the maturation of lung tissues in adults, have been recognized to estrogen [29]. The alveoli development in females is estrogens dependent and control alveologenesis by ERα and ERβ activations [217, 259]. Estrogen treatment tends to show increased surfactant production in the fetal lung [74], which correlates to more rapid lung maturation in the female fetus than in the male fetus [322]. Although the number of alveoli per unit area and alveolar volume has no difference between males and females, males develop larger lungs with larger conducting airways in adulthood [212]. However, women are more vulnerable than men to the occurrence of several lung diseases; further implicates an estrogen's role in this scenario. In connection to this, studies also reported that the levels of estrogen, in particular,

E_2 tend to increase in the bloodstream in pregnancy and may worsen lung disease such as asthma [22, 65].

Divergent sex-steroid signaling is described in the healthy and diseased condition of the lungs [294, 295, 328, 337]. Notably, female sex-steroid estrogen has a well-recognized function outside the reproductive system in different organs and tissues in both males and females, which includes cell growth, synthesis, and differentiation [81]. Recent advances in estrogen biology and a wide range of potential effects of estrogen on lung physiology in diseased conditions considerably increased our interest in exploring divergent estrogen signaling and its role in influencing the airway structure and function [325, 326, 328]. In humans, estrogen demonstrates its effect mainly via full-length ERα and ERβ, which comes under the nuclear receptor family of transcription factors [180, 238]. Earlier studies, including our own, have demonstrated the increased expression of both ERα and ERβ in asthmatic and nonasthmatic human airway smooth muscle (ASM) cells and found that ERβ expression is significantly greater in the inflammatory/asthmatic condition in human ASM from males and females [18, 85, 155, 164, 325].

The genomic effects of nuclear ERs activation or those translocating from the cytosol to the nucleus are well reported. But, the role of membrane-bound activated ERs, their nongenomic activities, and associated downstream transcription factors are not fully explored [18]. In addition to full-length ERα (ERα-FL), there are two shorter (truncated) isoforms of ERα (ERα-36 and ERα-46) which do not have the AF-1 domain and show complex effects on ERα-FL [18]. ERβ, on the other hand, has five known variants (V1-5), while the modulatory signaling mechanism of ERβ splice variants during inflammation/asthma [18, 144] remains unclear. The overall cellular effects of estrogens vary depending on the nature and effects of the ligand binding (genomic vs. nongenomic), which makes for more complicated signaling within the tissue [91, 137, 139, 312]. Recent studies, including our own in ASM, have shown that acute exposure of E_2 at physiological concentration inhibits plasma membrane influx via ERα, resulting in reduced ASM intracellular calcium ($[Ca^{2+}]_i$) [326]. Additionally, PKA and cAMP levels in ASM were increased due to acute exposure of E_2 [325]. Further, long-term exposure of E_2 revealed the divergent effects of ERs in regulating $[Ca^{2+}]_i$ in normal and asthmatic conditions. ERα activation showed increased $[Ca^{2+}]_i$ response, while ERβ activation tends to decrease $[Ca^{2+}]_i$ response in normal as well as asthmatic conditions. Here, ERβ activation effectively reduces $[Ca^{2+}]_i$ responses, particularly in asthmatic ASM, via enhanced sarcoplasmic reticulum Ca^{2+} ATPase 2 SERCA2 function and inhibition of pathways involved in activating voltage-gated L-type Ca^{2+} channels (LTCC) [36, 37]. Sex-steroid signaling on different calcium regulatory channels in ASM is represented schematically in Fig. 14.2. In addition to $[Ca^{2+}]_i$ regulation, studies showed differential ER signaling in regulating ASM proliferation and airway remodeling in the context of asthma [11, 13]. Here, ERβ activation downregulated platelet-derived growth factor (PDGF)-induced proliferation via ERK/Akt/p38 MAPK pathways in human ASM cells, while ERα did not show any significant changes [13]. Additionally, ERβ activation also dampened TNFα or PDGF-induced ASM-derived ECM production and deposition via suppressing NFκB signaling [11].

Less is known about the role of progesterone in asthma, as most of the studies related to women's sex-steroids are considerably focused on the regulatory effects of estrogen in lung diseases specific to asthma. Progesterone exerts its effect via activation of PR-A and PR-B with subsequent gene transcription [108, 294]. Both PR receptors are transcribed from the same gene, with minor changes in the truncated N-terminal domain of PR-A [97, 116, 249]. Typically, progesterone has a similar affinity to both PRs, with varied transcriptional activation. Here, PR-B is a strong promoter of transcriptional activities as reported in multiple cell types [116]. It has been shown that activation of PRs leads to recruitment of steroid receptor coactivator (SRCs; SRC-1, SRC-2, SRC-3), and CBP/p300, which are crucial regulatory proteins [226] that modulate histone acety-

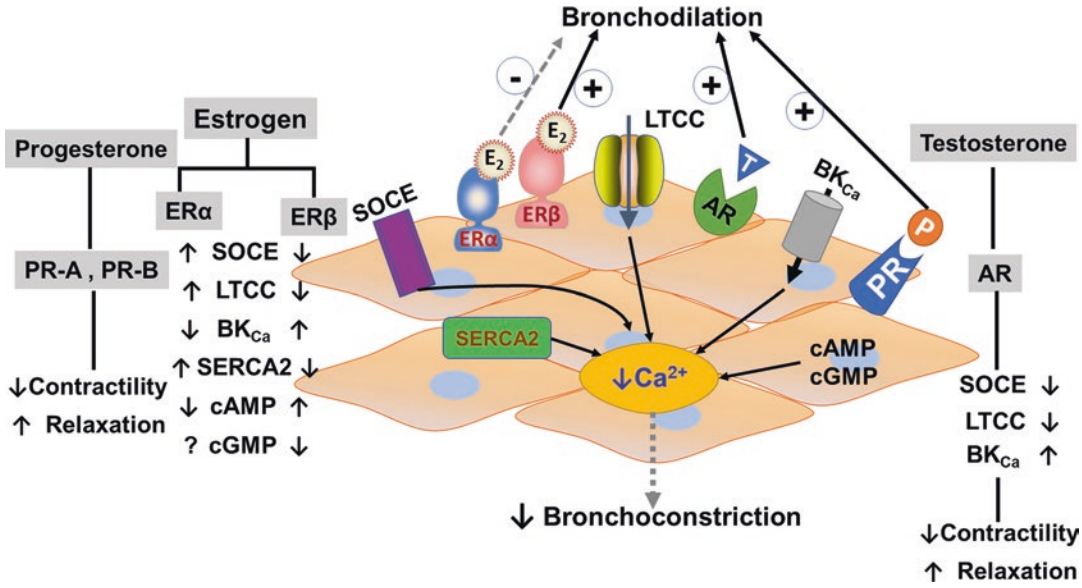

Fig. 14.2 Sex-steroid signaling and their airway smooth muscle cellular mechanism in the regulation of intracellular calcium in lung disease

lation/deacetylation and chromatin remodeling. In addition to nuclear receptor activation, PRs also promote and regulate numerous cellular signaling mechanisms independent of nuclear activation [294]. Limited studies have shown that progesterone decreases contractility and induces relaxation of bronchial smooth muscle [262]. Furthermore, progesterone has shown potent vasodilator effect than estrogen and testosterone in pulmonary arteries of both male and female rats [98]. Clinical studies reported a positive association of serum progesterone with peak expiratory flow rate during the luteal phase of the menstrual cycle, when the levels of progesterone are high [209] (Fig. 14.3).

In comparison to female sex-steroid hormones, the association of androgens in asthma prevalence is more apparent, albeit only by limited data that is available. Multiple studies involving androgens have shown anti-inflammatory activity by decreasing the Th2 cell response [69]. On the contrary castration in males exacerbates asthmatic symptoms [69]. Besides, the severity of asthma in men remains relatively constant from puberty to aging. However, when serum testosterone levels are declined due to aging, the increased asthma severity is observed [59, 294, 324], signifying the positive role for testosterone. Interestingly, even in women, testosterone improves asthma symptoms [344]. Androgens (testosterone and DHT) are well reported to modulate the contractility of various smooth muscle types by regulating the $[Ca^{2+}]_i$ levels [44, 100, 245, 261, 289, 293, 324]. DHT, an active metabolite of testosterone at acute exposure (nongenomically) has been found to relax the smooth muscle by decreasing Ca^{2+} influx through large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels, store-operated Ca^{2+} entry (SOCE) channels, LTCC, and myosin light chain (MLC) phosphorylation [106, 165, 188, 245, 261, 291, 304]. In a mouse model of asthma, testosterone and DHT have shown relaxant effects on tracheal smooth muscle cells via AR [69, 110, 224, 236]. Studies using guinea pig airway tissues showed that AR activation modifies SOCE and LTCCs [165] along with IP3 (inositol 1,4,5-trisphosphate) receptors [246], independent of epithelium and potassium channel [107]. Similarly, the dehydro-epiandrosterone (DHEA) was also shown the same types of effects in the asthma model of guinea pig [110]. Further stud-

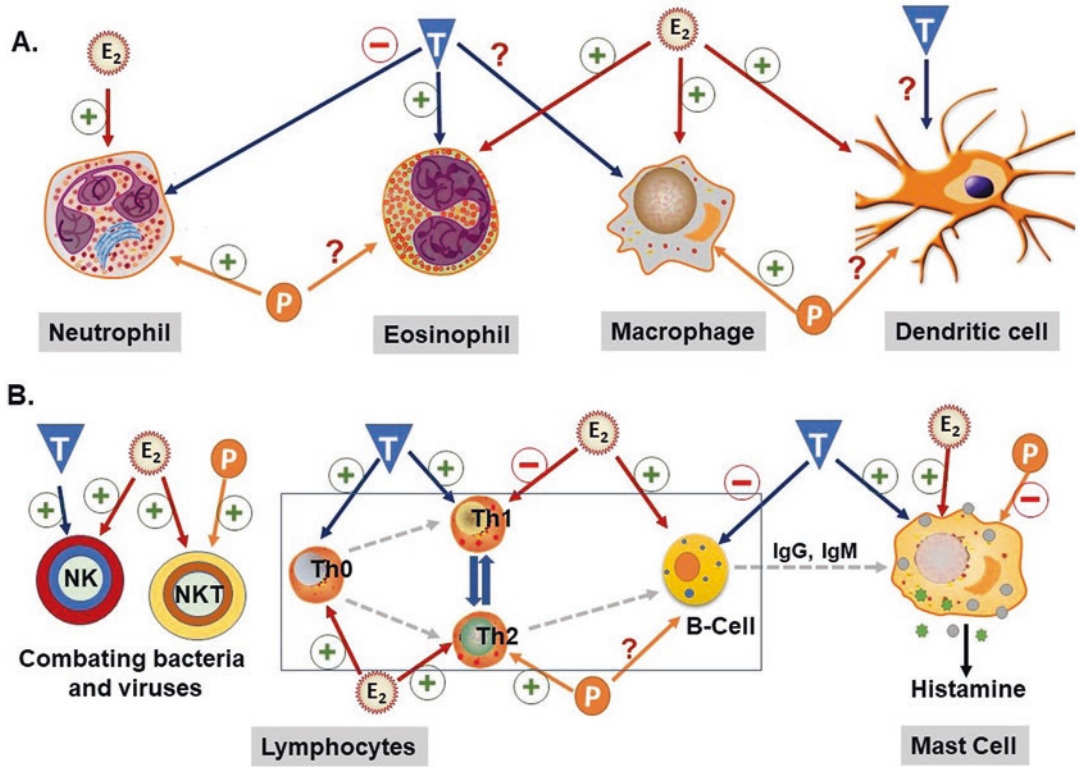

Fig. 14.3 Sex-steroid effects on the immune cells. The effects of estrogen (E), progesterone (P), or testosterone (T) on dendritic cell, neutrophils, eosinophils and macrophages depicted in (**A**). Many substantial inflammatory components like dendritic cells and macrophages/monocytes involved in various inflammatory lung diseases. These cells, particularly initiated after the first response of antigens, CD4+ lymphocytes, regulatory T-cells (Th0), B-lymphocytes, and other immune cells depicted in (**B**)

ies also reported the relaxant effect of testosterone on the epithelium in the rabbit trachea [188]. Studies using human ASM showed functional expression of AR in both males and females, which is upregulated during inflammation/asthmatic condition [171]. Furthermore, activation of AR significantly reduced Ca^{2+} influx via LTCCs and SOCE [171].

In contrast to human epidemiological data, animal studies relating to sex-steroids and asthma have been shown conflicting results. In a mouse model of asthma, estrogen appears to protect against airway hyperresponsiveness [92, 219], while progesterone aggravates inflammatory airway disease [146]. Furthermore, studies using male and female mice along with ovariectomization (OVX) mice, have reported that estrogen decreases AHR in an ovalbumin-induced model of allergic asthma [219]. Furthermore, spontaneous airway hyperresponsiveness has been reported in ERα knockout mice [61]. Additionally, *in vivo* studies from our group using a murine model of asthma showed that ERβ activation with specific pharmacological agonists downregulated AHR and airway remodeling [10]. These results were further confirmed by our subsequent study, where we evaluated receptor-specific effects of circulating endogenous estrogen in regulating AHR and remodeling using ERα and ERβ KO mice and found that ERβ KO upregulates airway remodeling and AHR [169, 170]. Interestingly, we found that ERα was either less effective in modulating airway structure/function in the mouse model, or in fact, worsened AHR and remodeling. Studies suggest progesterone has a stimulatory effect in the development of Th2

cells and inflammation with subsequent airway hyperresponsiveness in an allergic model of lung inflammation [87, 138]. In a study using a mouse model of influenza, progesterone treatment declined inflammation and improved lung function by restoring lung tissue homeostasis [138]. Overall, the above data, although limited, suggest very complex, somewhat synergistic, and opposing effects of female sex-steroids, with totally contrasting effects of male sex-steroids in asthma. In order to better understand the role of specific sex-steroids and their crosstalk in the context of asthma pathophysiology, detailed studies are warranted concerning specific cell types, life stages of a person, comorbidities, and the duration and concentration of exposure.

14.3.2 COPD

COPD is a chronic inflammatory lung disease that causes airflow blockage and difficulty in breathing [63]. The common symptoms associated with COPD are excess mucus (sputum) production, frequent coughing, wheezing, and shortness of breath [21]. In most cases, COPD is caused due to long-term exposure to irritating gases or particulate matter, most often from cigarette smoke [294]. Emphysema and chronic bronchitis are the most common conditions, which contribute to the progression of COPD. Emphysema is associated with damage to the alveoli at the end of smaller air passages (bronchioles) in the lungs. While chronic bronchitis is characterized by inflammation of the lining of the airways and subsequent cough and excess mucus production [26, 172, 278, 294, 295]. Historically, COPD was always considered to be a disease that mainly affects males due to the higher prevalence of cigarette smoking [26, 278]. However, epidemiological data in the last few decades showed steadily increasing rates of COPD in females [316, 343]; this could be due to increased smoking by females or other modulators.

In the past from 1999 to 2007, the rate of hospitalizations due to COPD significantly dropped for both men and women, but death rates associated with COPD were only lowered in men suggesting a relation between sex-steroids and pathophysiology of COPD [278]. The age-related deaths in U.S. men with COPD are approximately 30% higher than the rates for women [278]. However, as per the updated Centers for Disease Control and Prevention (CDC) data in the United States suggest that the overall death rates due to COPD in women are greater since the year 2000 [294]. This thinning gap in death rates represents a continuing shift of paradigm in the relative burden of COPD in women [4, 21]. In a recent clinical study with a large population of patients with COPD, females demonstrated more severe COPD with early-onset disease (COPD at age < 60 years) and greater susceptibility to COPD with lower tobacco exposure [237, 278, 307]. Additional evidence suggests a greater prevalence of emphysema in men than women [278]. However, the reason for the change in disease patterns in men and women is largely unknown. The airways of female lungs are relatively smaller than that of males with the same lung volume, so there may be a chance that women's lungs are exposed to a higher concentration of cigarette smoke per unit area of small airway surface [26, 233]. Additionally, the effect of sex-steroids on the prevalence of COPD is not yet explored. Reports exhibit that hormone replacement therapy in women did not affect the incidence of COPD [27]; however, another study showed improved lung function in postmenopausal women after hormone replacement therapy [64]. Interestingly, the metabolism of cigarette smoke may change in men and women since sex differences control the expression and activity of cytochrome P450 enzymes [305]. It is still not clear whether sex-steroids have a protective or detrimental role in COPD. As, studies reported estrogen via ERα activation promotes proliferation and thus potentiates the effect of cigarette smoke, toxic gases, and particulate matters on airway cells, leading to airway narrowing which is more common in women [294]. On the contrary, male sex-steroid testosterone relates to occurrence of greater emphysema due to alveolar destruction, while testosterone replacement

therapy (TRT) has shown to decline disease progression in men with COPD [24]. Furthermore, sex-steroids can also modulate various immune responses in a very complex way and contribute to the overall response of the lungs to cigarette smoke in the context of COPD. Overall, these data suggest that women may be more susceptible to developing COPD in response to less cigarette smoke exposure in their lifetime compared to men [26, 294].

14.3.3 Pulmonary Fibrosis

Pulmonary fibrosis (PF) is a progressive fibrotic lung disease-causing scarring in the lungs with unknown etiology [294]. When PF persists for a long time, the scar tissue starts destroying the normal lung tissue and makes it hard for oxygen consumption in the bloodstream. Eventually, low blood oxygen levels and the stiffness in lung tissue due to scarring can lead to shortness of breath, particularly when walking and exercising [138, 317]. Substantial epidemiological reports suggest that sexual dimorphism impact the prevalence and severity of idiopathic pulmonary fibrosis (IPF) [62, 140]. The prevalence of IPF is more common in men with a worse prognosis than women [128, 256, 330]. However, women showed a higher symptomatic burden of IPF when compared to men with worse health-related quality of life [140]. Yet, the role of sex-steroids in modifying these differences in prevalence and symptomatic changes of IPF in men and women are currently unknown.

Although in the recent period, the understanding of IPF biology has improved, few animal studies sought to understand the role of sex differences in animal models [114, 335]. Studies using a rat model of PF reported the role of gender difference in experimental PF, where female rats displayed a higher degree of fibrosis than males in bleomycin models of PF. Upon OVX, female rats showed improvement in fibrosis while estrogen treatment worsened the response, which concludes the detrimental effect of estrogen in PF [114]. Interestingly, these changes in PF severity due to sex differences are reversed in a mouse model of bleomycin-induced fibrosis [142]. From these conflicting data it's hard to interpret the exact role of sex-steroids in PF. A recent study showed the expression of PRs in the fibrotic areas of patients with usual interstitial pneumonia, but whether the PRs signaling mechanism exerts any role in these effects is still unclear [228].

14.3.4 Role of Sex-Steroids in the Pathophysiology of Rare Lung Diseases

Rare lung diseases (RLD) affect an estimated 2.5–5.5 million people in North America and Europe [222]. RLD includes a broad spectrum of pathophysiological conditions occurring either individually or as a cluster affecting every 1 in 2000 individuals [222]. Few of the widely known RLD's include alpha-1 antitrypsin deficiency (AATD), pulmonary lymphangioleiomyomatosis (LAM), pulmonary alveolar proteinosis (PAP), and lung sarcoidosis (LS). Research on RLDs is comparatively less due to their rare incidence and prevalence. Among the several RLDs, few have been extensively researched in LAM and AATD [222], while others are yet to be explored. Identifying the pathophysiology and epidemiology of these RLDs is quintessential to better understand the disease manifestation and to be able to manage these diseases with appropriate therapeutics. Few among these RLDs such as LAM, AATD, and LS show a gender disparity suggesting a plausible role for sex-steroids (Table 14.3). Here, we describe the role of sex-steroids in RLDs that have been evidenced to show gender disparities.

14.3.4.1 Pulmonary Lymphangioleiomyomatosis

The pathophysiology of lymphangioleiomyomatosis (LAM) involves abnormal invasion and proliferation of smooth muscle-like cells, leading to the destruction of lung parenchyma [148, 150, 223]. LAM almost exclusively affects women, especially during childbearing age, suggesting a crucial role of female sex-steroids [80, 123, 183].

Table 14.3 Reported roles of sex-steroids in rare lung diseases

	LAM	AATD	LS	Other RLDs
Estrogen	↑Disease progression ↑Proliferation Mechanistic evidence available (ERα ↑ severity and progression)	↑ A1AT levels Limited data No mechanistic studies	Females have a higher risk, suggesting a role for estrogen and progesterone	No apparent gender differences. Sex-steroid mechanisms yet to be explored
Progesterone	↑Disease progression ↑Proliferation Limited data with no concrete mechanistic evidence	–		
Testosterone	–	–	–	

Several studies demonstrated the expression of ER's (α and β) and PRs in LAM cells [35, 182], with no information on the expression of ARs. Here, several clinical studies indicate estrogen plays a predominant role in the development and progression of LAM. The progression of LAM is higher in pregnant women and in women taking exogenous estrogen via HRT, while it slows down after menopause. Another female predominant steroid that might contribute to LAM is progesterone. Furthermore, evidence from multiple studies suggests that estrogen positively regulates proliferation in tuberous sclerosis-2 (TSC2)-null AML cells, eker leiomyoma tumor-3 (ELT3) cells, and 621-101 AML cells via c-myc and ERK pathways [130, 149, 197]. Also, matrix metalloproteinase-2 (MMP-2) expression and activity are upregulated in LAM cells in the presence of estradiol [122, 197]. *In vivo* studies with TSC2 null cells as xenografts in mice showed enhanced tumor growth in the presence of estrogen, whereas in OVX and aromatase inhibited mice, the tumor growth slowed down indicating the importance of estrogen [76, 275, 276].

Although the theory of estrogen being the major cause of LAM is being aggressively pursued, one major caveat to consider is males produce progesterone albeit significantly less compared to females [218]. The fact that progesterone is high during the luteal phase of the menstrual cycle, during pregnancy and with oral contraceptives and its consistency with situations associated with LAM progression further strengthen the need for exploring progesterone [276]. Furthermore, few studies suggest a higher PR to ER expression ratio in LAM cells [112]. The overall role of progesterone in LAM is still inconclusive as research is still in early stages with contradicting findings. Another study suggests progesterone along with estrogen synergistically potentiates proliferation in ELT3 cells [315], while other studies suggest progesterone inhibits estrogen-mediated proliferation in the same cell type [121, 153]. Further, *in vivo* murine studies suggest, estrogen is required for tumor growth, while progesterone alone had no effect on the tumor or did not modulate estradiol-induced growth [275]. Overall, the role of estrogen in LAM has been extensively studied by multiple groups establishing its importance in the progression of LAM. However, the role of progesterone and testosterone in LAM is yet to be identified and will probably shed valuable insights into the mechanisms behind the incidence and progression of LAM and its gender specificity.

14.3.4.2 Alpha1-Antitrypsin Deficiency

Alpha1-antitrypsin (A1AT) deficiency (AATD) is a rare lung disease associated with declining levels of A1AT resulting in the destruction of the lungs by neutrophil elastase [88, 99, 266]. In a few cases, A1AT is produced, but functionally inactive due to abnormal folding of the proteins during their synthesis. Due to its rare incidence and prevalence, there is very limited progress in terms of understanding the epidemiology and pathophysiology of AATD. However, one study reported disparities in gender, age-related hormonal changes, and oral contraceptive use in regulating A1AT levels. According to this study,

premenopausal women and women undergoing HRT or using oral contraceptives had higher circulating levels of A1AT, which was reversed in postmenopausal women implicating a potential role for female sex-steroids in controlling circulatory levels of A1AT [301]. Here, the role of progesterone and androgens is yet to be explored. Overall, the direct association of female sex-steroids and A1AT levels suggest a protective role for female sex-steroids in AATD; however, the exact mechanisms are still not known.

14.3.4.3 Lung Sarcoidosis

Sarcoidosis is a systemic inflammatory disease that most commonly targets lymph nodes, lungs, skin, and eyes [157]. Studies suggest the risk of sarcoidosis in slightly higher in females than in males [147, 239, 345]. The current study at a tertiary referral center found that 65.5% of the patients were females, which suggests a plausible role for sex-steroids [166]. This percentage goes upward to 70.3% in the age group of 70 years or older [72]. Although, one study in the African American race suggests increased incidence and risk of sarcoidosis during menarche, menopause, and parity; however, it is still inconclusive when it comes to a wider population [82]. Currently, there is no information available on the role of estrogen, progesterone, and testosterone in sarcoidosis and the mechanisms involved.

14.4 Role of Sex-Steroids Signaling in Influencing Immune Responses in the Lungs

Being the most exposed organ, lungs are in a constant battle against exposure to a wide array of pathogens, allergens, toxins, air pollutants, and irritants [243]. Inflammation is the primary and key response to a multitude of insults such as hypersensitivity, infection, and trauma [243]. The constituents of the inflammatory response in the lungs vary depending on the type of pathogen mediated infection, allergens, or injury [1, 309]. During inflammation, numerous types of inflammatory cells are recruited in the lungs, which along with the resident lung cells orchestrate a plethora of inflammatory responses to address the specific need [33, 103, 201]. This orchestrated inflammatory milieu is skewed during disease conditions leading to an imbalance in T-cell mediated response [201]. Since, lungs are vital organs for gaseous exchange, prolonged or excessive inflammation leads to obstruction of the airways resulting in life-threatening conditions [243].

Inflammation is a direct result of the immune responses developed against a specific stimulus, either external or internal. These immune responses are divided largely into two categories, innate and adaptive, which play a pivotal role in host defense mechanisms [213]. Innate mechanisms include leukocytes and epithelium, which are the primary first-line barriers in the lung. On the other hand, adaptive immune mechanisms are far more complex and are driven and influenced by innate immune responses [213]. Here, we present the facts based on earlier and ongoing studies on the role of sex-steroids in regulating airway inflammation by modulating the function of various immune cells and resident lung cells.

14.4.1 Role of Sex-Steroids in Immune Cells

Epidemiological and clinical evidence suggests sex-steroids play a crucial role in regulating inflammatory milieu, especially in the lungs. The female predominance in allergic and autoimmune diseases has created a need for exploring sexual dimorphism and the role of sex-steroids in the immune system. This phenomenon is especially even more complicated in females as reproductive status affects the progression and severity of many diseases. Studies suggest sex-steroids have the potential to bind to various immune cells and resident lung cells influencing the overall immune responses and thereby inflammation [213]. Estrogen plays a pivotal role in regulating inflammation by regulating the immune responses in multiple cell types. The exact role of estrogen in inflammation is complex, as studies suggest it suppresses inflammation in chronic inflamma-

tory diseases, while on the other hand, it produces pro-inflammatory cytokines in autoimmune diseases [22]. In addition, the effect of estrogen on inflammation is highly varied based on the concentration. Allergic airway diseases often exhibit exaggerated inflammation as a result of immune responses elicited by T-cells, B-cells, dendritic cells, macrophages, eosinophils, and neutrophils. These cells release a complex network of inflammatory milieu, which is Th2 biased in allergic diseases such as asthma, resulting in eosinophilia, airway inflammation, and AHR. Here, we limit the discussion to characteristic roles of different immune cells and existing findings on the role of different sex-steroids on regulating the activity of these cells.

14.4.1.1 Dendritic Cells

Dendritic cells (DCs) originate in the bone marrow and reach the lung via circulation [201]. They typically reside around the airway epithelium, alveolar septa, pulmonary capillaries, and airway spaces [201]. DCs along with macrophages serve as the first line of defense against inhaled agents [243]. Further, DCs are antigen-presenting cells (APCs) that have the potential to stimulate naïve T cell proliferation and differentiation. The mechanism of DCs includes identification, ingestion, and processing of antigen followed by migration to lymph nodes leading to a specific immune response [201]. Studies suggest DCs express both ERα/β as well as PR-A, and their activity is modified by sex-steroids [118, 119]. One study in a mouse model of asthma suggests, female mice showed an increase in migratory DCs compared to males [230]. In studies not involving lungs, supraphysiological estradiol levels (pregnancy levels) downregulated the production of TNFα and IL-12 in mouse DCs [203].

14.4.1.2 Macrophages

Macrophages are essential in modulating inflammatory responses (acute vs. chronic) [184]. Although macrophages can proliferate in the lungs, their number is inadequate to fight pathogens, which is augmented by DCs [253]. Furthermore, macrophages are the mainstream source for cytokines, chemokines, and other inflammatory mediators, which collectively either aggravate or suppress immune response depending on the pathophysiological scenario [243]. Evidence suggests, macrophages express all 3 steroid receptors (ERs, PRs, and AR) and are modulated by sex-steroids [94, 118, 230]. Studies using murine models of asthma reported that female mice show exacerbated AHR and inflammation compared to male mice [10, 55, 169]. In a separate study, asthmatic mouse models have also shown elevated numbers of macrophages in the lungs of female mice compared to male mice, suggesting a potential role for female sex-steroids in modulating macrophage activity [230]. In addition, sex-steroids have also been implicated in influencing the polarization state of macrophages [334]. Here, estrogen shortened the pro-inflammatory phase of macrophages, while increasing the gene expression of proteins responsible for the resolution phase during asthma [334]. Our own *in vivo* studies using a mixed-alleregen (MA)-induced a mouse model of asthma showed elevated macrophages in females compared to males, which was abrogated in OVX mice, suggesting the importance of estrogen in regulating airway inflammation [10]. Further, we found that this change in macrophage numbers in lungs was largely dependent on specific ER, where ERα KO mice showed lower numbers of macrophages compared to ERβ KO mice [169]. In studies from other systems, estradiol inhibited lipopolysaccharide (LPS)-induced TNFα release and nitrite production in RAW 264.7 cells [350], while progesterone inhibits the release of pro-inflammatory membrane-bound vesicular microparticles from macrophages [267]. In contrast, limited studies on androgens suggest, testosterone downregulated TNFα and NO production in macrophages *in vitro* [285, 297], and downregulated airway inflammation *in vivo* in mouse model of asthma [69, 110]. Considering these findings, it is evident that sex-steroids regulate macrophage function in inflammation, and to understand the underlying mechanisms involved, further studies are warranted.

14.4.1.3 Neutrophils

Most forms of acute lung injuries showed neutrophils to play a central role in their pathogenesis [193]. They are the first type of cells to be recruited in the event of inflammatory stimuli, which serve as the second line of defense following DCs and macrophages [103, 118, 230]. Neutrophils ingest the foreign particles or debris via phagocytosis, followed by killing them with reactive oxygen species (ROS), antimicrobial proteins, and neutrophil elastase [243]. Furthermore, neutrophils are terminally differentiated cells, which are nonproliferative and synthesize minimal RNA and protein. Any deficiency in the amount or activity of neutrophils in the lungs predisposes individuals to lung infections.

Although the expression studies of sex steroid receptors in neutrophils are limited, multiple studies confirmed the role of gender and sex-steroids in regulating neutrophil numbers and activity [240, 244]. In studies from other systems, pregnancy and luteal phase showed increased granulocyte numbers compared to the follicular phase and normal ovarian cycle [16, 46–48, 101, 102, 254], suggesting a role for progesterone and estrogen in regulating neutrophil activity. However, the neutrophil count in males is similar to females in their menstrual cycle, which raises a question on the role of estrogen in neutrophil activity [48, 348]. In a separate study, estrogens decreased the chemotactic activity of neutrophils, while progesterone enhanced this activity [240]. The role of sex-steroids in regulating the free radical production activity of neutrophils is contradicting and needs further studies [31, 68, 244]. Overall, from the existing literature, estrogen seems to have an anti-inflammatory effect on neutrophils, while progesterone shows a pro-inflammatory effect. Finally, both gender and the reproductive condition seem to affect neutrophil numbers and activity, albeit the underlying mechanisms remain elusive [47].

14.4.1.4 Eosinophils

Eosinophil infiltration is a characteristic feature of asthma and serves as a key cellular component development of allergic airway diseases [57]. Eosinophils are the least common white blood cells in normal lung, which is changed during disease conditions such as asthma, where the increased number of infiltrated eosinophils is found in the lungs from murine models of asthma and in lungs from human asthmatic patients [10, 57, 134, 169, 280, 281, 347]. Further, it is an important source for many cytokines, lipid mediators, growth factors, and chemokines [177].

Studies suggest, estradiol binds to eosinophils [185] and enhances the adhesiveness and degranulation of eosinophils [118, 119]. There are no reports available on the expression of PRs and AR on eosinophils; however, one study reported that progesterone increases eosinophil infiltration and AHR in a murine model of asthma [286]. Here, an important fact to know is that progesterone is converted to estrogen, which might be the reason for this particular activity. On the contrary, limited studies suggest that androgens downregulate eosinophil adhesiveness and degranulation; however, the mechanisms behind this action are still unclear [145, 146]. Here, our recent studies suggested an elevated eosinophil levels in bronchoalveolar lavage (BAL) from female mice compared to male and OVX mice, suggesting a crucial estrogen in eosinophil recruitment [10, 169]. However, the mechanisms behind this effect are still unclear. Overall, further research into the mechanisms behind these effects is warranted to understand the comprehensive role of sex-steroids on eosinophils.

14.4.1.5 Lymphocytes

Lymphocytes are distributed across the lungs, starting from the airways all the way into the lung parenchyma. There are two major subsets of lymphocytes, thymus-dependent T-lymphocytes and bone marrow dependent B-lymphocytes, which are discussed in detail in the following subsections. Lymphocytes (Both B and T) play a pivotal role in autoimmune and allergic disorders due to their immune response regulatory role [48, 167, 218, 264, 339]. It is well documented that sex-steroids, especially estrogen and testosterone bind to lymphocytes and regulate their activity. Peripheral blood mononuclear cells (PBMCs) and thymic cells respond to estradiol, whereas only thymic cells are regulated by androgens in

humans [14, 73, 285, 349]. Here, we discuss the role of sex-steroids in regulating the function of T and B lymphocytes, thereby affecting the overall immune response in the lungs.

T-lymphocytes

T-lymphocytes are originated from thymus and are widely classified into two subsets: CD4+ and CD8+ cells [243]. CD4+ cells (T helper cells) are further divided into two subsets, Th1 and Th2 with different cytokine populations. Th1 cytokines (IFNγ, TNFα, etc.) produce pro-inflammatory responses and drive cellular immunity, Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) on the other hand drive humoral immunity resulting in antibody production, IgE and eosinophilic responses [243]. Historically, it is known that Th1 and Th2 mediated immune responses are antagonistic to one another and nullify the overall inflammation. However, in case of an imbalance between these immune responses, it often leads to prolonged inflammation that often translates to a key component of disease pathophysiology. In recent years, our understanding of the interactions and crosstalk between immune cells has significantly increased and has led us to consider, if this concept is far more complex than it is. In this context, recently identified Th17 mediated immune responses have gained considerable significance. Depending on the disease pathophysiology, multiple cell types trigger a coordinated T-cell mediated immune response, which can be widely classified into T-helper 1 (Th1), Th2, and Th17 mediated immune responses [167]. CD8+ cells on the other hand secrete toxic molecules that help in eliminating infected cells and tumor cells and are cytotoxic [167]. In addition to CD4+ and CD8+ cells, there are natural killer cells (NK cells) and natural killer like T cells (NKT cells), which have no specific antigen-specific receptors and are important in combating bacterial and viral infections [50, 181, 182].

All the subpopulations of T-cells express ERs (α and β) [265]; however, the expression profiles of PRs and ARs have not been examined systematically across the human anatomy [141, 154, 241], although studies suggest that sex-steroids (including progesterone and androgens) modulate T-cell numbers and function. Multiple studies suggest lower T-cell numbers in males compared to females [46–48], which might be due to the ability of testosterone to induce apoptosis in T-cells [70]. Evidence indicates higher estrogen levels skew the immune system toward Th2 response, increasing eosinophil recruitment and IL-4 and IL-13 levels in the lung [87, 309, 312]. This may possibly explain the higher incidence and severity of asthma in females compared to males. Further, *in vivo* studies show a higher allergic response in female mice compare to male mice, while OVX mice tend to develop relatively less airway inflammation [10, 169, 200]. Progesterone inhibits differentiation of Th1 cells [154, 264] while inducing IL-4 and IL-10 production [241, 264] and inhibiting TNFα action by antagonizing NFκB [141].

On the contrary, androgens tend to skew the immune response toward Th1. DHEA, a precursor to testosterone alleviated airway inflammation in murine models [349]. Interestingly, male patients with asthma tend to show relatively lower levels of DHEA compared to healthy controls [73]. Overall, existing evidence suggests female sex-steroids (estrogen and progesterone) are detrimental in allergic airway diseases in the context of T-cells due to their bias toward Th2 response; however, reports on androgens effects on T-Cell are contradicting and warrants further studies. Although the superficial role of sex-steroids on regulating T-cell function in surfacing, the underlying mechanisms responsible for these changes are still obscure.

B-lymphocytes

Activation of B-cells plays a crucial role in increasing the serum IgE levels. B-cells are activated by differentiated Th2 cells. Occasionally, antigen-activated B-cells differentiate into memory cells, which produce prolonged and long-lasting inflammation [135, 136]. More importantly, B-cells are the major mediators of antibody production in response to multiple stimuli [48, 70, 167, 181]. These B-cell mediated antibodies, especially IgE degranulates mast cells resulting in the release of histamine, IL-4, and

IL-13, which aggravate inflammation and AHR in the allergic airways.

Evidence from clinical blood panels suggests elevated serum antibody concentrations in females compared to males, suggesting a crucial role for female sex-steroids (estrogen and progesterone) in regulating B-cell activity [6, 120, 198]. Testosterone has been evidenced to inhibit IgG and IgM production, while estrogen increased it *in vitro* [174–176, 339]. Several studies in mouse models of allergic diseases reported elevated levels of IgE and IgG in female mice compared to male mice [79, 302]. Furthermore, another study in a murine model of allergic asthma reported downregulated bronchial inflammation in males compared to females, which was alleviated in castrated males, suggesting a pivotal role for androgens in allergic airway diseases [143]. Existing evidence suggests an important role for testosterone and estrogen in B-cell mediated antibody production; however, the role of progesterone is yet to be investigated.

14.5 Conclusion and Future Scope

To conclude all the above, the existing knowledge on the intrinsic sex difference and their association in controlling intracellular signaling to whole organ structure and function in normal and disease condition suggest the importance of sex-steroids. Convincing data from an array of studies also demonstrated the differential effects of sex-steroids in the management of lung structure and function, but there is limited knowledge on sex-steroid signaling during normal lung physiology and diseased conditions. Sex-steroids are known to produce their cellular and molecular effects via genomic or nongenomic activation of nuclear and membrane-associated receptors, respectively. However, there are multiple additional factors such as gender, age, effector cell types, receptor expression pattern, duration of exposure, disease type, and crosstalk between the sex-steroids, which further directs the sex-steroid signaling and their resultant effects on cellular function. Additionally, the emerging concepts of locally produced versus systemic steroids and their metabolites produced in tissue amplify the complexity of sex-steroid signaling in lung diseases. Another major issue that needs to be a highlight is the role of sex-steroids in regulating inflammatory milieu, especially in lung diseases. As studies exploring the mechanistic role of sex-steroids in airway inflammation by modulating Th1 and Th2 immune cell signaling has supported both protective versus detrimental effects of sex-steroids. Overall, multiple studies have recognized the divergent effects of sex-steroids with respect to their differential receptor expressions in respiratory diseases, but it remains to understand the sex-steroid signaling and how it controls the pathophysiology of the lung in normal and diseased conditions. A detailed exploration of sex-steroid signaling and their mechanisms in airway disease can open a new avenue to the effective management of lung diseases by developing a novel therapeutic approach.

Acknowledgments Supported by NIH grants R01-HL123494, R01-HL123494-02S1, and R01-HL146705 (Venkatachalem).

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15

Cytokines, Chemokines, and Inflammation in Pulmonary Arterial Hypertension

Shuxin Liang, Ankit A. Desai, Stephen M. Black, and Haiyang Tang

Abstract

According to the World Symposium Pulmonary Hypertension (WSPH) classification, pulmonary hypertension (PH) is classified into five categories based on etiology. Among them, Group 1 pulmonary arterial hypertension (PAH) disorders are rare but progressive and often, fatal despite multiple approved treatments. Elevated pulmonary arterial pressure in patients with WSPH Group 1 PAH is mainly caused by increased pulmonary vascular resistance (PVR), due primarily to sustained pulmonary vasoconstriction and excessive obliterative pulmonary vascular remodeling. Growing evidence indicates that inflammation plays a critical role in the development of pulmonary vascular remodeling associated with PAH. While the role of auto-immunity is unclear, infiltration of inflammatory cells in and around vascular lesions, including T- and B-cells, dendritic cells, macrophages, and mast cells have been observed in PAH patients. Serum and plasma levels of chemokines, cytokines, and autoantibodies are also increased in PAH patients; some of these circulating molecules are correlated with disease severity and survival. Preclinical experiments have reported a key role of the inflammation in PAH pathophysiology in vivo. Importantly, anti-inflammatory and immunosuppressive agents have further exhibited therapeutic effects. The present chapter reviews published experimental and clinical evidence highlighting the canonical role of inflammation in the pathogenesis of PAH and as a major target for the development of anti-inflammatory therapies in patients with PAH.

Keywords

Inflammation · Pulmonary arterial hypertension · Anti-inflammatory and immunosuppressive agents

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_15

Abbreviations

CCL	chemokine (C-C motif) ligand
CHD	congenital heart disease
COPD	chronic obstructive pulmonary disease
CTD	connective tissue disease
CXCL	chemokine (C-X-C motif) ligand
CX3CL1	chemokine (C-X3-C motif) ligand 1
DC	dendritic cell
EC	endothelial cell
ECE-1	endothelin converting enzyme 1
ET-1	endothelin-1
HIV	human immunodeficiency virus
LHD	left heart disease
MCP-1	monocyte chemotactic protein
MCT	monocrotaline
PAH	pulmonary arterial hypertension
PASMC	pulmonary artery smooth muscle cell
PVR	pulmonary vascular resistance
RV	right ventricle
SSc	systemic sclerosis
TGF-β	transforming growth factor β
Th	T-helper
TNF-α	tumor necrosis factor α

Table 15.1 Hemodynamic definitions of pulmonary hypertension (PH)

Definitions	Characteristics	Clinical groups[#]
PH	mPAP >20 mm Hg	All
Pre-capillary PH	mPAP >20 mm Hg	1, 3, 4, and 5
	PAWP ⩽15 mm Hg	
	PVR ⩾3 WU	
Isolated postcapillary PH (IpcPH)	mPAP >20 mm Hg	2 and 5
	PAWP >15 mm Hg	
	PVR <3 WU	
Combined pre- and postcapillary PH (CpcPH)	mPAP >20 mm Hg	2 and 5
	PAWP >15 mm Hg	
	PVR ⩾3 WU	

Adapted from 6th WSPH

mPAP mean pulmonary arterial pressure, *PAWP* pulmonary arterial wedge pressure, *PVR* pulmonary vascular resistance, *WU* Wood Units. #: group 1: PAH; group 2: PH due to left heart disease; group 3: PH due to lung diseases and/or hypoxia; group 4: PH due to pulmonary artery obstructions; group 5: PH with unclear and/or multifactorial mechanisms.

15.1 Introduction

Pulmonary hypertension (PH) is a progressive vascular disease that is defined as an increase of mean pulmonary arterial pressure (mPAP) >20 mm Hg at rest in the supine position measured by a right heart catheterization (RHC) (Table 15.1) [1]. PH is classified into five WSPH groups based on similar histopathology, clinical presentation, hemodynamic characteristics, and therapeutic management. Among these, pulmonary arterial hypertension (PAH), which includes diverse diseases that result in similar pathological changes within the pulmonary vasculature [1], constitutes the first Group of PH, referred to as Group 1 PAH. Group 1 PAH is progressive and, often fatal, in which increased pulmonary vascular resistance (PVR) leads to right ventricular remodeling and failure, increasing the risk of premature death. Hallmarks of PAH include sustained pulmonary vasoconstriction and excessive obliterative pulmonary vascular remodeling.

Histopathology analyses of the pulmonary vasculature in PAH have confirmed involvement of vascular remodeling, in situ thrombosis and vascular wall stiffness. Vascular remodeling in PAH is characterized by proliferation of smooth muscle cells (hypertrophy of the medial layer) and endothelial cells (forming the plexiform lesions), arteriolar muscularization, as well as accumulation of fibroblasts (fibrosis) with extracellular matrix deposition and inflammatory cell recruitment [2] (Fig. 15.1). Thrombotic lesions, or so-called in situ thrombosis, can be observed in eccentric intimal thickening that is reflected by proliferation and recruitment of fibroblasts and myofibroblasts (intimal fibrosis or obliteration) [3]. Pulmonary artery stiffening, which can occur in both the proximal and distal arteries, contributes to increased right ventricular afterload in PAH [4].

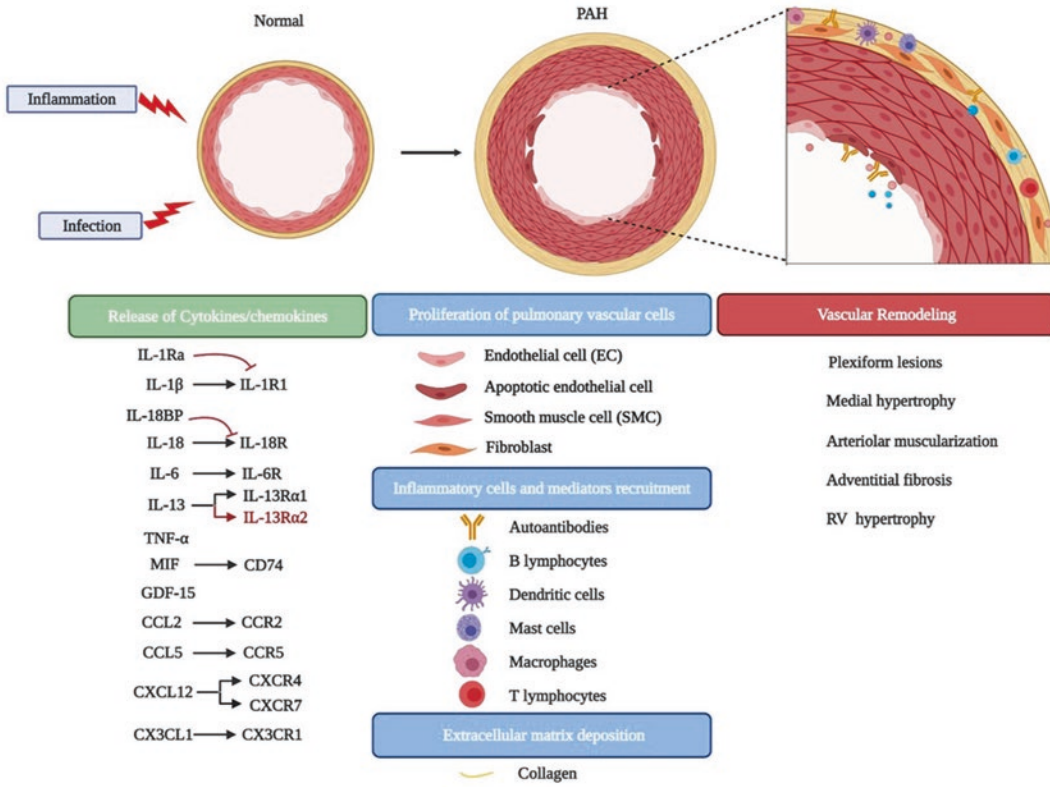

Fig. 15.1 Schematic illustration of inflammation-mediated pulmonary vascular remodeling**:** The pulmonary vascular cells produce and release inflammatory mediators (chemokines and cytokines) in response to infection and inflammatory stimulation, thereby recruiting the inflammatory cells. Under the coordination of inflammatory mediators, inflammatory cells can promote the release of cytokines and chemokines, which leads to vascular remodeling through vascular cell proliferation and collagen deposition. The progressive process generates plexiform lesions, arterial muscularization, and even RV hypertrophy

As a complex disease, understanding PAH pathobiology remains a significant challenge.

Since the first report in 1994 [5] of inflammatory infiltrates observed in the plexiform lesions of PAH patients, increasing lines of evidence demonstrate that inflammation plays a crucial role in the pathogenesis of PAH [6]. Indeed, preclinical data show that the degree of perivascular inflammation correlates with vascular remodeling [7, 8]. While the presence of inflammatory mediators and cells is well-established in diseased PAH tissue, the exact role of inflammation in the development of pulmonary vascular remodeling remains unclear. Nonetheless, drug studies targeting several inflammatory mediators in both animal models and in clinical trials have shown significant promise [9].

This chapter will summarize the current state of knowledge and data on the inflammatory processes involved in the development of PAH including the roles of inflammatory cells and mediators observed in PAH, as well as describe potential therapeutic drugs that target inflammation and immunity in PAH.

15.2 Evidence of Inflammation in PAH

15.2.1 Clinical Classification of PAH

Group 1 pulmonary arterial hypertension (PAH) is associated with diverse diseases, including idiopathic, familial, drug, and toxin induced-

Table 15.2 Clinical classification of PAH

PAH
1 Idiopathic PAH
2 Heritable PAH
3 Drug- and toxin-induced PAH
4 PAH associated with:
4.1 Connective tissue disease
4.2 HIV infection
4.3 Portal hypertension
4.4 Congenital heart disease
4.5 Schistosomiasis
5 PAH long-term responders to calcium channel blockers
6 PAH with overt features of venous/capillaries (PVOD/PCH) involvement
7 Persistent PH of the newborn syndrome

Adapted from 6th WSPH
PAH pulmonary arterial hypertension, *PVOD* pulmonary veno-occlusive disease, *PCH* pulmonary capillary hemangiomatosis

PAH and associated forms of PAH like systemic sclerosis, human immunodeficiency virus (HIV) infection, portal hypertension, congenital heart disease, among others. The recently updated clinical classification of PAH is presented in Table 15.2. These disorders all fall within Group 1 PAH, in part, because they share common histopathology features including hyperproliferative vasculopathy and obliterative vascular remodeling of the distal pulmonary arterioles. Group 1 PAH has received the most attention during the past four decades; the nomenclature and associated conditions within Group 1 PAH have evolved with time. The remaining chapter will discuss the role of inflammation and related cytokines within Group 1 PAH.

15.2.1.1 Group 1.1/1.2: Idiopathic and Heritable PAH

Idiopathic PAH (IPAH) corresponds to disease that is sporadic and in isolation, without apparent identifiable causes or family history. Approximately 70–80% of familial PAH (termed "heritable PAH") and ~15–20% of IPAH cases are caused by rare mutations in the bone morphogenic protein receptor type 2 (*BMPR2*) gene, which is a member of the transforming growth factor (TGF-β) superfamily [10]. While there are several additional mutations in a growing list of genes over the past two decades, observations of incomplete penetrance and female predominance add greater complexity to the genetic basis of PAH. Studies of inflammation can be confounded by genetic underpinnings that predispose to PAH and by systemic conditions that are associated with PAH; in this context, studying inflammation in IPAH without known mutations and associated conditions allows for a homogenous examination.

Table 15.3 Updated classification of drugs and toxins associated with PAH

Definite	Possible
Aminorex	Cocaine
Fenfluramine	Phenylpropanolamine
Dexfenfluramine	L-tryptophan
Toxic rapeseed oil	St. John's wort
Benfluorex	Amphetamines
Methamphetamines	Interferon-α and -β
Dasatinib	Alkylating agents
	Bosutinib
	Direct-acting antiviral agents against hepatitis C virus
	Leflunomide
	Indirubin (Chinese herb Qing-Dai)

15.2.1.2 Group 1.3: Drug- and Toxin-Induced Pulmonary Hypertension

Several drugs and toxins have been implicated in the development of PAH. In the current classification, the categorization of risk factors and the likelihood of developing PAH have been updated [11] and are listed in Table 15.3. "Definite association" includes drugs with data based on reported epidemics or large multicenter epidemiologic studies demonstrating an association between a drug and PAH. "Possible association" is defined as association of drugs with PAH reported in multiple case series or that share similar mechanisms of action.

15.2.1.3 Group 1.4 Associated with Systemic Conditions

PAH is frequently found in patients with systemic sclerosis [12, 13] and congenital heart disease (CHD) [14, 15], while it is recognized as a

rare complication of HIV infection [16]. In schistosomiasis, one of its most severe chronic complications is PAH [17, 18]. Other conditions include portopulmonary hypertension. All of these associated systemic conditions can have a profound impact on inflammatory signaling during PAH, increasing the difficulty of isolating origins of inflammation. Nonetheless, they provide gross insights into the role of inflammation in the development of PAH.

15.2.2 Clinical Evidence of Inflammation in PAH

15.2.2.1 Histological and Cytological Evidence in PAH

Histological data were some of the first to provide evidence that inflammation plays an important role in PAH pathobiology [5]. A varying degree of inflammatory infiltrates which comprise T- and B-lymphocytes, macrophages [5, 19, 20], dendritic cells [21], and mast cells [22–24] were observed around pulmonary vascular lesions including plexiform lesions in PAH patients and in animal models of PH. In IPAH, the formation of a tertiary lymphoid follicle composed of B-lymphocytes, T-lymphocytes, and dendritic cells appears to have connections to the remodeled pulmonary artery via a stromal network supplied by lymphatic channels [25]. In addition, significant correlation between marked perivascular inflammation and remodeling of the intima plus media has been reported [26]. Cumulatively, these published findings suggest that immune cells are implicated in the pathogenesis of PAH.

15.2.2.2 Inflammatory Mediators and Biomarkers in PAH

In IPAH patients, serum levels of certain cytokines and chemokines are abnormally elevated, including interleukin (IL)-1β, IL-6 [27], IL-8 [28], chemokine (C-C motif) ligand (CCL)2/monocyte chemotactic protein (MCP)-1 [29], CCL5/regulated upon activation, normal T cell-expressed and secreted (RANTES) [30], and CXC3CL1/fractalkine [31]. Increased tumor necrosis factor α (TNFα), C-reactive protein (CRP), MCP-1, and IL-6 have also been observed in CHD-PAH [32]. Elevated serum cytokines are also found in CTD-PAH [33], HIV-associated PAH [34] and PAH associated with sickle cell disease [35]. Other data suggest that levels of inflammatory cytokines [28] and CRP [36] correlate with survival rates in PAH patients and may serve as biomarkers of disease progression. Levels of IL-1β and TNF-α are also associated with an accumulation of extracellular matrix proteins such as fibronectin [37], observed in PAH lesions [38]. Beyond serving as possible biomarkers, in patients with PAH, smooth muscle cells have a stronger migratory and proliferative response to CCL2 [29] and IL-6 [39]. Thus, aside from observing local inflammation in PAH tissues, these biomarker data imply that PAH is also associated with a systemic inflammatory state that may correlate with disease severity.

15.2.2.3 Inflammatory Conditions Associated with PAH

There is increasing evidence to support a causal role of inflammation in the development of PAH, especially in patients with CTD, as well as in those with PAH-associated HIV infection or schistosomiasis. Among all CTD, systemic sclerosis (SSc) is most commonly associated with PAH, affecting approximately 40% of SSc patients [40–42]. SSc is a systemic autoimmune disease characterized by vasculopathy, inflammation, cytokine dysregulation and fibrosis, all combined to result in a poor prognosis. The preceding vascular damage and activation of the innate immune system leads to mobilization of the innate lymphoid cells and the upregulation of pro-fibrotic cytokines. For instance, the inflammasome is activated in systemic sclerosis and responds by producing copious amounts of IL-1β, which itself can induce pro-inflammatory and pro-fibrotic phenotype [43]. In addition, specific cytotoxic T cells are enriched in this disease and secrete high amounts of the pro-fibrotic cytokine, IL-13. IL-13 can directly activate fibroblasts to secrete extracellular matrix [44]. Finally, accumulated evidence suggests a specific role of various agonistic autoantibodies in SSc-PAH that

mediate activation of fibroblasts to a myofibroblast and remodeling of the vasculature [45, 46]. For example, PAH-SSc patients have been found to have antifibrillarin antibodies [47], antifibroblast antibodies [48], and poorly characterized antiendothelial cell antibodies, which correlate with digital infarcts [49].

PAH prevalence has been reported to be between 0.5% and 17% in systemic lupus erythematosus (SLE) [50, 51]. SLE is a chronic autoimmune disease affecting multiple organs characterized by autoantibody production, inflammation, and destruction of end-organs. The presence of antinuclear antibodies, rheumatoid factor, immunoglobulin G, complement fractions, cytokines and growth factors in pulmonary arteries reveals a role for an immunological mechanism in SLE-associated PAH [52]. In fact, the development of SLE-associated PAH is shown to be associated with antiribonucleoprotein antibodies [53], rheumatoid factor positivity [52], and elevated levels of endothelin-1 [54]. Studies have proposed that specific inflammatory factors could also serve as candidate biomarkers for SLE. Serum IL-34 levels are significantly elevated in SLE patients and are positively correlated with SLE disease severity indices, antidouble-stranded DNA antibody (anti-dsDNA) titers, C-reactive protein (CRP) levels, and inversely, with complement 3 (C3) levels [55].

HIV infection is an independent risk factor for PAH. The prevalence of PAH in the HIV-infected population is ~0.5% [56]. Clinical features of HIV-related PAH are similar to PAH of other etiologies. Chronic exposure to viral products, chronic inflammation, as well as upregulation of pro-inflammatory cytokines and growth factors might contribute to pulmonary vascular dysfunction. Indeed, inflammation and infection of the myocardium and intramural vessels is detectable in patients with HIV-associated PAH. The persistent release of inflammatory cytokines and/or viral proteins from an infected myocardium induces inflammatory damage of pulmonary vessels, progressing to intimal fibrosis and smooth muscle cell proliferation and ultimately, leading to plexiform vascular lesions [57]. The well-known vasoconstrictor endothelin 1 (ET-1) [58], vascular endothelial growth factors (VEGF) [59], and platelet-derived growth factors (PDGF) [34], which are all significantly elevated in a variety of PAH disorders, are also elevated in HIV-PAH. In addition, HIV protein negative factor (Nef) [60], transactivator of transcription (TAT) [61, 62], and glycoprotein120 (GP120) [63] are also able to induce endothelium growth factor and pro-inflammatory factor production such as IL-6 and ET-1, and proliferation, which result in endothelial injury.

More than 200 million people worldwide are infected with *Schistosoma*, and approximately 6.1% of those are believed to develop PAH. Schistosomiasis-associated PAH is more commonly linked with the species *Schistosoma mansoni*, which causes hepatosplenic infection with subsequent portal hypertension. Inflammation is one of the potential mechanisms underlying the pathogenesis of schistosomiasis-associated PAH. Inflammatory signaling originating in the peri-egg granulomas causing pulmonary vascular disease includes varied cytokines such as IL-4, IL-6, IL-13, and transforming growth factor (TGF)-β [64]. Of which, IL-13 is a key inducer of several type 2 cytokine-dependent pathologies and regulates inflammation, tissue remodeling, and fibrosis [8, 65]. Abnormal TGF-β family signaling has been extensively linked to remodeled pulmonary arteries and human PAH [66]. A prominent perivascular inflammatory infiltrate is also observed, characterized by T cells, mast cells, and dendritic cells; the dendritic cell density is greater than that observed in IPAH tissue [67]. Similarly, in infected mice, a diffuse mobilization of macrophages exists in the alveolar walls and medial fibrosis, intimal thickening, and destruction of elastic fibers appear in the pulmonary arterioles and arteries [68].

As suggested by these various systemic conditions associated with a higher risk of developing PAH, inflammation is an adaptive response that is

triggered by various stimuli and conditions, such as infection and tissue injury. The inflammatory mediators, including cytokines and chemokines and growth factors, can directly recruit inflammatory cells and convey inflammatory pathways that contribute to vascular cell proliferation, migration, and extracellular matrix deposition, thereby resulting in pulmonary vascular remodeling.

15.3 Inflammatory Cytokines and Chemokines in PAH

Preclinical (animal models of disease) and clinical data supporting roles for specific cytokines/chemokines in the pathogenesis of PAH are summarized in Table 15.4. The actions of the common cytokines and chemokines implicated in PAH are summarized in Fig. 15.1.

Table 15.4 Cytokines and chemokines implicated in pulmonary vascular inflammation

Cytokine/chemokine	Cellular source	Function in PH
IL-1β [27, 28]	Monocytes/macrophages, EC, SMC, fibroblasts, lymphocytes	As a growth factor for SMC, EC, and fibroblasts, promotes cell migration and thrombosis, pro-inflammatory
IL-4 [28, 69]	Th2 cells, mast cells	Growth and differentiation of B cells and Th2 cells
IL-6 [27, 28, 70]	Monocytes/macrophages, SMC, EC, fibroblasts, T- and B-cells	Pro-inflammatory, induction of SMC proliferation
IL-8 [28]	Many cells including endothelial and epithelial cells, T cells	Pro-inflammatory, chemoattractant for neutrophils
IL-10 [28]	TH1- and TH2-cells, cytotoxic T cells, B-cells, mast cells, macrophages	Anti-inflammatory, inhibits Th1 and Th2 response and Th activation, promotes differentiation and maturation of B-cells and Ig secretion
IL-12 [28]	B-cells, DC, neutrophils, and mast cells	Induces proliferation and cytotoxicity of NK- cells and lymphocytes
IL-13 [69, 71]	Many cells, especially Th1- and Th2-cells	Important in allergic and parasitic disorders
TNF-α [28]	Many cells including monocytes/macrophages, EC	Induces fibroblast and SMC proliferation, promotes thrombosis
TGF-β [28]	SMC, fibroblasts, monocytes, some T cells, B-cells,	Promotes fibrosis, wound healing and scar formation, angiogenesis, suppresses B- lymphocytes and TH- and cytotoxic- lymphocytes
MIF [72, 73]	Macrophages, lymphocytes, ECs, SMCs, and epithelial cells	Promotes angiogenesis, artery remodeling, vasoconstriction induces ICAM-1, VCAM-1, E-selectin, IL-6, and MCP-1
GDF-15 [74]	Cardiomyocytes, SMCs, and ECs	Prevents muscle loss
CCL2/MCP-1 [29]	Many cells including pulmonary EC, SMC	Promotes monocyte/macrophage activation, migration (especially in PAH EC) and pulmonary vascular remodeling, induces ET-1 and ECE-1
CCL5/RANTES [30, 75]	T cells, EC, SMC, macrophages, fibroblasts, epithelial cells	Promotes angiogenesis, increases ET-1, and ECE-1, chemoattractant for monocytes and T cells
CXCL10 [76]	Monocytes, keratinocytes, EC, SMC, and fibroblasts in response to IFN-γ	Inhibits angiogenesis, attracts activated T- lymphocytes
CXCL12 / SDF-1 [77, 78]	Bone marrow stromal cells, fibroblasts, and ECs	Promotes homing and mobilization of stem cells, neutrophils, and T cells, enhances SMC phenotypic switch and proliferation of SMC and pericytes, increases ICAM-1
Fractalkine/CX3CL1 [79, 80]	Neurons, epithelial cells, EC, DC, macrophages, T cells, SMC	Promotes adhesion of leukocytes to the endothelium, as an adhesion molecule and a soluble chemoattractant, as a growth factor for PASMC

15.3.1 Cytokines

Cytokines represent a large family of signaling proteins that are produced and secreted by cells of the immune system and other cells such as ECs, fibroblasts, and epithelial cells. They have pleiotropic effects and intricately participate in numerous biological processes including inflammation, immunity, and hematopoiesis [81]. There is also increasing evidence for the effect of inflammatory cytokines in the pathogenesis of PAH [82]. Several cytokines can directly regulate cell proliferation, migration, and differentiation of pulmonary vascular cells. In addition, cytokines might act as biomarkers both for diagnosis and clinical outcome of patients with PAH [83]. Here we review animal experiments and clinical results of the most relevant cytokines in PAH.

15.3.1.1 IL-1 Family

The IL-1 family is divided into 3 subfamilies on the basis of the IL-1 consensus sequence and the primary ligand binding receptor, including IL-1 subfamily, IL-18 subfamily, and IL-36 subfamily [84]. The IL-1 family is primarily associated with innate immunity and is unique due to shared functions with Toll-like receptor (TLR) signaling. Recent literature supports a close link between the IL-1 family and PAH development.

The elevated levels of IL-1β and IL-1R1 (a specific receptor of IL-1α, IL-1β, and IL-1Ra) have been found in lung tissues, remodeled vessels and serum of PAH patients and animal models of PH [27, 28, 85]. In a case report, anakinra, as a recombinant IL-1 receptor antagonist, was shown to alleviate inflammation and right ventricular failure in PAH and improve severe PH in a patient with Adult-Onset Still's Disease [86, 87]. Animal models also support the role of IL-1 subfamily in the progression of PH. For example, repeated injections of an IL-1 receptor antagonist (IL-1Ra) reduced pulmonary artery pressure and right heart hypertrophy in a monocrotaline (MCT) model but not in the chronically hypoxic rat [88]. Similarly, IL-1Ra protects against PH secondary to bronchopulmonary dysplasia (BPD-PH) in mice pups [89]. Moreover, both knockout of IL-1R1 or anakinra treatment protect mice from hypoxia-induced PH (HPH) and MCT-induced PH [85]. Other research indicates that high-fat diet-fed apoE knockout mice develop moderately severe PAH with obliterative pulmonary vascular remodeling in an IL-1 dependent manner, and via a lung-specific putative IL-1R1 receptor [90]; PAH patients have reduced expression of apoE [91]. In addition, interplay between BMP and IL-1 signaling has also been reported. Importantly, reduced functional BMPR2 expression (via mutant *BMPR2* mice) combined with IL-1β drives small pulmonary vessel muscularization in preclinical mouse models of PH and correlated positively with serum IL-6 levels [92].

IL-18, a member of IL-1 family, is activated by the endoprotease IL-1–converting enzyme, known as caspase-1. Plasma and serum IL-18 levels are highly expressed from medial SMCs of the pulmonary artery in patients with PAH, while its receptor IL-18Rα is expressed in medial SMCs, ECs, and mononuclear cells [93]. A strong correlation between serum IL-18 levels and intimal medial thickness has also been demonstrated [94]. Beyond associations, IL-18 signaling may also drive PAH pathology. Indeed, constitutive IL-18 overproduction in the lungs of transgenic mice results in dilatation of the right ventricle and mild pulmonary hypertension with aging [95]. In addition, the balance between IL-18 and its decoy receptor, modulates the activation status of IL-18 signaling. Among these IL-18-binding protein, IL-18BPa has been reported to be positively correlated with the parameters of PH and systemic inflammation in SSc patients [96]. These data suggest that IL-18 has deleterious effects in the development and progression of pulmonary hypertension. The exact mechanisms, however, remain unclear and therapeutic inhibition of IL-18 and other IL-1 family members is limited to anecdotal case reports precluding therapeutic use at present. Nonetheless, collectively, these data suggest that IL-1 family represents an unfavorable factor for the development and progression of PAH.

15.3.1.2 IL-6

IL-6 is a pleiotropic cytokine with broad biologic effects, which is mediated by two different path-

ways: classical or cis-signaling pathway and trans-signaling pathway. In the classical signaling pathway, IL-6 binds to membrane-bound IL6R (CD126), resulting in dimerization and activation of the signal-transducing protein gp130. In contrast, in the trans-signaling pathway, IL-6 binds to soluble IL6R (sIL6R) and activates gp130 [97].

Recently, IL-6 has been identified as playing a key role in the development of PAH. Elevated levels of IL-6 have been reported in patients with idiopathic PAH, familial PAH [28] and CTD-PAH [98]. Elevated circulating IL-6 in serum correlates with right ventricular function, worse clinical outcome, greater incidence of quality of life-related symptoms, and/or poorer prognosis of PAH patients [28, 99, 100]. Elevated IL-6 has also been observed in other WSPH Groups of PH, such as COPD-PH, CTEPH [101], and PH due to advanced heart failure [102]. Furthermore, an IL-6 promoter polymorphism (–572C/G [rs1800796]) is associated with increased serum IL-6 levels and risk of both IPAH [103] and COPD-PH [104], suggesting a pathogenic role for IL-6 in PH. Indeed, via in vivo animal experiments, IL-6 blockade or knockout and IL-21 (a downstream target of IL-6 signaling) receptor-deficient mice show resistance to development of HPH [105, 106], whereas lung-specific overexpressing IL-6 mice develop spontaneous PH and show distal arteriolar muscularization and plexogenic arteriopathy in chronic hypoxic conditions. Furthermore, delivery of recombinant IL-6 protein can cause vascular remodeling and augment the pulmonary hypertensive response to hypoxia [107]. IL-6 is also known to have effects on vascular cells. IL-6 induces PASMC and EC proliferation and increases pericyte migration elicited by pulmonary EC media and vascular pericyte coverage in a mouse model of retinal angiogenesis [107, 108]. In addition, there is now increasing evidence that the interaction between BMPR2 and IL-6 is implicated in the development of PH in both in vitro and in vivo models. Transgenic mice overexpressing a dominant-negative BMPR2 or BMPR2 deficiency in smooth muscle, for example, show an exaggerated inflammatory response, overexpression of IL-6 and increased susceptibility to PH [109, 110]. In turn, activity of the canonical BMP target gene, Id1, is increased in mice injected with an IL-6-expressing adenovirus [109].

IL6R as a membrane-bound receptor of IL-6, is ectopically upregulated in the smooth muscle layer of remodeled vessels in IPAH and experimental models of PH [111], implying a key role of IL6R in the pathological process of PAH. The relevance of IL6R to the pathogenesis of PAH has been demonstrated by a series of in vitro and in vivo experiments. For example, specific deletion of IL6R in the smooth muscle protected against chronic hypoxia-induced PH in the mouse model via reduced PASMC accumulation and inflammatory cell infiltration. Likewise, treatment with an IL6R-specific antagonist attenuates pulmonary vascular remodeling and prevented or reversed MCT or hypoxia plus SU5416-induced PH in rat models [111]. Notably, a humanized monoclonal antibody that recognizes IL6R, tocilizumab (currently approved for the treatment of rheumatoid arthritis), can disrupt both classical and trans-signaling, improved PAH symptoms in patients with mixed CTD and severe PAH [112].

15.3.1.3 IL-13

IL-13 is emerging as a regulator of cell proliferation and tissue remodeling [113] and an important mediator of granulomatous and vascular responses [114]. IL-13 cytokine has been observed in immune cells associated with plexiform lesions and inflammatory cell infiltrates in the lung of patients with IPAH [71]. Increased expression and activity of IL-13 has been identified in patients with systemic sclerosis-associated PAH [115], schistosomiasis-associated PAH [116] and animal models [114]. The role of IL-13 in the pathogenesis of PH remains ambiguous. On the one hand, studies have shown that depletion of IL-13 signaling ameliorates pulmonary vascular remodeling by regulating Th2 responsiveness [69, 114]. Similarly, combined IL-4 and IL-13 deficiency protects against *Schistosoma*-induced PH, with decreased right ventricular pressures, pulmonary vascular remodeling, and right ventricular hypertrophy [117].

Moreover, IL-13-overexpressing transgenic mice spontaneously developed PH phenotype by 2 months of age, enhancing the proliferation of PASMCs in an arginase 2-dependent manner [118]. In contrast, IL-13 inhibits PASMC proliferation through a G0/G1 checkpoint block [71].

IL-13 signaling is regulated by a complex receptor system, including the IL-4 receptor (IL-4R) and two IL-13 receptors, the low-affinity IL-13Rα1, and the high-affinity IL-13Rα2. Lacking the canonical IL-13 receptor (IL-13Rα1) leads to a loss-of-function of IL-13 signaling, and deficiency of the soluble and membrane-bound "decoy" IL-13 receptor (IL-13Rα2) results in a gain-of-function for IL-13 signaling [119]. IL-13Rα1 and IL-13Rα2 have been found in both plexiform and concentric vascular lesions in the small pulmonary arteries of IPAH patients. There is increased expression of IL-13Rα2 relative to that of IL-4R and IL-13Rα1 in lungs of patients with IPAH and in hypoxia-induced PH animal models [71]. Previous research also indicates that dysregulation of IL-13 receptor expression in IPAH may partially underlie smooth muscle hypertrophy. For example, ectopic expression of IL13Rα2 resulted in partial suppression of endothelin-1-dependent growth control of PASMC via blunting the antiproliferative effects of IL-13, whereas knockdown of IL13Rα2 had the opposite effects [71]. In addition, in *Schistosoma mansoni*-induced mice models, knockout IL-13Ra2 is sufficient to cause PH by enhancing IL-13 signaling, whereas loss of IL-13Rα1 function did not result in PH and instead, resulted in reduced pulmonary vascular remodeling [114]. In brief, IL-13 signaling appears to be involved in the development of PAH, although these apparently contradictory results demonstrate the need for further investigations.

15.3.1.4 TNF-α

TNF-α is a pro-inflammatory cytokine with potent modulatory effects on the pulmonary circulation. Increased expression levels of TNF-α have been shown in PAH patients [28, 120] and in PH animal models [121]. Higher TNF-α levels are significantly associated with certain quality of life-related symptoms in patients with PAH, such as increased bodily pain, better mental health scores [99], and worse survival [28]. In animal studies, TNF-α transgenic mice develop pulmonary vascular remodeling and severe PH [122]. The silencing of TNF-α in rats decreases cold-induced increases in expression of TNF-α and IL-6, prevents macrophage infiltration, and attenuates pulmonary arterial remodeling and phenotype of PH [123]. In addition, the TNF-α antagonist, recombinant TNF-α receptor II: IgG Fc fusion protein (rhTNFRFc)-treated rats have attenuated mean pulmonary artery pressure, pulmonary vascular remodeling, and pulmonary inflammation following MCT [124]. Consistent with these findings, etanercept, TNF-α-blocking agent, prevents and reverses MCT-PH in rats by reducing inflammatory cell infiltration [125], and also reverses PH in pigs with endotoxemia [126]. More interesting, etanercept administration reversed PAH progression in the Sugen/hypoxia-induced PH models via restoring unbalanced BMP/NOTCH signaling. Mechanically, TNFα, in the setting of BMPR-II deficiency, promotes the development of PAH by enhancing heightened PASMC proliferation through c-SRC family members and dysregulated NOTCH2/3 signaling [127].

15.3.1.5 MIF

Migration inhibitory factor (MIF), is a pleiotropic upstream pro-inflammatory mediator, which is expressed by various immune cell and vascular cells, including macrophages, lymphocytes, ECs, SMCs, and epithelial cells, as well as is involved in multiple biological processes. With its important role in activating the downstream signal cascade, MIF promotes the release of various other inflammatory cytokines such as IL-1β, IL-6, and TNF-α, ultimately triggering a chronic inflammatory immune response [128]. The elevated expression levels and excessive activity of MIF have been found in pulmonary artery and lung tissues of several forms of PAH [129–131] and animal models [73, 132]. Moreover, significant positive correlations between MIF and several angiogenic biomarkers [72] or clinical severity [129] have been described in PAH, suggesting

that MIF is associated with the angiogenesis and pathobiology of PAH. Once released, MIF can contribute to pulmonary vasoconstriction in response to hypoxia condition or phenylephrine administration, which ultimately results in pulmonary vascular remodeling. MIF inhibition with ISO-1 significantly attenuated delayed hypoxic vasoconstriction [133]. Notably, the removal of endothelium has no impact on vasoreactivity of MIF, implying that the effect of MIF on the nonsmall intrapulmonary arteries is independent of an intact endothelium. In addition to vasoconstriction, MIF deficiency or treatment with ISO-1 has been shown to reduce hypoxia-induced pulmonary artery remodeling and hypertension [134].

Consistent with the these findings, the MIF receptor, CD74, has been shown to be overexpressed in the endothelium of muscularized distal pulmonary arteries and pulmonary ECs from patients with IPAH and simultaneously contributes to an excessive adhesion and recruitment of peripheral blood mononuclear cells. ISO-1 or anti-CD74 neutralizing antibodies attenuates hypoxia and MCT-induced PH development and reduces the inflammatory phenotype via inhibiting expressions of endothelial ICAM-1, VCAM-1, E-selectin, levels of circulating IL-6 and MCP-1 [73]. In addition, MIF098 treatment, which blocks MIF binding to its cognate receptor CD74, ameliorated PH and inhibited distal pulmonary artery thickness, and collagen deposition in hypoxia-challenged mice, associated with an inhibition of PASMC proliferation, migration, and fibrosis [131].

15.3.1.6 GDF-15

Growth differentiation factor (GDF)-15, a multifactorial cytokine, is a stress-responsive member of the transforming growth factor (TGF)-β cytokine superfamily that was initially thought to derive from activated macrophages [135]. The expression of GDF-15 is strongly increased in cardiomyocytes, vascular SMCs, and ECs following oxidative stress, inflammation and tissue injury [136–138]. Interestingly, elevated levels of GDF-15 are also found in serums, plasma, lung and vascular ECs of IPAH, CHD-PAH, and SSc-PAH and correlate with the risk of death or transplantation in various forms of PAH patients [139–141], implying it could be used as a prognostic marker in PAH. In the MCT and Sugen/hypoxia-induced PH animal models, lung GDF-15 levels are elevated and enhance muscle dysfunction, a clinically important complication of PAH, via activating TGF-β-activated kinase 1 (TAK1). TAK1 inhibition with 5(Z)-7-oxozeaenol had no effect on hemodynamics, but prevents muscle loss. These results further suggest that GDF-15 may be a potential target for therapeutic intervention aimed at improving quality of life and mortality [74].

15.3.2 Chemokines

Chemokines can be divided into four subfamilies on the basis of positioning of the N-terminal cysteine residues, including C-C, C-X-C, CX3C, and C chemokine subfamily. The C-X-C and C-C subfamilies are, respectively, characterized by the separation of the first two cysteine residues through an amino acid (CXC) and an adjacent cysteine residues (CC). The two major families contain majority of the known chemokines. In addition, two other subfamilies have also been described: C and CX3C. The former lacks the first and third cysteine and contains a single cysteine residue in the conserved locus, while the latter is identified by three variable amino acids between the first two cysteines [142]. Recent studies have indicated dysregulated chemokine signaling may also participate in disease progression of several lung diseases, including PAH [143].

15.3.2.1 CCL2/MCP-1

CCL2 (also known as MCP-1) is a C–C chemokine produced by vascular cells that has effective chemotactic and activating effects for monocytes/macrophages, basophils, T lymphocytes, and NK cells [144, 145]. Elevated CCL2 protein levels have been observed in plasma and lung tissue of IPAH patients [29]. Within PAH lung tissues, PAECs, fibroblasts, and macrophages display increased expression of CCL2 [29, 146].

In the setting of PAH, CCL2 expression is induced in endothelial cells exposed to high pulsatility flow perfusion, along with increased adhesion molecule that contribute monocyte adhesion to endothelium [147]. Similarly, lung microvascular endothelial cells display higher CCL2 expression and release CCL2 in response to mechanical strain associated with PVH, thereby driving pulmonary structural remodeling [148]. The relevance of CCL2 to the pathogenesis of PAH has been shown in several studies. For example, delivery of potent dominant negative inhibitor of CCL2 prevents the progression of MCT-induced PH [149]. In patients with IPAH, increases in EC–derived CCL2 contribute to increased chemotactic activity, as well as induced stronger migratory and proliferative effects on PASMCs [29].

CCR2, as a cognate receptor for CCL2, is also increased in lung tissues of PAH patients and hypoxia-induced PH mice model [29, 150]. However, the impact of CCR2 deficient on progression of PH shows contradictory results. CCR2 deficiency promotes spontaneous PAH and displays more severe PAH in response to hypoxia due to Notch signaling activation [151]. But Florentin et al. demonstrates that CCR2-deficient mice have reduced pulmonary inflammation and diminished pulmonary vascular remodeling despite the lack of any impact on hemodynamics [152]. Another study found that, under hypoxia, mice deficient in CCL2 alone or as a double mutant for knockout of both, CX3CR1 and CCL2, develop a similar degree of PH and pulmonary vascular remodeling compared with WT counterparts [150]. In brief, the CCL2/CCR2 axis is an important signaling pathway involved in the pathological processes of PAH, although these apparently contradictory results emphasize the need for further studies.

15.3.2.2 CCL5/RANTES

The chemokine CCL5 (also known as RANTES), which is produced in various cells including T cells, ECs, SMCs, fibroblasts, macrophages, and epithelial cells, is an effective chemoattractant for T cells, granulocytes, monocytes, DCs, mast cells, and NK cells [153–155]. Several studies have reported an association of CCL5 (or RANTES) levels with PAH disease parameters. For example, elevated CCL5 expression has been observed in the pulmonary vascular walls of PAH and predominates in proliferative EC of vascular lesions in PAH patients [30, 156]. Similarly, increased expression of CCL5 is also detected in several PH animal models including hypoxia-induced PH mice [157], MCT-exposure/pneumonectomy rats [158], Sugen/hypoxia-induced PH rats [159], and hypoxia-induced mitogenic factor (HIMF)-treated PH mice [160]. Moreover, CCL5 deficiency reverses obliterative pathological changes, inhibits angiogenesis and alleviates development of Sugen/hypoxia-induced PH [75]. Lastly, CCL5 may also exert an indirect effect in PAH by inducing ECE-1 and ET-1, which have strong vasoconstrictive and mitogenic actions [161].

CCR5 is the major receptor for CCL5. In the setting of PAH, several studies have demonstrated that CCR5 expression is elevated in vascular cells and lung tissues [157]. Similarly, experimental animal data also show that CCL5 expression is increased in lungs from mice with hypoxia-induced PH [157]. Of note, studies suggest a role for CCR5 in the pathogenesis of PAH. For example, deficiency of the *CCR5* gene protects against the development of hypoxic PH in mice. Moreover, CCR5 blockade by maraviroc attenuates development of PH in CCR5 knock-in mice expressing human CCR5 [157].

15.3.2.3 CXCL12/SDF-1

CXCL12, also known as CXC-chemokine stromal cell-derived factor (SDF-1) or pre-B-cell-growth-stimulating factor (PBSF), belongs to the C-X-C subfamily of chemokines. CXCL12 is highly expressed in diverse cell types including bone marrow stromal cells, fibroblasts, and ECs but it is also constitutively expressed in various tissues/organs including the brain, heart, liver, lymph nodes, and lungs [162]. CXCL12 has two chemokine receptors, CXCR4 and CXCR7 (ACKR3) [163]. Given that CXCL12 and its receptors are involved in the homing and mobilization of stem/progenitor cells [164], neutrophils [165], and T cells [166] in the perivascular niche,

it may play an important role in vascular remodeling [167], angiogenesis [77], and neointimal formation [168].

In the setting of PAH, upregulation of CXCL12 has been documented in clinical and animal experiments [169–174]. And various pulmonary cells display high levels of CXCL12 including alveolar macrophages [105], fibroblasts [146], intimal ECs [174], and medial PASMCs [175]. In MCT-injected and Sugen/Hypoxia-treated rats, neutralization of CXCL12 partially reversed established PH, attenuates right ventricular hypertrophy, and pulmonary vascular remodeling [174]. In contrast, CXCL12 nanoparticles, but not free CXCL12, is found to protect against MCT-induced PH by recruiting circulating progenitor/stem cells to repair injured lung tissue [176]. In addition, under hypoxia, inhibition of CXCL12 or administration of CXCR4 antagonist, AMD3100, inhibits PASMCs proliferation and cell cycle progression via the PI3K/Akt pathway [177], as well as prevents the development of PH in neonatal mice by decreasing progenitor cell recruitment to the pulmonary vasculature and right ventricles [178]. Notably, elevated levels of CXCL12 in platelets and plasma of PAH patients is associated with poorer prognosis [172, 179].

Expression of CXCR4 is also elevated in pulmonary arteries, endothelium [180], and PASMCs [177, 181] of PH animals and patients with PAH. Increased expression of CXCR4 is found in lung tissues of PAH patients and is accompanied by increased circulating endothelial progenitor cells, suggesting a critical role of CXCR4 in the pathogenesis of PAH [180]. Indeed, transplantation with bone marrow cells electroporated with CXCR4 shRNA significantly decreased hypoxia-induced PH, vascular remodeling, and right ventricular hypertrophy in rats due to reduced bone marrow-derived progenitor cells recruitment to the lungs [167]. Moreover, administration of AMD3100, whether alone or combined with a CXCR7 antagonist (CCX771), attenuates PH pathology by reducing pulmonary c-kit+ hematopoietic progenitor cell accumulation [78]. In addition, there is complementary evidence that $CXCR4^{+}/PDGFR\beta^{+}$ progenitor cells can be induced to differentiate into SMCs, thereby mediating hypoxia-induced muscularization of alveolar arterioles [182].

CXCR7, as another receptor of CXCL12, also plays an important pathogenic role in PH. CXCR7 has been demonstrated to be highly expressed in ECs [183] and in SMCs [184], and implicated in endothelial cell regeneration, repair, and proliferation [183]. Notably, the CXCR7 antagonist, CCX771, reportedly decreases pulmonary vascular remodeling and attenuates PH in newborn mice exposed to chronic hypoxia [185]. However, CCX771 administration did not abrogate development of chronic hypoxia-induced PH in mice [78], suggesting that the role of CXCR7 in PAH can be context-dependent.

15.3.2.4 CX3CL1/Fractalkine

Fractalkine (CX3CL1) is a unique CX3C chemokine as a soluble form (chemotactic protein) and as a membrane-bound form with a mucin-like glycosylated stalk on endothelial cells (cell-adhesion molecule) [186]. CX3CL1 has been shown to participate in the development of PAH. According to several studies, expression of CX3CL1 is upregulated in different cells from lungs of PAH patients, such as ECs and perivascular inflammatory cells [79, 187]. In animal models, CX3CL1 is observed to be overexpressed in serum, as well as inflammatory cells and ECs surrounding pulmonary arterial lesions [80, 158, 188, 189]. In addition, other research supports the crucial role of CX3CL1 in the pathophysiologic changes of PH. For example, increased release of endothelial CX3CL1 promotes SMC phenotypic switching from the contractile to the proliferative state and enhances proliferation of pericytes, which lead to vessel remodeling and EC dysfunction [188]. CX3CL1 has a proliferative effect on SMC but not migration [80]. Notably, exposure of ECs to hypoxia and reoxygenation, release soluble CX3CL1, resulting in an exaggerated pro-inflammatory phenotype and increased intercellular adhesion molecule (ICAM)-1 via activation of the JAK–STAT5 pathway [190].

Effects of CX3CL1 are mediated through the cognate receptor, CX3CR1. Increased expression

of the CX3CR1 has been demonstrated both on the inflammatory cells surrounding pulmonary arterial lesions [150], ECs, SMCs, and pericytes isolated from PAH patients and various types of PH animal models. Furthermore, growing evidence indicates that CX3CL1 is implicated in the pathogenesis of PAH. In hypoxia-induced PH mice models, whether exposed to hypoxic conditions for 18 days or 4 weeks, knockout or inhibition of CX3CR1 diminishes PASMC proliferation and pulmonary vascular remodeling, as well as protects against PH [150, 188], yet a different study found that genetic deficiency of CX3CR1 results in decreased pulmonary inflammation and significantly diminished vessel remodeling but with a less robust hemodynamic effect, compared with hypoxic WT mice [152]. The CX3CL1/CX3CR1 axis may act as a growth factor for SMCs and thus play a role in pulmonary artery remodeling.

15.4 Inflammatory Cells in PAH

15.4.1 T-Lymphocytes

T cells are critical members of the adaptive immune response and are differentiated into many subtypes including: $CD4^+$ T-helper (Th) cells, T-regulatory (Treg) cells, and $CD8^+$ cytotoxic T (Tc) cells. $CD4^+$ Th-cells can be further divided into Th1, Th2, and Th17 based on their cytokine profiles. $CD4^+$ Th-cells stimulate B-cell differentiation and macrophage activation, which are important in initiating the immune response. $CD8^+$ Tc cells are recognized as "professional killers" in viral-infected cells and tumor cells. Treg cells play an important role in maintenance of immunologic tolerance and limitation of inflammatory responses. Several lines of evidence support the effects of T cells in PAH development. For example, Treg cells can protect against the development of PAH by inhibiting vascular inflammation and limiting the propagation of vascular injury [191]. The involvement of T cells in the development of PAH is further supported by recent studies showing that athymic nude rats, which lack mature T cells, develop more severe PH in response to either MCT or the vascular endothelial growth factor receptor blocker SU5416 [192, 193]. These data imply a protective role of Treg cells in PAH. Conversely, depletion of $CD4^+$ T cells and Th2 response significantly ameliorated pulmonary arterial muscularization [69]. Similarly, depletion of $CD4^+$ T cells or treatment with SR1001, an inhibitor of Th17 cells development, prevented increased right ventricular systolic pressure and pulmonary arterial remodeling under chronic hypoxia [194]. More recently, it has been shown that deleting $CD4^+$ T cells or inhibiting their Th2 function protects against *Schistosoma*-associated PH in mice models [195]. Thus, certain subtypes of T cells may protect against the development of PAH, whereas others like Th2 and Th17 may promote pulmonary arterial muscularization. In general, pulmonary vascular infiltration of T cells are increased in IPAH patients. It was also reported that $CD8^+$ T cells and Th17 cells are decreased and Treg cells are increased in the peripheral blood of IPAH patients [196]. The role of the immune system in regulating the progression of PAH has received growing attention and the future research in the field is required to elucidate the precise role of T-cell subtypes in PAH.

15.4.2 B-Lymphocytes

B-Lymphocytes (B-cells) are responsible for regulating humoral immunity response through the production of various antibodies to specific antigenic epitopes. Various autoantibodies are present in circulating blood of IPAH patients [197]. The prevalence of antibodies directed against pulmonary ECs and fibroblasts in PAH have also been demonstrated in several studies, suggesting the effect of B-cells in PAH [48, 198]. In addition, increased perivascular infiltration of B-cells and activation of peripheral blood B-cells have been reported in patients with IPAH [67]. Indeed, patients with IPAH have a distinct RNA expression profile of their peripheral blood B-cells compared with healthy groups with some clearly upregulated transcripts genes, which are involved

in vessel biology, vasomotor regulation, angiogenesis, and cell proliferation [199].

15.4.3 Dendritic Cells

Dendritic Cells (DCs) play an important role in the immune system as professional antigen-presenting cells, which display antigen for recognition by T cells and activate the adaptive immune response. DCs additionally initiate the inflammatory response and can differentiate into other cell phenotypes, including EC, thereby exerting effects in vascular disorders. An increase in the number of DCs is seen in lungs from patients with IPAH and higher numbers of infiltrating dendritic cells are also seen in the adventitia of pulmonary arteries compared with donor lungs [200]. Studies have also demonstrated the infiltration of immature DCs in vascular lesions of both IPAH patients and MCT-induced PH rat models [21]. Accumulation of professional antigen-presenting cells may present antibodies to ECs, fibroblasts, and nuclear antigens that are found in the serum of IPAH and scleroderma-related PAH patients [122]. However, compared with control subjects, the number of circulating monocyte-derived DCs is lower in peripheral blood of patients with IPAH, implying trafficking to the lung [201].

15.4.4 Macrophages

Macrophages constitute an important part of the innate immune system. They are phagocytic cells that can clear tissue debris and foreign materials, thus maintaining tissue homeostasis. A significant accumulation of macrophages in the vessel wall of the pulmonary arteries, even down to small-size vessels, has been observed in humans with PH and small and large PH animal models [200, 202]. Several studies have demonstrated that recruitment and accumulation of macrophage in the vessel wall is a key precondition for vascular remodeling and development of PH. Indeed, deletion of circulating monocytes in several model systems including hypoxia-exposed rats and bleomycin-treated neonatal rats prevented macrophage accumulation and development of PH [203, 204]. In addition, alveolar macrophages, another population of the macrophage, also contribute to HPH immunopathology. Clodronate-mediated selective depletion of alveolar macrophage attenuated the hypoxia-induced increase in pulmonary arterial pressure [205], which could be a potential target for the treatment of PH.

A critical discovery in patients and in experimental animal models of PH was that macrophage accumulation remained largely restricted to the adventitial/perivascular compartment of the vessel wall, implying an important effect of the adventitia and adventitial macrophages in the vascular remodeling and the pathogenesis of PH [200, 206]. It is reported that fibroblasts from animals and humans with PH are capable of activating naive macrophages toward an alternative activation phenotype, which is identical to the one observed within the adventitia of the PA. Interestingly, remodeled adventitial tissue explanted from PH animal models was also able to activate macrophages to this phenotype [207].

15.4.5 Mast Cells

Mast Cells (MCs) are critical in allergic and non-allergic immune responses. Upon activation, mast cells produce a vast amount of growth factors, vasoactive substances, pro-inflammatory cytokines, and proteases (tryptase and chymase). There are many reports of accumulation of MCs in lung tissues from several PAH groups [22–24]. Similarly, distribution and degranulation (e.g., histamine, serotonin, matrix metalloproteinases, tryptase, and chymase) of MCs are also found in rat models under acute or chronic hypoxia [208–210]. Compared with donors, the presence/expression of perivascular chymase+ MCs was higher in IPAH patients [211]. In addition, MCs/c-kit expressing cells infiltrate along the periphery/adventitial layer of remodeled pulmonary arteries in experimental PH and human IPAH [212–214]. These findings suggest the contribution of MCs to the development of PAH.

Considering their distribution and degranulation in remodeled pulmonary vessels, MCs are recognized as a manager for PAH development. Pharmacological inhibition of MC degranulation with PLX and disodium cromoglycate attenuate pulmonary vascular remodeling and inhibit the development of PH in rat models [215, 216]. Similarly, MC stabilizers, cromolyn sodium salt (CSS), and ketotifen, also attenuate MCT-induced vascular remodeling in lung tissues of rats [216, 217]. In addition, blockade of proteinase signaling such as chymase activity [218] or tryptase/protease-activated receptor 2 (PAR2) pathways [219] significantly protects against pulmonary vascular remodeling in PH. These studies, combined, have demonstrated that MCs may represent novel targets for treatment in PAH.

15.5 Role of Autoantibodies in PAH

Roles for autoimmunity have not yet been fully characterized in the pathogenesis of IPAH and SSc-PAH. Autoantibodies such as anti-RNP, anti-histone, anti-Scl70, antiendothelin-1 type A receptor, antiangiotensin II type 1 receptor, and antiannexin C antibodies are found in CTD and IPAH patients [197]. The specific role of these autoantibodies in PAH pathogenesis has not yet been elucidated.

15.5.1 Antifibroblast Antibodies

The presence of antifibroblast antibodies in the serum of IPAH and SSc-PAH patients suggests a significant pathogenic importance. Fibroblast dysfunction has been identified in the pulmonary vascular remodeling of PAH and is also observed in the remodeled vessel walls in both IPAH and SSc-PAH [220] subjects. Antifibroblast antibodies enhance activation of fibroblasts, production of adhesion molecules, and, in turn, induce collagen synthesis, which is potentially implicated in the remodeling process [45]. In addition, antifibroblast antibodies target different antigens including calumenin, tropomyosin 1, heat shock proteins, glucose-6-phosphate dehydrogenase, PI3-kinase, and DAP kinase, which are involved in cell contraction, oxidative stress, cell energy metabolism, cell growth, and cytoskeleton organization [221], resulting in increasing contractility of myofibroblasts as observed in IPAH and SSc-PAH.

15.5.2 Antiendothelial Cell Antibodies

Antiendothelial cell antibodies have been identified in patients with SSc with and without PAH and in IPAH patients. Dib et al. [222] demonstrated that target antigens of anti-EC antibodies include lamin A/C, tubulin β-chain, and vinculin. The anti-EC antibodies can activate ECs, induce the expression of adhesion molecules, and trigger apoptosis [223]. Moreover, studies have suggested that anti-EC antibodies could be related to vascular injury and could reflect EC damage [224]. However, evidence to support whether anti-EC antibodies play a role in the pathogenesis of PAH remains scarce and further work is needed.

15.5.3 Anti-Inflammatory and Immunosuppressive Agents in PAH

15.5.3.1 Anti-Inflammatory Agents

Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide with potent anti-inflammatory and immunomodulatory effects. VIP displays antiproliferative, strong vasodilatory and high bronchiectasic properties through direct action on vessels and modulation of ET-1-mediated vascular constriction. In preclinical models, treatment with VIP, almost totally prevented PAH pathology and, combination therapy with VIP plus bosentan exhibits a synergistic effect [225]. Conversely, deficiency of VIP increases perivascular inflammatory cell infiltrates, vascular and right ventricle (RV) remodeling as well as the development of PH [226]. In IPAH patients, a reduction of VIP with a concomitant increase of

VIP-mediating receptors in serum has been reported [227]. A pharmacological study with VIP in 20 patients with PH during right heart catheterization demonstrated that a single dose of VIP temporarily improves hemodynamics and oxygenation without side-effects [228].

Rituximab, another anti-inflammatory pharmacologic agent, is a chimeric monoclonal antibody targeting B-cell surface protein CD20. It is currently used in the therapy of autoimmune disorders, lymphomas, and leukemias. Considering the potential pathogenic role of inflammation and immune abnormalities in PAH, the therapeutic use of rituximab is appealing, specifically in SSc-associated PAH. Effects of 24 weeks of rituximab in 80 patients with SSc-PAH was previously assessed in a phase II, randomized, double-blind, placebo-controlled clinical trial, with a change in PVR as the primary study endpoint and it is expected to be completed at the end of 2019 [229].

15.5.3.2 Immunosuppressive Agents

Immunosuppressive agents such as glucocorticoids, mycophenolate mofetil, dexamethasone, cyclosporin, and etanercept have previously demonstrated improvements in PH in animal models [230]. An IL-6 receptor antagonist, Tocilizumab, is currently established for the treatment of rheumatoid arthritis [231] and Castleman's disease [232]. As there have been case reports of regression of PAH [233, 234] with this drug, tocilizumab is being considered for further investigation for the treatment of some forms of PAH. Sirolimus (or rapamycin), as an antiproliferative immunosuppressive drug, prevents T cells and B-cells activation through obstructing their response to IL-2 [235].

Currently, rapamycin is employed clinically in transplantation medicine as an immunosuppressant [236] that prevents proliferation of T cells, and in cardiovascular medicine [237] as an antiproliferative agent to reduce local restenosis. Rapamycin has also been shown to attenuate the development of PH, right ventricular hypertrophy, and pulmonary vascular neointimal formation in experimental models [238–240]. In addition, rapamycin is also recognized as an anticancer agent [241–243]. Considering the similarities between PAH and cancer, rapamycin may provide a novel therapeutic strategy for PAH. But several lessons recently show that targeting inflammation may also have unexpected consequences including compensatory mechanisms that may need to be addressed separately. Recently, in our preclinical work, administration of rapamycin resulted in an unexpected upregulation of PDGFR in SMCs that is involved in the development of PAH/PH by enhancing PASMC proliferation [244]. Combination therapy with both rapamycin and imatinib (an inhibitor of PDGFRs) may hold promise as a novel approach to these observations.

15.6 Inflammation in Other Groups of PH

Current classification of PH categorizes clinical conditions associated with PH based on similar pathophysiology, etiologies, clinical presentation, hemodynamic characteristics, and therapeutic management. Group 2 PH is secondary to the left heart diseases like heart failure, valvular diseases, and others. Group 3 is associated with lung diseases and/or hypoxia. Group 4 PH is due to chronic thromboembolic pulmonary hypertension (CTEPH) and other pulmonary artery obstructive processes. Group 5 includes diseases with unclear and/or multifactorial mechanisms (Table 15.5).

Although the critical roles of inflammation in the pathophysiology of WSPH Group 1 PAH has received considerable attention, knowledge of inflammation in the other WSPH PH group remains scarce. Notably, recent reports document that inflammation may trigger the development of vascular remodeling in Group 2 PH. For example, in a supracoronary aortic banding rat model combined with metabolic syndrome-induced LHD-PH, macrophage accumulation, increased IL-6 levels, and STAT3 activation were all observed in whole lung tissues. Metformin and anti-IL-6 antibodies further improved hemodynamic parameters and adverse pulmonary vascular remodeling [245]. Moreover, the presence of inflammation has also

Table 15.5 Clinical classification of PH

Group 2 PH due to left heart diseases
2.1 PH due to heart failure with preserved LVEF
2.2 PH due to heart failure with reduced LVEF
2.3 Valvular heart disease
2.4 Congenital/acquired cardiovascular conditions leading to postcapillary PH
Group 3 PH due to lung diseases and/or hypoxia
3.1 Obstructive lung disease
3.2 Restrictive lung disease
3.3 Other lung disease with mixed restrictive/obstructive pattern
3.4 Hypoxia without lung disease
3.5 Developmental lung disorders
Group 4 PH due to pulmonary artery obstructions
4.1 Chronic thromboembolic PH
4.2 Other pulmonary artery obstructions
Group 5 PH with unclear and/or multifactorial mechanisms
5.1 Hematological disorders
5.2 Systemic and metabolic disorders
5.3 Others
5.4 Complex congenital heart disease

Adapted from 6th WSPH
LVEF left ventricular ejection fraction

been reported in Group 3 PH. There are many abnormalities in the expression of inflammatory mediators in PH associated with lung disease. For example, (protein or mRNA) expression levels of MIF, CD74, and CXCR4 are significantly elevated in whole lungs from both IPF-PH patients and bleomycin-injected mice. Treatment of bleomycin-injected mice (to induce pulmonary fibrosis) with an MIF inhibitor attenuates pulmonary arterial muscularization, and reduces both, pulmonary inflammatory infiltration and right ventricular systolic pressures [246]. Speculation suggests close interactions between hypoxia, inflammation, and epigenetic changes may result in chronic persistent inflammation and irreversible pulmonary vascular remodeling [247]. In addition, several reports have suggested the prevalence of an inflammatory disease is higher in chronic thromboembolic pulmonary hypertension (CTEPH) patients [248–250]. The plasma level of CCL2 is also significantly correlated with pulmonary vascular resistance in CTEPH [251]. To sum up, inflammation also seems to play a considerable role in the other Group PH.

15.7 Summary

It is now evident that inflammation is inextricably linked to the initiation/development of PH. The inflammatory cells and their cytokines/chemokines participate in vascular remodeling of PAH (Fig. 15.1). Treating the inflammation in animal models and in human PAH associated with strong inflammatory disorders (e.g., SLE, mixed CTD, and POEMS syndrome) has been shown to improve pulmonary vascular remodeling and clinical and hemodynamic responses. Nevertheless, mechanisms of inflammation in the initiation/propagation of vascular remodeling remain still unclear. Recognition of the inflammation in PH may provide innovative and promising therapeutic strategies for the treatment of this devastating disease.

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16 Interactive Roles of CaMKII/Ryanodine Receptor Signaling and Inflammation in Lung Diseases

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Abstracts

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional protein kinase and has been recently recognized to play a vital role in pathological events in the pulmonary system. CaMKII has diverse downstream targets that promote vascular disease, asthma, and cancer, so improved understanding of CaMKII signaling has the potential to lead to new therapies for lung diseases. Multiple studies have demonstrated that CaMKII is involved in redox modulation of ryanodine receptors (RyRs). CaMKII can be directly activated by reactive oxygen species (ROS) which then regulates RyR activity, which is essential for Ca^{2+}-dependent processes in lung diseases. Furthermore, both CaMKII and RyRs participate in the inflammation process. However, their role in the pulmonary physiology in response to ROS is still an ambiguous one. Because CaMKII and RyRs are important in pulmonary biology, cell survival, cell cycle control, and inflammation, it is possible that the relationship between ROS and CaMKII/RyRs signal complex will be necessary for understanding and treating lung diseases. Here, we review roles of CaMKII/RyRs in lung diseases to understand with how CaMKII/RyRs may act as a transduction signal to connect prooxidant conditions into specific downstream pathological effects that are relevant to rare and common forms of pulmonary disease.

Keywords

Ca^{2+}/calmodulin-dependent protein kinase II · Ryanodine receptors · Reactive oxygen species · Inflammation

Abbreviations

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
Asp	*Aspergillus fumigatus*
Ca	Calcium
CaMK	Ca^{2+}/calmodulin-dependent kinases
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CFs	Cardiac fibroblasts
CICR	Ca^{2+}-induced Ca^{2+} release

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_16

ER	Endoplasmic reticulum
Erk1/2	Extracellular signal-regulated kinase 1/2
IP3Rs	Inositol triphosphate receptors
MCT	Monocrotaline
Mt-ROS	Mitochondrial ROS
OVA	Ovalbumin
ox-CaMKII	Oxidative activation of the Ca^{2+}/calmodulin-dependent protein kinase
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary vascular smooth muscle
ROS	Reactive oxygen species
RyRs	Ryanodine receptors
SCLC	Small cell lung cancer cells
Ser/Thr	Serine/threonine

16.1 Introduction

Chronic lung diseases such as asthma [1, 2], chronic obstructive pulmonary disease (COPD), and pulmonary hypertension [3] are a major and increasing global health burden with a high unmet need. Drug discovery efforts in this area have been largely disappointing and new therapeutic targets are needed [4]. Reactive oxygen species (ROS) generated from multiple mechanisms play a key role in the pathogenesis of chronic lung diseases [5]. Specifically, new studies have shown that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and ryanodine receptors (RyRs) play a pivotal role in ROS generation [6–9] and contribute to airway smooth muscle and pulmonary vascular smooth muscle (PASMC) phenotype transitions and remodeling. The aim of this review is to highlight the recent insights into the physiological role of CaMKII/RyRs signaling in lung diseases.

16.2 Structure and Activity of CaMKII

CaMKIIs are a family of multifunctional serine/threonine protein kinases [10] that respond to increases in intracellular [Ca^{2+}] which is the major second messenger inside the PASMCs and indispensable for the cellular contraction and pulmonary vasoconstriction [11]. An increase of [Ca^{2+}] inside the cardiomyocyte leads to activation (potentially lethal overactivation) of calcium-dependent signaling. As a result, overall CaMKII activity is increased approximately threefold in human heart failure [12], and the expression rate of CaMKIIδ was shown to be upregulated approximately twofold [13]. CaMKII protein expression is significantly upregulated in right ventricles of PH rats [14], but its role in lung diseases is not clearly elucidated.

CaMKII functions as a homo- or heteromultimer consisting of 12 subunits, each consisting of three conserved domains: an amino-terminal catalytic domain, a central autoregulatory domain, and a carboxy-terminal association domain [15]. The catalytic domain contains the ATP and substrate binding pockets, providing the catalytic activity of the protein. The autoregulatory domain contains an inhibitory pseudosubstrate sequence, several sites for posttranslational modification, and the calmodulin-binding region. The association domain is responsible for oligomerization of the subunits to form the holoenzyme, and contains variable regions that are alternatively spliced to form different splice variants of CaMKII [16].

CaMKII has four differentially expressed but highly homologous isoforms encoded by separate genes (α, β, δ, γ) [17].CaMKII structure and function have been most thoroughly studied in the brain, where holoenzymes composed of α and β isoforms are very abundant and involved in regulating postsynaptic signaling complexes, neurotransmission, and memory [18]. Substantial progress has also been made defining functions of CaMKII in cardiac homeostasis and pathophysiology where it contributes to heart failure secondary to chronic pressure overload [19]. Major CaMKII isoforms in VSM have been identified as mixtures of alternatively spliced products from δ and γ genes [20–22].

16.3 Structure and Activity of RyRs

Ryanodine receptors (RyRs), the intracellular release channels localized on the endoplasmic reticulum (ER), are the major cellular players of

Ca^{2+}-induced Ca^{2+} release (CICR) in mammalian cells [23]. Three ryanodine receptors (RyRs), RyR1, RyR2, and RyR3, are expressed in mammalian cells; each is encoded by a distinct gene [24]. The RyR1 isoform is dominant in skeletal muscles, whereas the RyR2 represents the cardiac RyRs isoform. The RyR3 isoform is found only at low expression levels in certain skeletal muscle types and brain [25, 26]. The RyR release channels have a critical function among the many molecules involved in cellular signaling. Through a stimulation in activity in response to an increase in $[Ca^{2+}]_i$, the RyR, and to a minor extent the inositol trisphosphate receptors, give rise to CICR, a universal cellular mechanism that allows amplification and propagation of the signals initially generated by entry. RyR-mediated CICR is a necessary requisite for cardiac muscle contraction and has an important role in neuronal function.

16.4 Redox Signaling and CaMKII/RyRs

16.4.1 Ca^{2+}-Dependent Activation of CaMKII

CaMKII is activated by increases in intracellular $[Ca^{2+}]_i$ [19]. These increases in intracellular $[Ca^{2+}]_i$ are sensed by the EF hand domain-containing protein calmodulin (CaM) and result in calcified CaM (Ca^{2+}/CaM) that binds to CaMKII, releasing the catalytic domain from constraint by the pseudosubstrate sequence embedded within the CaMKII regulatory domain. This allows the substrate and ATP to access the catalytic domain and results in activation of the kinase [15, 19]. Sustained activation of CaMKII results in autophosphorylation across subunits at Thr^{287} [27]. This phosphorylation event leads to a 1000-fold increase in affinity for CaM and prevents the reassociation of the catalytic domain resulting in persistence enzyme activity even in the absence of Ca^{2+}/CaM [28]. Recent reports show that activation of CaMKII subunits by Ca^{2+}/CaM and subsequent intrasubunit phosphorylation may also stimulate subunit exchange between holoenzymes leading to further activation of inactive holoenzyme [29].

16.4.2 ROS-Dependent Activation of CaMKII

Evidence for an alternative mechanism for increases in CaMKII activity in the absence of Ca^{2+}/CaM has been ascribed to generation of ROS. CaMKII can be oxidized at methionine 281 and 282 in the presence of ROS. H_2O_2 enhanced CaMKII activity was lost when this methionine pair (281/282) was replaced by valine (MMVV) [9]. This oxidation resistant mutant of CaMKII retained normal activation by Ca^{2+}/CaM and Thr^{287} autophosphorylation, strongly suggesting that CaMKII activity can be generated by a combination of Ca^{2+}/CAM and ROS-mediated oxidation of M281/282. Increased methionine oxidation of CaMKII, determined by immunoblotting, does not correlate with a decrease in the total amount of CaMKII protein present [30–32], but with an increase in kinase activity [8]. Thus, autophosphorylation and oxidation both have the capacity to "lock" CaMKII into a Ca^{2+}/ CaM-autonomous conformation with sustained activity. Oxidized, constitutively active CaMKII has been linked to heart failure, arrhythmias, vascular injury, asthma, and cancer, suggesting that CaMKII is an important ROS sensor for transduction of oxidative stress into clinically important disease phenotypes [19].

16.5 Oxidative Modification of RyRs

Using whole cell patch-clamp and confocal imaging of cardiomyocytes derived from ascending aorta thoracic aortic banding (TAB) hearts (TCMs), Kim et al. found that mitochondrial ROS increases oxidation of reactive cysteines in RyR in TCMs, which ultimately leads to destabilization of RyR-mediated release and induce dependent arrhythmia in hypertrophic rat hearts [33].

Redox status has a role in the age-related increase of RyR activity. Cooper et al. found that an age-associated increase of ROS production by mitochondria leads to the thiol-oxidation of RyR, which underlies the hyperactivity of RyR and thereby shortened refractoriness of release in cardiomyocytes from the ageing heart [34].

Redox modification of RyR affects alternans in a canine model of sudden cardiac death. Belevych et al. revealed abnormal RyR function in ventricular fibrillation cells was indicated by increased fractional release for a given amplitude of current and elevated diastolic RyR-mediated SR leak. VF myocytes had an increased rate of reactive oxygen species production and increased RyR oxidation. Treatment of VF myocytes with reducing agents normalized parameters of handling and shifted the threshold of alternans to higher frequencies. It means oxidation modulation of RyR promotes generation of alternans by enhancing the steepness of the release–load relationship and thereby providing a substrate for post-MI arrhythmias [35].

RyRs have 360 cysteine residues per tetrameric channel; 80 of these cysteines are estimated to be in a reduced free thiol state [36]. For the single RyR2 subunit, about 21 of 90 cysteine residues are in the free thiol state and available for redox modifications [36, 37]. Thus, RyR2 has been considered as the highly redox-sensitive ion channel. Each free thiol residue can be a target for various oxidative modifications, including disulfide bond formation, *S*-nitrosylation, and *S*-glutathionylation [38]. During oxidative stress, sulfhydryl groups of cysteine residues on RyR2 can be oxidized by ROS producing sulfenic, sulfinic, and sulfonic acids [39]. While there is no proof to support the functional significance of sulfinic and sulfonic acids, sulfenic acid can react with sulfhydryl groups, RNS, and GSH, yielding disulfide bridges, *S*-nitrosylation, *S*-glutathionylation, respectively. A lot of in vitro studies have pointed out that both ROS and other free radicals can induce changes in RyR channel activity. Lipid bilayer and single cell experiments have shown that ROS activates the single RyR channel function [40, 41]. However, the effects of oxidative agents on RyR2 largely depend on experimental conditions [42]. It has been demonstrated that low concentrations of oxidizing agents activate RyR2, whereas prolonged exposure or high concentrations of oxidants lead to irreversible RyR2 inhibition [43]. Different cysteine residues have been suggested to play a critical role in activation or inhibition of RyR2 by oxidative stress. Furthermore, an increase in overall oxidation of RyR2 with abnormal SR Ca^{2+} release has also been observed in lung and heart diseases. In the canine model of chronic heart failure (HF), the increased SR Ca^{2+} release has been attributed to the redox modification of RyR2 by ROS [44]. Similar to HF studies, cardiomyocytes isolated from the canine postmyocardial infarction (MI) model have been well characterized by increased levels of ROS production and RyR2 oxidation [35]. Using multiple complementary state-of-the-art approaches, we have very recently demonstrated the functional importance of RyR2 in the development of pulmonary artery hypertension (PAH) in both animals and human samples [45]. Specifically, our findings unveil that hypoxia dissociates FK506-binding protein 12.6 (FKBP12.6), the endogenous RyR2 stabilizer, from this Ca^{2+} release channel, which causes Ca^{2+} leak from the sarcoplasmic reticulum and then increases $[Ca^{2+}]_i$ in PASMCs, thereby leading to subsequent pulmonary artery remodeling, vasoconstriction, and hypertension. In this study, we have further discovered that these cellular events occur due to the mitochondrial Rieske iron–sulfur protein (RISP)-dependent ROS-mediated RyR2 protein oxidation. Moreover, genetic and pharmacological inhibition of RyR2, stabilization of FKBP12.6/RyR2 complex by treatment of S107, and in vivo lentiviral shRNA-mediated knockdown of SMC-specific RISP all can block the development of PAH, providing effective strategies to prevent and treat this devastating pulmonary disease in humans.

16.6 The Relationship Between CaMKII and Ryanodine Receptors

16.6.1 Regulation of Ryanodine Receptors by CaMKII

Strong evidence suggests that RyRs are important for Ca^{2+}-dependent processes in lung dis-

eases [46–49]. RyR2, a member of the ryanodine receptor (RyR) family, has been implicated as a substrate of CaMKII [50]. CaMKII phosphorylation is critical for regulating RyR2 activity [23, 51–53]. CaMKII phosphorylates RyR2 on Ser-2808 and Ser-2814. Specifically, Ser-2808 phosphorylation was mediated by muscarinic receptor subtype 2 and activation of protein kinase G (PKG), whereas dephosphorization of Ser-2814 involved activation of muscarinic receptor subtype 3 and decreased ROS-dependent activation of CaMKII. The overall effect of these changes in RyR2 phosphorylation is an increase in Ca^{2+} release even with low sarcoplasmic reticulum [Ca^{2+}] content and a reduction in aberrant Ca^{2+} leak [23, 51–53].

16.6.2 Regulation of CaMKII by Ryanodine Receptors

There is no strong evidence for RyRs directly regulating CaMKII, but crosstalk between CaMKII and RyRs may still exist. It has been suggested that increases of RyR2 activity can initiate increased sarcoplasmic reticulum Ca leak [24]. This increased intracellular [Ca^{2+}] could lead to activation of CaMKII. Thus, CaMKII could be indirectly regulated by RyRs through Ca^{2+} signaling.

16.7 Oxidative Stress and Redox Regulation of Lung Inflammation

Inflammation is an integral part of lung diseases and arises as a result of the persistent exposure of the respiratory tract to microbes, allergens, pathogens, etc. In response to such stimuli, the lung employs a number of defense mechanisms that produce various inflammatory mediators, such as ROS and cytokines (e.g., interleukin 6, tumor necrosis factor, and interleukin-1β) [54]. Persistent inflammation within the respiratory tract underlies the pathogenesis of numerous chronic pulmonary diseases including COPD, asthma, pulmonary fibrosis, and lung cancer. For example, COPD is a debilitating irreversible inflammatory lung disease associated with cigarette smoking. Asthma is characterized by excessive airway inflammation and accumulation of eosinophils. Pulmonary fibrosis maintains a significant inflammatory component throughout the course of the disease [55].

ROS, which could be produced during the inflammation as mentioned above, may play a role in enhancing inflammation through the activation of stress kinases (c-Jun activated kinase, extracellular signal-regulated kinase, p38) and redox-sensitive transcription factors, such as nuclear factor (NF)-κB and activator protein-1. Activation of these signaling molecules results in increased expression of a battery of distinct proinflammatory mediators [10]. Oxidative stress activates NF-κB-mediated transcription of proinflammatory mediators either through activation of its activated inhibitor I kappa B kinase (IKK) or the enhanced recruitment and activation of transcriptional coactivators. Enhanced NF-κB-coactivator complex formation results in targeted increases in histone modifications, such as acetylation leading to inflammatory gene expression [10].

16.8 Inflammatory Cellular Responses and CaMKII/RyR2 Signaling in the Lung

16.8.1 Inflammation and CaMKII

Recently, CaMKII has been shown to promote inflammatory responses in various cell types, including cardiomyocytes and airway smooth muscle (ASM) [56], by regulating expression of proinflammatory genes and a TLR-mediated NF-κB inflammatory pathway [57, 58]. Inhibition of CaMKII activity using molecular/genetic approaches or by inhaled KN93, a CaMKII inhibitor, strongly attenuated airway hyperresponsiveness (AHR) and inflammation in the mouse asthma model. Spinelli et al. found a spe-

cific function for the CaMKIIδ isoform in promoting ASM proinflammatory function in vivo in a murine model of allergen-induced AHR [56]. Ovalbumin sensitization and challenge resulted in induced CaMKIIδ upregulation relative to CaMKIIγ in tracheal smooth muscle, correlating with marked AHR and extensive airway inflammation. CaMKIIδ is also found to mediate inflammatory gene expression and inflammasome activation [59].

16.8.2 Inflammation and RyR2

All isoforms of the ryanodine receptor, including RyR2, are downregulated in inflammatory airway after exposure under cigarette smoke [49]. Furthermore, ryanodine can attenuate the duration of the high $K^{(+)}$-evoked $Ca^{(2+)}$ transient in inflamed rats. However, there was no significant impact of inflammation on the potency or efficacy of ryanodine-induced block of the caffeine-evoked $Ca^{(2+)}$ transient, or the impact of sarcoendoplasmic reticulum ATPase inhibition on the high $K^{(+)}$-evoked Ca^{2+} transient [60].

16.9 Role of CaMKII/RyR2 in Lung Diseases

CaMKII decodes the frequency and amplitude of intracellular transients [61]. As mentioned before, CaMKII phosphorylates a variety of Ca^{2+}-handling proteins such as RyR2 after being activated by higher concentrations of intracellular [62–64]. The effects of CaMKII phosphorylation on the proteins include greater influx, larger SR release, and prevents SR depletion by increasing SR loading [62, 63]. Based on these reports, we conclude that the crosstalk between CaMKII and RyR2, i.e., CaMKII-dependent RyR2-mediated Ca^{2+} release and RyR2-mediated Ca^{2+} release-induced CaMKII activation, may form a positive reciprocal loop in modulating intracellular Ca^{2+} homeostasis.

16.9.1 Pulmonary Artery Hypertension (PAH)

16.9.1.1 CaMKII in PAH

Pulmonary arterial hypertension (PAH) is a progressive disease of excess vasoconstriction and vascular cell proliferation that results in increased pulmonary vascular resistance and right heart failure. Recent studies in other vascular smooth muscle beds support the concept and provide potential mechanisms, whereby signaling through CaMKII promotes vascular smooth muscle phenotype transitions and vascular remodeling in the vascular pathology of PAH.

Luo et al. found augmentation of CaMKII phosphorylation level was caused by hypoxia in pulmonary arteries, lung tissues, and PASMCs [65]. Blocking the activation of CAMKII by specific inhibitor KN62 attenuates the proliferation of PASMCs under hypoxic conditions This suggests that CaMKII may contribute to hypoxia-mediated proliferation of PASMCs [65, 66]. In cardiac fibroblasts (CFs) isolated from hypertrophied right ventricles of monocrotaline (MCT)-induced PAH model rats, CaMKII is also significantly enhanced. The enhanced migration of MCT-CFs was prevented by pharmacological inhibition of CaMKII pathway. These results suggest that MCT-CFs exhibit proliferative and migratory phenotypes through a CaMKII signaling pathway [67]. Additionally, thrombin/fibronectin stimulated autophosphorylation of CaMKII in pulmonary microvascular endothelial cells, and inhibitors of CaMKII blocked thrombin-induced migration on fibronectin [68]. Conversely, there was no significant CaMKIIδ phosphorylation in right ventricular samples from PAH patients undergoing heart–lung transplantation as compared to nonfailing donors suggesting that CaMKII's involvement in pulmonary pathology is not universal nor completely understood [69].

16.9.1.2 RyR2 in PAH

Extensive experimental studies indicate that RyR plays an important role in the development of

hypoxia-induced pulmonary vasoconstriction (HPV) [70–73]. Oxidative modification of RyR is also related to HPV. Du et al. indicated that redox activation of RyR by ROS may transduce HPV. By reverse transcriptase-polymerase chain reaction, the authors found that all three RyR isoforms are expressed in rat pulmonary arteries and in PASMCs. The sustained phase, but not the transient phase, of HPV can be prevented by pretreating pulmonary arteries with RyR inhibitors ryanodine (200 μmol/L) or dantrolene (50 μmol/L). The addition of dantrolene, ryanodine, or the thiol-reducing agent dithiothreitol (1 mmol/L) during the sustained phase of HPV reversed the hypoxic vasoconstriction. In contrast, the superoxide scavenger nitroblue tetrazolium (500 nmol/L) prevented further hypoxic pulmonary vasoconstriction during the sustained phase of HPV but did not reverse it. Redox activation of RyR by ROS has an important role in transducing the sustained contraction of pulmonary arteries under hypoxia [74].

16.9.2 Asthma

16.9.2.1 CaMKII in Asthma

Asthma is a highly prevalent chronic lung disease with an associated high burden of disease [75]. Exposure of the respiratory epithelium to allergens is the initiating event in allergic asthma which is characterized by excessive pulmonary inflammation, airway hyperreactivity, and mucus production. Despite this complexity, there is a clear and established connection of enhanced oxidative stress and asthma [76, 77]. However, the potential mechanisms and pathways for increased ROS to affect asthma have remained unclear. Recently, more and more research has shown that CaMKII plays an important role in ROS generation [6, 78, 79] and contributes to asthma phenotypes in asthmatic patients and in vivo models of allergic asthma [9, 80].

Sanders and colleagues showed [80] that enhanced oxidative activation of the Ca^{2+}/calmodulin-dependent protein kinase (ox-CaMKII) in bronchial epithelium positively correlates with asthma severity and that epithelial ox-CaMKII increases in response to inhaled allergens in patients. The mouse models of allergic airway disease induced by ovalbumin (OVA) or *Aspergillus fumigatus* (Asp) further validated the relationship between ROS and ox-CaMKII in asthma progression [80]. Mice lacking functional NADPH oxidases due to knockout of p47 and mice with epithelial-targeted transgenic expression of a CaMKII inhibitory peptide or wild-type mice treated with inhaled KN-93, an inhibitor of CaMKII, were protected against airway hyperreactivity to inhaled methacholine.

Furthermore, Sebag and colleagues located CaMKII in the mitochondria (Mt-CaMKII) and found Mt-CaMKII of pulmonary airway epithelial cells contributes to an increase in mitochondrial ROS (Mt-ROS) and induction of hallmark features of allergic asthma [81]. Utilizing a novel transgenic mouse model in which bronchial epithelial cells conditionally express a potent CaMKII inhibitor directed to mitochondria, they found that mitochondrial CaMKII inhibition significantly reduced *A. fumigatus* and OVA-induced AHR, eosinophilic inflammation, and cytokine expression compared with WT controls.

CaMKIIδ and γ isoforms are primarily expressed in differentiated airway smooth muscle and have similar expression patterns as differentiated vascular smooth muscle [82]. Ovalbumin sensitization and challenge resulted in induced CaMKIIδ upregulation relative to CaMKIIγ in tracheal smooth muscle, correlating with marked AHR and extensive airway inflammation [56]. OVA-induced AHR and airway inflammation were absent in mice with smooth muscle conditional CaMKIIδ knockout. This effect was specific for the CaMKIIδ isoform as conditional deletion of CaMKIIγ had no significant effects on OVA-induced AHR, indicating nonequivalent functions of closely related CaMKIIδ and γ isoforms in this system.

16.9.2.2 RyR2 in Asthma

Spontaneous sparks were described in tracheal myocytes, and these were associated with the Ca^{2+}-induced release from RyRs [83].

Subsequently, in mouse airway smooth muscle, these sparks were characterized as the elementary release from RyRs, occurring predominantly through RyR2 [84]. In this context, studies on the pulmonary artery revealed that sparks are activated by release via ITPR [85], as well as in airway smooth muscle [86]. The physiological role of these sparks in guinea pig tracheal myocytes was well established. Essentially, they produce spontaneous transient outward currents caused by large-conductance Ca^{2+}-activated K^+ channels; they also induce spontaneous transient inward currents accomplished through Ca^{2+}-activated Cl-channels [83]. Therefore, all these components may serve an important role in the basal state regulation of the airway smooth muscle by stabilizing the membrane potential, the basal $[Ca^{2+}]_i$, and the basal contractile tone [87].

16.9.3 Lung Cancer

16.9.3.1 CaMKII in Lung Cancer

CaMKII hyperactivation has emerged as a signaling node for promoting lung cancer. CaMKII was aberrantly expressed in lung cancer cells and was correlated with poor prognosis in human lung cancer [88]. Perhaps this is not surprising given that CaMKII targets the action of proteins important for cell cycle control, cell survival, metastasis, and metabolism [89]. Chai et al. showed that CaMKIIγ promoted cell proliferation via direct activation of NF-κB and multiple oncogenic signaling pathways in non-small-cell lung cancer (NSCLC). Specifically, CaMKIIγ phosphorylates IκBα kinase β (IKKβ) at Ser177/181 and functions as a mediator of IKKβ activation in NSCLC. Furthermore, CaMKIIγ may also directly or indirectly upregulate multiple signaling pathways such as extracellular signal-regulated kinase 1/2 (Erk1/2), protein kinase B (Akt1), Stat3, and β-catenin and involve in regulating the survival and proliferation of NSCLC cells [90]. It has also been shown that CaMKII may suppress cell cycle progression by stabilization of p53 via CaMKII-dependent phosphorylation of the RING-H2 type E3 ligase (Pirh2) in lung cancer cell lines [91]. Evidence suggests that CaMKII also regulates small cell lung cancer cells (SCLC) as inhibition of CaMKII has antiproliferative effects on SCLC [92].

16.9.3.2 RyRs in Lung Cancer

ER and SR are the major intracellular storage organelles in nonexcitable and excitable cells, respectively. While RyRs are predominantly expressed in excitable cells, IP_3Rs are the main intracellular release channels in nonexcitable cells, including many cancer cells. Through the ER/mitochondrial crosstalk, IP_3Rs can further determine cell fate by controlling the mitochondrial elevation and metabolism [93]. That being said, there is limited evidence linking implicating RyRs to lung cancer but further research is still warranted.

16.10 Summary, Open Questions, and Future Research Directions

ROS and inflammation have been shown to play a key role in the pathogenesis of chronic lung diseases. The discovery that CaMKII/RyRs is configured to coordinate and transduce upstream, ROS and inflammation signals into physiological and pathophysiological downstream responses has potentially broad implications for understanding lung diseases (Fig. 16.1). However, their role in the pulmonary physiology in response to ROS is still an ambiguous one, especially the relationship between CaMKII and RyR2 in lung diseases. Because CaMKII and RyRs are important in pulmonary biology, cell survival, and cell cycle control, it is possible that the relationship between CaMKII and RyRs will be important for understanding and treating lung diseases and cancer. Future research in this area will allow us to parse the contributions of the various CaMKII/RyRs signal activation mechanisms to specific physiological processes in the lung.

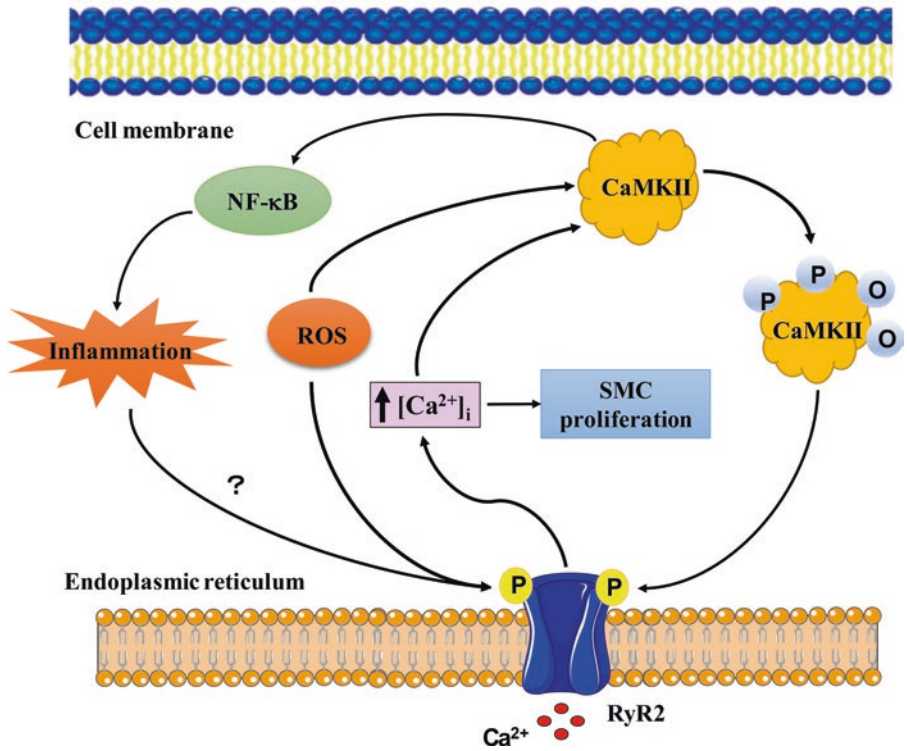

Fig. 16.1 The relationship between Redox signaling and CAMKII. CaMKII can be directly activated by ROS as oxidation or activated by increased intracellular as phosphorylation. CaMKII activation is critical for regulating RyR2 phosphorylation. The effect of the changes in phosphorylation of RyR2 is an increase in systolic Ca release at the low sarcoplasmic reticulum content. CaMKII has been shown to promote inflammatory responses by regulating NF-κB inflammatory pathway. Inflammation may downregulate RyR2

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17

Reciprocal Correlations of Inflammatory and Calcium Signaling in Asthma Pathogenesis

Ryan Okonski, Yun-Min Zheng, Annarita Di Mise, and Yong-Xiao Wang

Abstract

Asthma is a chronic disease characterized by airway hyperresponsiveness, which can be caused by exposure to an allergen, spasmogen, or be induced by exercise. Despite its prevalence, the exact mechanisms by which the airway becomes hyperresponsive in asthma are not fully understood. There is evidence that myosin light-chain kinase is overexpressed, with a concomitant downregulation of myosin light-chain phosphatase in the airway smooth muscle, leading to sustained contraction. Additionally, the sarco/endoplasmic reticulum ATPase may be affected by inflammatory cytokines, such as IL-4, IL-5, IL-13, and TNF-α, which are all associated with asthmatic airway inflammation. IL-13 and TNF-α seem to promote sodium/calcium exchanger 1 overexpression as well. Anyhow, the exact mechanisms beyond these dysregulations need to be clarified. Of note, multiple studies show an association between asthma and the ORMLD3 gene, opening new perspectives to future potential gene therapies. Currently, several treatments are available for asthma, although many of them have systemic side effects, or are not effective in patients with severe asthma. Furthering our knowledge on the molecular and pathophysiological mechanisms of asthma plays a pivotal role for the development of new and more targeted treatments for patients who cannot totally benefit from the current therapies.

Keywords

Asthma · Airway hyperresponsiveness · Allergen · Cytokine · Calcium signaling · Ion channels

Ryan Okonski and Yun-Min Zheng both made an equal contribution

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Abbreviations

$[Ca^{2+}]_i$	Intracellular Calcium Concentration
AHR	Airway Hyperresponsiveness
ASM/ASMC	Airway Smooth Muscle/Airway Smooth Muscle Cell
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II

Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_17

CICR	Calcium-Induced Calcium Release
CRTH2	Chemoattractant receptor-homologous molecule
DAG	Diacylglycerol
DHPR	Dihydropyridine Receptor
FKBP12.6	FK-506 Binding Protein
GPCR	G-Protein Coupled Receptor
IL	Interleukin
IP_3	Inositol Triphosphate
K_{Ca}	Calcium-Dependent Potassium Channel
MLCK	Myosin Light-Chain Kinase
MLCP	Myosin Light-Chain Phosphatase
NCX	Sodium/Calcium Exchanger
PGD2	Prostaglandin D2
PMCA	Plasma Membrane Calcium ATPase
ROCK	RhoA-kinase
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptors
SBB	Superficial Buffer Barrier
SERCA	Sarco/endoplasmic Reticulum ATPase
SR	Sarcoplasmic Reticulum
TMEM16A	Transmembrane Protein 16A
TNF-α	Tumor Necrosis Factor-Alpha
UPR	Unfolded Protein Response

17.1 Introduction

Asthma is a chronic inflammatory disease characterized by acute bronchial obstruction combined with various manifestations, such as shortness of breath, wheezing, cough, and chest tightness [1]. These symptoms emerge consequently to airflow restriction due to chronic airway inflammation which arises in response to certain factors, usually inhaled allergen(s), such as house dust mites (HDMs), tobacco smoke, chemical irritants, air pollution and viral infections, causing airway hyperresponsiveness (AHR), airway remodeling, and mucus hypersecretion [1–3].

Asthma is estimated to affect 25 millions of Americans and 334 million people worldwide [4, 43]. Despite the patients categorized as having severe asthma represent a minority, 1 out of 250 deaths is associated with asthma [3], resulting in annual direct medical and indirect economic costs of about €34 billion for the European Union (EU) [5] and over $80 billions for the United States [6, 7]. To this respect, it is important to carry on the deepening of our understanding about the molecular and physiological mechanisms that contribute to asthma pathogenesis.

On the basis of the immunological mechanisms involved, asthma can be divided into Th2 inflammation-related and non-Th2 inflammation-related asthma [1]. Th2-type asthma can be further classified as allergic, in which case it is associated with the production of Th2 cytokines, such as IL-4, IL-5, and IL-13, or nonallergic inflammation [8]. Non-Th2 inflammation is mainly mediated by Th1 and Th17 pathways, which lead to IL-17, ILC3, and other cytokines secretion to activate alveolar macrophages and neutrophils, causing neutrophil inflammation [9].

In addition to inflammation, two other important asthma features are airway hyperresponsiveness (AHR) and airway remodeling [1]. AHR is mainly caused by chronic airway inflammation, predominantly due to genetic factors, and characterized by high sensitivity of airway to various stimuli, whose exposure cause severe and premature contraction response resulting in excessive narrowing [1, 44]. Airway remodeling exhibits mucous metaplasia of airway epithelial cells, hyperplasia, and hypertrophy of smooth muscle, and distant subepithelial deposition of collagen [1, 10, 11].

Increased contractility of airway smooth muscle (ASM) contributes to airway hyperresponsiveness [12]. Calcium acts as a crucial secondary messenger for cellular contraction, hence dysregulations of its intracellular handling in ASM represent one of the molecular bases of asthma development. Moreover, it has been postulated that Ca^{2+} dysregulations may lead, at least in part, to changes in both the architecture and function of the lung [13]. Therefore, Ca^{2+}

handling mechanisms are finely tuned to regulate basal intracellular Ca^{2+} concentrations. It is conceivable that alterations in any of these processes may render airway smooth muscle susceptible to develop hyperresponsiveness that is observed in diseases, such as asthma [14]. Understanding the molecular mechanisms underlying these pathways is essential to gain insights into various respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF).

17.2 Ca^{2+} Homeostasis and Function in ASM

17.2.1 Smooth Muscle Contraction

Smooth muscle has a central function in the regulation of airway tone. It plays an important role in the development of some pathologies generated by alterations in contraction, such as hypercontractility and the airway hyperresponsiveness observed in asthma [15]. The mechanism of contraction in smooth muscle cells differs from skeletal or cardiac muscle cells [45, 53], being centered around myosin light chain (MLC) phosphorylation elicited by myosin light chain kinase (MLCK), whose activation is calcium-dependent [15]. There are three mechanisms that increase intracellular calcium concentration. The first consists in voltage-gated calcium channels activation by membrane depolarization, allowing calcium to enter the cell [16]. The second mechanism is initialized by hormones or neurotransmitters which can open ligand-gated channels expressed on the plasma membrane [16]. Lastly, the phospholipase-C pathway, triggered by hormones and neurotransmitters such as norepinephrine and angiotensin II, causes an increase in intracellular inositol triphosphate IP_3, which binds the IP_3 receptors on the membrane of the sarcoplasmic reticulum (SR) and induces Ca^{2+} release, resulting in calcium-induced calcium release (CICR) from the ryanodine receptors (RyRs), expressed on the SR membrane. This increase in $[Ca^{2+}]_I$ initiates the contraction process. Specifically, the augmented $[Ca^{2+}]_i$ saturates the binding sites on the calmodulin (CaM) protein and leads to the formation of a complex between the C-terminal domain of CaM and the N-terminal domain of MLCK. MLCK is then activated and phosphorylates MLC20, the regulatory myosin light chain (MLC) [15, 17], in the neck region of myosin, which is necessary for myosin-actin cross-bridge cycling and smooth muscle contraction and shortening [53]. Inhibition of MLC20 dephosphorylation depends on the decrease in myosin light chain phosphatase (MLCP) activity, whose downregulation seems to be strictly correlated with AHR [15].

As long as there is calcium bound to calmodulin, and the MLC is still phosphorylated, the smooth muscle will remain contracted. The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) then sequesters Ca^{2+} back into the SR, leading to muscle relaxation.

17.2.2 Ryanodine Receptors

The Ryanodine Receptors (RyRs) are expressed on the SR membrane of skeletal, cardiac, and smooth muscle cells, in addition to the brain. In mammals three isoforms of RyRs (RyR1-3) are known, all expressed in ASMCs [70]. Calcium increase in ASM cells can be generated endogenously or experimentally by stimulation with contractile agonists such as muscarinic, histamine, cysteinyl leukotrienes, and purinergic agonists, activating G-protein coupled receptors (GPCRs) associated with $G_{\alpha q}$, as well as membrane depolarizing agents [13]. The G_q in particular, when activated, induces phospholipase C activation, which cleaves phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG. IP_3 then binds the IP_3Rs on the SR, inducing Ca^{2+} release. RyRs can colocalize with IP_3Rs on the sarcolemma membrane, and can be reciprocally activated, inducing further CICR [98]. Ca^{2+}-bound calmodulin then acts as a negative feedback inhibitor on RyRs, thereby inhibiting CICR from the SR [69]. RyR2 is stabilized by FK-506 bind-

ing protein 12.6 (FKBP12.6), and dissociation of FKBP12.6 from RyR2 has been shown to increase $[Ca^{2+}]_i$ in ASMCs [98].

Calcium release from RyRs is associated to Ca^{2+} sparks, which can be either evoked or spontaneous [49], and consists in localized releases of Ca^{2+} from the SR, specifically from adjacent RyRs [98]. These Ca^{2+} sparks are an extremely localized release of Ca^{2+}, albeit a large release, which induce a local $[Ca^{2+}]$ increase on the micromolar scale, while their contribution to the global cellular Ca^{2+} increment is on a nanomolar scale [46]. In guinea-pig tracheal myocytes, it has been shown that Ca^{2+} sparks produce spontaneous transient outward currents caused by large-conductance Ca^{2+}-activated K^+ channels. Moreover, they also induce spontaneous transient inward currents accomplished through Ca^{2+}-activated Cl-channels [18]. On this basis, all these components appear to serve an important role in the basal state regulation of the ASM by stabilizing the membrane potential, the $[Ca^{2+}]_i$ and the basal contractile tone [14].

17.2.3 Sarco/Endoplasmic Ca^{2+}-ATPase

It has been suggested that RyRs are crucial to the initiation but not maintenance of calcium oscillations [13, 19]. A proposed model is that at the start of the Ca^{2+} oscillations, when SR calcium levels are sufficiently high, the open probability of RyR can be increased via calcium-induced calcium release (CICR), following activation of IP_3R [13, 20]; this causes the SR depletion. The sarcoendoplasmic reticulum calcium ATPase (SERCA) actively pumps cytosolic Ca^{2+} back into the SR, triggering muscle relaxation while maintaining SR Ca^{2+} stores. The primary SERCA involved with Ca^{2+} regulation in ASMCs is SERCA2b [56]. However, SERCA2 is unable to fully restore SR calcium to basal levels during continual IP_3R activation [21]. This inhibits RyRs opening but has less effect on the IP_3R [13]. On this basis, after the initial $[Ca^{2+}]_i$ increment, mainly due to RyRs activation, it is clear that the periodic increase in $[Ca^{2+}]_i$ may entirely depend by calcium flux through the IP_3R [13].

The replenishment of the SR operated by SERCA2 is crucial for the maintenance of Ca^{2+} oscillations. Pharmacological SERCA2 inhibition as phospholamban treatment, the most studied regulator of SERCA2 activity, has been shown to disrupt cardiac contractility by reducing its affinity with calcium [22]. SERCA2 regulation in human ASM appears to be quietly different as phospholamban protein is not expressed and it has been suggested the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) as the primary SERCA2 activity regulator [23].

Basal $[Ca^{2+}]_i$ is restored both by the activity of SERCA2, which pumps Ca^{2+} into the SR, and by calcium extrusion out of the cells operated by the sodium–calcium exchanger (NCX) and plasma membrane calcium ATPase pump (PMCA) [13]. Therefore, for Ca^{2+} oscillations to persist, SR content of calcium has to be replenished from extracellular sources. When SR Ca^{2+} content decreases below a certain threshold, DAG activated receptor operated mechanisms (ROCE) [24, 25] and calcium entry via store-operated Ca^{2+} channels (SOCE) [26, 27] are activated [24, 25], thus sustaining airway contraction during prolonged contractile agonist stimulation [13, 26].

17.2.4 Na^+/Ca^{2+} Exchanger and the Plasma Membrane Ca^{2+}-ATPase

The sodium/calcium exchanger (NCX) is a membrane protein which extrudes one Ca^{2+} ion while introducing 3 Na^+ ions. This flux, which allows the active calcium extrusion thanks to the passive Na entry, is active when the exchanger is in its forward mode. By contrast, in its reverse mode (NCXREV), it introduces Ca^{2+} and extrudes Na^+ [14, 27]. Three isoforms of NCX (NCX1-3) are known to date. NCX1 is extensively distributed in mammalian cells, NCX2 is mainly expressed in the brain, spinal cord, gastrointestinal and kidney tissues, while NCX3 is present in the brain

and skeletal muscle [28]. The primary isoform present in ASM is NCX1.3 [29].

The reverse mode occurs under higher intracellular [Na^+] conditions. In smooth muscle cells, Ca^{2+} depletion of the SR causes [Na^+] influx [51], providing a plausible mechanism for the NCX reverse mode to refill SR stores, although it has been given a minor role in Ca^{2+} homeostasis [30]. A selective inhibitor of the NCX reverse mode, KB-R7943, has been shown to reduce spasmogen-induced contractions of tracheal ASM. Moreover, KB-R7943 treatment reduced Ca^{2+} fluxes after caffeine administration [52], clearly demonstrating the involvement of NCX reverse mode in SR stores refilling. Moreover, in a murine chronic model of allergen-induced airway hyperresponsiveness, it was shown that the levels of NCX1 were significantly augmented, and that NCXREV activity was increased [14, 31].

The plasma membrane Ca^{2+}-ATPase (PMCA) is also responsible for the extrusion of intracellular Ca^{2+}, albeit through an active process. According to an extensive number of studies, NCX is predominantly expressed in areas of the plasma membrane that are in direct contact with the SR, while the PMCA seems to be distributed throughout the whole membrane [50]. This implies a unique physiological function of NCX compared to the PMCA in the ASM.

Furthermore, in human myocytes, the addition of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin IL-13, also increased the expression of NCX1 and favored NCXREV activity [14, 31]. These findings suggest that, during inflammation, NCXREV could significantly contribute to an increase in the $[Ca^{2+}]_i$, which would predispose airway smooth muscle to hyperresponsiveness [14].

17.2.5 Ca^{2+}-Activated Potassium/ Chloride Channels

Ca^{2+}-dependent potassium channels (K_{Ca}) are voltage-gated ion channels which open in response to local [Ca^{2+}] increase, causing hyperpolarization. Three classes of these channels have been described, BK, IK, and SK [71]. BK channels are large-conductance potassium channels, which have been found to react to Ca^{2+} sparks. Ca^{2+} sparks seem to play a role in Ca^{2+}-dependent potassium (K_{Ca}) channel activation in smooth muscle cells, inducing an outward potassium current, which leads to hyperpolarization of the plasma membrane and eventually to relaxation [46–48]. These spontaneous transient outward K^+ currents (STOCs) are counterbalanced by spontaneous transient inward currents (STICs), which result from activation of Ca^{2+}-dependent chloride channels. STICs have been recorded in both murine and equine ASMCs, causing plasma membrane depolarization by efflux of chloride ions, leading to Ca^{2+} influx and subsequent contraction [90, 91]. Kotlikoff and Wang demonstrated that STOCs are favored at more positive resting membrane potentials, while STICs at more negative resting potentials [92]. In both cases, STICs and STOCs are involved in membrane potential regulation.

17.3 Ca^{2+} Dysregulations in the Asthmatic Airway

17.3.1 MLCK and MLCP Dysregulations

Several studies have addressed the relationship between the role of MLCP and airway smooth muscle hypercontractility and airway hyperresponsiveness [15]. Alterations in the asthmatic airway lead to increased mass, which results from both hypertrophy and hyperplasia [54, 55].

However, the exact mechanisms through which this occurs have still to be elucidated. One proposed contribution comes from evidence of MLCK upregulation in the asthmatic ASM, compared to healthy controls [58]. Measuring single cell shortening capacity and velocity, it has been shown that MLCK upregulation is associated with increased contractility [58]. MLCK upregulation occurs consequently to MLCP inhibition [15], whose activity is regulated by RhoA-kinase (ROCK), a serine/threonine protein kinase which is a downstream effector of RhoA, a monomeric G-protein.

Specifically, RhoA is a small GTPase that binds several surface receptors which activate ROCK [32]. ROCK expression is increased in airways of patients with asthma, and importantly its inhibitors, such as Y27632, Y39983, H1152, and fasudil, have been demonstrated to induce airway smooth muscle relaxation [33]. In 2015, a study performed in ROCK knockout mice showed that ROCK expression is directly correlated with the increase in hyperresponsiveness caused by exposure to ozone. Similarly, reduced expression of either ROCK isoform, ROCK1 or ROCK2, prevented hyperresponsiveness development [34]. An interesting work performed in ovalbumin-sensitized mice, a murine model of allergic asthma, showed an increase in both total RhoA and RhoA-GTP compared to controls [59]. The degree of hyperresponsiveness is directly associated to increased ROCK expression in the airway, which in turn results in the increased phosphorylation, and thus inhibition, of MLCP which leads to MLCK upregulation [15]. Moreover, the RhoA pathway mediates increased Ca^{2+}-sensitivity in ASM. If MLCP activity is downregulated, net myosin phosphorylation in response to a given change in $[Ca^{2+}]_i$ will be enhanced and/or prolonged, resulting in greater contraction: in other words, the Ca^{2+}-sensitivity of the contractile apparatus is increased [35].

17.3.2 SERCA Activity Downregulation

The downregulation of SERCA2 plays a critical role in the dysregulation of ASM Ca^{2+} homeostasis in asthma and the consequent alterations in phenotype [36]. It has been reported that SERCA2 protein expression in both native and cultured ASM from endobronchial biopsies of patients with mild and moderate/severe asthma is diminished compared with that of healthy subjects [36] and this effect correlates with the disease severity [37]. Of note, suppression of SERCA2 by small interfering RNA (siRNA) in ASM cells derived from donors without asthma, recapitulated the asthmatic phenotype, with similarly slowed recovery of $[Ca^{2+}]_I$ to baseline and increased rates of cell proliferation [36].

Moreover, a study performed by Espinosa and colleagues showed that the cytokine IL-13 alone was not sufficient to produce ASM cell proliferation, but after 24-h exposure to IL-13, subsequent treatment with leukotriene-D4, a member of the leukotriene family shown to be a potent contractor of smooth muscle, resulted in a significant increase in cell proliferation [67]. Multiple studies using both cultured human ASM cells and animal models have shown that inflammatory cytokines impact SERCA activity. Both human and rat pancreatic islet cells showed decreased SERCA2b expression after exposure to IL-1B, due to altered protein half-life [68]. A study by Sathish and co-workers demonstrated that overnight exposure of human ASM cells to either TNF-α or IL-13 induced a lower $[Ca^{2+}]_i$ decline after treatment with both bradykinin and acetylcholine, which indicates impaired SERCA function [72]. The mechanism causing SERCA activity impairment was elucidated by Mahn et al. by investigating the role of SERCA expression after IL-13 exposure and the subsequent release of eotaxin-1 [57], an eosinophil chemotactic protein whose production is stimulated by IL-13 [62, 63]. In ASM cells from asthmatics and healthy controls, Mahn et al. demonstrated an increased release of eotaxin-1 in response to IL-13 in the asthmatic ASM cells compared to control. The same result was also obtained after knocking down SERCA2 expression in both asthmatic and healthy ASM cells, revealing higher eotaxin-1 levels in the asthmatic ASM cells [57]. These results suggest that SERCA2 reduced expression and activity in the ASM may potentiate eotaxin-1 release. As eotaxin-1 production induces eosinophil maturation and migration, it has been reported a high correlation between eosinophilia and asthmatic disease severity [60, 61]. Increased eotaxin-1 release resulting from SERCA downregulation represents a possible mechanism which correlates Ca^{2+} dysregulation to the heightened inflammatory signaling observed in the asthmatic airway.

As SERCA2 plays a pivotal role in the generation of Ca^{2+} oscillations [38], its reduced expres-

sion and/or function results in increased basal levels of $[Ca^{2+}]_i$, enhanced Ca^{2+} influx-induced elevations of $[Ca^{2+}]_i$, and altered dynamics of Ca^{2+} oscillations [36]. Consistent with this, an increased resting $[Ca^{2+}]_i$ and slowing of Ca^{2+} oscillations has been observed in ASM with downregulated SERCA activity [23].

17.3.3 NCX1 Overexpression

Similar to SERCA, NCX1 expressed in ASM has been shown to be affected by inflammatory cytokines. Sathish et al. showed augmented NCX1 expression in human ASM cells under overnight exposure to TNF-α or IL-13, compared to controls. Using a combination of protein synthesis and transcriptional inhibitors with simultaneous exposure to cytokines, Sathish et al. demonstrated decreased NCX1 production, suggesting that TNF-α and IL-13 treatments upregulate both transcription and synthesis of NCX1 protein [73]. This is corroborated by a study from Yoo and colleagues, which showed increased protein and mRNA expression of NCX1 in human ASM cells after TNF-α, IL-33, and IL-13 treatment. Additionally, in a murine ovalbumin model of asthma, they showed increased NCX1 expression in the asthmatic mice, with respect to controls [74]. Currently, it needs to be still elucidated whether NCX1 overexpression is involved in asthma pathogenesis by the reverse mode activity, or is merely a compensatory response in order to extrude the increased $[Ca^{2+}]_i$ in the attempt of restoring basal Ca^{2+} levels.

17.3.4 Effect of Inflammation on Ryanodine Receptors

Mutations or post-translational modifications in RyRs cause intracellular Ca^{2+} leak, leading to SR Ca^{2+} depletion, and activation of Ca^{2+}-dependent enzymes with cell-type specific downstream effects [39]. Proximity of mitochondria to the SR can result in mitochondrial Ca^{2+} overload and impaired energy metabolism, inducing an oxidative state that further damages RyR channels, creating a vicious cycle [39]. Moreover, during the inflammatory response, eosinophil migration toward the tissues has been shown to be associated with the production of reactive oxygen species (ROS), possibly through interaction with adhesion molecules [95, 96]. On this basis, being eosinophilia associated with the asthmatic airway inflammation, ROS can potentially alter the molecular machinery of the ASM. In murine pulmonary artery smooth muscle cells, it has been demonstrated that ROS production caused enhanced Ca^{2+} release from RyR2 [97]. Specifically, RyR2 oxidation causes the dissociation of FKBP12.6, which stabilizes the RyR2 channel in the closed state and reduces its activity, leading to increased Ca^{2+} release. The significance of FKBP12.6 on the activity of the RyR2 channel was demonstrated in a guinea-pig ovalbumin model. FKBP12.6 was showed to normalize the AHR of asthmatic rats when overexpressed [75]. Similarly, another RyR2-stabilizing protein, calstabin2, was showed to dissociate from RyR2 after acute exposure to interleukins involved in the asthmatic inflammatory response (IL-5, IL-13, and TNF-α), resulting in increased Ca^{2+} release [39].

17.4 Genetic Causes of Ca^{2+} Dysregulations in Asthma

In a genome-wide study, it was found that a single nucleotide polymorphism of the ORMLD3 gene, which encodes transmembrane ER proteins, has been strongly associated with the childhood development of asthma [64]. Furthermore, a later study showed that the ORMLD3 gene product influences the development of the unfolded protein response (UPR), an ER-mediated intracellular signaling process, which can trigger inflammation [65, 66]. This study demonstrated augmented basal $Ca^{2+}{}_i$ levels in response to ORMLD3 overexpression, and a slower return of $Ca^{2+}{}_i$ content to baseline, suggesting an impaired SERCA activity. Conversely, silencing the ORMLD3 gene, the authors observed an attenuated UPR suggesting a potential cause and effect relation [65].

Another recent study by Huang et al. showed increased mRNA expression of TMEM16A, an important Ca^{2+}-activated chloride channel in ASM, in Th2-high human asthma patients, as well as in an ovalbumin-induced asthmatic mouse model [93]. Moreover, they showed that by inhibiting TMEM16A in both murine and human ASMCs, the contraction in response to methacholine, a well-known muscarinic agonist, was markedly reduced. This was corroborated by Zhang et al., who also showed increased expression of TMEM16A in the ASMCs of OVA-sensitized mice [94]. Using two different inhibitors of TMEM16A in vivo, they demonstrated prevention of AHR, showing a 50% reduction in agonist-induced cell shortening in TMEM16A knockout mice. Considering that TMEM16A depolarizes the cell membrane in response to Ca^{2+} sparks, it makes sense that overexpression of this protein in the asthmatic ASMCs would increase the propensity for airway hyperresponsiveness. Furthermore, a very recent work demonstrated that Eact, a TMEM16A agonist, modulated ASM contraction in both ex vivo and in vivo models, suggesting that agonism of TMEM16A may lead to clinically relevant bronchospasm [40]. Thus, the demonstrated airway hyperresponsive effects of inhaled Eact in vivo could be of high clinical significance [40].

17.5 Asthma Therapies

17.5.1 Current Treatments

Asthma therapies are currently classified into two groups: short-term treatments for immediate relief of acute bronchoconstriction episodes, and preventive longer-term therapies daily administrated. The first category consists of short-acting inhaled β2-agonists and anticholinergics, while the second includes longer-acting β2-agonists, inhaled or oral corticosteroids, antileukotrienes, allergy shots, and others immunomodulators [77].

Short-acting β2-agonists, such as albuterol, work by binding and activating β2-adrenergic receptors in the lungs, leading to bronchodilation. However, these effects are only temporary, lasting from 3 to 6 h for the oral inhalation route [78]. Besides ASM, β2-adrenergic receptors are also expressed in many other districts, and are associated with sympathetic nervous system activation. Therefore, side effects can also include tachycardia, shakiness, and induction of arrhythmias, as well as hypokalemia [78].

Anticholinergic drugs, such as ipratropium bromide, lead to bronchodilation through nonselective antagonism of M2 and M3 muscarinic receptors in the lungs, primarily of the larger-caliber airways. Their action lasts up to approximately 6 h, with a peak of efficacy at roughly 1–2 h [79].

Long-acting β2-agonists, such as salmeterol, are often used in conjunction with inhaled corticosteroids. Inhaled corticosteroids act as a potent anti-inflammatory agent that works through epigenetic pathways, reversing histone acetylation of certain inflammatory genes [80]. Despite the efficacy of corticosteroids, particularly when combined with long-acting β2-agonists [80, 81], patients affected by severe asthma tend to have a poor response to these drugs [80]. Antileukotrienes, which block 5-lipoxygenase or antagonize the leukotriene receptor, represent a relatively new class of drugs, with some failures early-on, although direct comparison studies are still needed [81], especially for the poor known clinical efficacy and safety compared to mainstay treatments. Allergen-specific immunotherapy, commonly referred to as allergy shots, is effective, albeit only for those patients reporting specific IgE antibody reactions to known allergens [82]. This makes their widespread clinical use more difficult, as only a subset of asthmatic subjects who choose to undergo testing can benefit from this treatment.

17.5.2 Potential Future Therapies

As the knowledge about asthma pathophysiology is deepened, more genetic and molecular targets are unveiled. Gene therapy might play a curative

role by fixing the single nucleotide polymorphism of the ORLMD3 gene and dampening the UPR. The discovery of overexpression of FKBP12.6 reducing the asthmatic response in mice opens the door for potential gene therapy. Even though further studies on humans are needed, upregulation of the gene promoting FKBP12.6 is expected to provide a long-term therapy by decreasing $[Ca^{2+}]_i$.

A study performed by Deshpande et al. examined the use of inhaled bitter tastants, which created a paradoxical bronchodilation in human ASM cells, despite increased intracellular Ca^{2+}. This occurred due to a localized Ca^{2+} increase, presumably Ca^{2+} sparks, which were found to activate BK_{Ca} channels, leading to hyperpolarization of ASM cells membrane. Interestingly, compared to inhaled β2-agonists, the inhaled bitter tastants produced a threefold higher bronchodilation, which was also reversible [76]. Further research is needed on the effect of inhaled bitter tastants, which might be administered as a standalone therapy or a combined treatment.

Rho-associated protein kinase inhibitors also could be explored for treatment of asthma. As mentioned above, RhoA kinase acts by phosphorylating and then inactivating MLCP, leading to MLCK upregulation. Currently, HA-1077, a RhoA kinase inhibitor, is approved in both China and Japan for use in cerebral vasospasm, thanks to its effect in inducing arterial smooth muscle relaxation [83]. Therefore, it is conceivable that this drug could be beneficial in asthmatics by inducing bronchorelaxation.

As discussed before, there are numerous inflammatory cytokines associated with the airway inflammation in asthma, including IL-4, IL-5, IL-13, IL-33, and TNF-α. All of these are potential targets for controlling asthmatic inflammation, particularly IL-4 [84, 85], IL-5 [86], IL-13 [87], and TNF-α [88, 89]. Because all of these cytokines are associated with eosinophilia, targeting them either through monoclonal antibody therapy or targeted fusion proteins might limit the spread of eosinophil-derived inflammation of the airway. This could also result in downstream effects on SERCA and NCX1 expression.

A promising drug, currently employed in phase II studies, is represented by fevipiprant, an antagonist of the CRTH2 receptor of prostaglandin D2 (PGD2) [41]. CRTH2 is a chemoattractant receptor-homologous molecule expressed on Th2 cells, associated with inhibitory G-protein (G_i), whose activation leads to a decrease in intracellular cAMP concentration and to a concomitant elevation of $Ca^{2+}{}_i$ levels [42]. Since PGD2 is a pleiotropic mediator which in asthma exerts relevant actions on many immune/inflammatory and airway structural cells via stimulation of CRTH2, this receptor may represent a suitable molecular target for novel anti-asthma treatments [41]. The phase II studies have preliminarily shown that fevipiprant is characterized by a good efficacy and safety profile. If these findings will be further corroborated and extended by ongoing phase III trials, fevipiprant could become a valid option for add-on asthma therapy. In particular, when compared to the currently available anti-asthma drugs, fevipiprant shows several advantages such as a lower cost, and especially the oral route of administration [41].

17.6 Summary

The pathogenesis of asthma is complex and still not fully understood. As diagrammed in Fig. 17.1, rising evidence from studies using both ASM cells and animal models support the downregulation of SERCA in the asthmatic airway, leading to prolonged $[Ca^{2+}]_i$. The origin of the attenuated SERCA expression and/or activity may be a result of an altered ORMLD3 gene expression, whose product is implicated in the UPR and has been shown to impair SERCA activity. Whether the UPR apparent effect on SERCA expression contributes to further inflammatory response, such as eosinophilia or the dissociation of FKBP12.6 from RyR2, still needs to be explored. Additionally, overexpression of MLCK results in an increased myosin light chain phosphorylation. These effects lead to sustained ASM contraction, which would normally be counteracted by MLCP, and results to be exacerbated by the upregulation of RhoA, dampening MLCP activity. An impor-

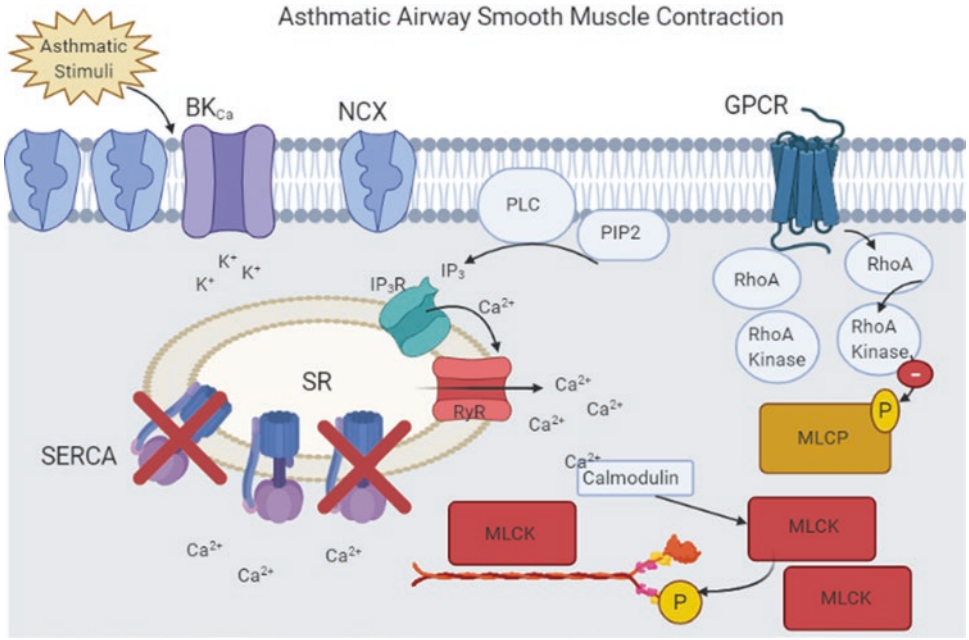

Fig. 17.1 A diagram of the signaling pathway for increased contractile responses in asthmatic airway smooth muscle cells**.** Evidently, asthmatic stimuli cause upregulation of RhoA/RhoA kinase, MLCK, and NCX, along with a concomitant down regulation of SERCA. *SERCA* sarcoendoplasmic reticulum calcium ATPase, *SR* sarcoplasmic reticulum, *NCX* sodium–calcium exchanger, *MLCK* myosin light-chain kinase, *MLCP* myosin light-chain phosphatase, *RyR* ryanodine receptor, IP_3 inositol triphosphate, IP_3R inositol triphosphate receptor, *GPCR* G-protein coupled receptor, *PLC* phospholipase c, *PIP2* phosphatidylinositol 4,5-biphosphate, BK_{Ca} calcium activated potassium channel

tant role is also played by immune cells and cytokines. As the knowledge of the underlying genetic causes of asthma is deepened, and gene therapy becomes more popular, targeting patient-specific phenotypes will lead to more individualized therapy, perhaps with a decreased incidence of systemic side effects. This will be important especially for patient categories that respond poorly to mainline therapies.

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18

Crosstalk Between Lung and Extrapulmonary Organs in Infection and Inflammation

Zhihan Wang, Qinqin Pu, Canhua Huang, and Min Wu

Abstract

Acute and chronic lung inflammation is a risk factor for various diseases involving lungs and extrapulmonary organs. Intercellular and interorgan networks, including crosstalk between lung and brain, intestine, heart, liver, and kidney, coordinate host immunity against infection, protect tissue, and maintain homeostasis. However, this interaction may be counterproductive and cause acute or chronic comorbidities due to dysregulated inflammation in the lung. In this chapter, we review the relationship of the lung with other key organs during normal cell processes and disease development. We focus on how pneumonia may lead to a systemic pathophysiological response to acute lung injury and chronic lung disease through organ interactions, which can facilitate the development of undesirable and even deleterious extrapulmonary sequelae.

Keywords

Pneumonia · Lung inflammation · Intercellular networks · Interorgan networks · Acute lung injury · Chronic lung disease

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List of Abbreviations

ACE2	Angiotensin-converting enzyme 2
AH	Alcoholic hepatitis
AIS	Acute ischemic stroke
ALD	Alcoholic liver disease
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BBB	Blood–brain barrier
CAP	Community-acquired pneumonia

Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_18

CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus Disease 2019
CoV	Coronavirus
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DPP4	Dipeptidyl peptidase 4
GIT	Gastrointestinal diseases
HI	*Haemophilus influenzae*
HPA	Hypothalamic–pituitary–adrenal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICH	Intracerebral hemorrhages
ICU	Intensive care units
LPS	Lipopolysaccharide
MC	*Moraxella catarrhalis*
MERS-CoV	Middle east respiratory syndrome CoV
MOF	Multiple organ failure
MV	Mechanical ventilation
NK	Natural killing
NPE	Neurogenic pulmonary edema
NTHI	Non-typeable *Haemophilus influenzae*
PA	*Pseudomonas aeruginosa*
PNS	Parasympathetic nervous system
SA	*Staphylococcus aureus*
SAH	Subarachnoid hemorrhage
SAP	Stroke-associated pneumonia
SARS-CoV	Severe acute respiratory syndrome CoV
SCFA	Short-chain fatty acids
SFB	Segmental filamentous bacteria
SIDS	Stroke-induced immunodepression syndrome
SIRS	Systemic inflammatory response syndrome
SNS	Sympathetic nervous system
SP	*Streptococcus pneumoniae*
TBI	Traumatic brain injury
T_H	T helper cells
T_H17	T helper 17
Treg	Regulatory T cells
VALI	Ventilator-associated lung injury
VAP	Ventilator-associated pneumonia

18.1 Introduction

With the rapid development of technologies and advances in scientific research, such as the Human Microbiome Project, microbial DNA has been detected in the lungs of healthy individuals [1, 2]. The perception of sterility of healthy lungs has been thoroughly revised by discovering varied microbiota in healthy and diseased lungs. A plethora of lung microbes provides a unique perspective for understanding mechanisms in lung diseases [3]. Most human bacterial pathogens colive with us and do not cause diseases, or cause subclinical or asymptomatic infection, depending on multiple factors, including the microbes and their interaction with the host.

Acute lung infection often affects the lung parenchyma (pneumonia), a common and severe acute lower respiratory tract infection. Pneumonia, such as the ongoing pandemic COVID-19, is also the leading cause of global health problems. During acute infection, the lungs can coordinate complex interactions with many extrapulmonary organs, to boost resistance and resilience to diseases and transmit pathological impact. Indeed, respiratory host defense failure leads to acute and chronic complications to influence other organ systems [4–6].

Various underlying diseases cause chronic lung infections. Bronchiectasis is formed by tuberculosis, scarring associated with acute infection, cystic fibrosis, etc., resulting in deterioration of the lung parenchyma [3]. During exacerbations, viral infections are considered to be a trigger [7]. In addition, due to the complex exchanges between host cells and microorganisms, such as bacteria, viruses, and fungi, chronic obstructive pulmonary disease (COPD) may deteriorate dramatically [8].

Acute and chronic inflammation is potentially attributed to the development and progression of many diseases, in which organ interactions play a role in the pathogenesis. Organ crosstalk mediates pulmonary inflammation, resulting in damage to multiple target organs, such as the brain, gut, heart, liver, and kidney (Fig. 18.1).

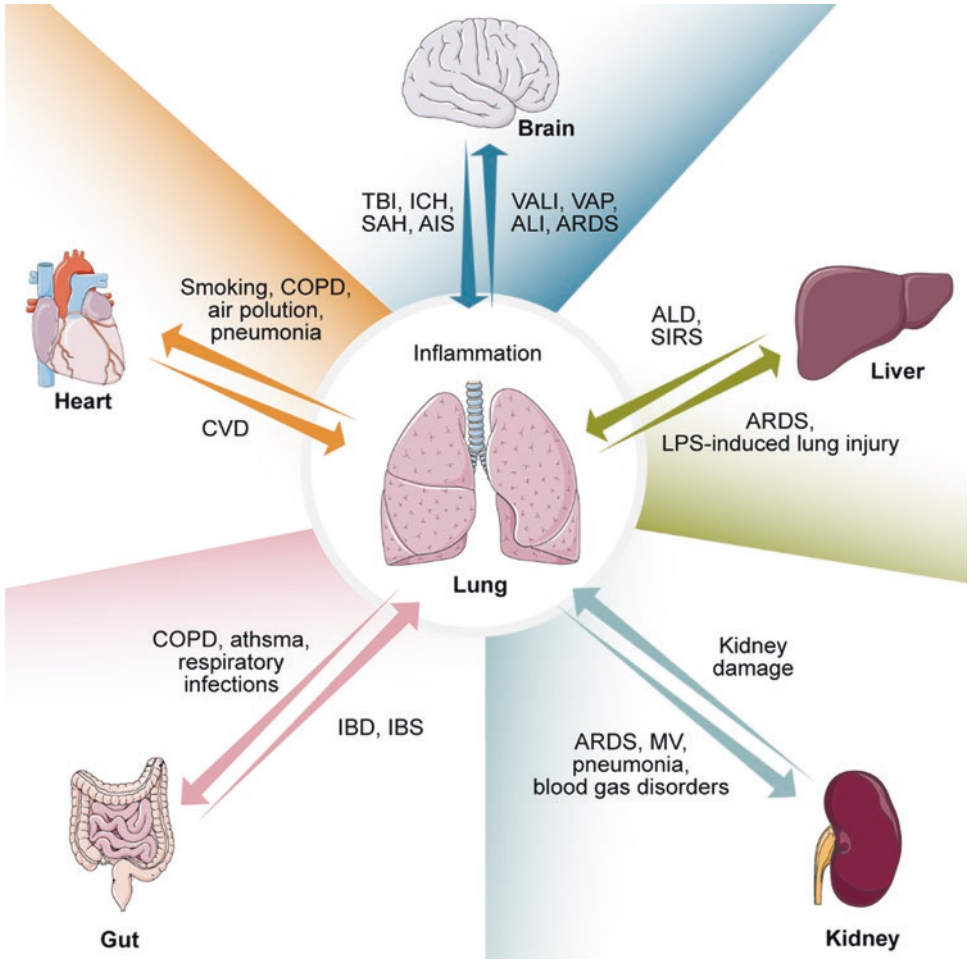

Fig. 18.1 Schematic diagram of broad-scale crosstalk between the lungs and extrapulmonary organs, and the effects of lung inflammation on the vital organs. This crosstalk is bidirectional and may impact both lungs and other involved organs; Abbreviations: *AH* alcoholic hepatitis, *AIS* acute ischemic stroke, *ALD* alcoholic liver disease, *ALI* acute lung injury, *ARDS* acute respiratory distress syndrome, *COPD* chronic obstructive pulmonary disease, *CVD* cardiovascular disease, *IBD* inflammatory bowel disease, *IBS* irritable bowel syndrome, *ICH* intracerebral hemorrhages, *LPS* lipopolysaccharide, *MV* mechanical ventilation, *SAH* subarachnoid hemorrhage, *SIRS* systemic inflammatory response syndrome, *TBI* traumatic brain injury, *VALI* ventilator-associated lung injury, *VAP* ventilator-associated pneumonia

This chapter aims to discuss the established and proposed communications between the lung and other organ systems. These communications may profoundly impact the development and progression of lung inflammation and other organ diseases. The potential mechanisms of interaction between organs are also discussed.

18.2 Microbiome in the Lung

Pathogens in healthy lungs may originate from the oropharynx and airways, but similar species and abundancy of pathogens may cause different diseases [9, 10] (Fig. 18.2). Importantly, the abundance of *Prevotella*-affiliated taxa in the lung is decreased compared to that in the surrounding tissues, while *Proteobacteria* increased, especially *Enterobacteriaceae*, *Ralstonia* spp.,

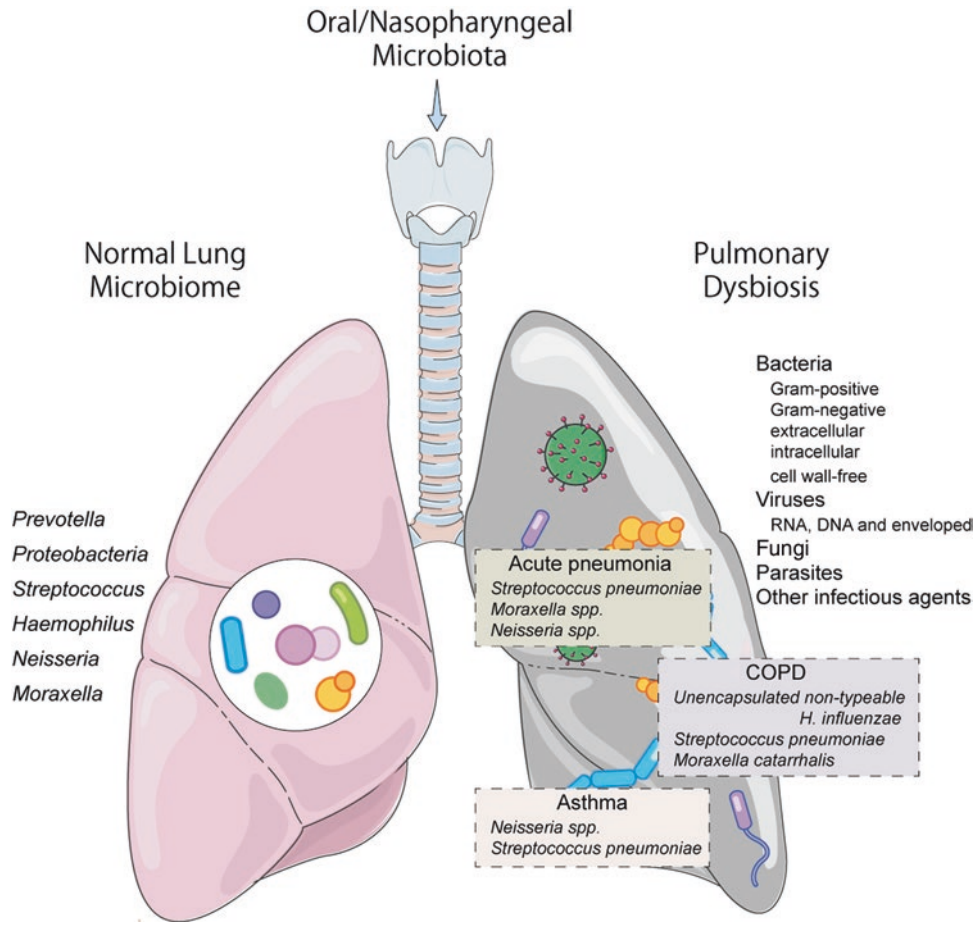

Fig. 18.2 Healthy lung microbiome and dysbiosis in disease states. In healthy lungs (left; pink lobe), *Prevotella, Proteobacteria, Streptococcus, Haemophilus, Neisseria*, and *Moraxella* are commonly found. The lungs can be affected by many different pathogens, including bacteria, viruses, fungi, parasites, etc. Enhanced bacterial reproduction, ciliary dysfunction, and mucus production may lead to increased microbial density, reduced microbial clearance and trapping, and, ultimately, lung diseases (right: gray lobe). As shown starting from the middle right, different respiratory diseases are characterized by dominant bacterial species: acute pneumonia is usually characterized by *S. pneumoniae*, *Moraxella* and *Neisseria*; chronic obstructive pulmonary disease (COPD), by unencapsulated non-typeable *H. influenzae*, Streptococcus, and *Moraxella catarrhalis*; asthma, by *Neisseria* and *Streptococcus*

and *Haemophilus* spp. [1]. The microbiome in the lung helps establish immune homeostasis and maintains an immune response between tolerance and inflammation [11]. For example, *Streptococcus pneumoniae* (SP), *Haemophilus influenzae* (HI), *Neisseria* spp., and *Moraxella* spp. are commonly found in healthy lung microbiota, but can sometimes cause diseases [9].

The microbes that can cause pneumonia are numerous, diverse, and poorly understood, often sharing nothing in common, making it difficult to deduce a conventional approach to control the disease. Pathogens include bacteria (Gram-positive, Gram-negative, extracellular, intracellular, and cell wall-free), viruses (RNA, DNA, and enveloped), fungi, parasites, and other infectious agents. Analyses of clinical data by Jain et al. showed that among adult patients hospitalized with community-acquired pneumonia (CAP), rhinoviruses (9%), influenza viruses (6%), and pneumococci (5%) are the three most common microorganisms, although there are still

many species and types undetected (62%) [12]. Different populations (children, patients in or outside of the hospital, etc.) involve various microorganisms [13]. SP is the leading cause of acute pneumonia, followed by *Moraxella* spp. and *Neisseria* spp. [3]. HI is often found in smoking-related lung diseases and causes bronchopneumonia [3]. Unencapsulated non-typeable *H. influenzae* (NTHI) along with SP and *M. catarrhalis* (MC) can cause recurrent airway infections in patients with COPD [14]. *Proteobacteria* (*Neisseria* spp.) and *Firmicutes* (SP) are commonly present in asthmatic airways [15]. NTHI, SP, and MC cause chronic infections of the middle ear. In addition, SP, and *N. meningitidis* and capsulated forms of HI, are the most common cause of bacterial meningitis [3]. Overall, the microbiome in the lung is diverse and may be associated with diseases of the lung and other organs.

The occurrence and severity of pneumonia depend on microbial factors, the delicate balance between microbial exposure and elimination, and the complex interaction between the host and the environment. Impaired ciliary clearance or enhanced bacteria propagation and migration may contribute to increasing growth in certain species and influence lung inflammation [16]. The regulatory network of non-coding RNAs (ncRNAs) and inflammasomes may also play roles by modulating inflammatory responses, tissue remodeling, and innate and adaptive immunity [17, 18]. For example, oral infection of *Helicobacter pylori* protects against asthma symptoms by NLRP3 inflammasome activation [18]. These findings help to explore more potential mechanisms of lung inflammation, especially behind the gut–lung axis.

18.3 Brain–Lung Crosstalk

Recently, the interaction between the brain and lungs has received intense attention, and increasing evidence points out that crosstalk between them exacerbates inflammation [19–21] (Fig. 18.3). People with severe brain injury, such as severe traumatic brain injury (TBI), intracerebral hemorrhages (ICH), subarachnoid hemorrhage (SAH) or acute ischemic stroke (AIS), are likely to develop lung injury, including respiratory failure, ventilator-associated lung injury (VALI) and ventilator-associated pneumonia (VAP), acute respiratory distress syndrome (ARDS), and neurogenic pulmonary edema (NPE) [22–25]. On the other hand, pulmonary infection leads to increased neuroinflammation (IL-1β, TNFα, and NOX2) and neurological deterioration [26]. Ischemia, hypoxia, and sepsis resulted from a variety of pulmonary disorders can cause acute lung injury (ALI) and ARDS [27]. ALI can cause brain dysfunction [28], and ARDS survivors experience cognitive impairment [29].

18.3.1 Pneumonia Occurring after Traumatic Brain Injury

Traumatic brain injury (TBI), an insult to the brain by external mechanical forces, is one of the leading causes of disability and death involved in trauma and may dysregulate systemic immune responses, making TBI patients more susceptible to infection in the post-acute injury period [30]. The nosocomial infection rate of patients with severe TBI is as high as 50%, which is higher than that of other patients in intensive care units (ICU) (about 30%) [31, 32]. The frequent use of mechanical ventilation in severe TBI patients increases the risk of VAP, which is related to the intranasal carriage of nosocomial pathogens [i.e., *Pseudomonas aeruginosa* (PA) [33], *Staphylococcus aureus* (SA) [34], *Enterobacteriaceae* [35], SP [36], and HI [37]]. In addition, patients with severe TBI often require urinary catheters and are accompanied with long-bone fractures, expanding additional access to infection [38]. Moreover, trauma patients with sepsis may have increased propensity of an excessive immune response to primary and secondary infections and the subsequent onset of immunodeficiency (called TBI-induced immunosuppression), impairment of the systemic immune defense system, especially natural killer cells (NK), T helper cells (T_H), regulatory T cells (Treg), and neutrophils [39].

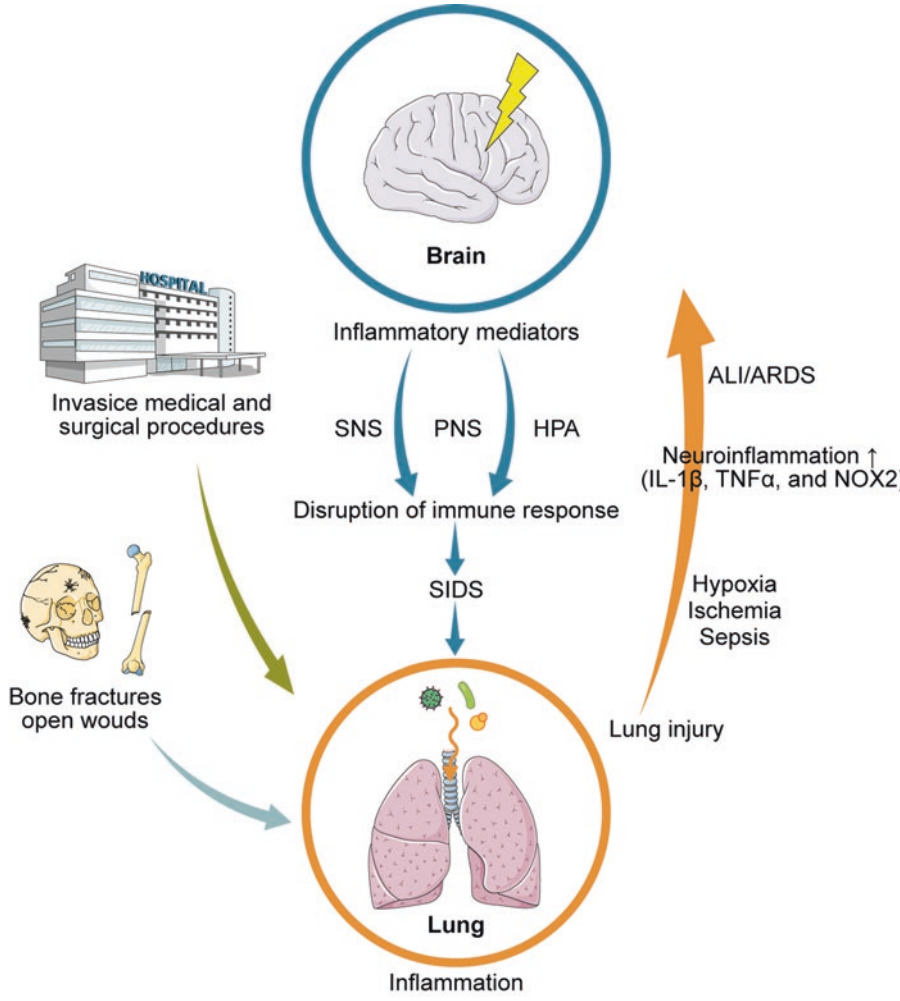

Fig. 18.3 Pathophysiology of brain injury and its influence on the systemic immune system contributing to lung infection; *ALI* acute lung injury, *ARDS* acute respiratory distress syndrome, *HPA* hypothalamus–pituitary–adrenal axis, *PNS* parasympathetic nervous system, *SIDS* stroke-induced immunodepression syndrome, *SNS* sympathetic nervous system

Further understanding the complex common and interactive biological mechanisms between this "double-hit" insult of TBI and infection may shed light on novel therapeutic strategies for TBI patients [40]. This two-phase injury involves primary brain injury, followed by neuroinflammation, oxidative stress, necrosis, and apoptosis [41], and then secondary "hit" injuries, such as secondary lung injury [42]. TBI induces the activation of glial cells (microglia and astrocytes), the destruction of the blood–brain barrier (BBB), the release of inflammatory cytokines and chemokines (IL-1β, IL-12, TNF-α, CCL2, and CXCL9, etc.), as well as the recruitment and migration of blood-derived leukocytes into the brain parenchyma, leading to widespread immunosuppression in systemic immunity [43, 44]. Independent of TBI, systemic infection also activates the host immune system. PA is the most common cause of VAP in ICU patients, producing endotoxin lipopolysaccharide (LPS) that can be detected by TLR4 of innate immune cells, including neutrophils, to trigger the production of pro-inflammatory cytokines, such as IL-1β,

TNF-α, and IL-6 [45]. During sepsis, the number of circulating neutrophils is reportedly increased, but the function of these cells is impaired [46], including reduced pathogen clearance [47] and chemotaxis dysfunction (reduced CXCR2) [48], increased production of anti-inflammatory cytokines (IL-10) [49], and delayed apoptosis [50]. When the neutrophil function is severely impaired, patients may have much increased risk of nosocomial infection [51], which is confirmed by an experimental sepsis model to make mice more susceptible to SA pneumonia secondary infection [52]. Besides, the nervous system may communicate with immune systems through two main routes: the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS), and then a series of neuromodulators are released, including catecholamine, norepinephrine, acetylcholine, and glucocorticoid [53]. These events lead to immunosuppression in systemic immunity, making patients susceptible to secondary lung infections due to impaired immunity.

18.3.2 Stroke-Associated Pneumonia (SAP)

Significant progress has been made in elucidating the pathophysiological mechanisms of stroke-induced immunodepression syndrome (SIDS) and stroke-associated pneumonia (SAP) [54]. SAP is associated with increased morbidity, mortality, and medical costs after acute ischemic stroke (AIS) [55]. The inflammatory response after stroke can promote tissue healing, but an excessive inflammatory response can cause secondary damage. The immunosuppression caused by stroke reduces inflammation to avoid brain tissue damage, but weakens the body's resistance to pathogens, and potentially resulting in infections.

Currently, the pathophysiological mechanism of SAP is an aspiration theory and stroke-induced immunosuppression theory [54]. Risk factors related to aspiration, such as impaired levels of consciousness and dysphagia, are important risk factors for SAP [56]. Systemic immunosuppression not only is unique to stroke but also occurs after brain trauma, brain surgery, spinal cord injury, and injuries of other central nervous system (CNS) [57]. The main clinical manifestations of SIDS are a continuous and rapid decline in cellular immune function, inactivation of monocytes and Th1 cells, decrease in Th-mediated lymphocytes, and increase of apoptosis of immune cells in the spleen, thymus, and lymph nodes [58, 59]. Post-stroke activation of three systems, such as SNS, HPA axis, and parasympathetic nervous system (PNS), leads to immunosuppression [60]. Stressors can initiate brain damage from any organs. For example, the cholesterol-fed rabbits showed extensive neurodegenerative changes, particularly DNA-base excision repair disorders [61]. A deep understanding of the pathophysiological mechanisms leading to SAP will be crucial for developing new treatment strategies to improve stroke patients' outcomes.

18.3.3 Neuroinvasive Potential of Coronaviruses

Coronaviruses (CoVs) are large enveloped positive-sense single-stranded RNA viruses that primarily cause mild respiratory disease (such as HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1), but three recent outbreaks of CoVs, namely severe acute respiratory syndrome CoV (SARS-CoV), middle east respiratory syndrome CoV (MERS-CoV), and current SARS-CoV-2 [62], can develop into life-threatening conditions. SARS-CoV-2 causes Coronavirus Disease 2019 (COVID-19), which has been spreading globally, as of today (July 9, 2020) causing more than 10 million cases and more than 300,000 death globally.

There is increasing evidence that coronaviruses may also invade CNS inducing neurological diseases [63]. A report shows that 36.4% (78/214) of COVID-19 patients experienced neurological symptoms, including headaches, unconsciousness, and paresthesia [64]. Autopsy analyses indicate that the deceased patient had brain tissue edema and degeneration of some

neurons [65]. Also, genome sequencing analysis reveals the presence of SARS-CoV-2 in cerebrospinal fluid [66]. CoVs infection may cause viral encephalitis (referring to inflammatory lesions in the brain parenchyma) [66, 67], infectious toxic encephalopathy (referring to a type of reversible brain dysfunction syndrome) [64, 65], and acute cerebrovascular disease [68, 69]. These findings suggest that respiratory failure caused by SARS-CoV-2 may be associated with the neurological abnormality, and dissecting the viral neuroinvasion may be of significance for the prevention and treatment of COVID-19.

However, the pathobiology of these neuroinvasive viruses is not fully understood. The exact mechanisms underlying the brain–lung association remain to be determined, including angiotensin-converting enzyme 2 (ACE2), neuronal pathways, blood circulation pathways, hypoxia, immune injury, and so on (Fig. 18.4).

SARS-CoV enters human host cells mainly through cell-mediated receptor ACE2 [70, 71], while MERS-CoV through dipeptidyl peptidase 4 (DPP4) [72]. ACE2 is expressed in the airway epithelium (including the oral cavity and nasal mucosa), the lung parenchyma, and also widely expressed throughout the CNS (neurons, astrocytes, and oligodendrocytes) [73, 74] (Fig. 18.4a).

Respiratory viruses, such as neurotropic influenza A virus, invade the nasal cavities and upper airways through the trigeminus and the vagus nerves [75]. Increasing evidence shows that CoVs may invade peripheral nerve terminals and enter the CNS through synaptic connections [75–77] (Fig. 18.4b). CoVs may spread retrogradely via transsynaptic transfer through the mechanism of endocytosis or exocytosis [78].

Another example is olfactory neurons' transportation, including the unique anatomy of the olfactory nerve and the olfactory bulbs in the nasal cavity and forebrain, which effectively forms a channel between the nasal epithelium and the CNS [79] (Fig. 18.4c). As a result, CoVs can enter the brain through the olfactory tract at the early stages of infection or nasal vaccination [80]. Several studies have explored the possible involvement of the olfactory pathway in the route of SARS-CoV-2 to the CNS [73, 81, 82].

Furthermore, when a virus causes diffuse alveolar and interstitial inflammatory exudation and edema in the lungs, in turn, it can cause abnormal gas exchange in the alveoli, leading to hypoxia in the CNS, causing headaches and acute cerebrovascular diseases, such as AIS [83] (Fig. 18.4d).

The immune system can also mediate damage to the nervous system [84] (Fig. 18.4e). In severe pneumonia, SIRS or SIRS-like immune disorders following virus infection may be abnormally triggered and cause multiple organ failure (MOF) [68]. Neurotropic viruses can activate glial cells and induce a pro-inflammatory state [85]. Therefore, patients with CoV infection should promptly subject to necessary tests, including cerebrospinal fluid and consciousness, for potential neurological complications, which is crucial to improve the prognosis of critically ill patients.

18.4 Gut–Lung Axis

Chronic respiratory inflammatory diseases (such as COPD and asthma) are highly prevalent airway diseases and often associated with chronic gastrointestinal diseases (GIT), for example, inflammatory bowel disease (IBD, up to 50% of adults) or irritable bowel syndrome (IBS, 33% of patients) [86–89]. Moreover, COPD patients manifest increased intestinal permeability and are 2–3 times more likely to be diagnosed with IBD. Asthma patients also show changes in intestinal mucosal function and structure [87, 89, 90]. It is expected that other lung and airway disorders have an intestine manifestation component, which should take into consideration for basic and clinical scientists.

The microbiome mediates the arguably most important interaction between the gut and the lungs. Although many intestinal microbiomes have not been cultivated, the "core" community includes up to 14 bacterial genera and 150 bacterial species, four major bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Actinobacteria*) [91–93]. For microbiome, the vast numbers herald the importance and diversity of its functions and the complexity of its interac-

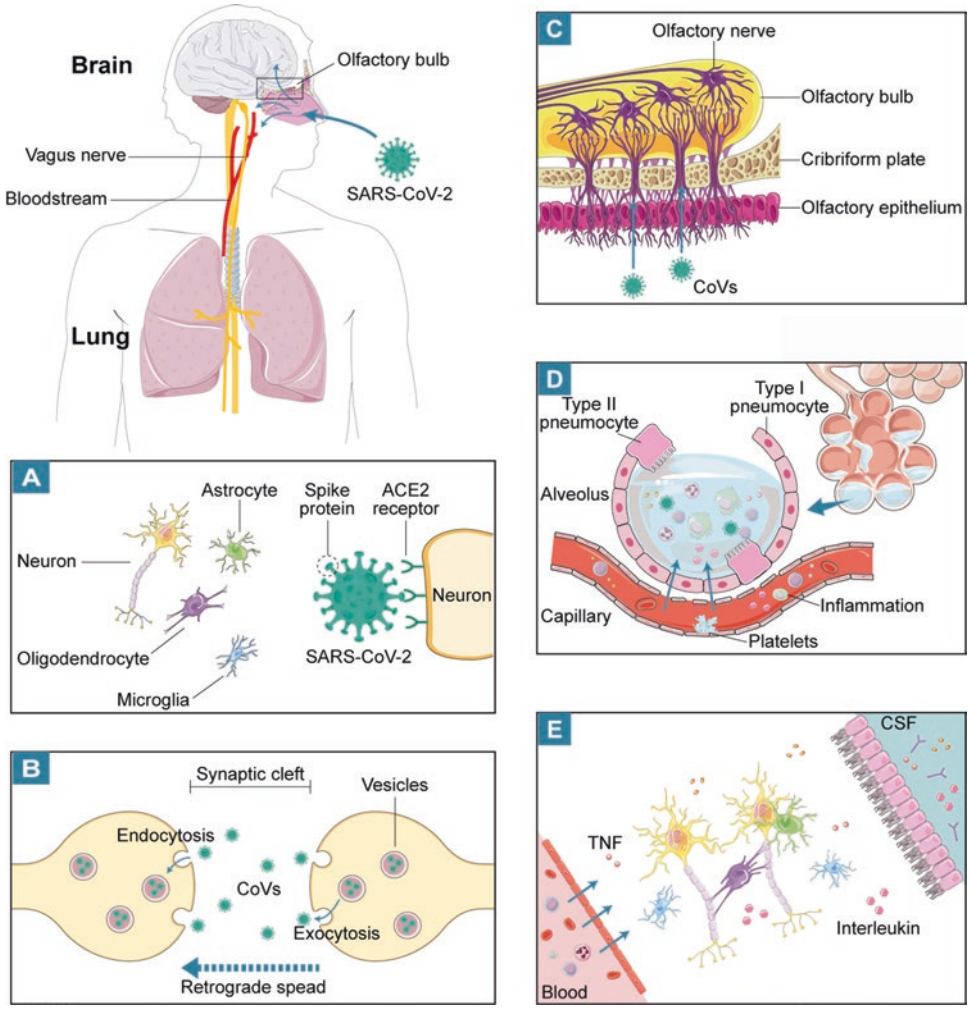

Fig. 18.4 Schematic illustration of the potential neuromechanisms of (SARS-CoV-2). (**a**) Angiotensin-converting enzyme 2 (ACE2) receptors are expressed in the critical cell types of the central nervous system (CNS), including neurons, microglia, astrocytes, and oligodendrocytes, binding to the spike protein on SARS-CoV-2. (**b**) Coronavirus (CoV) may spread retrogradely via transsynaptic transfer. (**c**) SARS-CoV-2 spreads from the olfactory epithelium along the olfactory nerve to the olfactory bulb in the CNS. (**d**) SARS-CoV-2 attacks the lung tissue and causes a series of lung lesions such as hypoxia damage. (**e**) Severe pneumonia caused by CoV infection may trigger systemic inflammatory response syndrome (SIRS) and even lead to multiple organ failure (MOF). Neurotropic viruses can activate macrophages and glial cells, secrete many inflammatory factors, and eventually cause chronic inflammation and brain damage. *CSF* cerebrospinal fluid

tion with the immune system in different organs. For example, intestinal epithelial cells and immune cells directly obtain information from microbes to trigger local cytokine responses to regulate inflammatory responses, thereby affecting immune responses at distant sites (e.g., the lungs) [94]. Segmental filamentous bacteria (SFB) in the intestine can stimulate pulmonary T helper 17 (T_H17) responses and protect mice from *S. pneumoniae* infection when introduced by probiotic administration or co-housing or present naturally in mice [95]. The transfer of bacteria from GIT to the lungs has been observed in sepsis and ARDS; however, there is lesser evidence that microorganisms are transferred directly between sites [96]. It is important to

understand that many of the crosstalk is bidirectional. Cecum-ligation puncture (CLP) is a classical animal sepsis model, whose damage in the gut can induce significant subsequent pathophysiology in the lung, liver, kidney, and other organs [97]. Therefore, it is crucial to assess the structure–function relationship between the gut and lung microbiota and host immunity (Fig. 18.5).

18.4.1 Asthma

Asthma is one of the most common chronic diseases in children, with over 30% prevalence, affecting more than 300 million people worldwide [98]. Microbial abnormalities at the beginning of life may be the critical factor that may render the tendency toward the development of asthma [99]. The colonization of *Clostridium difficile* in infants at one-month-old is associated with asthma after 6–7 years of age [100]. In adults, the overall composition of fecal microbiota in allergic asthma patients is not different from healthy individuals, but there are differences in specific taxa, such as the enrichment of *Bifidobacterium adolescentis* is negatively correlated with the time since asthma diagnosis [101, 102]. *Lachnospira, Veillonella, Clostridium faecalibacterium,* and *Rothia* in the gut have also been shown closely related to infant asthma [103]. Although there are still many mysteries in the relationship between intestinal microbes and asthma, many studies have shown that the microbe-based diagnosis and treatment and probiotics' prevention can potentially treat asthma and other related allergies in children.

18.4.2 COPD

COPD manifests with chronic bronchitis and/or emphysema characterized by airflow obstruction,

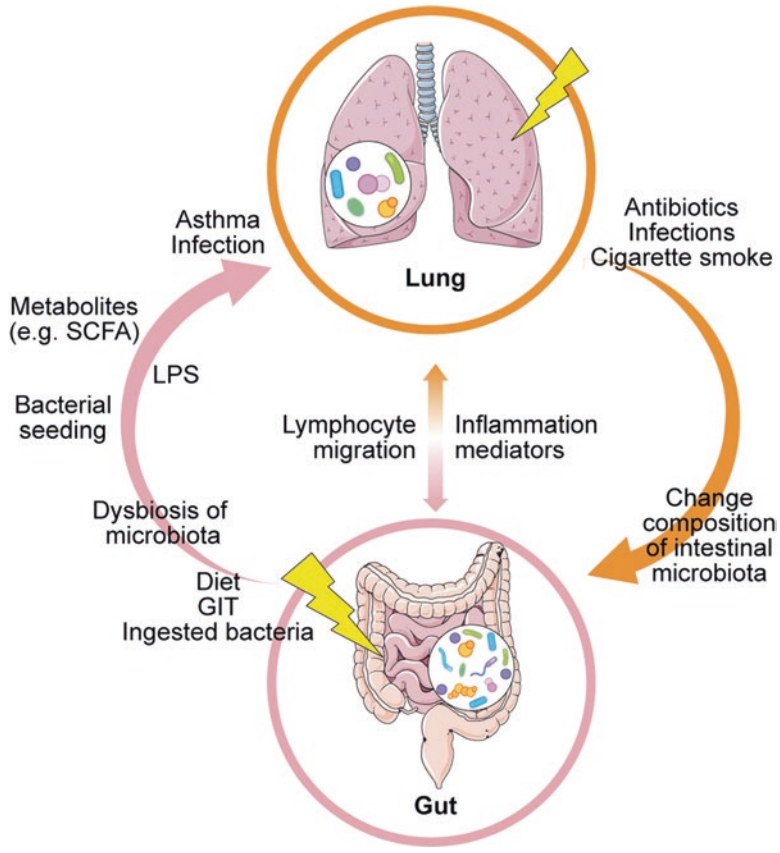

Fig. 18.5 Factors affecting the crosstalk between the intestine and the lung. The interaction between the lung and the gut is bidirectional. Many stressors from various diseases or other oxidants (smoking) put pressure from the lung to the gut, while the gut also has multiple mechanisms to transmit signals to feedback to the lung; *GIT* gastrointestinal diseases, *LPS* lipopolysaccharide, *SCFA* short-chain fatty acids

which can further develop into common chronic diseases of pulmonary cardiovascular disease and respiratory failure. Smoking is the leading cause of COPD. Cigarette smoke can directly affect the virulence of bacteria [104] and fungi [105] and alter the growth and exopolysaccharide structure of known intestinal bacteria (e.g., *Bifidobacterium animalis* [106], which can cause dysbiosis). "Healthy" smokers and non-smokers have similar lung microbiota, but the oral microbiota is very different [2]. In addition, compared to "healthy" smokers, COPD patients have significant differences in lung microbiota [107, 108]. These suggest that respiratory microbiota may be useful for early diagnosis of COPD. Furthermore, the fecal microbiota of "healthy" smokers exhibits an increase in the abundance of *Bacteroides-Prevotella* [109] and a decrease in the ratio of *Firmicutes*/*Bacteroidetes* [110] versus non-smokers, which were also related to intestinal inflammation and IBD [111, 112]. The recent meta-analyses suggest that *H. pylori* infection is positively associated with an increased incidence of COPD and other chronic bronchial diseases [113].

However, due to the lack of longitudinal or interventional studies used to study changes in COPD patients' gut microbiota, it is difficult to determine whether the changes are the direct cause or result of COPD. Combination changes in the environment, host, and microorganisms may be responsible for smoking-associated changes in the composition of gut microbiota [89], including intestinal and immune destruction, reduced ability to clearance of pathogens [114, 115], acidification of gastric contents [116], and intake bacteria in cigarettes [117]. It is imperative to determine whether a causal role of microbiota alteration in COPD development and progression exists or not, which needs to be thoroughly dissected in the future in clinical settings.

18.4.3 Respiratory Infections

Gut microbiota offers a broad protective effect on respiratory tract infections. NOD-like receptors and TLR agonists (including peptidoglycan, LPS, lipoprotein acids, and CpG DNA [118, 119]) exposed to GIT greatly enhance the innate immune response to bacteria in the lungs. Stimulation of TLRs by cell wall components and gut bacterial flagellin is necessary for arousing effective adaptive immune responses against influenza [120, 121]. At the same time, oral administration of short-chain fatty acids (SCFA) has anti-inflammatory effects of reducing pathological changes following pulmonary bacterial [122, 123] and viral [124] infection in mice. In turn, the microbiome can also drive the intestinal pathology of lung infections. Influenza virus infection in mice leads to the growth of *Escherichia coli* and causes abnormal T_H17 responses and intestinal damage [125]. The study of microbiota in influencing respiratory infectious diseases is at the infancy, which will be better understood with time.

18.5 Other Organ–Lung Interactions

18.5.1 Cardio–Pulmonary Interactions

Lung injury and inflammation are recognized risk factors for cardiovascular disease (CVD), and furthering research in these aspects may help discover and develop successful future treatments and biomarker targets [126]. The rapid progress shows that small airway and alveolar exposure to such as smoking [127, 128], air pollution [129, 130], diesel exhaust [131], bacterial pneumonia [132, 133], and viral respiratory infections [134] plays a significant role in lung-related cardiovascular morbidity and mortality. According to recent statistics, new or worsening heart failure during adult CAP hospitalization is as high as 33%, arrhythmia is 11%, and acute coronary syndrome is 11% [135].

Streptococcus pneumoniae is the leading cause of CAP, bacteremia, and sepsis [136]. SP infection has a direct cardiotoxic effect, and people who experience adverse cardiac events during pneumonia have a much higher risk of death than

pneumococcal pneumonia alone [137]. SP can translocate into the myocardium, forming microscopic lesions filled with pneumococci [136]. The replication of SP in the alveolar cavity can damage myocardial cells by releasing pneumolysin (pore-forming toxin) and lead to the activation of innate immunity [138]. Pro-inflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) mediate the inflammatory response and lead to ventilation–perfusion mismatch and subsequent hypoxemia. At the same time, pro-inflammatory mediators (such as platelet-activating factor) increase pulmonary vascular resistance, increase right ventricular afterload, and reduce left ventricular preload. This high dynamic state ultimately leads to hypotension and triggers the development of myocardial arrhythmia [138]. Because of the proximity relationship between the heart and lungs, it is evident that a dreaded lung would interfere with the heart function and vice verso.

18.5.2 Liver–Lung Interactions

Alcoholic liver disease (ALD), including steatosis (fatty liver), alcoholic hepatitis (AH), and cirrhosis, is a global health burden that is highly related to alcohol consumption and is considered a systemic disease on end-stages of illness [139]. AH patients are also prone to SIRS, and over 30% of them developed multiple organ failure, including respiratory, circulatory, kidney, and neurological complications [140]. Clinical data indicate that patients who have been diagnosed with alcohol use disorder have increased sensitivity to bacterial infections, increased incidence of ARDS, and cause hepatopulmonary syndrome [141, 142]. LPS-induced lung injury can be altered by mediators released from the liver (such as TNF-α) and required liver perfusion [143]. Thus, a link between lung pathology and liver health is hypothesized, which warrants further studies.

18.5.3 Lung–Kidney Inter-relationship

Many kidney injury incidents occur with various lung diseases, such as blood gas disorders, pneumonia, and mechanical ventilation (MV) [144]. As the lung and kidney are major organs that perform acid–base balance and fluid maintenance, kidney damage can profoundly affect the lungs by disturbing the acid–base or fluid balance. The kidney can also play a causal and regulatory role in acute and chronic lung disease by producing or reducing mediator clearance [145, 146]. In turn, potential lung injury (the most common being ARDS) and its mistreatment can worsen kidney function through hemodynamics, biological trauma, neurohormonal disorders, cell signaling pathways, and remote oxidative stress [147]. Quite ironically, the molecular mechanisms by which regulate and crosstalk between lungs and kidneys are mostly unknown and may be understudied. Therefore, we call for researchers from both fields to look into the many unknown biochemical, cellular mechanical features, which may ultimately facilitate the control of renal and pulmonary disorders with newly characterized pathways, therapeutic targets, and biomarkers.

18.6 Conclusions and Future Directions

The burden of pathological lung situations and extrapulmonary diseases is very high and rapidly growing worldwide. Emerging data strongly suggest that there is a direct link between lung injury and inflammation with extrapulmonary conditions. The lungs communicate with other organs (such as the brain, intestines, heart, liver). Moreover, with the development of highly sensitive molecular and genetic technologies (such as single-cell sequencing) for detecting bacterial and fungal organisms, scientists and civilian people are increasingly aware of the rich and complex lung microbial flora. It would be essential to assess the possible role of the lung microbiome in the pathogenesis of lung inflammation and its

connection with extrapulmonary sequelae. A better understanding of the mechanisms and molecular links between chronic and acute lung inflammation and extrapulmonary diseases may help discover new therapeutic targets and biomarkers to protect one or all of them, which will benefit the overall biomedical entity immensely.

Acknowledgments This work was supported by the National Institutes of Health (NIH) grants R01 AI138203, AI109317, P20 GM103442, and GM113123. Figures were created by modifying illustrations provided by Sevier Medical Art (SMART), licensed under a Creative Commons Attribution 3.0 Unported License.

Conflicts of Interest The authors declare that there is no conflict of interest, financial or otherwise regarding the publication of this paper.

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Inflammation in Pulmonary Arterial Hypertension

19

Timothy Klouda and Ke Yuan

Abstract

Pulmonary artery hypertension (PAH) is a devastating cardiopulmonary disease characterized by vascular remodeling and obliteration of the precapillary pulmonary arterioles. Alterations in the structure and function of pulmonary vessels result in the resistance of blood flow and can progress to right-sided heart failure, causing significant morbidity and mortality. There are several types of PAH, and the disease can be familial or secondary to an underlying medical condition such as a connective tissue disorder or infection. Regardless of the cause, the exact pathophysiology and cellular interactions responsible for disease development and progression are largely unknown.

There is significant evidence to suggest altered immune and vascular cells directly participate in disease progression. Inflammation has long been hypothesized to play a vital role in the development of PAH, as an altered or skewed immune response favoring a proinflammatory environment that can lead to the infiltration of cells such as lymphocytes, macrophages, and neutrophils. Current treatment strategies focus on the dilation of partially occluded vessels; however, such techniques have not resulted in an effective strategy to reverse or prevent vascular remodeling. Therefore, current studies in human and animal models have attempted to understand the underlying pathophysiology of pulmonary hypertension (PH), specifically focusing on the inflammatory cascade predisposing patients to disease so that better therapeutic targets can be developed to potentially reverse or prevent disease progression.

The purpose of this chapter is to provide a comprehensive review of the expanding literature on the inflammatory process that participates in PH development while highlighting important and current studies in both animal and human models. While our primary focus will be on cells found in the adaptive and innate immune system, we will review all potential causes of PAH, including cells of the endothelium, pulmonary lymphatics, and genetic mutations predisposing patients. In addition, we will discuss current therapeutic options while highlighting potential future treatments and the questions that still remain unanswered.

Keywords

Pulmonary arterial hypertension · Innate and adaptive immune response · Immune cells

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_19

Abbreviations

CRTH2	Chemoattractant receptor homologous molecule expressed on Th2 cell
CTD-PAH	Connective tissue disease-associated pulmonary artery hypertension
CTEPH	Chronic thromboembolic pulmonary hypertension
DC	Dendritic cell
ET1	Endothelin-1
GM-CSFR	Granulocyte macrophage colony stimulating factor receptor
HCV	Hepatitis C virus
HHV	Human Herpes virus 6
IPAH	Idiopathic pulmonary arterial hypertension
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte-chemoattractant protein-1
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PH	Pulmonary hypertension
PL	Plexiform lesion
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
SU5416	Sugen 5416 selective inhibitor of the vascular endothelial growth factor receptor
Tc	Cytotoxic T cells
Th	T helper cells
TLO	Tertiary lymphoid organ
TNFSRF11B	Tumor necrosis factor receptor superfamily member 11B
Treg	T-regulatory cells
VEGF-2	Vascular endothelial growth factor-2

19.1 Introduction

In this chapter, we will review the literature in animal and human models supporting the role of immune cells and how they interact with vascular cells in the development of pulmonary arterial hypertension (PAH) and provide future perspectives on therapeutic application.

Pulmonary arterial hypertension (PAH) is a devastating cardiopulmonary disorder characterized by narrowing and/or disorganization of the terminal pulmonary arterioles. If untreated, PAH can progress to right ventricular failure, causing significant morbidity and mortality. Pathogenic drivers contributing to the extensive obliterative changes seen in PAH throughout the vasculature are still under investigation, but there is evidence to suggest that inflammation is a major contributor.

Immune cells such as T and B lymphocytes have been hypothesized to play a role in PAH development for more than 50 years. Inflammatory cell aggregates composed of T and B lymphocytes, macrophages, dendritic cells, and mast cells have been documented to surround pulmonary vasculature in both animal and human models with PAH [1]. There is ongoing debate whether inflammatory processes are the cause and propagation of vascular remodeling or just a consequence. Regardless of the etiology, several animal and human models have revealed that high levels of inflammatory mediators are predictive of worse clinical outcomes, highlighting their clinical significance and therapeutic potential [2, 3].

Intriguingly, idiopathic pulmonary arterial hypertension (IPAH)-associated tertiary lymphoid tissues (organized ectopic lymphoid follicles) suggest that impaired or hyperpermeable infiltration of the lymphatic collection system is associated with elevated immune cell recruitment. Animal models of PH, which partially recapitulate human pathology with a variable degree of vascular remodeling, have dissected various inflammatory pathways and identify a variety of proinflammatory mediators. PAH is

associated with several infectious and autoimmune diseases, including HIV, HHV-8, HCV, and schistosomiasis. These associations suggest that an off-target inflammatory response can inadvertently damage the pulmonary vasculature [4]. Inflammatory dysregulation and PAH are also well-documented in patients with autoimmune disorders. Ten percent of patients with systemic sclerosis have diagnosed PH. A lower incidence is seen in patients with other connective tissue disorders, such as systemic lupus erythematosus and Sjogren's syndrome [5, 6].

Additionally, IPAH patients can present with Raynaud's phenomena and scleroderma. Increased levels of antinuclear/endothelial cell antibodies and autoantibodies can also be detected in IPAH serum [6–9]. While it has been recognized that autoimmunity is highly associated with PAH, we still lack definitive evidence to show that autoimmunity itself is a direct cause of the cellular changes leading to PAH development in animal or human models. For instance, sex, age, functional class, duration of symptoms, or hemodynamic status has no significant impact on the expression level of autoimmune-related antibodies in patients with PAH.

19.2 T Cells

T cells are essential components of the adaptive immune response. In general, three subsets, T helper cells (Th, CD4+), T-regulatory cells (Treg, CD4 + $CD25^{high}$FoxP3+), and cytotoxic T (Tc, CD8+) cells, are required for equilibrium and homeostasis [10]. Th cells are further subdivided into Th1, Th2, and Th17 cells, named after the proinflammatory cytokines they secrete. Each subtype has a specific role and response in the inflammatory cascade. Th and Tc cells produce a proinflammatory response, while Treg cells exert a balancing response, for self-tolerance and preventing autoimmunity. When an imbalance between the T-cell subtypes occurs, an exaggerated response can be seen secondary to the overexpression of proinflammatory cytokines released by Th cells or the anti-inflammatory cytokines due to Treg cell products. Numerous animal and human models have investigated the relationship between T-cell dysregulation and PAH. The balance and homeostasis of T cells along with their cytokines are vital to prevent the loss of self-tolerance, which may predispose patients to inflammation and the development of PAH [11] (Table 19.1).

19.3 Treg Cells

Treg cells play a vital role in regulating the inflammatory response of Th cells to self and foreign antigens. The downregulation of Tregs and their associated anti-inflammatory cytokines can lead to increased proliferation of Th17 cells. Tregs maintain immune homeostasis by suppressing CD4, CD8, and NK Cells [12]. They can accomplish this by interfering with the major histocompatibility complex II (MCHII) complex on T cells via interleukin 10 (IL-10), inhibiting dendritic cells antigen recognition process, and suppressing T-cell expansion [13, 14]. The Treg cell surface molecule, galectin-1, can also bind to effector T cells and dendritic cells, causing cell cycle arrest and apoptosis [1].

Regulating immune dysfunction is critical to preventing the progression of PAH. Human and animal models show that the imbalance of the Treg/Th17 ratio correlates with PAH disease severity in those with idiopathic, genetic, and PAH secondary to connective tissue disease, highlighting the role of Treg in PAH [15]. Athymic rats have a T-cell immunodeficiency that renders them particularly sensitive to developing severe PH. To some extent, these animals can recapitulate severe human pulmonary arterial hypertension [16].

Athymic rats injected with SU5416, a vascular endothelial growth factor-2 (VEGF-2) inhibitor, have been shown to develop significant right ventricle (RV) remodeling, perivascular inflammation, smooth muscle hypertrophy, and occlusive arteriolar lesions [16]. However, when CD4 + CD25hi Treg populations were restored in these inbred models prior to vascular injury by SU5416 injection, the development of pulmonary disease was prevented and revealed reduced tumore necrosis factor a (TNF-a)/IL-6.

Table 19.1 Immune cells role in PAH

Cell	Role in PAH development	PH findings in animal models
Treg cells (CD4 + CD25 + FoxP3+	Downregulation of Treg cells lead to TH17 proliferation and proinflammatory cytokines [22] Imbalance of Treg/TH17 ratio correlated with PAH disease severity in patients with PAH [15] Treg cells improve EC function and limit proinflammatory cytokine release from SMCs [21] Proinflammatory cytokine release from SMCs decreased with Treg cell injection [19] Treg cells can enhance pulmonary artery EC nitric oxide synthase activity [154] Inhibition of Rho1/RhoA kinase pathway protects Treg/TH17 imbalance and decreased SMC hypertrophy [20] Treg activity decreased collagen accumulation by suppressing TGF-b1 and FGF-9 [23]	Athymic rats injected with SU5416 showed reversal of PAH when Treg cell function restored [16, 17] Mouse hypoxic models injected with Treg cells not reductions in RVSP and pro-inflammatory cytokines [15] Athymic female rates with decreased Treg activity exhibit greater inflammation and PAH severity compared to male counterparts due to Treg estrogen receptor isoform [18]
Cytotoxic T cells (CD8+)	Low CD8 cells associated with mortality and decreased 6-min walk test in PAH patients [37].	
TH2 cells (CD4+)	Known to exacerbate PAH in patients with Schistosomiasis [34]	Disruption of CRTH2 suppressed TH2 cells and improved vascular remolding in animal and human models [35] CHRTH2 deficiency in mouse models suppressed TH2 activation, decreased IL-4 and IL-13 secretion alleviated established PAH [35]
TH17 cells	Increased TH17 cells and associated cytokines in patients with PAH secondary to CTD [85]. Increased TH17 cells in patients with PAH lead to GM-CSFR+ release and recruitment of inflammatory cells and vascular remodeling [31]	Mouse models exposed to chronic hypoxia and treated with SR10001, a TH17 inhibitor, had decreased vascular remodeling compared to controls [33]

This finding suggests that Tregs may be important immune regulators preventing the propagation of vascular injury by limiting inflammation [17]. Intriguingly, the same inbred athymic female rats lacking normal Treg activity exhibited greater inflammation and developed more significant PH than their male counterparts, revealing that the Treg estrogen receptor isoform associated with inflammation activation pathways [18]. In the hypoxic mouse model of PH, injection with Treg cells significantly reduces right ventricular systolic pressure (RVSP) and proinflammatory cytokine expression compared to controls [15].

While the immune imbalance between Treg and Th has been documented in PAH, it is unclear if the development of PAH is due to the T-cell subset imbalance itself or the inflammation from vascular damage. Further studies are needed to identify the exact role of Treg cells in PAH development. Furthermore, the process of CD4 + CD25- conversion to CD4 + CD25+ subsets, as well as its origin and function still need more characterization. The protective effects

against PAH seen in Treg-restored animal models suggest a potential for therapeutic strategies.

Treg cells and their anti-inflammatory byproducts play a vital role in vascular remodeling, via (1) inhibiting smooth muscle proliferation, (2) protecting endothelium function, and (3) preventing extracellular matrix proteins deposition. Numerous studies in human and animal models have demonstrated the regulatory effects that Treg cells exhibit on smooth muscle proliferation. Levels of proinflammatory cytokines, such as IL-6 and IL-1b, in smooth muscle cells from humans with PAH decrease with recombinant Treg cell injection compared to controls [19]. Human smooth muscle cells incubated with Treg cells in hypoxic conditions have also been shown to have reduced densities when compared to control groups. This further suggests that Treg cells regulate growth and hypertrophy of the vasculature and smooth muscle cells under inflammatory conditions.

The proposed mechanism by which Tregs control pulmonary artery smooth muscle cells is via suppressing Akt/ERK, the pathway responsible for triggering cell growth, survival, and motility [19]. The inhibition of the Rho1/RhoA kinase pathway has shown the potential to correct the imbalance between Th17 and Treg cells. The correction of this ratio and re-establishing immune cell homeostasis could lead to decreased smooth muscle growth and inflammation [20]. Regardless of the exact mechanism, it is clear from experimental studies that Tregs may prevent the hypertrophy of smooth muscle cells in pulmonary arterioles.

Treg dysfunction also has direct interaction with endothelial cells and can decrease inflammatory effects known to aggravate PH progression [21]. Circulating Treg cell function is downregulated in patients with idiopathic PAH, favoring inflammatory proprieties and resulting in the development of PAH in an endothelium leptin-dependent manner [22]. IL-10, an anti-inflammatory cytokine produced by Treg cells, is protective against the development of PAH by enhancing nitric oxide synthase phosphorylation in pulmonary arteriole endothelial cells.

Treg cells have suppressive effects on collagen accumulation by suppressing TGF-b1 and fibroblast growth factor 9 (Fgf-9) secretion [23]. These cytokines directly inhibit the excessive activation of fibroblasts, decreasing collagen and extracellular matrix proteins deposited within arterioles [23–25]. In addition to regulating fibroblasts in pulmonary arterioles, Treg cells downregulate cardiac fibroblasts via the secretion of IL-10, contributing to the control of ventricular modeling and the development of right ventricular hypertrophy (RVH) seen in PAH [26] (Fig. 19.1).

It remains unclear how immune reconstitution regulates endothelial bone morphogenic protein receptor 2 (BMPR2) and by what mechanism endothelial injury is attenuated. Of note, several reports have described increased Tregs in the peripheral circulation of idiopathic PH patients [27, 28]. Thus, Treg abnormalities in certain PH patient groups should be further investigated.

In summary, these findings suggest that Tregs may reverse vasculature changes during the progression of PAH. Thus, restoring of Treg function may provide a path for future therapeutic strategies.

19.4 TH17 Cells

Treg cell suppression induces the generation of TH17 cells from naÏve T cells, leading to increased inflammatory response. This response is secondary to the release of proinflammatory cytokines, such as IL-17 IL-21 and IL-22, which provide defense against foreign pathogens. Most studies have investigated and described the relationship between Treg cells and PAH, and TH17 overexpression has also been described in both animal and human models of PH.

The TH17 dominant proinflammatory state has been documented in several diseases to predispose patients to PAH. Patients with PAH secondary to a connective tissue disorder have an increase in peripheral TH17 cells, cytokines, and mRNA levels [29]. Furthermore, using immunophenotyping on monocyte-driven dendritic cells,

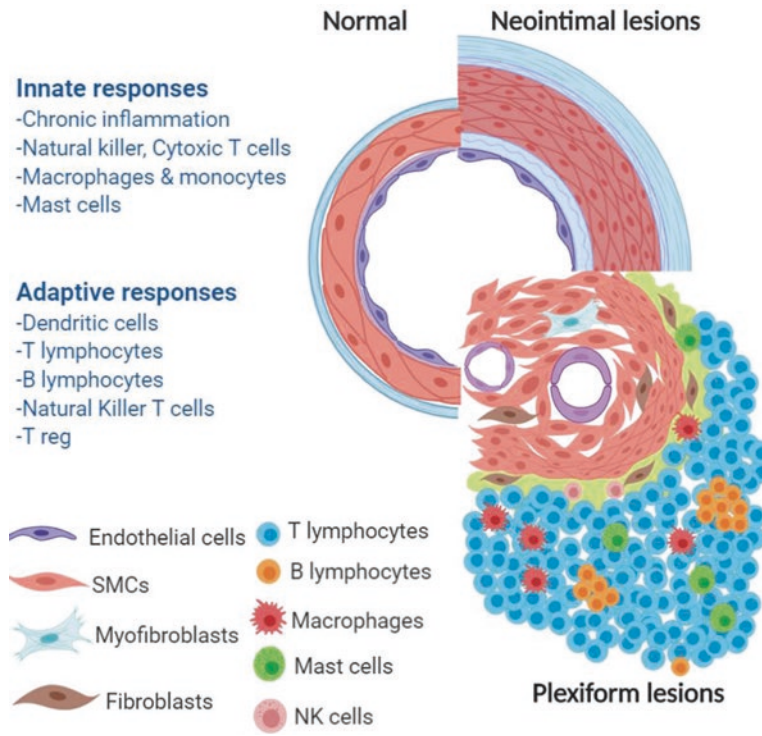

Fig. 19.1 The role of the inate and adaptive response leading to PAH and vascular remodeling

IPAH patients were noted to have higher levels of the T-cell activating molecules CD86 and CD40 after dexamethasone pretreatment [30]. These studies suggest an increase in TH17 cell number in patients with PAH. When immune dysfunction leads to a skewed TH17 reaction, increased cytokines, such as IL-6 and TNF-a, stimulate the release of granulocyte macrophage colony stimulating factor receptor(GM-CSFR). GM-CSFR release results in the recruitment of macrophages. Studies show that leukotriene B4 (LTB4) signaling elicits increased GM-CSF levels [31]. The increased macrophages can directly induce vascular injury and remodeling via endothelial cell apoptosis and smooth muscle proliferation.

Cytokines derived from TH17 cells, including IL-6 and IL-21, potentially play a role in the pathophysiology of PAH and provide possible therapeutic targets for patients. IL-6 blockade through the receptor antibody MR16-1 ameliorated hypoxia-induced PH in mice models and prevented accumulating of macrophages and TH17 cells in lungs [32]. In animal models, mice depleted of CD4 cells and those treated with SR10001, a TH17 cell inhibitor, prevented vascular remodeling when exposed to chronic hypoxia [33].Overall, experimental data appear to be associated with a dysregulated immune response and increased TH17 expression. However, it is unclear if this overexpression is a cause or result of PAH.

19.5 Th2 Cells

Th2 cells interact with the innate immune system to activate a humoral response to pathogens. They play a major role in the promotion of eosinophilic responses, IgE production, atopy, and combating extracellular pathogens, such as parasites and worms. Th2 cells produce IL-4 and IL-13, which stimulate B-cell proliferation and antibody class switching. Originally described as being an antagonist to the development of autoimmune pathology, Th2 cells and their related cytokines have been shown to exacerbate PH pro-

gression in certain diseases, such as Schistosomiasis. In experimental models, mice with insufficient or inhibited CD4+ Th2 cells were protected against PH development after Schistosoma exposure. CD4+ Th2 cells are recruited from the circulation of Schistosoma egg-sensitized mouse model and necessary for the development of PH [34].

Experimental models have suggested type 2 inflammatory cytokines released by TH2 cells, specifically IL-4 and IL-13, which participate in PH development. This is demonstrated by reduced inflammation and alleviated RVSP in mouse models after inhibition of IL-4 and IL-13. Human and rodent models have increased expression of chemoattractant receptor homologous molecule expressed on Th2 cell (CRTH2) in circulating CD3 + CD4+ T cells. The disruption of CRTH2 was further shown to suppress Th2 cells, including IL-4 and IL-13. Thus, results in improved vascular remodeling and PH suggest that Th2 cells directly participated in the progression and development of IPAH [35]. While these models suggest that Th2 cells are involved with PAH development, more clinical studies are needed before causation is proven.

19.6 T Cytotoxic Cells

CD8+ T lymphocytes, also known as cytotoxic T cells, identify cells marked for destruction via interacting with antigens bound to class 1 MHC molecules and promote cell death in response to the release of perforin, granzymes, and other molecules. Decreased levels of cytotoxic T cells have been well documented as a consequence of aging, as well as various chronic diseases.

A decreased number of circulating CD8 cells have been recognized as an adverse prognostic marker in cancer and chronic viral infections such as HIV [36]. This response is known as cytotoxic T-cell depletion, which has also been documented in patients with PAH. It is hypothesized that the abnormalities are in response to a self or foreign antigen, causing depletion of cytotoxic T cells or T-cell exhaustion. Studies have demonstrated that deficiencies in cytotoxic T cells and NK cells may be independently associated with death and a decreased 6 min walk test in patients with PAH [37]. Increased percentage of Treg cells ($CD25^{high}$ $FoxP3^{+}CD4^{+}$) along with decreased CD8+ T cells in peripheral blood has been detected in IPAH patients [38].

While it is thought the imbalance of cytotoxic T cells described is in response to surrounding immune dysregulation, immunohistochemistry of lung tissue from PAH patients revealed increased perivascular CD8+ compared to controls, suggesting that CD8+ T cells potentially have an active role in vascular remodeling [39]. However, phenotype and functional analyses of CD8 cells through the different stages of PAH are needed to further understand the mechanism and contribution of these cells in disease progression to determine their exact role.

19.7 Neutrophils

Neutrophils are the predominant circulating leukocyte in the body and a staple of the immune system. They act as first responders to infection and inflammation through phagocytosis to clear debris and recruit cells of the innate and adaptive immunity. While few studies have explored the role of neutrophils in the development of PAH, some have found an increased neutrophil presence and activity in animal and human models.

Increased neutrophil accumulation in the lungs has been seen in monocrotaline (MCT)-induced rats and hypoxic mice [40–42]. Furthermore, an increased neutrophil-to-lymphocyte ratio has also positively correlated with the New York Heart Association PAH functional class and predicted survival, suggesting neutrophils have prognostic capabilities of disease severity [43].

While it is not well understood why the increase in neutrophils is associated with PAH progression, a wide range of neutrophil byproducts may theoretically contribute. Neutrophils produce neutrophil elastase (NE), which is one of the primary proteolytic enzymes responsible for

the release of neutrophil extracellular traps (NETs). A relationship between neutrophil elastance activity and vascular changes in PAH experimental rat models has been documented [44]. There are anecdotal documents that showed augmented NE release both from neutrophils isolated from PAH patients and PAH smooth muscle cells (SMCs) and in rat models of PH [45–48]. NETs assist in microbial trapping and elimination as its normal function [49], but the emergence of excessive NETs has been identified in vascular pathologies in PAH, which promotes SMC proliferation, induces EC apoptosis, and increases matrix deposition [50, 51]. The degradation of the extracellular membrane leads to the release of growth factors and proinflammatory cytokines, promoting extracellular membrane remodeling and disease progression. In addition, markers of NETs were identified in proximity to plexiform lesions in PAH lung tissues.

The numerous mechanisms of neutrophils contribute to PAH that has led to studies to look into therapeutic options. Interestingly, the inhibition of neutrophil elastase has been noted to reverse disease in experimental models, specifically in monocrotaline mice and the Sugen/hypoxia rat model following treatment with elafin, an endogenous elastance inhibitor [40]. These findings have been replicated in human models. Explanted lung tissue with PAH treated with elafin displayed regression of intimal changes and increased vascular lumen size [52]. In addition to tissue destruction leading to PH, neutrophil elastase activates IL-1b, a proinflammatory cytokine released by endothelial cells, which can reciprocally promote the neutrophil survival and increase matrix deposition.

The role of intrinsic neutrophil abnormalities and alterations in NET and NE release in different stages of PH and during progressive vascular remodeling remain elusive. Nevertheless, a treatment targeting neutrophil elastance is a challenging therapeutic strategy to treat PAH, as they have a vital role in host defense and would leave recipients exposed to infection. More clinical research is needed to determine the exact role and mechanisms of neutrophils in PAH development and progression.

19.8 Macrophages

Macrophages are white blood cells whose primary role is to engulf pathogens and foreign particles found throughout the body via phagocytosis. They are a key component of the innate immune system, presenting manufactured antigens to T cells for differentiation and activation of the adaptive immune system. The infiltration of macrophages is prominent in the inflammatory infiltrate of plexiform lesions in experimental and different forms of clinical PAH [39, 41, 53, 54]. Additionally, depletion of macrophage prevents experimental PH and portopulmonary hypertension [55, 56].

Elevated levels of monocyte-recruiting chemokines in the lung, along with increased peripheral blood monocytes, have been reported in human and animal models with PH. After migration of the monocytes into the pulmonary vasculature, these cells may differentiate into perivascular macrophages through Ccl2 and Cx3cl1 activation [57]. Inhibition of Cx3cI1/Cx3cr1 signaling leads to decreased interstitial macrophage expansion and reduced pulmonary vasculature remodeling and inflammation in rodent models with PH [57].

Without Treg cell suppression, macrophages are activated after an insult and participate in vascular remodeling leading to PAH. This has been suggested by the protective effects seen against PAH development in animal models lacking alveolar macrophages [54]. The depletion of alveolar macrophages in rats exposed to chronic hypoxia has a protective effect against pulmonary artery pressure changes, suggesting macrophages participate directly in the development of PH. Rats exposed to chronic hypoxia with decreased alveolar macrophages had no change in serum monocyte-chemoattractant protein-1 (MCP-1), suggesting that MCP-1 is likely not involved in the recruitment and differentiation of macrophages leading to PAH development [58]. Regardless of how macrophage recruitment occurs, their presence in the distal arterioles of both human and animal models with PH is more sophisticatedly documented.

Humans and SU5416 rat models have shown LTB4, a proinflammatory molecule derived from arachidonic acid and released from macrophages is a key component in PH progression. It was found that LTB4, through inhibition of the endothelial nitric oxide synthetase, causes endothelial cell apoptosis leading to proliferation and hypertrophy of human pulmonary artery smooth muscle cells. Blocking macrophage-derived LTB4 biosynthesis or signal transduction reverses experimental PH, and depleting CD68+ macrophages prevents PH from developing in Sugen-treated athymic rats [56]. Activation of macrophages is closely linked to epigenetic changes that stimulate fibroblast proliferation in PAH patients and experimental models [59].

Although the exact mechanisms of how macrophage participation in vascular remodeling and how their interaction with vascular cells are unknown, targeting macrophages to decrease PAH progression may be promising therapeutic strategies.

19.9 Dendritic Cells

Dendritic cells (DCs) act as professional antigen-presenting cells and are key players in the activation of naïve T cells. Their main function is to act as an intermediate between the innate and adaptive immune system, processing foreign antigens for T-cell presentation and differentiation [60]. They have recently been found to be a key modulator in many disorders, including asthma, autoimmunity, and tumorigenesis [61–63].

In experimental PH and clinical PAH, immature dendritic cells accumulate in remodeled pulmonary vessels, suggesting their involvement in the immunopathology of pulmonary hypertension [64]. Besides their T-cell activating function, DCs are crucial for the presence and preservation of tertiary lymphoid organs (TLOs) seen near pulmonary blood vessels, which consist of other myeloid cells, T-, B cells, and monocytes [65, 66].

Intriguingly, multiple DC subsets can be found in steady states, such as conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Under inflammatory conditions, monocytes can differentiate into monocyte-derived DCs (mo-DCs). In IPAH patients, the proportion of circulating cDCs is decreased compared to controls, potentially as a result of an increased cDC migration toward lung TLOs [67]. In IPAH lungs, pDC numbers are enhanced and pDCs are specifically located around the pulmonary vessels, while circulating pDC numbers are unaltered [68]. During the inflammation, pDCs produce type-I IFN and chemokine secretion such as CXCL10 and promote activation of immune cells [69]. Inflammation and chemokines can also attract monocytes to lung of IPAH and CTD-PAH patients, and gave rise to mo-DCs, further exacerbate inflammation and trigger influx of inflammatory cells [70].

In summary, DC subset distribution and activation status play important roles in the pathobiology of autoimmune diseases and most likely in the development of IPAH and CTD-PAH. However, little is known about DC subset distribution and function in IPAH, CTD-PAH, and autoimmune diseases.

19.10 Mast Cells

Classically, mast cells are identified in tissues by their unique products of chymase and tryptase. These products were originally identified during tumor angiogenesis and later reported to increase and correlate with the severity of pulmonary hypertension and pulmonary vascular remodeling [71–74].

Rats with mutations in mast cell growth factor receptor c-kit develop less PH and vascular remodeling when exposed to MCT [75]. Rats with flow associated PAH that treated with mast cell stabilization attenuated pulmonary vascular remodeling and had a lower chymase activity, correlating with more favorable hemodynamics and pulmonary vascular remodeling [76]. Human tissues with IPAH have shown elevated levels of chymase-positive mast cells, with its product chymase to convert angiotensin 1 to angiotensin 2 and activate cytokines TGF-b and IL-18. These cytokines are noted to directly contribute to the development of hypertension and atherosclerosis [77]. In addition, tryptase was shown to induce

pulmonary artery smooth muscle cell proliferation and migration, as well as the synthesis of matrix protein deposition [78]. Although these findings strongly indicate the role of mast cell in pulmonary hypertension, the underlying molecular mechanism is not yet understood.

Interestingly, after intervention with mast cell inhibitors, cromolyn and fexofenadine, PAH patients showed a decrease in tryptase/leukotriene LTE4/VEGF, along with increase in exhaled nitric oxide, a commonly used vasodilator [74]. In a small clinical study, imatinib, a tyrosine kinase inhibitor that targets c-Kit and some subtypes of PH revealed decreased circulating progenitor cells/mast cells and in parallel a decrease in pulmonary vascular resistance [79].

These findings suggest that potential therapies targeting mast cells may show promise for future treatment strategies. Recent studies have also indicated that mast cells directly participate in the formation of bronchus-associated lymphoid tissue (BALT), and c-Kit+ cells were found locally surrounding these structures, suggesting mast cells contribute to their formation and development [66, 80]. Lymphoid structures found in patients with chronic lung disease provide a structure and base for inflammatory cells to congregate and accelerate the progression of vascular remodeling [81]. Mast cells can recruit and activate B cells by the release of IL-6, promoting the formation of this tertiary lymphoid tissue. Additionally, mast cells can induce T-cell activation, proliferation, and cytokine secretion [82]. Conversely, mast cells can suppress Tregs that were reported to protect against hypoxia-induced PH[19].

Although mast cell therapy may present a testable and promising strategy, further studies are needed to determine if preventing mast cell migration early in the disease course can prevent vascular remodeling or in the late disease stage.

19.11 Cytokines

Cytokines represent a large group of signaling proteins that have important roles mediating systemic biological responses, including inflammation and immunity. These molecules are typically produced by cells of the immune system, but can also be released from endothelium and vasculature in response to stressful environments and triggers [83]. Specific cytokines are elevated in PAH patients and are known to correlate with disease severity [84]. Identifying the temporal relationship and effects cytokines have on the development of PAH is essential to understanding pathophysiology and disease progression. Therapies targeting specific cytokine responses and pathways are a high yield area for potential treatments and therapeutic strategies.

19.12 IL-6

IL-6 is a potent cytokine with a wide range of proinflammatory properties affecting immune regulation, inflammation, and metabolic pathways. It plays a vital role in regulating the balance between TH17 cells and Treg cells. Oversecretion can cause an immune response favoring Th17 cells and place subjects at risk for PAH [85].

The exact mechanism that IL-6 contributes to PH development is unclear. Studies suggest that unopposed mitogen-activated protein kinase (MAPK) intracellular signaling and decreased TGF-b expression result in proliferative and anti-apoptotic signaling pathways [86]. IL-6 also has prognostic value in patients with PH. Serum levels have been shown to be an independent predictor of survival in patients with PH, and in conjunction with other markers of disease severity, can predict patient outcome [87]. Animal models have demonstrated the direct effect IL-6 has on the development of PH. Rats injected with recombinant human IL-6 display increased RVH and under normoxic conditions develop PH [88]. Conversely, IL-6 knockout mice exposed to hypoxia were found to be resistant to the development of increased RVP [89]. The most convincing study for IL-6 inducing PH revealed that transgenic mice overexpressing IL-6 developed muscularization of the proximal arterial tree, distal arteriolar vessels, and were found to have occlusive proliferative lesions consisting of endothelial cells and T lymphocytes [90].

Potential therapies targeting IL-6 and its signaling pathways may help decrease progression or even reverse the vascular remodeling seen in PH. However, more research is needed to determine how to target specific pathways activated by IL-6 contributing to PH development and to identify which patients would benefit.

19.13 IL-1b

IL-1b is a proinflammatory cytokine that, like IL-6, is shown to correlate with worse outcomes in PH patients. Levels of IL-1b decrease prostacyclin PGI2, a metabolite of arachidonic acid, which possesses vasodilatory and antiproliferative effects protective against the development of PH.

Murine models of PH also demonstrate high serum levels of IL-1b, and the initiation of an IL-1b receptor antagonist has been shown to decrease PH and RVH [91]. In addition, pulmonary artery smooth muscle cells treated with IL-1b display increased levels of COX-2 mRNA, a key enzyme in prostacyclin synthesis [90].

While IL-1b itself has properties leading to PAH, it can also activate other proinflammatory cytokines and molecules. Cleavage of IL-18 by IL-1b converting enzyme generates the biologically active IL-18, which is elevated in patients with pulmonary vascular disease. Vascular injury releases IL-18 from smooth muscle cells and causes the local proliferation and recruitment of smooth muscle cells, leading to hypertrophy and PAH progression [92].

19.14 Other Secreted Factors

Other cytokines, such as IL-4, IL-5, IL-8, IL-10, IL-13, VEGF, and TNF-a, have been shown to be abnormal in human and animal models of PH. IL-8 plays a role in the early vascular remodeling and development of PH via its proangiogenic and antiapoptotic properties that act as a growth factor for endothelial cells [93]. Injections of TNF-a into rat models can cause increased vascular activity and remodeling [94].

CXCL12a, a potent proangiogenic chemokine, is also elevated in patients with PH compared to those without. The chemoattractant has been shown to cause endothelial proliferation through the CXCR7 and CXCR4 receptors on endothelial cells [95]. Levels also correlate with disease severity, as they have been found to be an independent risk factor for earlier death and correlate with mean pulmonary arterial pressure [96]. IL-10, a potent anti-inflammatory cytokine released by T cells, is increased in patients with PAH, likely due to counter-regulatory mechanisms as levels have been found to inversely correlate with prostacyclin therapy and be decreased in patients following cardiopulmonary bypass [97].

Abnormal levels of other biological markers, such as osteoprotegerin, also known as tumor necrosis factor receptor superfamily member 11B (TNFSRF11B), have been noted in patients with PAH. Increased expression and secretion of TNFSRF11B have also been identified in patients with PH. Multiple studies have also found that elevated serum osteoprotegerin levels correlate with hemodynamic markers predictive of disease severity [98]. It is hypothesized that multiple different pathways contributing to PAH development can cause increased osteoprotegerin, including increased BMPR2 expression [99].

Whether over or under expression of specific cytokines leads to the development of PH or if the abnormalities detected occur secondary to disease progression remain unclear. Growing evidence suggests that levels of cytokines can distinguish PAH into four distinct immune phenotypes [100]. Independent of underlying etiology, these four immune phenotypes may play a role in the development of future therapies, as patients can be categorized into an immune phenotype and treated with appropriate therapy. Immune clusters found that groups had different signals for various pathways contributing to PAH development. Some groups skewed toward a TH1 response while others toward a TH17 response or adaptive immunity.

Immune phenotyping could offer a framework for therapies in clinical settings. Therapies targeting immunity would be beneficial in patients who lack an increased inflammatory response based

on their phenotype and vice versa. The potential of combined treatments based on a patient's phenotype is a potential future therapeutic option. Targeted treatment toward cells, cytokines, and other molecules overexpressed in an immune phenotype could provide multitargeted treatment strategies personalized for a patient's inflammatory phenotype.

19.15 Other Cell Types

BMPR2 is a serine/threonine receptor kinase that binds to bone morphogenic proteins, which are a type of TGF-b ligand. BMPR2 is involved in various cellular functions, including osteogenesis, cell growth, and differentiation. It functions to inhibit the proliferation of vascular endothelial and smooth muscle cells, preventing arterial damage and local inflammatory response. Inhibition of BMPR2 gene expression leads to unregulated proliferation and survival of endothelial cells through disordered TGF-b signaling, contributing to vascular remodeling [101]. BMPR2 mutations are the most common genetic mutations associated with PAH. Roughly 80% of patients with HPAH and 30% of IPAH patients have a mutation within the BMPR2 gene [102]. In response to a reduction in BMPR2 function in the endothelium, it is hypothesized that the integrity of the endothelium barrier may be compromised leading to apoptosis, the release of TGF-b, and the development of apoptosis-resistant clones [103]. In contrast, smooth muscle cells proliferate due to TGF-b signaling and undergo an exaggerated growth response leading to vascular remodeling [104]. It is reasonable to speculate that BMPR2 deficiency can increase vascular–immune interaction, with increased immune cells infiltration to the subintimal blood or lymphatic vascular structures.

19.16 Lymphatics

The lymphatic vascular system transports fluid, immune cells, and wastes through the body to help prevent edema within tissues, facilitate an immune response, and remove harmful toxins. Lymphatic vessels are found abundantly in the lung, help maintain a fluid balance, and precipitate an immune response. Small lymphatic vessels around distal bronchus drain into larger vessels and eventually into the right and left lymphatic ducts. Lymph nodes, the major site of T and B cells, filter foreign particles and house an immune response to pathogens [105].

Disrupted lymphatic function and flow can lead to an inflammatory state and alveolar damage characterized by the formation of tertiary lymphoid organs (TLOs) in chronic lung disease [106]. Thus, perivascular lymphatic infiltrates may be a major source to form TLOs seen in severe PAH. After lung transplantation, interrupted lymphatic vessels are associated with the induction of allograft tolerance as well as rejection [107]. There is very little pulmonary research focusing on lymphatic circulation. The characterization and examination of the lymphatic system in PH need more investigation.

19.17 Endothelial Cells

Endothelial cells (ECs) play a key role in maintaining vascular homeostasis in response to various stimuli and regulate inflammation through mediators such as nitric oxide (NO), endothelin-1 (ET1), cell adhesion molecules, cytokines, and chemokines. Endothelial cell dysfunction has been shown to contribute to the development of multiple cardiac and vascular diseases, including PAH and heart failure [108].

Under pathological conditions such as inflammation and hypoxia, pulmonary artery endothelial cells decrease the production of vasodilators such as nitric oxide and vascular growth factors, favoring vasoconstriction of the distal pulmonary arteries [109]. Unregulated endothelial cell proliferation and neoangiogenesis can result in the formation of plexiform lesions (PLs), glomerular like vascular structures, seen in severe PAH [108, 110]. PLs consist of disorganized endothelium channels as well as a uniform myogenic origin cells and immune cells. Rat model exposed to chronic hypoxia and SU5416 has been shown to

form PL-like structures that partially resemble human pathology [111, 112]. Bioinformatics analysis further validates the mixture of cell identities. The cellular contribution to the process of PL formation and EC recruitment of inflammatory cells need more clarification.

There is evidence that pulmonary arteries also have a permeability defect in animal models of PH [113, 114]. The pulmonary endothelium aids in the passage of circulating immune cells through alveolar capillaries and closely associated with distal air sacs. The inflammatory and immune cells that migrate into the lung parenchyma of PH can be from multiple resources, such as the pulmonary arterioles, the vasa vasorum, perivascular capillary network, or even the lymphatic vasculature. Following initial tethering at the endothelial cell surface (as known as the classic paradigm of the leukocyte adhesion cascade [115]), leukocytes start to roll and firmly arrest on the vessel surface to ultimately migrate into the subendothelial space, typically via a paracellular route but occasionally also via a transcellular route.

Some studies have analyzed circulation soluble adhesion molecules and their role in cell migration into the lung. For example, one of circulating soluble adhesion molecules P-selectin was shown to be elevated in the plasma of PAH patients or CTEPH, as well as in animal models [116–118]. A few other studies have addressed the expression of endothelial adhesion molecules, ICAM-1, VCAM-1, and E-selectin, in PAH tissues that could be related to BMPR2 mutations [119, 120]. Taken together, adhesion molecule expression correlates with leukocyte interaction within the pulmonary endothelium in PH.

However, the mechanisms of these molecules impact on endothelium permeability are still lacking. Circulating endothelial cells (CECs) may participate in processes of vascular injury and tumorigenesis or interaction with immune cells. Additionally, endothelial progenitor cells (EPCs) are bone marrow-derived cells involved in homeostasis, but also physiological and pathological angiogenesis. The increase in proinflammatory cytokines also favors platelet adherence and activation of coagulation cascades, leading to further arteriole occlusion [121].

The role of these cells in the pathobiology of PH is yet to be elucidated. Although bone marrow-derived endothelial progenitor cells have been tested as a therapeutic option in animal and human models with promising results, with much of the measured benefit attributed to gene manipulation [122–124]. Regardless, inhibiting endothelial cell apoptosis and migration may stop a necessary initial key step in PAH pathogenesis and provide future therapeutic options.

19.18 Pericytes

Pericytes are mesenchymal-derived mural cells that wrap around endothelial cells throughout the entire capillary vasculature in all organs**.** Due to its controversy and lack of unique cellular markers, pericytes are largely ignored and under investigation.

Our groups recently discovered that impaired endothelial–pericyte interaction contributed to small vessel loss in PAH [125]. Intriguingly, SDF1 (aka CXCL12), an inflammatory cytokine, regulated pericyte migration and lineage and potentially associated with pulmonary arterial muscularization [126, 127]. In addition to their vascular functions, pericytes regulate different aspects of immune responses, though most of our understanding of pericyte-related immune responses was elucidated from brain or placenta pericytes.

Some studies suggested that central nervous system microvascular pericytes may display macrophage-like/nonprofessional antigen-presenting cell characteristics and involve in several possible immune responses [128]. A clear distinction of pericytes versus macrophages was lacking and the conclusions need to be extensively tested on multiple lineages tracing models. Whether lung pericytes represent the same phenotype seen in the brain and participate in inflammatory processes has remained entirely unclear.

In rat lung, pericytes were demonstrated to upregulate TLR4, increase vessel permeability, and produce of IL-1b upon LPS exposure [129–

131]. LPS has been shown to generate NO in pericytes, leading to vasodilation. In addition, an iNOS-independent pathway was associated with lung pericyte contractility [132]. Vascular endothelial growth factor (VEGF) may relate to the induction of endothelial nitric oxide, inducing vascular leakage and inflammation. VEGF also modifies the contractile response of lung pericytes. This mechanism may play a role in the increased permeability demonstrated in inflammatory conditions [133].

In the capillary, dynamic changes in response to proinflammatory signals are necessary for the efficient recruitment of leukocytes. Sequential interactions of endothelium-expressed cytokines with circulating immune cells can initiate extravasation during a series of processes, as discussed above, known as the leukocyte adhesion cascade [134].

Endothelium was extensively studied, much more so than pericytes, during acute inflammation. For example, in distal microvessels, the regions with partial coverage of mural cells appear to be preferential inflammatory sites for the transmigration of neutrophils [135]. A follow-up study further characterized the low matrix expression region and identified pericyte partial coverage that could be preferred sites for monocytes and neutrophil migration [136]. Intriguingly, increased neutrophil recruitment and transmigration into extravascular tissue are more associated with EC–pericytes bilayers than a monolayer, suggesting the cytokine released by pericytes could promote vascular inflammation [137].

Subsequent studies showed that pericyte generated basement membrane as a cellular matrix composite model could be the front line barrier to affect leucocyte recruitment upon TNF-a exposure [138]. Pericytes triggered the chemotactic migration of interstitial neutrophils and macrophages after extravasation from capillary [139]. Thus, pericytes can be crucial for the efficient navigation of cells of the innate immune system, which can execute their effector functions at the local inflammation. Using confocal intravital microscopy, pericytes facilitated leukocyte trafficking into sites of inflammation *in vivo* [140]. Increased PDGFRb signaling induced a panel of immune response genes in pericytes. Lung pericyte-like cells release proinflammatory molecules following epithelial injury and promote acute inflammatory responses by recruiting leukocytes [141].

All studies support the vital role of pericytes in mediating inflammatory and immune signaling, whether lung specific pericytes can recapitulate the same physiological function still require further characterization.

19.19 Other Vascular Mural Cells

In response to hypoxia and other stimuli, the pulmonary vessels undergo proliferation and hypertrophy secondary to enhanced cytokine production. This complex remodeling of the vasculature includes all layers of the pulmonary vasculature but especially affects the medial layer. The increased medial thickness of the pulmonary arteries is well documented in both human and animal models. The process is driven by increased numbers and hypertrophy of its principal cellular constituent, smooth muscle cells (SMCs).

It was proposed that inflammation could recruit SMC populations and enhance their contribution to pulmonary vascular remodeling because hyperproliferative SM-like cells are observed in local occluded vessels. Several studies have also shown that sustained hypoxia induces the recruitment of mesenchymal progenitor cells in the perivasculature. The recruitment of these cells is critical to the development of PAH [142, 143]. It is possible that the inflammatory vasculature changes seen are due to these migrating cells, as well as resident smooth muscle cells, which resume the capabilities needed for vascular remodeling.

While the exact timing and role of smooth muscle cells in PAH development is unclear, therapies targeting the proliferation of smooth muscle cells in animal models have shown success. This includes inhibiting the receptors tyrosine kinase, mTOR, p38 and CDK4/6 [144]. Imatinib, discussed previously, has shown promising results in improving exercise capacity and hemodynamic in patients with advances PAH;

however, its clinical use is limited by adverse effects [145]. Future studies are needed to determine if therapies targeting different phenotypes of smooth muscle cells will be of benefit.

Therapeutic strategies targeting dendritic cell migration have also shown some promise in the treatment of PAH. Imatinib, a platelet-derived growth factor receptor antagonist STI571, has been demonstrated to reverse vascular remodeling in monocrotaline exposed rats through effects on smooth muscle cells [146]. However, imatinib has multiple other mechanisms of action, including targeting the common Ras/MAPK and Jak/STAT pathway. Before the effects of imatinib on dendritic cells are confirmed, more studies in human models are needed to better understand the exact roles of dendritic cells have in the progression of PAH.

Fibroblasts, the most common cell found in connective tissue, are responsible for providing the framework and structure of the extracellular matrix. They play a critical role in wound healing and the surrounding environment of pulmonary vessels, reacting to local inflammation and stress, and in return, activating the innate immune system. The release of proinflammatory cytokines and growth factors from stimulated fibroblasts is a potential contributor to the development of PAH, and has been shown in animal and human models to potentially contribute to disease progression.

Activated fibroblasts found in the pulmonary artery adventitia have been documented in experimental and human models with PAH to display antiapoptotic, hyperproliferative, and proinflammatory features [59, 147, 148]. They have also been shown to recruit cells of the innate immune system to participate in disease progression. Fibroblasts of PH patients were shown to recruit and activate naïve macrophages [149]. The exact mechanism of fibroblast hyperactivity and resistance to apoptosis is unclear. Recent studies have shown that hyperactive fibroblasts may be regulated by a pro-oxidase status secondary to complex I deficiency in mitochondrial oxidase phosphorylation [150].

Furthermore, experimental results in mice exposed to hypoxia with mutations in FGFR1 and FGFR2 are consistent with PH compared to controls. This suggests that endothelial receptor activation leads to endothelial cell survival and decreased apoptosis protecting against the development of PH [151]. More studies are needed to confirm that cells exhibiting this mitochondrial abnormality exist during the development of PH before they are targeted for potential therapeutic strategies.

Until recently, it was thought that smooth muscle-like cells expressing a-SMactin accumulate in arterioles due to expansion of resident cells. New literature suggests that circulating progenitor cells, such as fibrocytes from the bone marrow, migrate to the pulmonary vasculature and are responsible for vascular remodeling. These cells continually produce extracellular membrane component-modifying enzymes that alter the structural composition of the lung [152]. These cells can differentiate into myofibroblasts in the presence of TGFb. Activated myofibroblasts are included in the organized thrombotic tissues of Group 4 CTEPH [153]. The differentiation between fibroblast and myofibroblast can mediate adventitial remodeling that found in larger sized pulmonary artery (Fig. 19.2).

19.20 Conclusions

There is increasing evidence that immune dysregulation plays an important role in the pathogenesis and progression of PAH. Inflammatory cell recruitment, cytokine and autoantibody production, and enhanced vascular wall remodeling lead to the abnormal interplay between the immune system and the pulmonary vasculature. Current therapies for PAH mainly target vasodilators NO and prostacyclin, or vasoconstrictors ET-1. Other combined strategies include optimizing cardiac function, such as the use of diuretics and calcium channel blockers. While these medications have transformed PAH management in the past few decades, new studies and a better understanding of PAH pathophysiology will improve therapies by targeting immune cells and inflammation, preventing cellular and vascular changes. Targeting inflammatory cascades before cellular changes are seen can not only treat PAH, but also prevent its progression altogether.

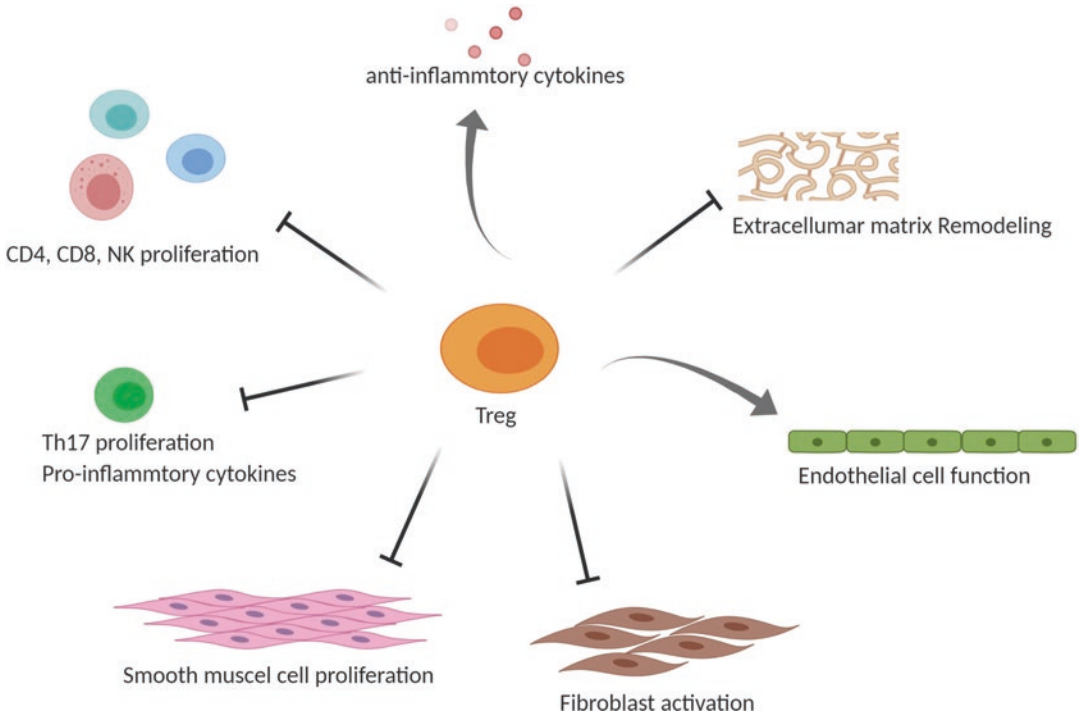

Fig. 19.2 The role of Treg cells in PAH development

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Lysophospholipids in Lung Inflammatory Diseases

20

Jing Zhao and Yutong Zhao

Abstract

The lysophospholipids (LPLs) belong to a group of bioactive lipids that play pivotal roles in several physiological and pathological processes. LPLs are derivatives of phospholipids and consist of a single hydrophobic fatty acid chain, a hydrophilic head, and a phosphate group with or without a large molecule attached. Among the LPLs, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are the simplest, and have been shown to be involved in lung inflammatory symptoms and diseases such as acute lung injury, asthma, and chronic obstructive pulmonary diseases. G protein–coupled receptors (GPCRs) mediate LPA and S1P signaling. In this chapter, we will discuss on the role of LPA, S1P, their metabolizing enzymes, inhibitors or agonists of their receptors, and their GPCR-mediated signaling in lung inflammatory symptoms and diseases, focusing specially on acute respiratory distress syndrome, asthma, and chronic obstructive pulmonary disease.

Keywords

Lysophospholipids · Lysophosphatidic acid · Sphingosine-1-phosphate · G protein–coupled receptors · Signaling pathway · Lung inflammation

Abbreviations

AGK	Acylglycerol kinase
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
ATX	Autotaxin
BALF	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary disease
DGK	Diacylglycerol kinase
DMS	Dimethylsphingosine
DTD	DL-threo-Dihydrosphingosine
ECMO	Extracorporeal membrane oxygenation
Edg	Endothelial cell differentiation gene
EMT	Epithelial–mesenchymal transition
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterases family member 2
G3P	Glycerol 3-phosphate
GPAT	Glycerol 3-phosphate acyltransferase
GPCR	G protein–coupled receptor
HLMVEC	Human lung microvascular cell

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1_20

HPAEC	Human pulmonary arterial endothelial cell
IP	Intraperitoneal
IT	Intratracheal
IV	Intravenous
LPA	Lysophosphatidic acid
LPAATs	LPA acyltransferases
LPL	Lysophospholipid
LPP	Lipid phosphatase
LPS	Lipopolysaccharide
MAG	Monoacylglycerol
MLC	Myosin light chain
NOX2	NADPH oxidase type 2
ORMDL3	OR-like protein isoform 3
PA	Phosphatidic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phosphatidylserine
S1P	Sphingosine-1-phosphate
SphK	Sphingosine kinase
Spns2	Spinster homolog 2
SPPase	S1P phosphatase

20.1 Introduction

Lysophospholipids (LPLs) are derivatives of phospholipids. Much attention has been paid to phospholipids and their roles in maintaining biological membrane structure. Most phospholipids contain a glycerol backbone that has three carboxyl positions (*sn*). Two fatty acid chains are esterified to positions 1 (*sn*-1) and 2 (*sn*-2), while a phosphate group is attached to *sn*-3. Phosphatidic acid (PA) is the simplest phospholipid with only a phosphate group attached to its *sn*-3 position. Some other organic molecules can link to the phosphate group to generate phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), etc. The other phospholipid form is based on a sphingoid backbone, such as sphingomyelin. LPLs differ from phospholipids due to lack of a fatty acid chain in either *sn*-1 or *sn*-2. Lysophosphatidic acid (LPA) is the simplest LPL, consisting of one fatty acid chain and a hydroxyl group in *sn*-1 or *sn*-2, and a phosphate in the *sn*-3 position. Depending on the molecules attached to the phosphate, LPLs may contain LPC, lysoPS, LPG, sphingosine-1-phosphate (S1P), etc. Numerous enzymes are involved in the metabolism of LPA and S1P, which contribute to homeostatic regulation of LPLs. In this chapter, we will focus on LPA and S1P, while the role of other LPLs in lung inflammatory diseases will be briefly discussed.

Changes of LPLs in biological fluids, including plasma, have been reported (reviewed in [1–7]). Extracellular LPLs may trigger intracellular signaling pathways and a wide spectrum of biological activities through ligation to specific plasma membrane receptors. G protein–coupled receptors (GPCRs) mediate LPL-induced biological responses, although not every receptor for LPLs has been identified. So far, receptors for LPA and S1P have been well characterized and extensively studied. LPA receptors consist of $LPA_{1–6}$, and S1P receptors consist of $S1P_{1–5}$. $LPA_{1–3}$ and all S1P receptors belong to the endothelial cell differentiation gene (Edg) family. $S1P_1$ (also called Edg1), $S1P_2$ (Edg5), $S1P_3$ (Edg3), $S1P_4$ (Edg6), and $S1P_5$ (Edg8) bind to S1P, whereas LPA_1 (Edg2), LPA_2 (Edg4), and LPA_3 (Edg7) are specific receptors for LPA. Three non-Edg receptors ($LPA_{4–6}$) are members of P2Y family. Agonists and antagonists of LPA and S1P have been developed to activate or interfere LPA/LPA receptors or S1P/S1P receptors. In addition, intracellular LPA is an endogenous ligand for peroxisome proliferator-activated receptor gamma (PPARγ) (reviewed in [8–10]).

The lungs, as the pivotal organs in the respiratory system, facilitate gas exchange between the atmosphere and blood stream. Lungs consist of a conducting zone and respiratory zone. Trachea, bronchi, and bronchioles function as airways for bulk air flow. In addition, airway epithelial cells clear away inhaled pathogens or irritants through mucus secretion and cilia movement. Alveolus in the respiratory zone is the site for gas diffusion between lungs and capillaries. Alveolar epithelial cells release surfactant to maintain alveolar space. Capillary endothelial barrier integrity is important to prevent plasma and erythrocyte leakage into alveolar spaces. Interstitial fibro-

blast cells in the respiratory zone function as structural cells, and play a role in lung repair and remodeling after injury [11]. Impaired oxygen diffusion is a hallmark of lung disorders [12].

In this chapter, we will discuss role of LPLs in the pathogenesis of lung inflammatory diseases, such as mild acute respiratory distress syndrome [mild acute respiratory distress syndrome (ARDS), previously called acute lung injury (ALI)], asthma, and chronic obstructive pulmonary disease (COPD). LPA and S1P have gained attention due to their pathogenic role in pulmonary fibrosis; thus, we will include pulmonary fibrosis in this chapter.

20.2 Metabolism of LPA and S1P

20.2.1 Biosynthesis of LPA

LPA contains one fatty acid chain in either the *sn*-1 or *sn*-2 position. Commonly, saturated and monounsaturated fatty acids are in the *sn*-1 while polyunsaturated fatty acids are linked to the *sn*-2 position. LPA can be synthesized both inside and outside of cells.

Inside cells, three pathways regulate LPA synthesis that is achieved through breaking down PA, adding a phosphate group to monoacylglycerol (MAG), or de novo synthesis. Phospholipases are a group of enzymes that hydrolyze phospholipids. In the first pathway, phospholipase A1 (PLA_1) or PLA_2 converts PA to LPA by removing one of fatty acid chains at either the *sn*-1 or *sn*-2 positions of PA. It has been shown that two membrane-bound PA-specific $mPLA_1\alpha$ and $mPLA_2\beta$ regulate LPA synthesis [13, 14]. Both enzymes are localized in the plasma membrane, especially in lipid rafts, suggesting $PLA_{1/2}$-mediated intracellular LPA generation's involvement in signaling within protein-enriched membrane domains. Thus, increase in PA levels may lead to upregulation of intracellular LPA. Activations of phospholipase C (PLC), phospholipase D (PLD), or diacylglycerol kinase (DGK) generate PA, thus leading to the formation of LPA [15]. Another pathway for LPA production is phosphorylation of MAG by acylglycerol kinase (AGK), which is a novel lipid kinase in the mitochondria [16]. The molecular mechanism regarding LPA exports from mitochondria is not clear. De novo synthesis of LPA is the third pathway. LPA is formed by linking a fatty acid to glycerol 3-phosphate (G3P) in the *sn*-1 position by glycerol 3-phosphate acyltransferases (GPATs) [17, 18].Out of these three pathways, conversion from PA is the major contributor to intracellular LPA generation.

LPA can be generated outside of cells. Extracellular LPA triggers intracellular signaling pathways and is attributed in a wide range of cellular responses. LysoPLD is also known as autotaxin (ATX) or ectonucleotide pyrophosphatase/phosphodiesterases family member 2 (ENPP2) (reviewed in [19, 20]. ATX heterozygous knockout mice exhibit significantly reduced plasma LPA levels [21, 22], while recombinant ATX demonstrates increased extracellular LPA levels [23]. ATX generates LPA through hydrolyzing LPC, which is enriched in plasma and bronchoalveolar lavage. ATX is a secreted glycoprotein and its level changes in various human disorders including lung diseases and cancer [1, 24, 25]. Another source of extracellular LPA is from activation of secretory PLA_2 [26].

20.2.2 Catabolism of LPA

Two major pathways have been identified to limit LPA levels either inside or outside of cells. Inside the cells, a reversible reaction allows LPA conversion to PA by LPA acyltransferases (LPAATs), which include four isoforms [27, 28]. This pathway facilitates increases of intracellular PA levels, which is also a bioactive lipid and regulates signaling pathways.

Another limiting pathway is to eliminate extracellular LPA levels. A group of lipid phosphatases (LPPs) catalyze LPA dephosphorylation to generate MAG. LPP1–3 are three major isoforms of LPPs, which are transmembrane proteins with their enzymatic activity domain lying outside of the plasma membrane ([29], reviewed in [30, 31]).

20.2.3 Synthesis of S1P

S1P is based on a sphingoid backbone and is synthesized intracellularly. Sphingosine kinases (SphKs) utilize sphingosine as a substrate and transfer a phosphate to synthesize S1P. SphKs belong to the group of lipid kinases. Two isoforms of SphKs (SphK1 and SphK2) have been identified and well characterized (reviewed in [32, 33]). SphK1 is predominately localized in the cytoplasm, while SphK2 mainly resides in nucleus [4, 34, 35]. S1P is also generated in mitochondria by SphK2, suggesting that SphK2 is also expressed in mitochondria [36]. Both SphK1 and SphK2 are expressed in lungs [37, 38]. The two kinases compensate for each other to maintain normal S1P levels. S1P can be released outside of cells by membrane transporters. ABC transporters (ABCA1, ABCB1, ABCC1, and ABCG2) and spinster homolog 2 (Spns2) export intracellular S1P ([39], reviewed in [40, 41]. A recent study revealed that a new S1P transporter (named Mfsd2b) plays a critical role in export of S1P within erythrocytes and platelets. Deletion of Spns2 or Mfsd2b in mice significantly eliminates plasma S1P levels [42, 43].

20.2.4 Catabolism of S1P

Similar to LPA, catabolism of S1P occurs intracellularly and extracellularly. S1P lyase (SPL) is localized within the endoplasmic reticulum membrane and its activity site is on the cytoplasmic side. SPL plays a major role in the elimination of cellular S1P levels by degrading S1P to phosphoethanolamine and hexadecenal [44, 45]. Lack of SPL expression in erythrocytes and platelets causes higher concentrations of S1P compared to other cells. S1P is degraded by SPL to ethanolamine phosphate and hexadecenal [46]. This reaction is irreversible and dependent on pyridoxal phosphate.

In addition to dephosphorylating LPA, LPPs convert S1P to sphingosine. LPP functions are not only to eliminate extracellular S1P levels, but also to facilitate uptake of sphingosine by cells, sequentially increasing intracellular S1P by phosphorylation via SphKs. In addition to LPP1–3, two S1P phosphatases (SPPases) have been identified to dephosphorylate S1P and other sphingolipids ([47, 48], reviewed in [49]). Though both SPPases and LPPs catalyze the catabolism of S1P, there is very little homology between these two groups, except the conserved active sites.

20.3 LPA- and S1P-Mediated Signaling Pathways

20.3.1 LPA and S1P Receptors in Lungs

Extracellular LPA triggers a wide range of biological functions through ligating and activating its specific GPCRs. Among the Edg and non-Edg receptors, LPA_1 and LPA_2 have been well studied in various lung cell types. Heterotrimeric G proteins, including Gα, Gβ, and Gγ, couple to the intracellular potion of LPA receptors. Among the G proteins, Gα plays a major role in determining downstream signaling. Depending on which G subunit is bound to LPA receptors, LPA may exhibit various, even opposite, biological functions (reviewed in [6, 8, 50]). PPARγ has been identified as an intracellular LPA receptor [10], while the LPA/PPARγ pathways in lung diseases have not been revealed. This chapter will focus on LPA_1 and LPA_2 in lung diseases. LPA_1 and LPA_2 are potential targets for treating lung diseases. Several antagonists of $LPA_{1–3}$, such as AM966, ki14625, BMS-986278, H2L5186303, VPC32183, and VPC12249, have been developed to inhibit LPA receptor-mediated signaling.

All S1P receptors ($S1P_{1–5}$) belong to the Edg family. Similar to LPA receptors, S1P receptors are coupled with G proteins [8]. Expression of S1P receptors in different cell types of lungs has been determined. Most studies have been focused on the role of S1P receptors in lung endothelial cells and immune cells. FTY720-phosphate, a phosphorylated chemical of a fungus metabolite, is a S1P mimetic. FTY720 has been approved by FDA as an immunomodulator drug in treating

multiple sclerosis as it can be phosphorylated by SphK2 and activate $S1P_1$ [51–53].

20.3.2 LPA and S1P Receptor-Mediated Signaling

As discussed above, LPA and S1P receptors both utilize Gα-mediated signaling, thus, parts of LPA- and S1P-triggered signaling and biological functions are comparable. The role of LPA and S1P in tumorigenesis has been well documented (reviewed in [54–56]). They activate Ras-Raf-Erk1/3 and PI3K-AKT pathways, increase cell proliferation, and function as pro-oncogenic factors (reviewed in [54–56]). S1P antagonizes the proapoptotic action of ceramide and impairs the caspase-dependent proapoptotic pathway [57]. LPA reduces proapoptotic Bax protein levels in the cytosol and increases anti-apoptotic Bcl-2 expression [58]. In addition to the activation of GPCRs, LPA and S1P trigger cross-talking between their receptors with epidermal growth factor recetpor (EGFR) [59] and platelet-derived growth factor recetpor (PDGFR) [60], which contribute to cancer progression.

Both LPA and S1P regulate inflammatory responses. Transcriptional factor nuclear factor kappa B (NF-κB) is critical for cytokine gene expression (reviewed in [61]). Activation of LPA and S1P receptors induces phosphorylation of I-κB and degradation, thus leading to NF-κB nuclear translocation and transcriptional activation [29, 62–64]. Other transcriptional factors, such as AP1, p38 MAPK, and cAMP-response element binding protein (CREB), are common downstream molecules of LPA and S1P receptors (reviewed in [65–67]. LPA and S1P treatment induces cytokine release and MMPs expression in a variety of cell types including lung cells.

Although both LPA and S1P exposure triggers activation of the Rho family of GTPases, including Rho, Rac, and Cdc42 in various cells [67–70], the immediate effects on the cytoskeleton may differ between different cell types. It has been shown that S1P enhances endothelial barrier integrity through activation of Rac1 [71, 72], while LPA increases endothelial permeability through activation of myosin light chain (MLC) via RhoA-mediated phosphorylation [73, 74]. In contrast, in bronchial epithelial cells, LPA induces E-cadherin accumulation at cell–cell contacts and reduces cells' permeability [75]. Despite the distinct effects on cell–cell contact in different cell types, both LPA and S1P have shown to increase cell migration in most cell types, including lung epithelial, endothelial, and fibroblast cells.

The molecular regulation of LPA and S1P receptors has not been well studied. Gene regulation of these receptors has been reported in lung diseases, but the molecular mechanisms remain unclear. Protein stability and internalization of LPA and S1P receptors were demonstrated. Ubiquitin E3 ligases, Nedd4L and WWP2, are responsible for LPA_1 and $S1P_1$ ubiquitination and degradation [76], while deubiquitinating enzyme USP11 stabilizes LPA_1 [76].

20.4 LPA and S1P in Acute Respiratory Distress Syndrome (ARDS)

20.4.1 Pathogenesis of ARDS

ARDS is a severe condition characterized by acute inflammation and alveolar-capillary barrier disruption, leading to edema and gas exchange failure in the lungs. ARDS can be induced by inhalation of airborne pathogens such as bacteria or viruses. SARS-CoV2-induced COVID-19 has a high association with ARDS. Systemic inflammatory diseases such as sepsis also lead to ARDS. There are no effective treatments for ARDS, and the mortality rate of ARDS remains 30–40% [77]. For the past several decades, supportive therapies such as mechanical ventilation through extracorporeal membrane oxygenation (ECMO) have been essential treatments for ARDS [77]. Acute lung injury is a mild form of ARDS. Researchers have focused on investigating the pathogenesis of acute lung injury and are seeking new therapeutic strategies to treat this severe lung disease.

20.4.2 Pro- and Anti-inflammatory Roles of ATX-LPA-LPA Receptor Axis in Experimental Acute Lung Injury

The role of ATX/LPA in the pathogenesis of acute lung injury is controversial; however, there is solid evidence that LPA receptors are pro-inflammatory in experimental acute lung injuries. The functional disconnect between LPA and its receptors is not clear. It is possible that other ligands for LPA receptors have not been identified.

The levels of LPA and ATX in bronchoalveolar lavage fluid (BALF) are increased in experimental acute lung injuries caused by inhalation of lipopolysaccharide (LPS), bleomycin, or exposure to hyperoxia [78–81]. ATX plays distinct roles in different lung injury models. Pulmonary NKT cells have been shown to be a source of ATX and LPA in hyperoxia-induced lung injury [82]. Injection of Brp-LPA, which is an ATX inhibitor and LPA receptor antagonist, significantly improved survival and alleviated lung injury [82]. Another study confirmed that ATX levels were increased in hyperoxia-challenged 4-day-old rat pups. These studies indicate the ATX/LPA/LPA receptor axis plays a proinflammatory role in hyperoxia-induced lung injuries [81]. However, this conclusion is not supported by studies in LPS-induced experimental acute lung injury. Mouratis M-A et al. showed that lung epithelial cells are a source of ATX in BALF in response to inhalation of LPS [78]. Overexpression of ATX in lung epithelial cells increased two-fold of LPA levels in BALF [78], suggesting that ATX released from epithelial cells plays a role in LPA generation within BALF during periods of inflammation. The release of ATX in lung epithelial cells was confirmed in an in vitro study [83]. Interestingly, modulation of ATX levels in lung epithelial cells did not seem to contribute to the pathogenesis of LPS-induced lung injury [83]. In contrast, the overexpression of systemic ATX increased susceptibility to LPS-induced lung injury, evidenced by increased BALF cellularity, protein levels, and neutrophil infiltration into lungs. However, pharmacologic targeting of ATX had minor effects in lung injury severity [83]. These data indicate that ATX plays a role in the process of LPS-induced lung injury independent of LPA generation. This conclusion has been confirmed by another study showing that increased ATX activity is not required for BAL LPA production following bleomycin-induced lung injury [84]. An explanation for the phenomenon is that the extracellular ATX can interact with LPA_1 and trigger LPA_1-mediated signaling in an ATX activity-independent manner [83]. Thus, the proinflammatory effects of ATX may be due to directly ligation to LPA_1. As discussed above, extracellular LPA also can be generated via the activation of secretory PLA_2 [26]. Secretory group V PLA_2 has been shown to play a critical role in LPS-induced acute lung injury [85], indicating that the secretory PLA_2/LPA axis plays a role in the pathogenesis of acute lung injury. Secretory PLA_2's potential as a limiting enzyme in BAL LPA needs to be explored in the future. Evidence directly supporting the proinflammatory effect of LPA is that IT LPA induced neutrophil influx into lungs, though the effect is not comparable with the effects of IT LPS [86].

In addition to its proinflammatory properties, the role of LPA in attenuation of acute lung injury has been revealed. One study in which LPA was directly injected into murine lungs following LPS-induced acute lung injury showed that post-treatment LPA played a protective role [75]. Consistent with this conclusion, LPA administration has been reported to demonstrate a protective role in acute liver injury, and the effects of LPA were reported in an LPA receptor-independent manner [87]. The effect of LPA on lung epithelial barrier enhancement may explain the protective role of LPA in experimental acute lung injury.

In addition to changes in LPA and ATX, LPA receptor levels were increased in lung cells in both hyperoxia- and LPS-induced experimental acute lung injuries [79, 81]. The studies using LPA receptor-deficient mice or antagonists provided solid data to support that LPA receptors, especially LPA_1, act as proinflammatory GPCRs during the progression of hyperoxia- or LPS-induced acute lung injury and sepsis [79, 88, 89].

Interestingly, LPA_1 seems to have no effects on alveolar-capillary integrity as no changes of LPS-induced BAL protein were detected in LPA_1-deficient mice or ki16425-treated mice [79]. This may be due to the opposite effects of LPA on epithelial and endothelial barrier integrity [75]. Notably, all the studies used whole body LPA receptors knockout mice. Use of lung epithelial or endothelial cell specific knockout mice will clearly demonstrate in which cell type LPA receptors play roles in lung injury.

20.4.3 Role of ATX-LPA-LPA Receptor Axis in Biological Functions in Acute Lung Injury-Related Lung Cells

LPA treatment increases cytokine release in bronchial and alveolar epithelial cells. The proinflammatory effect of LPA occurs through activation of Gαi-coupled LPA receptors. Activation of transcriptional factors, NF-κB, AP-1, and p38 MAPK, by LPA regulates the expression of cytokines and MMPs. A variety of intracellular signaling pathways, such as activation of PLD or PKCδ, which results in increases of intracellular calcium, are involved in LPA-induced activation of transcriptional factors (reviewed in [6]). Intriguingly, LPA1 activation leads to phosphorylation of EGFR, and this crosstalk between GPCR and tyrosine kinase receptor contributes to IL-8 release through the activation of CREB [59].

Other major findings regarding the role of LPA in lung epithelial cells are that LPA induces lung epithelial repair and remodeling, such as epithelial barrier enhancement [75] and cell migration [83, 90]. PKCδ, PKCζ, and focal adhesion kinase (FAK) regulate LPA-induced E-cadherin accumulation at cell–cell contacts [75]. Rac1 is involved in LPA-induced lung epithelial cell migration [90]. The effects of LPA are consistent with its properties as a growth factor.

Vazquez-Medina JP et al. showed that LPA increases oxidant generation through the activation of NADPH oxidase type 2 (NOX2) in pulmonary microvascular endothelial cells [91]. A study demonstrated that LPA increases permeability in human pulmonary arterial endothelial cells (HPAECs), but not in human lung microvascular cells (HLMVECs) [92]. Cai J et al. showed that HLMVECs reduce barrier integrity in response to LPA treatment. Intriguingly [74], they also revealed that AM966, an LPA_1 antagonist, exhibits similar effects to LPA. Both LPA and AM966 activate RhoA and induce phosphorylation of MLC and VE-cadherin, which are critical factors for endothelial barrier dysfunction [74]. This warning study raised caution for using AM966 as an LPA receptor antagonist.

20.4.4 Protective Role of S1P in Experimental Acute Lung Injury

S1P levels in lung tissues and BALF, not in plasma, were upregulated in intratracheal (IT) LPS-induced acute lung injury [93]. Plasma S1P was increased in a two-hit model induced by intraperitoneal (IP) LPS combined with ventilation [94]. A recent study found that *Pseudomonas aeruginosa* challenge increases S1P levels in lungs and BALF [95]. However, a study using human samples showed an opposite phenomenon. Analysis of serum S1P from 121 ARDS patients and 100 healthy individuals revealed that serum S1P levels were decreased in ARDS patients [96]; however, the BAL S1P levels in patients were not measured in this study. The role of S1P in acute lung injury was studied by modulating expression of S1P metabolism enzymes or injection of S1P in various models of lung injury induced by LPS, *P. aeruginosa*, mechanical ventilation, or radiation [4, 93, 95, 97, 98]. As we discussed above, S1PL is a limiting enzyme of S1P degradation. S1PL expression is enhanced by LPS challenge and mechanical ventilation. S1PL heterozygous knockout mice increased S1P levels in lung tissues and BALF while reducing LPS- or mechanical ventilation-induced lung injury and inflammation [93, 98]. The effect in S1PL heterozygous knockout mice was confirmed by administration of S1PL inhibitor THI (2-acetyl-5-tetrahydroxybutyl imidazole) in

an LPS-induced a murine model of acute lung injury [93].

Synthesis of S1P is catalyzed by SphK1/2. Severe *Plasmodium falciparum* malaria causes lung edema and an increase in SphK1 in lung tissues [99], suggesting a role of the SphK1/S1P axis in lung edema. The effects of SphK1/2 on acute lung injury were examined using SphK1/2 deficient mice. IT LPS increased SphK1/2 expression in lung tissues. SphK1 knockout mice exhibited more susceptibility to LPS-induced lung injury [100]. This was rescued by SphK1 overexpression, but not by overexpression of SphK2 [100]. In addition to its anti-acute lung injury property, SphK1 has been reported to contribute to the pathogenesis of lung injury. IP administration of SphK1 inhibitor exhibited protective effects on the two-hit (ventilation + IP LPS)-induced acute lung injury [94]. Gutbier B et al. reported that SphK1-deficient mice had reduced lung hyperpermeability in a *P. aeruginosa*-induced murine model of acute lung injury [101]; however, another controversial study demonstrated that SphK1 knockout had no effects in *P. aeruginosa*-induced acute lung injury [95]. Ebenezer DL et al. revealed that SphK2 deficiency attenuated *P. aeruginosa*-induced acute lung injury, indicating a role of nuclear S1P in the pathogenesis of lung injury as SphK2 is localized in the cell nuclei [95].

Similar to the effects of SphKs, controversial conclusions regarding the effects of administration of S1P on acute lung injury have been drawn by different studies. Intravenous (IV) or IT S1P reduces IT LPS-induced edema and neutrophil influx into lungs [102, 103]. In the canine model, IV S1P attenuated IT LPS- or ventilation-induced edema and neutrophil infiltration into the lungs without altering BAL cytokine profile [103, 104]. Intriguingly, IV S1P increased serum proinflammatory cytokines [104], which raised concerns about the use of S1P as a therapy.

With the controversial studies in SphK1/2 and administration of S1P, it is possible that S1P may exhibit distinct effects due to ligation to different receptors. $S1P_1$ heterozygous mice potentiated LPS-induced lung injury, while $S1P_2$ knockout mice or downregulation of $S1P_3$ exhibited an opposite response compared to $S1P_1$ heterozygous mice [102], indicating that $S1P_1$ exhibits a protective role, while $S1P_{2/3}$ promotes LPS-induced lung injury. FTY720, an analog of sphingosine, is an FDA-approved drug for treating multiple sclerosis. FTY720 can be phosphorylated to phospho-FTY720 by SphK1 and has an endothelial barrier protective effect (reviewed in [105]). In a hindlimb ischemia reperfusion (IR)-induced acute lung injury model, pretreatment with FTY720 attenuated lung injury [106]. However, similar to S1P, FTY720 caused barrier disruption at higher concentrations and increased airway hyper-responsiveness [107]. (S)-FTY720-phosphoate, an analog of FTY720, prolongs $S1P_1$ levels on the cell surface and exhibited more protective effects compared to FTY720 [108]; thus, (S)-FTY720-phosphate might be developed as a potential therapy to treat acute lung injury.

20.4.5 S1P Regulates Biological Functions in Acute Lung Injury-Related Lung Cells

The role of S1P in the regulation of lung endothelial barrier integrity has been well investigated. S1P treatment increases transendothelial monolayer resistance, indicating that S1P is an enhancer of lung endothelial barrier integrity (reviewed in [105]. Rac1 plays a major role in the process through increasing cell spreading. Rac1 is activated by TIAM-1 (T-cell lymphoma invation and metastasis 1), the guanine exchange factor for Rac1. Various signaling pathways including PI3K/AKT, PKCζ, PKCε, and PLD are involved in S1P-activated Rac1 [109].

Several studies demonstrate that S1P could affect lung epithelial cell functions. Exogenous S1P induces intercellular adhesion molecule 1 (ICAM-1) expression in alveolar epithelial cells through activation of NF-κB [110]. $S1P_3$-mediated S1P signaling activates cytosolic $PLA_2\alpha$ in lung epithelial cells [111], which may explain the proinflammatory role of $S1P_3$ in lung injury. Plasma $S1P_3$ level is considered as a biomarker for acute lung injury since it is increased in human and experimental acute lung injury and

associated with mortality rate [112]. Nuclear S1P is generated by SphK2. *Pseudomonas aeruginosa* treatment induced phosphorylation of SphK2 and association with HDAC1/2 in the nuclei, resulting in acetylation of histone 3 and 4 [95].

Neutrophil influx into lungs is a hallmark of pathogenesis of acute lung injury. S1P has been shown to increase IL-8 release in bronchial epithelial cells, and the effect was mediated by S1P ligation to $S1P_2$ [113], indicating that S1P/$S1P_2$ pathway contributes to lung inflammation. However, pretreatment with S1P reduced IL-8- or fMLP-induced neutrophil chemotaxis, suggesting an anti-inflammatory effect of S1P. The S1P effects on neutrophils may be through ligation to $S1P_4$ in the neutrophil [114]. The neutrophil specific S1P receptor knockout mice may identify that S1P receptor is responsible for the effect of S1P on neutrophil migration.

20.5 LPA and S1P in Asthma

20.5.1 Pathophysiology of Asthma

Asthma is a chronic airway inflammatory disease affecting at least 300 million people and causes more than 380,000 deaths per year worldwide. Asthma is characterized by reversible airflow obstruction in association with airway hyperresponsiveness, increased mucus generation, eosinophilia, and increased Th2 cells and Th2 cytokines. An increase in airway smooth muscle (ASM) mass and mucus glands lead to airway wall thickening and airway constriction. Th2-dominant inflammatory responses are a hallmark of allergic asthma. Lung IL-4, IL-5, IL-13, and IL-33 levels are increased in both human and experimental asthma models (reviewed in [115–117]). Other cytokines including IL-9, IL-17A, and tumor necrosis factor (TNF)-a are involved in the pathogenesis of asthma (reviewed in [116, 117]). Treatments for asthma focus on dilation of airways and suppression of lung inflammation. While most inflammatory responses can be diminished by corticosteroids, while severe asthma is resistant to such and has no effective treatment (reviewed in [116, 117]).

20.5.2 Role of LPA in Asthma

Several studies have shown that ATX protein and LPA species in BALF are increased in segmental allergen challenge of allergic subjects and asthma patients [118–120]. Park GY et al. showed that ATX-overexpressing transgenic mice exhibited increased severity of asthmatic responses, while ATX heterozygous knockout mice or administration of ATX inhibitor (GWJ-23) significantly attenuated Th2 cytokines and allergic lung inflammation in a triple-allergen murine model of asthma [119]. This study suggests that the ATX/LPA axis is a potential target for treating asthma.

To investigate the role of LPA in the pathogenesis of allergic asthma, LPA receptor deficient mice were sensitized and challenged with an allergen. LPA_1 heterozygous deficient mice did not show dramatic changes compared to wild-type mice, while LPA_2 heterozygous deficient mice showed reduced eosinophil influx into lungs and mucus glands in bronchi [86], suggesting that the LPA/LPA_2 pathway is implicated in the development of allergic asthma. Further, this conclusion was confirmed by another study showing that LPA_2-deficient mice exhibited reduced BAL total cell numbers, IL-4 and IL-5 levels within BAL, and severity of lung inflammation [119]. Interestingly, both studies revealed that downregulation of LPA_2 significantly diminished LPA levels in BAL [86, 119]. The molecular regulation of LPA_2 on LPA generation remains unclear; it is possible that LPA/LPA_2 reduces LPA synthesis enzymes, such as ATX or secreted PLA_2, by a negative feedback mechanism. On the contrary, in a murine model of ovalbumin (OVA)-sensitized and challenged allergic lung inflammation, LPA_2 promoted lung inflammation, evidenced by increased BAL eosinophil numbers and hyper-reactivity compared to wild-type mice [121]. The reason for the controversial conclusion from the distinct effects of LPA_2 in the different allergic challenges has not been well

understood. Administration of LPA_2 specific antagonist is needed to understand the role of LPA_2 in the pathogenesis of asthma.

An interesting study by Jendzjowsky NG et al. showed that increased plasma LPA levels are implicated with carotid body activation-mediated vagal activity, which has been shown to trigger bronchoconstriction [122]. Administration of Brp-LPA, an ATX inhibitor and LPA receptor antagonist, prevents bradykinin-induced asthmatic bronchoconstriction [122], suggesting a role of LPA/LPA receptors in regulation of bronchoconstriction by activation of carotid bodies. Hashimoto T et al. showed that inhalation of oleoyl LPA induced airway hyper-responsiveness to acetylcholine, possibly through increasing release of histamine and activating the Rho/ROCK-mediated pathway [123]. LPA receptor deficient mice and ATX transgenic mice may be useful to investigate the role of LPA in activation of carotid body-regulated bronchoconstriction during an asthmatic attack.

20.5.3 Molecular Mechanisms by which LPA/LPA Receptors Contribute to the Pathogenesis of Allergic Asthma

Increase in ASM mass is implicated in airway bronchoconstriction in asthmatic patients. Proliferation of ASM isolated from asthmatic patients is increased compared to ASM from nonasthmatic patients [124]. LPA has been considered as a plasma growth factor that induces cell proliferation in a variety of cell types, including ASM. Coculture with EGF exhibited a markedly synergistic mitogenesis in human ASM [125]. Activation of Erk, Rho, and AP-1 is required for LPA-induced ASM cell growth [125]. In addition to increasing cell growth, LPA treatment facilitates methacholine-induced ASM contractility, attenuates isoproterenol-induced relaxation of ASM, and increase IL-6 release [126]. Notably, LPA alone did not increase ASM contraction [125].

The effects of LPA on immune cells have been reported. LPA increases Th17 differentiation in obesity [127], but the effect of LPA on IL-17A production in allergic asthma has not been studied. Here we focus on discussing the role of LPA in the release of Th2 cytokines and their signaling in the development of allergic asthma. Cocultured with T-cell activators, LPA induces IL-13 but not IL-4 production in human T cells [128]. In vitro chemotaxis assays showed that LPA induces CD4+ T cell and eosinophil migration [129]. Bronchial epithelial cells regulate Th2 cytokine expression and are also affected by Th2 cytokines. LPA treatment increased decoy receptors of Th2 type cytokine, such as IL-13Ra2 and soluble ST2 (sST2) [130], suggesting that LPA may attenuate IL-13- and IL-33-mediated signaling in bronchial epithelial cells, further suggesting an anti-Th2 response property of LPA in bronchial epithelial cells. As discussed by Kim S et al., these in vitro studies used LPA18:1, which is not the major LPA species in asthmatic patients (reviewed in [131]). The major species, LPA22:5 and 22:6, should be used to test and evaluate their effects on IL-13- and IL-33-mediated signaling in airway epithelial cells. In addition to regulating Th2 cytokine signaling, LPA induces cyclooxygenase (COX)-2 expression and prostaglandin E2 (PGE2) release in human bronchial epithelial cells [59]. Inhibition of COX-2 is an effective therapy to treat asthma, indicating that LPA receptors are therapeutic targets for treating asthma. Lundequist A and Boyce JA demonstrated that LPA_5 is highly expressed in human mast cells. LPA induces calcium flux, MIP-1β, and histamine release from mast cells [132]. Activation of mast cells is a hallmark of an allergic response. The LPA/LPA_5 axis is a potential target for diminishing IgE-mediated allergic responses.

20.5.4 Role of S1P in Asthma

S1P levels in BALF are increased in ragweed-allergic asthmatics and a murine model of OVA-challenged allergic asthma [133]. Exogenous S1P administration into isolated lungs increased

mast cell number, IL-4, IL-13, and IL-17 production, as well as contraction of isolated bronchi [134], suggesting a proasthmatic role of S1P. Inhibition of SphK1 reduces both intracellular and extracellular S1P levels. Increased SphK1 and SphK2 levels in bronchial tissues were found in OVA-sensitized mice compared to control mice [135]. An inhibitor of SphK1, SK1-I, reduced activation of human and murine bone marrow-derived mast cells, as well as OVA challenge-induced cellular infiltration into lungs, goblet cell hyperplasia, and pulmonary eosinophilia in mice [133]. Consistent with these findings, administration of another potent SphK inhibitor, *N,N*-dimethylsphingosine (DMS), or SphK1 siRNA exhibited anti-inflammatory effects in OVA-challenged mice [37]. DL-threo-Dihydrosphingosine (DTD), another SphK inhibitor, inhibited acetylcholine-induced contraction of isolated bronchi harvested from OVA-sensitized mice [135].

$S1P_2$ and $S1P_3$, but not $S1P_1$, were increased in lung tissues from OVA-sensitized mice [135]. The effects of S1PR antagonists on attenuation of asthmatic responses have been studied. Administration of JTE013 ($S1P_2$ antagonist) attenuated Th2 type cytokines and eosinophil numbers in BALF of OVA-challenged asthmatic mice [136]. In support of the conclusion from the JTE013 treatment, $S1P_2$-deficient mice were found to have reduced IL-4, IL-5, and IL-13 expression in both lung tissues and inflammatory cells in BALF [136]. Polymorphism analysis showed that functional variants of the $S1P_1$ gene are associated with asthma susceptibility [137]. IT FTY720 reduced features of airway remodeling in an OVA-induced rat model of asthma. IT FTY720 diminished OVA challenge-induced increase in airway smooth muscle mass, airway hyper-responsiveness, BAL eosinophil and lymphocytes, and IL-5 and IL-13 expression in lung tissues [138]. Consistent with this study, Oyeniran C et al. demonstrated that intranasal FTY720 administration reduced OR-like protein isoform 3 (ORMDL3) expression, airway inflammation, and mucus hypersecretion in HDM-challenged mice. ORMDL3 is considered as a gene associated with susceptibility to asthma [139]. In contrast to these two studies, Ble F-X et al. showed that intranasal FTY720 had no effect on OVA challenge-induced immune cell influx into lungs, while it inhibited allergen-edema [140]. In the same study, the authors demonstrated that intranasal administration of S1P1-selective agonist, AUY954, prior to OVA challenge reduced lung edema without altering BAL eosinophil influx into lungs [140]. These controversial conclusions raise concerns in using FTY720 to treat allergic asthma. The role of $S1P_1$ in the development of allergic asthma can be further evaluated using $S1P_1$-deficient mice in the future.

20.6 LPA and S1P in COPD

20.6.1 Pathogenesis of COPD

COPD is a chronic lung inflammatory disease characterized by poorly reversible airflow obstruction, emphysema, and bronchiolitis. Cigarette smoking is the leading cause of the progressive airway inflammatory disease (reviewed in [141]). Neutrophils, macrophages, and T lymphocytes release inflammatory mediators including lysophospholipids in COPD. Airflow obstruction is caused by mucus hypersecretion and hypertrophy of smooth muscle and connective tissues. Imbalances of protease and antiprotease disrupt alveolar structure, leading to abnormal enlargement of alveolar spaces. Major medications for COPD open the airway through inhaled bronchodilators and diminish inflammatory responses via use of corticosteroids, phosphodiesterase-4 inhibitors, and so on (reviewed in [141]).

20.6.2 Role of LPA in COPD

Unlike the role of LPA in acute lung injury and asthma, the role of LPA in COPD has not been well demonstrated. An increase in plasma LPA was revealed in a tobacco smoke-induced rat model of chronic bronchitis [142]. Naz S et al. discovered that both serum LPA (16:0) and LPA (18:2) are increased in smokers with COPD

[143]. Interestingly, they found that the levels of LPA are correlated with lung function in males with COPD, but not females [143]. The effect of increased LPA in the development of COPD remains unclear. LPA induces cytokine release, such as IL-8 and IL-6 in human bronchial epithelial cells, suggesting a proinflammatory role of LPA in COPD (reviewed in [6, 65]. Blanque R et al. attempted to reveal if reduction of LPA levels by inhibition of ATX alleviates severity of COPD. They showed that post-treatment with GLPG1690, an ATX inhibitor, dose-dependently reduced inflammatory cell influx into lungs [144], suggesting that ATX inhibitors can be developed as a novel therapeutic strategy to treat COPD. However, LPA has been shown to increase lung epithelial cell migration [90]. Analysis of LPA_1-deficient mice during lung development indicates a role of LPA/LPA1 in alveolarization [145]. To investigate whether LPA and LPA receptors contribute to the development of COPD, LPA receptor isotype deficient mice and antagonists should be used in experimental COPD models.

20.6.3 Role of S1P in COPD

The role of sphingolipids, including S1P, sphingosine, and ceramide, in the pathogenesis of COPD has been well studied. In this chapter, we will focus on discussing the discoveries of S1P, SphK, and S1P receptors in COPD. S1P levels are increased in lungs cigarette smoke-induced COPD mice [146]. The balance of S1P/ceramide in COPD has been well discussed [147]. Expressions of SphKs, S1P receptors, and SPL1 in lung tissues and alveolar macrophages were examined. Barnawi J et al. showed that SphK1/2, SIP_2, $S1P_5$, and SPL1 mRNA levels were increased in COPD patients compared to healthy controls [148]. The changes in mRNA expression within macrophages were confirmed in cigarette smoke extract-treated THP-1 macrophages [148], indicating that S1P signaling in macrophages may be involved with the development of COPD. Consistent with this conclusion, activation of $S1P_2$ and $S1P_3$, but not $S1P_1$, has been shown to stimulate macrophage migration [146]. In the study by Cunto GD et al., S1P challenge significantly increased contraction of isolated bronchi from cigarette smoke-exposed mice, and the effect was abrogated by pretreatment with $S1P_2$ and $S1P_3$ antagonists. Inhibition of SphK reversed carbachol-increased contractions in bronchi of mice challenged with cigarette smoke [146]. Taken together, these studies suggest that the inhibition of S1P signaling may alleviate the severity of COPD.

However, another study revealed that plasma S1P levels tended to be negatively correlated with emphysema and COPD exacerbations, supporting that S1P/ceramide ration plays a critical role in the pathogenesis of COPD [147]. Tran HB et al. showed that cigarette smoke extract exposure reduced activity of SphK1 but not SphK2 [149]. S1P is an endothelial barrier integrity enhancer, and FTY720 agonists attenuated nicotine-increased endothelial hyperpermeability [150]. Further, SEW2871, an S1P1 agonist, exhibited an antiapoptotic effect and blocked vascular endothelial growth factor (VEGFR) inhibition-induced emphysema [57], suggesting that S1P signaling suppresses the development of emphysema by maintaining lung epithelial and endothelial integrity. The dissimilar effects of S1P signaling on the development of COPD in the different studies will be investigated by using S1P receptor or SphKs global or cell-specific knockout mice.

20.7 LPA and S1P in other Lung Diseases

Both LPA and S1P exhibit pro-oncogenic properties by increasing cell proliferation, migration, antiapoptosis, and epithelial–mesenchymal transition (EMT) (reviewed in [151–153]). Thus, it is not surprising that LPA and S1P signaling play roles in the development of lung cancer. However, there is no clear preclinical evidence showing that targeting LPA and S1P signaling diminishes lung cancer. Serum S1P and LPA levels and their metabolic enzymes may be used as biomarkers

for both prognosis and prediction for lung cancers.

The profibrotic effects of LPA and S1P have been well documented. Tager AM et al. demonstrated that LPA and LPA receptors are increased in lungs in pulmonary fibrosis patients and in a bleomycin-induced murine model of pulmonary fibrosis [80]. LPA_1 knockout mice exhibited significantly reduced severity of pulmonary fibrosis, indicating that LPA/LPA_1 signaling is a potential target for treating pulmonary fibrosis [80]. Many restudies followed the discovery and showed that activation of $LPA_{1/2}$ and ATX contributes to the pathogenesis of pulmonary fibrosis (reviewed in [154, 155]). Similar to LPA, a large amount of preclinical experimental pulmonary fibrosis studies showed that reduction of S1P levels and inhibition of S1P signaling alleviate the severity of pulmonary fibrosis (reviewed in [156, 157]). A recent study indicates that intracellular S1P may exhibit an opposite effect on the development of pulmonary fibrosis compared to extracellular S1P [158]. Thus, precise regulation of extracellular S1P levels needs to be further investigated to better understand the pathogenesis of pulmonary fibrosis.

20.8 Perspective

LPA and S1P, the simple bioactive lipid mediators, have been implicated in normal physiological functions and in the pathogenesis of a variety of human disorders. Thus, their metabolic enzymes and receptors have been targeted for drug development to treat lung inflammatory diseases. Since both LPA and S1P play a variety of cellular functions, completely blocking synthesis of LPA and S1P may cause unexpected side effects. To improve our understanding of the roles of LPA and S1P signaling play in the development of lung inflammatory diseases, cell-specific LPA and S1P receptor isotype knockout mice need to be developed to investigate their role in preclinical models of lung inflammatory diseases. Regulation of GPCRs homeostasis by the ubiquitin-lysosome or ubiquitin-proteasome systems has been given more attention. The molecular mechanisms of LPA and S1P receptors

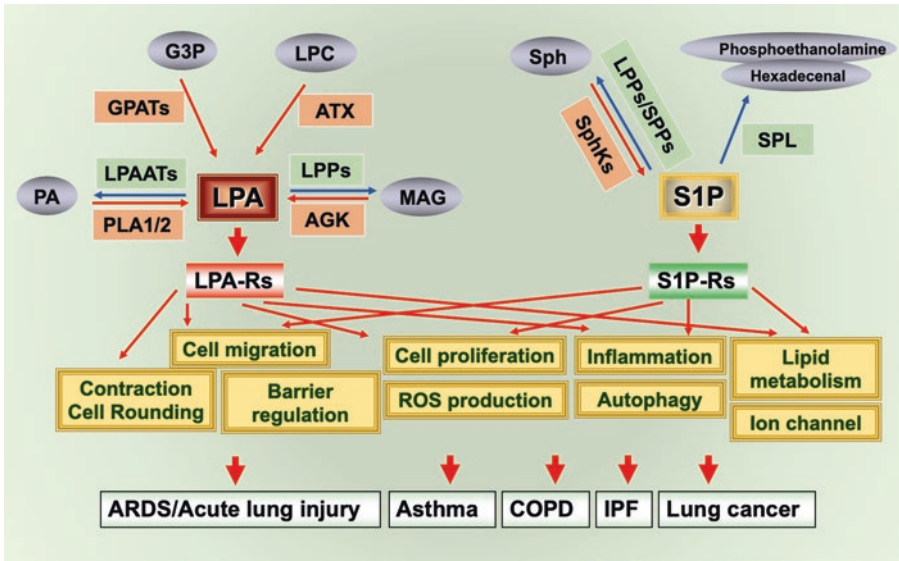

Fig. 20.1 Mechanisms of LPA and S1P generation and degradation and receptor-mediated cellular responses in lung diseases

internalization and degradation are a new focus, with the intention to discover new targets in regulating LPA and S1P signaling. As discussed above, both LPA and S1P levels are increased in most lung diseases. Reduction of extracellular lysophospholipids is a potential therapy strategy that has not been tested. LPPs degrade both extracellular LPA and S1P, and LPPs are transmembrane proteins. Thus, LPPs are druggable targets for treating human disorders, including lung inflammatory diseases. Cell-specific LPP isoform knockout mice need to be used in the preclinical models of lung inflammatory diseases (Fig. 20.1).

Acknowledgements Part of this work was supported by grants from National Institutes of Health (R01HL131665, HL136294 to Y.Z., R01 GM115389, R01HL151513 to J.Z.). We thank Kevin C Tran and Sarah J Taleb for editing the manuscript.

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Y. X. Wang (ed.), *Lung Inflammation in Health and Disease, Volume I*, Advances in Experimental Medicine and Biology 1303, https://doi.org/10.1007/978-3-030-63046-1