

# Pathobiology of Cigarette Smoke-Induced Chronic Obstructive Pulmonary Disease

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**Yoshida T, Tudor RM.** Pathobiology of Cigarette Smoke-Induced Chronic Obstructive Pulmonary Disease. *Physiol Rev* 87: 1047–1082, 2007; doi:10.1152/physrev.00048.2006.—Chronic obstructive pulmonary diseases (COPD), comprised of pulmonary emphysema, chronic bronchitis, and structural and inflammatory changes of small airways, is a leading cause of morbidity and mortality in the world. A better understanding of the pathobiology of COPD is critical for the developing of novel therapies, as the majority of patients with the disease have little therapeutic options at the present time. The pathobiology of COPD encompasses multiple injurious processes including inflammation (excessive or inappropriate innate and adaptive immunity), cellular apoptosis, altered cellular and molecular alveolar maintenance program, abnormal cell repair, extracellular matrix destruction (protease and anti-protease imbalance), and oxidative stress (oxidant and antioxidant imbalance). These processes are triggered by urban and rural air pollutants and active and/or passive cigarette smoke and modified by cellular senescence and infection. A series of receptor-mediated signal transduction pathways are activated by reactive oxygen species and tobacco components, resulting in impairment of a variety of cell signaling and cytokine networks, subsequently leading to chronic airway responses with mucus production, airway remodeling, and alveolar destruction. The authors provide an updated insight into the molecular and cellular pathobiology of COPD based on human and/or animal data.

## I. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) represents the fourth leading cause of morbidity and mortality in North America, in excess of 110,000 yearly deaths. After decades of a relative lack of interest in the disease, there is increased realization that we are in the midst of an epidemic, with a major impact on the health care resources, involving both the developed world and developing countries. The realization of the urgent need of novel therapies, new clinical end points, and insights into its natural history led to a major mobilization of resources of the National Institutes of Health of the United States directed to COPD research (57). It is clear that these urgent needs will be addressed by a better understanding of the pathobiology of COPD. It is our intent to provide the readers with an updated review of the pathogenesis of COPD, with a special focus on molecular mechanisms and novel overarching concepts developed in the past few years. The readers can access the summaries of recent meetings in the field in References 263, 335, 345.

COPD classically involves two spectra of clinical or pathological presentations, chronic bronchitis, emphysema, and small airway disease. While chronic bronchitis is defined clinically based on mucus production leading to cough with expectoration, emphysema is a pathological process of alveolar destruction with no apparent fibrosis (297). Both chronic bronchitis and emphysema eventually share a reduction of amount of air leaving the lung in the first second of a forced expiration, also known as forced expiratory volume at 1 s (or FEV<sub>1</sub>). The reduction of FEV<sub>1</sub> characterizes the airflow limitation in the disease, and probably occurs because of small airway disease. Its immense utility for screening, early diagnosis, and clinical follow-up overshadows the complexity of COPD, characterized by the existence of several clinically important phenotypes (potentially with underlying distinct pathogenesis), and most importantly, the lack of a clear understanding of the pathophysiological mechanisms underlying the decrement of FEV<sub>1</sub> in a given patient or even in a population with similar COPD phenotypes. It is clear that COPD is also a systemic disease with involvement of the cardiovascular system, skeletal muscle, bone marrow, and metabolism, among others (40). The lung compartment that is critically involved in the disease consists of airways and parenchyma. The large airways are the main site of chronic bronchitis, while the parenchymal changes underlie the process of emphysema. Notwithstanding this relatively simplistic regional stratification of the disease, it is clear that the small airways, particularly the terminal bronchioles (structures <2 mm in diameter and with extensive overall surface area given its branching pattern through 20–23 generations), are a critical site of disease involvement, with a potential role in the pathogenesis of both chronic bronchitis and emphysema. However, there

is a lack of understanding of how the small airway disease interfaces with the involvement of large airways or the destruction of the lung parenchyma, and the extent to which large and small airways contribute to airflow limitation.

The pathogenesis of COPD involves several pathogenetic processes such as inflammation, alterations of cell growth, cellular apoptosis, abnormal cell repair, extracellular matrix destruction, and oxidative stress, caused by air pollutants, including cigarette smoke, and modified by genetic factor (polymorphism), senescence, and infection. These processes are mediated by a growing number of molecular players, many of whom affect more than just one of these pathogenetic processes. This review focuses on specific pathobiologically relevant processes and molecules that ultimately mediate these processes, based on human or animal data and cell culture experimentation.

## II. RISK FACTORS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

### A. Direct Cigarette Smoking and Environmental Tobacco Exposure: The Main Causes of COPD

Active exposure to cigarette smoke causes the vast majority of COPD cases and contributes to increased incidence of related pulmonary diseases such as asthma and allergic rhinitis. Passive or environmental smoking increases the risk of smoking-related lung diseases, particularly of cancer and possibly of COPD. Cigarette smoking likely accounts for ~80–90% of COPD cases in the United States (283). Mainstream smoke, which represents 45% of the total biomass in the burning cigarette smoke, is inhaled by puffing, while ~55% of the content of the burning cigarette constitutes the side stream smoke, which is released into the environment. Environmental tobacco exposure (ETS) or involuntary smoking is defined as exposure to a combination of exhaled mainstream smoke and side stream smoke released from the smoldering end of a cigarette (249, 283). The effect that household ETS exposure has on children has been a growing focus of concern given its broad epidemiological impacts. Eisner et al. (74) evaluated the association between lifetime ETS exposure and the risk of developing COPD using data from a population-based sample of 2,113 United States adults aged 55–75 yr (74). The prevalence of prior prenatal ETS exposure was higher among adults with COPD than in those without the disease, consistent with multiple hits to potentially injurious agents throughout the life of a patient. Moreover, the prevalence of any subsequent lifetime home or workplace ETS exposure was higher among those with COPD than among those without COPD. In school children residing in as geographically distant regions as Southern California communities

or in Russian cities, maternal smoking was a risk factor for subsequent decreased lung function, airflow limitation, and chronic bronchitis (93, 138). Similar findings were reported by the European Community Respiratory Health Survey (306). The impact of cigarette smoke in the newborns and in children can be significant. In vivo experiments showed that rhesus monkeys when treated with nicotine or cigarette smoke during pregnancy and/or postnatally have compromised offspring, displaying emphysematous alterations and apoptosis, with caspase-3 activation in the lung (281, 372).

Smokers with congenital deficiency of serine protease inhibitor (or serpin)  $\alpha$ -1 antitrypsin are susceptible to emphysema largely because  $\alpha$ -1 antitrypsin is the main inhibitor of neutrophil elastase (303). School children with low levels of  $\alpha$ -1 antitrypsin were at risk of developing pronounced decrements in pulmonary function, particularly if exposed to ETS (337). Collectively, the results suggest that home and work ETS, including maternal smoking, synergistically contributes to the risk of COPD.

## B. The Adjuvant Role of Air Pollution

The outdoor and indoor air pollution from an urban environment provides a common ground to respiratory diseases, such as asthma, allergy, and COPD. Driscoll et al. (72) reported an estimated 386,000 deaths (asthma, 38,000; COPD, 318,000; pneumoconiosis, 30,000) and nearly 6.6 million of disability-adjusted life years (DALYS) (asthma, 1,621,000; COPD, 3,733,000; pneumoconiosis, 1,288,000) due to exposure to occupational airborne particulates worldwide in the year 2000 (72). A case control-crossover study carried out in 36 United States cities evaluated the effect of ozone and particulate matter with an aerodynamic diameter of less than 10  $\mu$ m ( $PM_{10}$ ) on respiratory hospital admissions during unseasonably warm weather spells between 1986 and 1999. This study showed that the combination of a 2-day exposure to 5 parts per billion (ppb) of ozone or the exposure to an increase of  $PM_{10}$  of 10  $\mu$ g/m<sup>3</sup> in particulate matter resulted in 0.27 and 1.47% increase in COPD rates, respectively (211). In consecutive cross-sectional studies conducted in the Rhine-Ruhr Basin of Germany between 1985 and 1994, an increase of 7  $\mu$ g/m<sup>3</sup> in 5-yr means of  $PM_{10}$  carried a 5.1% decrease in  $FEV_1$  for COPD women. Furthermore, women living <100 m from a busy road also had a significantly decreased lung function, and COPD was 1.79 times more likely than for control women (278). Decrements in lung function indices were associated with increasing concentrations of  $PM_{2.5}$ ,  $NO_2$ , and some metals (especially zinc and iron) in COPD cases in a study reported by the Catholic University Hospital in Rome, Italy (170).

The impact of the indoor environment also remains a major public health issue in COPD. A cross-sectional

assessment of indoor air quality in Nepal and its health effects revealed that solid biomass fuels (animal dung, crop residue, and wood) were the main sources of indoor air pollution affecting women's health (295). The average smoke level ( $PM_{10}$ ) in kitchens using biomass fuels was about three times higher than in those using cleaner fuels (kerosene, liquified petroleum gas, and biogas). The prevalence of respiratory illnesses and symptoms was considerably higher in those living in mud and brick houses when compared with concrete houses, and higher in those living on hills and in rural areas when compared with flatland and urban areas. Regalado et al. (260) reported that women who used a stove burning biomass fuel showed moderate airflow obstruction with COPD at stage GOLD >II, in the village of Solis, close to Mexico City. Orozco-Levi et al. (240) found that most of their study population composed by women with COPD were exposed to wood and charcoal smoke during their childhood and youth, but remained free of exposure for more than 25 years prior to presenting with symptoms of the disease, in Barcelona, Spain between 2000 and 2003. The follow-up data at the COPD Clinic of the National Institute of Respiratory Diseases, Mexico, between 1996 and 2003 demonstrated that women exposed domestically to biomass developed COPD with clinical characteristics, quality of life, and increased mortality similar in degree to that of tobacco smokers (257). Particles originated from biomass may synergize with cigarette smoke in the causation of airway diseases, and in fact replace cigarette smoke as the main culprit of COPD with the present populational trends of decreased smoking.

Although there is ample epidemiological and clinical evidence that ongoing exposure to cigarette smoke or environmental pollutants is clearly the main cause of COPD, these toxic agents recruit cell signaling pathways, which eventually get amplified, becoming self-perpetuating and highly destructive. Once lung destruction has ensued and patients have become airflow limited, interruption of cigarette smoke exposure may not significantly prevent further lung damage. The elucidation of these pathophysiological processes is the main hope for the development of biomarkers and targets of novel therapies.

## C. Tobacco Components and Nicotine Class Acetylcholine Receptors

Cigarette smoke contains thousands of chemical components, including  $\sim 10^{15}$  reactive species in the gas phase alone (199), particularly of high levels of nitric oxide. The tar phase has an equally abundant number of reactive oxygen and nitrogen species (ROS, RNS), including phenols and quinone. Obot et al. (237) quantified several smoke constituents in samples collected from experimental exposure chambers (237). The mean ratios of

nicotine/total particulate matter (TPM) (0.07), formaldehyde/TPM (0.002), acetaldehyde/TPM (0.1), acrolein/TPM (0.01), and propionaldehyde/TPM (0.005) were consistently present at all exposure concentrations. TPM levels of 250–600  $\mu\text{g}/\text{l}$  increased neutrophils and/or cytokines in bronchoalveolar lavage (BAL) in cigarette smoke-exposed mouse lungs.

The tobacco components, nicotine, nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and *N'*-nitrosonornicotine (NNN) stimulated signal transduction through the nicotine class acetylcholine receptors (nAChRs) (9, 279). Functional nAChR are composed of homopentamers of  $\alpha 7$ –10 subunits or heteropentamers derived from  $5\alpha(\alpha 2$ –6) and  $3\beta(\beta 2$ –b4) subunits. By RT-PCR, small airway epithelial cells expressed nAChR $\alpha 2$  and  $\alpha 4$ , while human normal bronchial epithelial cells (HBEC) expressed  $\alpha 3$  and  $\alpha 5$ ; the subunits  $\alpha 7$ ,  $\alpha 9$ ,  $\alpha 10$ ,  $\beta 2$ , and  $\beta 4$  of nARCHRs were expressed in both cells (321, 349). In the developing lung of monkeys, the  $\alpha 7$ -subunit was detected by immunohistochemistry in alveolar type II cells, pulmonary neuroendocrine cells, submucosal cells, smooth muscle cells, fibroblasts, and alveolar macrophages (281). By in situ hybridization, the subunits  $\alpha 3$ –5,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  were demonstrated in human vascular endothelial cell (198) and accounted for a significant nicotine-triggered cell signaling (111). These tobacco components activated nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) in several cell lines, mediated partly by extracellular signal-regulated kinase 1 and 2 (ERK1/2) through the nAChR (56, 118). Heeschen et al. (111) involved nAChR  $\alpha 7$ -dependent signaling in angiogenesis, based on testing the effects of the nAChR  $\alpha 7$ -antagonist  $\alpha$ -bungarotoxin in endothelial cells.  $\alpha 7$ -Mediated endothelial cell angiogenic networking depended partly on vascular endothelial cell growth factor (VEGF) and was completely reliant on signaling by phosphoinositide 3-kinase (PI3K), ERK, and p38 mitogen-activated protein kinase (MAPK), finally resulting in NF- $\kappa\text{B}$  activation.

nAChR form ion channels permeable to either calcium or sodium. Carlisle et al. (35) showed that HBEC have functional receptors of muscle-type heteropentamer  $\alpha 1/\beta 1/\gamma/\epsilon$  and neuronal homopentamers  $\alpha 7$  or  $\alpha 9$ , neuronal heteropentamers  $\alpha 3/\alpha 5/\beta 2$ ,  $\alpha 3/\alpha 5/\beta 4$ ,  $\alpha 6/\beta 2$ , or  $\alpha 6/\beta 4$  receptors, while airway fibroblasts had muscle-type heteropentamers  $\alpha 1/\beta 1/\gamma/\epsilon$  and neuronal homopentamers  $\alpha 7$  (35). Nicotine-induced calcium influx was mediated by protein kinase C (PKC) and p38 MAPK in HBEC and activated ERK1/2 in airway fibroblasts. Additionally, cigarette smoke increased the expression of subunit  $\alpha 5$  protein in HBEC and  $\alpha 3$  in a panel of airway fibroblasts obtained from active smokers, never smokers, and ex-smokers. It is noteworthy that  $\alpha 3$ -containing receptors, a sodium channel, undergoes inactivation upon long-term exposure to nicotine, leading to thickening of the airway

wall in chronic bronchitis because of an imbalance of apoptosis and cell proliferation.

Recent studies indicated that the vagus nerve, which is the longest of the cranial nerves and innervates most of the peripheral organs, can modulate immune responses and control inflammation through a “nicotinic anti-inflammatory pathway” dependent on the  $\alpha 7\text{nAChR}$  (329). Nicotine inhibited more efficiently than acetylcholine proinflammatory cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, and IL-18, but not the anti-inflammatory cytokine IL-10 through a posttranslational mechanism in human peripheral blood mononuclear cells (23, 342). Nicotine also inhibited the production of prostaglandin  $\text{E}_2$  (PGE $_2$ ), monocyte inflammatory factor (MIP)-1 $\alpha$  and MIP-1 $\beta$  in these cells (309, 365). These in vitro data are likely consistent with in vivo data showing that cigarette smoke reduced bacterial clearance in female C57BL/6 mice after infection with *Pseudomonas aeruginosa* (71). Furthermore,  $\alpha 7\text{nAChR}$ -deficient mice produced greater amounts of proinflammatory cytokines than wild-type mice when treated with lipopolysaccharide (LPS), consistent with an anti-inflammatory role of nicotine/ $\alpha 7\text{nAChR}$  signaling (342). Prolonged presence of bacteria and persistent inflammation is a critical event in the course of COPD, a central component leading to exacerbations.

Given the complexity of cell signaling triggered by components of cigarette smoke, it is often difficult to isolate the effects of a specific component with regards to pulmonary pathophysiology. Therefore, most of the studies rely on the pathobiological effects of cigarette smoke rather than on isolated components.

### III. AIRWAY DISEASE

#### A. Chronic Bronchitis

The two main characteristics of chronic bronchitis are excessive mucus production and chronic inflammatory cell infiltration of the bronchial wall. The excessive mucus production with increased expectoration correlates but poorly with increased mucus gland mass in the wall of large airways, which is documented by the Reid index that rates the level of airway wall occupied by mucus glands in relation to total airway thickness (261). The increase in mucus gland cells is not specific to COPD as it also occurs due to smog exposure, asthma, or cystic fibrosis. There is both an increase in mucus cell numbers via proliferation (hyperplasia) and enhanced mucus synthesis and secretion (hypertrophy). Inflammation caused by cigarette smoke is a critical factor affecting both pathobiological processes. The dominant site of the air-flow obstruction is now thought to reside in the smaller airways measuring  $<2$  mm in diameter. In contrast, the



dominant site of clinically relevant mucus secretion remains the larger airways. A distinction is often made between disease of the larger airways (chronic bronchitis) and disease of the noncartilagenous membranous bronchioles and terminal airways (small airway disease or remodeling; small airway disease is addressed subsequently in this review). In fact, recent evidence generated by three-dimensional computer tomography documented that FEV<sub>1</sub> values in COPD patients correlate with luminal area and inversely with percent wall area, particularly of smaller generation airways (3rd to 6th, i.e., closer to 2 mm in diameter airways) rather than larger airways (<3rd generation airways) (105). Animal models of experimentally induced chronic bronchitis were reviewed elsewhere (234).

## B. Airway Inflammation

Chronic bronchitis is defined as a CD8<sup>+</sup> cytotoxic T cell-dominant airway disease. CD8<sup>+</sup> T cells were observed in sputum (47, 328), and infiltrating the bronchial tree, including epithelium (269), submucosa (172), bronchial glands (270), smooth muscle layer (14, 268), and around lymphoid follicles (121). Morphometric analysis of bronchial biopsies showed that the ratios of CD8<sup>+</sup> to CD4<sup>+</sup> T cells were 1.3, 11.8, and 4.3 (mean/mm<sup>3</sup>) in healthy smokers, patients with stable chronic bronchitis, and patients with exacerbated chronic bronchitis, respectively (373). The lower ratio of CD8<sup>+</sup>/CD4<sup>+</sup> in patients with exacerbation might occur due to an enhanced recruitment of CD4<sup>+</sup> cells by RANTES, and/or potential lysis of CD8<sup>+</sup> cells due to enhanced viral infection. The role of CD8<sup>+</sup> T cells is well documented in respiratory virus infections, a common complication of COPD patients. CD8<sup>+</sup> T cells are important contributors to viral clearance, utilizing contact-dependent effector functions, mediated by perforin and Fas ligand (CD95L/FasL) as well as interferon (IFN)- $\gamma$  and TNF- $\alpha$ . These latter two cytokines are primary mediators of T-cell-mediated lung injury, particularly TNF- $\alpha$  (28). CD8<sup>+</sup> T cells isolated from the sputum of smokers with COPD were highly activated based on the expression of perforin (47), which is synthesized upon T-cell activation, and released through intercellular channels formed through the target cell membrane, causing activation of mediators of cell death (294). Cigarette smoke increased CD95L expression in bronchioles of male Wistar rats, in association with phosphorylation of c-Jun NH<sub>2</sub>-terminal kinase (JNK) (355). In humans, the role of FasL is unclear because one study reported that plasma-soluble Fas was increased in severe COPD (362), while another group reported that serum-soluble FasL and plasma-soluble Fas were not changed in patients with COPD, when compared with controls (308). Increased levels of CD95L could be potentially linked to

CD95-producing inflammatory cell influx, which could affect airway epithelial or alveolar cell survival by activating CD95 cell death receptor.

Cytokines and chemokines, their receptors, and functions described in this review are summarized in Table 1, and the pathways involved in cytokine regulation of mucin production are shown in Figure 1. The involvement of neutrophils containing high levels of myeloperoxidase (MPO) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is also a typical feature of chronic bronchitis, influencing disease progression and the risk of acute bacterial exacerbations (55, 95, 115). The airway epithelium is a rich source of cytokines/chemokines that recruit both neutrophils and macrophages. Chung (48, 49) summarized in detail the cytokines overexpressed in COPD (48, 49). IL-6, IL-1 $\beta$ , TNF- $\alpha$ , growth-related gene- $\alpha$  (Gro- $\alpha$ )/keratinocyte-derived chemokine (KC) (CXCL1), monocyte chemoattractant protein-1 (MCP-1), and IL-8 were increased in sputum, with further increases during exacerbations of COPD, and the bronchiolar epithelium overexpressed MCP-1, its receptor CCR2, MIP1 $\alpha$ , and IL-8. MCP-1 and CCR2 were involved in the recruitment of macrophages and mast cells into the airway epithelium in COPD (62). Airflow limitation (i.e., decreased FEV<sub>1</sub>) correlated with increased expression of IL-8, MIP-1 $\alpha$ , MCP-1, and the CCR2 in airway epithelium (85). Although IL-8 is thought to be a chemoattractant for neutrophil and CD8<sup>+</sup> T cells via binding to receptors CXCR1 and CXCR2, epithelial IL-8 expression did not necessarily correlate with the numbers of these cells in airways of smokers (62). Aqueous cigarette smoke extract (CSE, i.e., cigarette smoke-bubbled into PBS/medium) induced the release of IL-8 from human airway smooth muscle cells, and the effect was enhanced by TNF- $\alpha$  (238). Mice, which lack the IL-8 gene, have two neutrophilic CXC chemokines, MIP-2 and Gro- $\alpha$ /KC (356), which were significantly elevated in the BAL of smoke-exposed female C57BL/6 mice (314).

Eosinophils have been linked with exacerbations of chronic bronchitis (78, 373). Serum levels of eosinophilic cationic protein (ECP or EG2) were significantly increased during exacerbations and correlated with levels of the neutrophil myeloperoxidase (78). Increasing numbers of EG2<sup>+</sup> cells (i.e., activated eosinophils) correlated with the expression of the eosinophil chemoattractant RANTES (regulated on activation, normal T-cell expressed and secreted) mRNA but not with eotaxin or MCP-4 mRNA in bronchial biopsy samples from chronic bronchitis (373). Interestingly, peripheral blood eosinophils isolated from patients with chronic bronchitis were positive for IL-12 (235), which facilitates specific CD8<sup>+</sup> T-cell responses, promotes the development of T helper type 1 (Th1) cells, enhances the lytic activity of natural killer (NK) cells, and induces the secretion of IFN- $\gamma$  by both T cells and NK cells (273). These results suggest that eosinophils might play a role in promoting Th1-response

TABLE 1. *Cytokine/chemokines, their receptors, and functions*

Cytokines/Chemokines	Receptors	Functions	Reference Nos.
<i>Cytokines</i>			
TNF- $\alpha$	TNF- $\alpha$ R1 (p55/p60, CD120a), TNF- $\alpha$ R2 (p75/p80, CD120b)	Initiation and maintenance of inflammation, activation of endothelial and epithelial cells	217
IL-1 $\beta$	IL-1R1, IL-1RAcP	Initiation and maintenance of inflammation, activation of endothelial and epithelial cells	68
IL-4*	IL-4R1 (IL-4R $\alpha$ , common $\gamma$ -chain), IL-4R2 (IL-4R $\alpha$ , IL-13R $\alpha$ 1)	Th2/Tc2 cytokine, IgE production, eosinophil growth, endothelial cell VCAM-1 expression	101, 292
IL-5	IL-5R $\alpha$ , IL-5R $\beta$	Th2/Tc2 cytokine, stimulation and differentiation of B cells and eosinophils	310
IL-6	IL-6R $\alpha$ , gbl30	Multifunctional cytokine (inflammation, vascular permeability, cell proliferation)	117, 277
IL-10	IL-10R1 (IL-10R $\alpha$ ), IL-10R2 (IL-10R $\beta$ )	Suppression of Th1 and Th2 responses, stimulation of B cells and antigen-specific cytotoxic T cells	216
IL-11	IL-11R $\alpha$ , gbl30	Multifunctional IL-6 type cytokine (T- and B-cell proliferation, IgE production, fibroblast activation)	117
IL-12(p35, p40, p70, p40 <sub>2</sub> )	IL-12R $\beta$ L, IL-12R $\beta$ 2	Th1 cytokine	346
IL-13	IL-4R2 (IL-4R $\alpha$ , IL-13R $\alpha$ 1), IL-13R $\alpha$ 2	Th2/Tc2 cytokine, IgE production, activation of eosinophils, endothelial cell VCAM-1 expression, activation and maturation of dendritic cells and macrophages	292, 350
IL-15*	IL-15R $\alpha\beta\gamma$ , IL-15R $\alpha$ , IL-15 $\alpha\beta$ , IL-15 $\beta\gamma$	Proliferation and activation of NK cells, CD8+ T cells, and nonimmune cells	29
IL-17(A-F)	IL-17R, IL-17RH1(IL-17BR), IL-17 R-like protein (IL-17RL or IL-17RC), IL-17RD (SEF or IL-17RLM), IL-17RE	Proinflammatory cytokine, activation of endothelial and epithelial cells, Th1 and Th2 responses	228
IL-18	IL-18R $\alpha$ (IL-1Rrp), IL-18R $\beta$ (IL-1RAcP)	Th1 and Th2 responses, activation of NK cells and monocytes	232
IFN- $\gamma$	IFN- $\gamma$ R1(IFN- $\gamma$ R $\alpha$ ), IFN- $\gamma$ R2(IFN- $\gamma$ R $\beta$ ), IFN- $\gamma$ R2(IFN- $\gamma$ R $\beta$ )	Th1/Tc1 cytokine, activation of macrophages, NK cells, endothelial, and epithelial cells	44, 91
GM-CSF	GM-CSFR( $\alpha$ , $\beta$ c)	Apoptosis and activation of eosinophil, regulation of hematopoietic cells, endothelial cell migration	89, 316
<i>CC chemokines</i>			
MCP-1 (CCL2) JE(mouse)	CCR2	Acquisition of Th2 cell phenotype, monocyte, NK cell and basophil chemotaxis, mast cell mediator release	11, 20, 48, 49, 123, 145, 179, 215, 267, 288
MIP-1 $\alpha$ (CCL3)	CCR1, CCR5	Th1 chemokine, monocyte, NK cell and basophil, eosinophil chemotaxis, activation and maturation of dendritic cells	
MIP-1 $\beta$ (CCL4) RANTES (CCL5)	CCR5, CCR8 CCR1, CCR3, CCR5	Th1 chemokine, monocyte and NK cell chemotaxis Th1 chemokine, neutrophil, eosinophil, and NK cell chemotaxis, activation and maturation of dendritic cells	
Eotaxin (CCL11)	CCR3	Th1 chemokine, neutrophil and eosinophil chemotaxis	
MCP-4 (CCL13)	CCR2, CCR3	Monocyte, eosinophil, and T cell chemotaxis	
TARC (CCL17)	CCR4, CCR8	Th2 chemokine	
MIP-3 $\alpha$ /LARC/exodus-1 (CCL20)	CCR6	Recruitment of immature dendritic cells, T cells, and B cells	
<i>CXC chemokines</i>			
GRO $\alpha$ (CXCL1) <sup>†</sup> KC/MIP-2 (mouse/rat)	CXCR1 (human), CXCR2 (human, mouse)	Neutrophil chemotaxis, regulation of endothelial cell function	11, 20, 48, 49, 123, 145, 179, 215, 288
MIP-2 $\alpha$ /GRO $\beta$ (CXCL2) <sup>†</sup>	CXCR2 (human, mouse)	Neutrophil chemotaxis, regulation of endothelial cell function	
MIP-2 $\beta$ /GRO $\gamma$ (CXCL3) <sup>†</sup>	CXCR2 (human, mouse)	Neutrophil chemotaxis, regulation of endothelial cell function	
IL-8 (CXCL8) <sup>†</sup> (human)	CXCR1, CXCR2	Neutrophil, eosinophil, CD8+ T cell, basophil chemotaxis, regulation of endothelial cell function	
Mig (CXCL9)	CXCR3	IFN- $\gamma$ -inducible chemokine, eosinophil inhibitor, Th1 and Th2 responses	
IP-10 (CXCL10)	CXCR3	IFN- $\gamma$ -inducible chemokine, Th1 response, NK cell chemotaxis	

\* IL-2 family. <sup>†</sup> Glu-Leu-Arg (ELR motif) CXC chemokines. TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; GM-CSF, granulocyte-macrophage colony stimulating factor; MCP, monocyte chemoattractant protein; MIP, monocyte inflammatory factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TARC, thymus and activation-regulated chemokine; KC, keratinocyte-derived chemokine; GRO, growth-related oncogene; MIG, monokine induced by interferon- $\gamma$ ; IP-10, interferon-inducible protein 10. Readers can also access the reviews of cytokines in Refs. 48, 49.

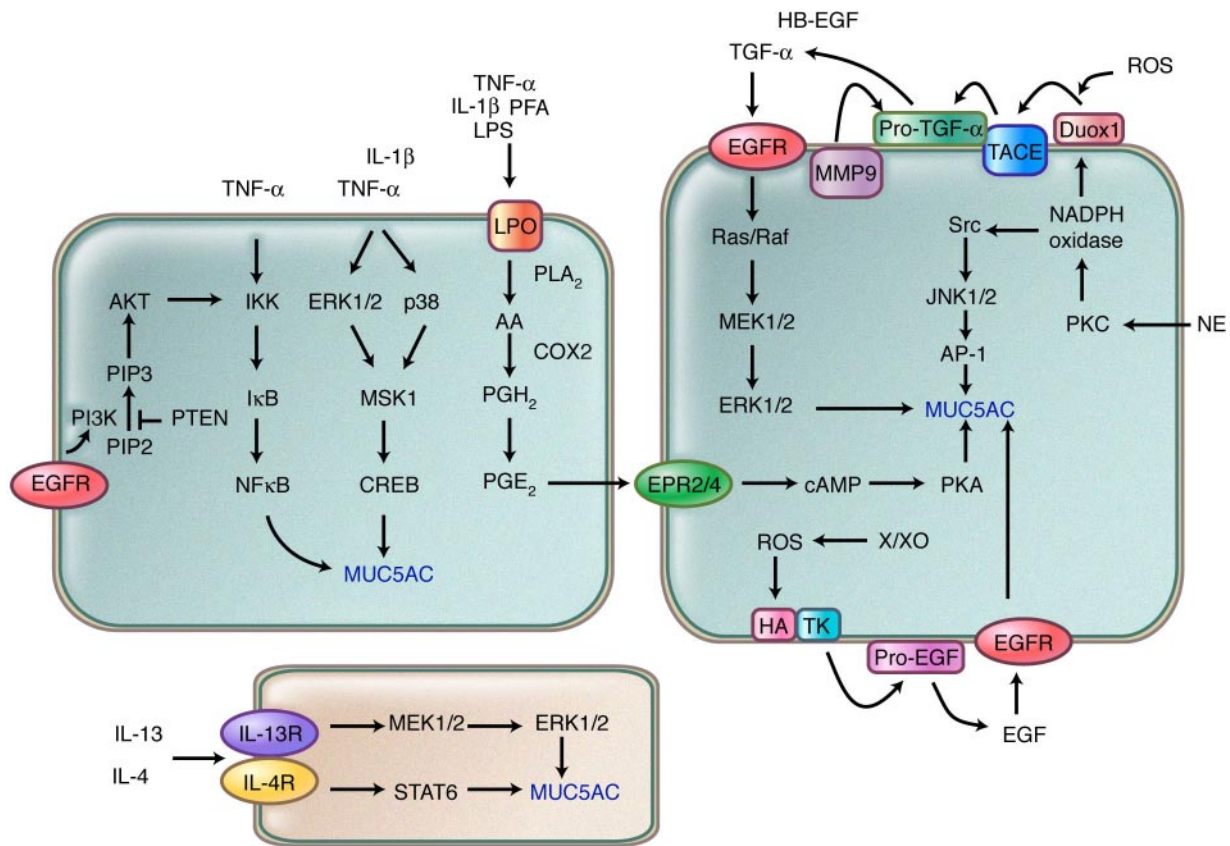


FIG. 1. Signal transduction involved in the regulation of mucin production. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; PFA, platelet activating factor; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B, inhibitor of NF- $\kappa$ B; IKK, I $\kappa$ B kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; AA, arachidonate; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; COX2, cyclooxygenase-2; PGH<sub>2</sub>, prostaglandin H<sub>2</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; EPR2/4, E-prostanoid 2 and 4 receptors; PKA, protein kinase A; MSK1, mitogen- and stress-activated protein kinase 1; CREB, cAMP-response element-binding protein; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; TACE, TNF- $\alpha$ -converting enzyme; Duox1, dual oxidase 1; NE, neutrophil elastase; PKC, protein kinase C; MEK1/2, ERK kinase 1/2; HA, hyaluronan; TK, tissue kallikrein; X/XO, xanthine/xanthine oxidase; ROS, reactive oxygen species; MMP-9, metalloproteinase-9; JNK1/2, c-Jun NH<sub>2</sub>-terminal kinase 1/2; AP-1, activator protein-1; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; STAT6, signal transducers and activators of transcription 6.

stimulated by cigarette smoke (rather than Th2-responses characteristic of asthma). The finding that IL-15<sup>+</sup> neutrophils and macrophages were evident in bronchial biopsies from chronic bronchitis suggests that IL-15 contributes to Th1-mediated inflammatory disease as well (230).

### C. Mucus

The mucus layer forms a film coating the apical portion of the airway lining. The coordinated movement of cilia propels the mucus layer directionally from the periphery of the lung into the upper airways. Mucus glycoproteins (mucins), water, and peptides are the main constituents of these layers, all playing key roles in the clearance of foreign materials and infectious agents. In chronic bronchitis, there is an increase in luminal mucus due to enhanced production of mucins, increased secretion from goblet cells, goblet cell hyperplasia and/or metaplasia, accumulation of cell debris, and inflammatory cells.

At least 12 human mucin genes (MUC1, 2, 4, 5AC, 5B, 7, 8, 11, 13, 15, 19, and 20) are expressed at the mRNA level in the lower respiratory tract from healthy individuals. Goblet cells typically express MUCs 5AC and 2, while glandular mucosal cells express MUCs 5B, 8, and 19. MUC5AC and 5B, which are large-molecular-mass glycoproteins, and MUC19, a newly identified mucin, are secreted and have cysteine-rich motifs. MUC7, expressed in serous cells, corresponds to a low-molecular-mass mucin lacking a cysteine-rich domain (265). Airway mucus obstruction is shared among cystic fibrosis, asthma, and COPD. COPD is associated with increased expression of MUC5B in the bronchiolar lumen and of MUC5AC in the bronchiolar epithelium (33).

Human bronchial mucins consist of highly polydispersed O-glycosyl proteins, which protect the respiratory surface from inhaled particles and microorganisms. The diversity of the mucins is enhanced by posttranslational modifications, such as O-glycosylation and sulfation (on



serine/threonine residues; 339), thus contributing to highly diverse O-linked carbohydrate chains (265). There is a distinct pattern of mucins in chronic bronchitis with and without bacterial infection. Di-sialylated oligosaccharide and sialylated or sulfated oligosaccharide bearing sialyl Lewis X epitope were not found in patients with chronic bronchitis with no evidence of heavy bacterial or viral infection (63, 191). In contrast, mucins from infected patients with chronic bronchitis were more sialylated and contained more sialyl-Lewis X epitopes (61). TNF- $\alpha$  increased  $\alpha$ 2,3-sialyltransferase activity, which mediated the sialylation of the weakly sialylated mucins in a human respiratory glandular cell line MM-39 (64). Thus the degree of sialylation of bronchial mucin might indicate the protection status against inhaled particles and microorganisms in chronic bronchitis.

A variety of stimuli such as neutrophil elastase, LPS, TNF- $\alpha$ , IL-1 $\beta$ , cigarette smoke, or oxidative stress causes goblet cell metaplasia and mucus hypersecretion (339) (Fig. 1). Neutrophil elastase increased MUC5AC mRNA levels by enhancing mRNA stability (340) mediated by ROS in HBEC (79). Male Balb/c mice showed MUC5AC mRNA and protein expression, and goblet cell metaplasia after 8 days of intratracheal instillation of pancreatic elastase. These changes were mediated by elastase proteolytic activity and subsequent initiation of an inflammatory process documented by increases in Gro- $\alpha$ /KC and IL-5 (338). *Pseudomonas* LPS induced MUC5AC expression, associated with neutrophil infiltration (359), and metalloproteinase (MMP)-9 expression (152). LPS also increased MUC7 mRNA and glycoprotein products in HBEC (188). TNF- $\alpha$ , IL-1 $\beta$ , and LPS-induced MUC5AC synthesis were mediated by 1) IKK- $\beta$  and NF- $\kappa$ B signaling (192), 2) mitogen- and stress-activated protein kinase 1 (MSK1), 3) signaling cascades leading to activation of the cAMP-response element by the cAMP-response element binding protein (CREB) via ERK and p38 MAPK (299), 4) COX-2 activation of E-prostanoid receptor (EP) 2 and/or 4, and 5) cAMP-PKA-mediated signaling (96) (Fig. 1).

Signaling initiated by epidermal growth factor receptor (EGFR) tyrosine phosphorylation (induced by cigarette smoke-derived ROS), epidermal growth factor (EGF), transforming growth factor (TGF)- $\alpha$ , or heparin-binding (HB)-EGF has been found to play an important role in mucin production in human airway epithelial cells. Hyaluronan fragmentation due to ROS generated by the xanthine/xanthine oxidase system activated the serine protease tissue kallikrein, which then cleaved the transmembrane precursor of EGF. Thus the EGF-EGFR-dependent stimulation of Ras-MAPK/ERK kinase (MEK)/ERK seems to be central in goblet cell hyperplasia and increased MUC5AC gene expression (36, 37). Cigarette smoke caused EGFR activation and mucin production via ROS and activation of TNF- $\alpha$ -converting enzyme (TACE), which shed pro-TGF- $\alpha$  in human airway epithelial cell

NCI-H292 (285). The same group showed that the TACE-EGFR pathway was dependent on dual oxidase 1, a homolog of glycoprotein p91phox, activated by PKC (284). Acrolein, also a component of cigarette smoke, induced MUC5AC expression via an early ligand-dependent activation of EGFR, mediated by TACE and MMP-9. A prolonged effect of acrolein may be mediated by altering MMP-9 and tissue inhibitor of metalloprotease (TIMP)-3 balance (67). Human airway trypsin-like protease, which is a novel serine protease purified from the sputum of patients with chronic bronchitis and bronchial asthma, induced the production of amphiregulin by the action of the protease-activated receptor-2. Amphiregulin was then released by TACE, resulting in activation of EGFR signaling (46).

Furthermore, ROS derived from cigarette smoke activated JNK, via a Src-dependent signaling cascade. This downstream signaling triggered transcriptional upregulation of MUC5AC, mediated by the binding of the activator protein 1 (AP-1) response element by JunD and Fra-2 (90). Additionally, exposure to cigarette smoke significantly decreased the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), thus increasing AKT and EGFR-specific signaling, leading to MUC5AC mucin production (185). The finding that aqueous CSE synergized with LPS or TNF- $\alpha$  in the induction of MUC5AC expression suggests that cigarette smoke potentially amplifies the expression of respiratory mucins by proinflammatory stimuli relevant to COPD pathogenesis (12).

The close interplay between inflammation, oxidative stress, and growth factors in the lung airways has been progressively unraveled using state of the art approaches in transgenic mice (Tables 2 and 3, and Figs. 1 and 2). CD4<sup>+</sup> Th2 cells and their cytokines, including IL-4, -10, and -13, play a crucial role in the development of goblet cell hyperplasia/metaplasia in animal models (182, 195, 371). IL-13 overexpressing mice had increased expression of MUCs 5AC, 1, and 4, and Gob-5 (42, 184). In signal transducers and activators of transcription 6 (STAT6)-deficient mice, in which both STAT6 and IL-13 signaling were impaired, allergen-induced goblet cell metaplasia was largely inhibited (184). Administration of recombinant IL-13 to non-immunized mice induced goblet cell hyperplasia with mucus hypersecretion (3, 296), via EGFR signaling (291). IL-13 altered ciliated cell differentiation and increased the proportion of secretory cells in human nasal epithelial cells (174). Using a mouse model of Sendai virus infection, Tyner et al. (327) proposed a dual signaling model driving ciliated cell differentiation into goblet cells in chronic mucus-producing airway epithelium (327). First, EGFR signaling activated AKT/PI3K, and thus inhibited apoptosis of ciliated cells. Subsequently, ciliated cells responded to IL-13/IL-13R signaling and activated MEK1/2, ERK1/2, and STAT6 to produce mucin, including MUC5AC. Furthermore, IL-13



TABLE 2. *Genetically altered mice prone to pulmonary emphysema*

Genes	Findings	Reference Nos.
Nrf2	Nrf2 KO mice showed more extensive emphysema caused by both cigarette smoking and elastase instillation than WT mice.	126, 132, 258
SP-D	SP-D KO mice developed air space enlargement at 3 wk and progressive with age (6–7 mo). Accumulation of foamy macrophage with upregulation of MMP-2, -9, and -12.	348
PIGF	PIGF TG mice showed emphysema and enhanced pulmonary compliance at 6 mo of age and became predominate at 12 mo. Increased apoptosis (type II cells and endothelial cells).	317
VEGF	Air space enlargement and loss of lung elastic recoil were noted after 5 and 8 wk of intratracheal delivery of adeno-associated cre recombinase virus. VEGF and VEGFR-2 levels were reduced at 5 wk and recovered by 8 wk. There were increased alveolar and bronchial cell apoptosis, and neither cell proliferation nor inflammation	312
LOX1L	LOX1L KO mice showed emphysematous change with elastic fiber defect.	190
Fibulin-4	Fibulin-4 KO mice showed markedly enlarged air spaces at birth.	210
Fibulin-5	Fibulin-5 KO mice showed markedly enlarged air spaces with elastin fragmentation at 2 wk of age.	360
STAT3	STAT3 KO mice developed severe air space enlargement with increased apoptosis (type II cells) from 1 to 7 days after intratracheal delivery of adenovirus.	209
Itgb6	Itgb6 KO mice developed emphysema with MMP-12 expression at 6 and 14 mo of age.	226
Smad3	Smad3 KO mice developed emphysema with MMP-9 expression at 1 and 4.5 mo of age.	41
Ltbp-3	Ltbp-3 KO mice showed air space enlargement with increased cell proliferation (type II cell) and apoptosis, noted at 10 and 23 days after birth. TGF- $\beta$ signaling was temporally decreased at days 4–6.	53
Ltbp-4	Ltbp-4 KO mice showed air space enlargement at birth and worsened with age (6–8 mo). Multiple patches of fragmented and condensed elastic fibers.	302
Pallid*	Pallid mice showed more extensive emphysema caused by cigarette smoke exposure, along with CD4+ T cell infiltration and decreased elastin content.	39, 311
IL-1 $\beta$	IL-1 $\beta$ TG mice developed emphysema when provided doxycycline from 3 to 8 wk of age. There were neutrophil, macrophage, and lymphocyte infiltration; fibrosis; mucous production; and KC, MIP-2, MMP-9 and -12 expressions.	175
IL-4	IL-4 TG mice showed eosinophil infiltration, airway fibrosis, and emphysematous change when provided 1 mo of doxycycline treatment. The lesions were accompanied with increased expressions of TGF- $\beta$ 1; MMP-2, -9, -12, and -14; cathepsin B, K, L, and S; and adenosine and its receptors as well as decreased expressions of TIMP-2-4, $\alpha$ 1-antitrypsin, and adenosine deaminase activity.	195
IL-13	IL-13 TG mice showed alveolar enlargement, and enhanced pulmonary compliance, mucous metaplasia, and macrophage, lymphocyte, and eosinophil-rich inflammation, when provided 1 month of doxycycline treatment. Expressions of MMP-2, -9, -12, and -13, cathepsins B, S, L, H, and K, and inhibition of $\alpha$ 1-antitrypsin.	371
IFN- $\gamma$	IFN- $\gamma$ TG mice showed alveolar enlargement and enhanced pulmonary compliance, macrophage- and neutrophil-rich inflammation, when provided 1 and 3 mo of doxycycline treatment. Expressions of MMP-12; cathepsins B, H, D, and S; and inhibition of secretory leukocyte proteinase inhibitor.	344
TLR4	TLR4 KO mice developed emphysema at 3 mo of age and progressed with age (6 and 12 mo). The lesions were accompanied by increased elastin degradation, oxidative stress, and apoptosis, with neither inflammation nor MMP and cathepsin expressions.	369
TIMP-3	TIMP-3 KO mice developed emphysema at 2 wk of age and progressed with age (18 mo). Degradation of collagen was evident.	181
SMP30	SMP30 KO mice developed emphysema with age and more sensitive to cigarette smoke-induced emphysema than in WT mice. Lipid peroxidation and glutathione (total and oxidized forms) were increased in KO mice after smoking.	276
FGF23	FGF23 KO mice showed emphysematous change at 3 wk of age.	259
SAM2	SAM2 mice spontaneously developed emphysema with age and were more sensitive to smoke-induced emphysema than in senescence-resistant strain.	313
Klotho	Klotho KO mice developed emphysema at 4 wk of age and progressed with age (10 wk). Neutrophil influx in BAL and increased MMP-9 expression and decreased TIMP-1 expression.	86, 304

KO, knockout; Nrf2, nuclear factor E2-related factor 2; SP-D, surfactant protein D; PIGF, placenta growth factor; VEGF, vascular endothelial cell growth factor; LOX1L, lysyl oxidase-like 1; STAT3, signal transducers and activators of transcription 3; Itgb6,  $\beta$ 6 subunit of  $\alpha$ v $\beta$ 6 integrin; Ltbp-3, latent transforming growth factor- $\beta$  binding protein 3; Ltbp-4, latent transforming growth factor- $\beta$  binding protein 4; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-4, interleukin-4; IL-13, interleukin-13; IFN- $\gamma$ , interferon- $\gamma$ ; TLR4, Toll-like receptor 4; TIMP-3, tissue inhibitor of metalloproteinase-3; SMP30, senescence marker protein-30; FGF23, fibroblast growth factor 23; SAM2, senescence-associated mouse 2. \* Low  $\alpha$ 1-antitrypsin mice.

made by CD8+ T cells (214) induced emphysematous changes in the lungs of mice (371).

Similarly to cystic fibrosis, chronic bronchitis is characterized by an excess of mucin on the airway surface. This alteration is associated with loss of the periciliary liquid layer and adhesion of the mucus layer to the cell surface, reducing mucociliary clearance and enhancing

retention of pollutants and pathogens (25). Aqueous CSE inhibited chloride secretion in HBEC without affecting sodium transport, indicating that there is sodium absorption out of proportion to chloride secretion in the setting of increased mucus secretion (165). The mechanism of CSE inhibition of chloride secretion remains unknown. Recently, Cantin et al. (32) found out that cigarette smoke

TABLE 3. *Genetically altered mice resistant to cigarette smoke-induced pulmonary emphysema*

Genes	Findings	Reference Nos.
CuZnSOD	CuZnSOD TG mice were protected from cigarette smoke-induced emphysema and had decreased expression of oxidative stress markers (3-nitrotyrosine, 8-hydrodeoxyguanosine, and lipid peroxidation).	80
MMP-12/MME	MMP-12 KO mice were protected from cigarette smoke-induced emphysema and showed no increase of macrophages.	107
TNF- $\alpha$ R	TNF- $\alpha$ R KO mice were protected from cigarette smoke-induced emphysema and had reduced numbers of macrophage and neutrophils. MMP-2, -9, -12, and -13 were smaller expressed in KO mice than in WT mice.	51
TNF- $\alpha$ R2	TNF- $\alpha$ R2 KO mice were protected from cigarette smoke-induced emphysema and had decreased apoptosis (type II cells) and reduced numbers of macrophages, neutrophils, dendritic cells, and CD4+ and CD8+ T cells.	58
NE	NE KO mice were protected from cigarette smoke-induced emphysema and had reduced numbers of neutrophils and macrophages, MMP-12 expression, and increased TIMP-1 expression.	287
CCR6*	CCR6 KO mice attenuated smoke-induced emphysema and reduced MIP-1 $\alpha$ and MIP-3 $\alpha$ levels and numbers of dendritic cells, CD8+ T cells, and granulocytes in BAL.	27

CuZnSOD, copper zinc superoxide dimustase; MMP-12/MME, matrix metalloproteinase-12/macrophage elastase; TNF- $\alpha$ R, tumor necrosis factor- $\alpha$  receptor; TNF- $\alpha$ R2, tumor necrosis factor- $\alpha$  receptor 2; NE, neutrophil elastase. \* A receptor for MIP-3 $\alpha$ /CCL20.

inhibited the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in human airway epithelial cell Calu-3 and colon adenocarcinoma cell T84. Because CFTR is a cAMP-regulated chloride channel, decreased CFTR might result in impaired anion transport. It is likely that loss of CFTR also leads to an increase in cellular Ca<sup>2+</sup> and activation of NF- $\kappa$ B signaling (197). Smokers have altered nasal transepithelial potential defenses, which is suggestive of decreased CFTR function (32).

#### D. Small Airway Remodeling

The progressive increase in connective tissue in the smoker's bronchioles has been documented in numerous studies (1, 122, 208, 233). The group led by Hogg et al. (120) recently provided the most compelling evidence for remodeling and fibrous thickening, based on the analysis of lungs obtained by cancer resections or lung volume reduction surgery (120). With increased severity of COPD based on GOLD criteria (244), small airways have increased thickness, and heightened inflammatory infiltrate, including infiltration by neutrophils, macrophages, T lymphocytes (CD4+ and CD8+ T cells), and B lymphocytes. Lymphoid follicles accumulated within the walls of the affected bronchioles. The lumen of these airways was also obliterated more often by mucus.

The interaction between inflammation and small airway remodeling has been mechanistically addressed in rodent models. Overexpression of the Th2 cytokine IL-10 caused mucus cell metaplasia, B- and T-cell-rich inflammation, and subepithelial fibrosis of airways (182). Interestingly, these responses were mediated by multiple mechanisms. Mucus metaplasia was dependent on IL-13/IL-4 receptor- $\alpha$ /STAT6 signaling, while inflammation and fibrosis were independent of these signaling pathways.

Furthermore, overexpression of IL-1 $\beta$  induced peribronchial fibrosis (175). In rat trachea organ culture, cigarette smoke released active TGF- $\beta$ 1 and induced nuclear localization of phospho-smad2 in epithelial and interstitial cells, subsequently leading to upregulation of the TGF- $\beta$ -dependent procollagen gene (343).

Fibroblast growth factor (FGF) and FGF receptor (FGFR) signaling seems to be associated with airway and vascular remodeling in chronic bronchitis. Immunohistochemical studies of lung tissues from COPD patients showed that FGF-1 and its receptor FGFR-1 are detected in vascular and airway smooth muscle as well as airway epithelial cells. Basic FGF/FGF-2 was localized in the cytoplasm of airway epithelium and in nuclei of airway, vascular smooth muscle, and endothelial cells (163). FGF-1 and/or FGF-2 increased steady-state mRNA levels of FGFR-1 and induced cellular proliferation of cultured human airway smooth muscle cells (164). Smokers with chronic bronchitis and airflow limitation had increased expression of FGF-2 in the central airways, which was mainly due to an enhanced expression in the bronchial gland compartment, suggesting that FGF-2 may have a role in promoting mucus hypersecretion in smokers (98).

Based on the assessment of bronchial biopsies of patients with airflow limitation, small airway remodeling and obstruction may be caused by submucosal hypercellularity of endothelial cells in response to overexpressed VEGF (30, 164). Enhanced vascularity of the inner region of medium-sized airways might contribute to airflow limitation in asthma as well (106). The molecular mechanisms accounting for the effects of VEGF-regulated vascular permeability may include Src-mediated signaling and an increase in both nitric oxide and prostacyclin. VEGF-overexpressing transgenic mice showed enhanced angiogenic responses in airways associated with inflam-

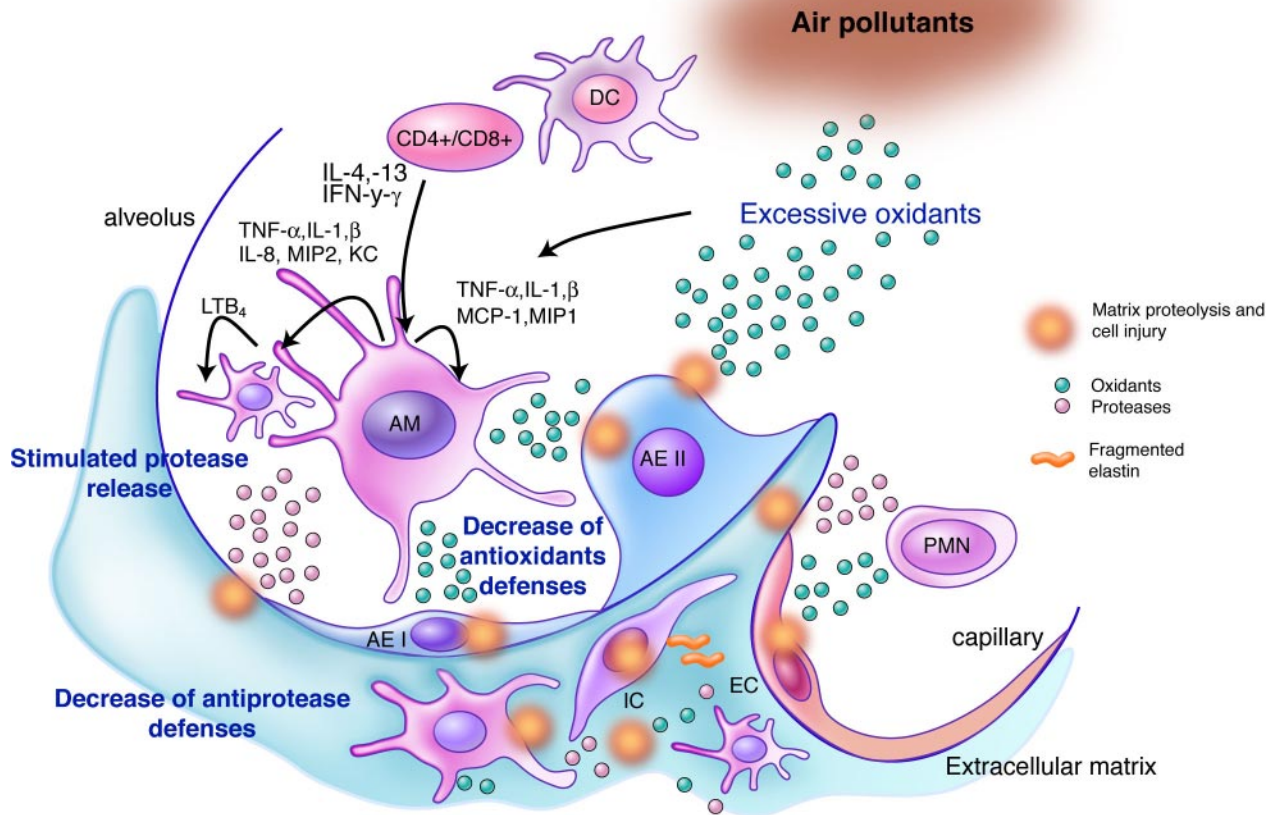


FIG. 2. Inflammation, extracellular matrix proteolysis, and oxidative stress: the classical hypothesis. Air pollutants, including cigarette smoke, are a rich source of oxidants (i.e.,  $O_2^-$ , NO), leading to the recruitment of macrophages and neutrophils. These inflammatory cells might be the source of cytokines (i.e., TNF- $1\alpha$ , IL- $1\beta$ , IL-8, MCP-1, MIP-1), oxidants (i.e.,  $O_2^-$ , NO, HOCl), and proteases (i.e., MMPs and neutrophil elastase). By stimulating dendritic cells, CD4+ and CD8+ T cells (Tc1/Th1-dominant) also release chemokines (i.e., IFN- $\gamma$ , IL-4, IL-13) and perforins. These stimuli result in alveolar wall cell breakdown and matrix proteolysis. Further inflammation might be triggered by elastin fragments. Cell injury decreases antioxidant and antiprotease defense so as to favor protease and oxidant-mediated alveolar wall destruction. AEI and AEII, alveolar type I and II epithelial cells; EC, endothelial cells; IC, interstitial cells; AM, alveolar macrophages; PMN, neutrophils; DC, dendritic cells; CD4+/CD8+, CD4+/CD8+ T cells.

mation, and mucus accumulation, dependent to some extent on enhanced production of nitric oxide (18).

#### IV. EMPHYSEMA

Emphysema has been defined pathologically as an irreversible destruction of alveolar structures with airspace enlargement, not associated with significant amount of fibrosis (297). The central elements of this definition are the concepts of destruction and irreversibility of the pathological process. The statement regarding the lack of fibrosis was warranted by the common finding of pericatricial emphysema around areas of interstitial scarring. The following discussion highlights that several destructive mechanisms do not act in isolation, but rather converge and ultimately account for the destruction of alveolar structures. The term *destruction* implies that

alveolar cells die, either by necrosis or apoptosis, the two prototypic terminal modes of cell death. But how do alveolar cells ultimately die in emphysema? Although alveolar lung tissue is exposed to similar environmental toxins facing the proximal airways, the responses of these two anatomic compartments are clearly distinct. Airways respond with cell proliferation, while alveolated lung tissue disappears. Three critically important pathophysiological processes interact in the periphery of the lung in COPD: oxidative stress, alveolar cell apoptosis, and extracellular matrix proteolysis. Inflammatory cells also actively participate in the pathogenesis of alveolar destruction. Furthermore, novel concepts pertaining to senescence and aging are currently of growing interest in this field. Current genetically altered mice prone to and resistant to pulmonary emphysema are summarized in Tables 1 and 2.



## A. Inflammation

Lung inflammation continues to dominate the present thinking of the pathophysiology of COPD, including emphysema (Fig. 2). Cigarette smoke inhalation incites acute and chronic inflammatory responses, which potentially cause alveolar destruction. Lung retention of activated neutrophils has been documented after inhalation of cigarette smoke (200). Short exposures to cigarette smoke were associated with increased desmosine detection in BAL, indicative of elastin fiber breakdown in mouse lungs (50). Chronic cigarette smoke exposure was associated with an increase in inflammatory cells in the BAL and lung tissue of humans and experimental models (324). Most importantly, an increase in CD8<sup>+</sup> T cells has been linked to worsened COPD (76), an effect that potentially overshadows the potential role for neutrophils. However, neutrophils and eosinophils predominate with progression of COPD, particularly during exacerbations, and may participate in the acceleration of tissue destruction or airway disease (243). The enhanced infiltration of inflammatory cells in COPD has been attributed to either cigarette smoke/pollutants or to oxidative stress as the result of the exposure to environmental hazards, causing enhanced recruitment of inflammatory cells. However, potential ground-breaking concepts were recently introduced that suggest that the inflammatory cells could be a result of an autoimmune attack to the lung tissue (2), or potentially linked to an associated lung aging process (325).

## B. Inflammatory Cells

The smoker's lung has increased numbers of neutrophils, lymphocytes, and macrophages (352). The specific pathophysiological roles of each of these inflammatory cells in emphysema remain to be determined. Neutrophils could be a source of oxidants and elastases, while macrophages produce oxidants and potentially destructive extracellular matrix proteases. On the other hand, some lung injuries require neutrophils for proper repair (114). Whether inflammatory cells play a similar role (i.e., protective or destructive) early versus late in the disease also remains unknown. The finding that ex-smokers have persistent inflammation despite discontinuation of smoking argues for a proinflammatory environment set by cigarette smoke-induced chronic lung damage (176). Recent ground-breaking studies indicate that parenchymal inflammation in advanced emphysema contains oligoclonal CD4<sup>+</sup> and CD8<sup>+</sup> T cells, perhaps the first indication in support of a potential autoimmune attack (161, 305). A potential target to infiltrating lymphocytes is the adenovirus latent infection antigen, which can amplify inflammatory responses to cigarette smoke in the guinea pig

model (212), and whose expression was enhanced in advanced emphysematous lungs compared with milder forms of emphysema (264).

## C. Cytokines and Chemokines

The cytokines, their receptors, and functions involved in COPD are listed in Table 1. In exhaled breath condensate, healthy smokers had increased levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70, and TNF- $\alpha$  when compared with nonsmokers, and all cytokines were increased on COPD (GOLD stages III and IV) exacerbations when compared with patients with stable COPD (92). Several rodent models exhibit increased cytokine profiles after acute and repeated (chronic) cigarette smoke. Guinea pigs had increased transcript levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-8, and MCP-1 and decreased IL-5, granulocyte-macrophage colony stimulating factor (GM-CSF), TGF- $\alpha$ , and eotaxin mRNA after a single exposure and/or 4 wk of cigarette smoke. These changes were associated with increases of mononuclear cells and neutrophils in the BAL (166). ICR and C57BL/6 mice had increased TNF- $\alpha$ , IL-1 $\beta$ , IL-5, IL-13, GRO $\alpha$ /KC, IL-17, RANTES, MCP-1/JE, and thymus and activation-regulated chemokine (TARC) expression in BAL, along with an increase of neutrophils, but not macrophages when exposed to 7 consecutive days of cigarette smoke. These alterations were prominent after 2-h exposure and partly recovered after 12 h (237). After 6-mo exposure to cigarette smoke, AKR mice increased TNF- $\alpha$ , IL-12 (p35 and p40 subunits), IL-10, MIP-1 $\beta$  and -1 $\alpha$  mRNA expression, indicative of Th1-adaptive response, while C57BL/6 mice only had an increase in IL-10 (99). These AKR mice had a marked inflammatory infiltrate comprised of CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$  T cells, neutrophils, and macrophages in the lung. Despite these studies, the interpretation of cytokine changes in rodents is complicated by the discrepant response among different strains to the same stimulus (i.e., cigarette smoke) and a significant variability of responses by individual mice of the same strain (99). Recently, Bracke et al. (27) demonstrated that mice lacking CCR6, a receptor of MIP-3 $\alpha$ /CCL20, have attenuated cigarette smoke-induced emphysema, accompanied by a decrease of dendritic cells, CD8<sup>+</sup> T cells, and granulocytes in the lung (27). These knockout mice showed lower levels of TNF- $\alpha$  and no increase of MCP-1 in BAL, suggesting that MCP-1-CCR2 interaction was affected by CCR6 deficiency (27).

TNF- $\alpha$  has been a leading cytokine linked to cigarette smoke-induced emphysema because of extensive documentation of increased levels of TNF- $\alpha$  in smoker's serum and sputum samples (150). TNF- $\alpha$  signals through the TNF- $\alpha$  receptor type 2 (p55, CD120b) and the type 1 receptor (p75, CD120a). Transgenic overexpression of TNF- $\alpha$  led to emphysema and exaggerated alveolar in-

flammation (82), while TNF- $\alpha$  receptor knockout mice showed significant protection against cigarette smoke-induced emphysema (51). Enhanced TNF- $\alpha$  can explain partly several of the specific airway and airspace pathologies seen in COPD. With relevance to emphysema pathogenesis, TNF- $\alpha$  stimulated MMP synthesis by alveolar macrophages (49). In cultured macrophages, aqueous CSE induced TNF- $\alpha$  via ERK1/2 in differentiated U937 cells (66). Furthermore, circulating TNF- $\alpha$ , soluble receptor p55, and p75 were significantly increased in COPD patients compared with healthy controls (308). The link between TNF- $\alpha$  and alveolar cell apoptosis remains unexplored. The TNF- $\alpha$  receptor p55, rather than the p75, is critical to the development of cigarette smoke-induced emphysema (58). Lack of TNF- $\alpha$  receptor p55 protected against cigarette smoke-induced emphysema, along with decreases of neutrophils, macrophages, and CD4+ and CD8+ T cells. Induction of alveolar wall apoptosis (predominantly involving type II epithelial cells) appeared to be dependent on the activation of both receptors. IL-1 $\beta$  also plays a role in the development of emphysema because lung-specific induction of human IL-1 $\beta$  caused emphysema, accompanied by expression of Gro $\alpha$ /KC and MIP-1 (175). Inhibition with anti-IL-1 $\beta$  antibody attenuated alveolar macrophage influx in BAL after 7 days of cigarette smoke exposure (38). Furthermore, the double IL-1 receptor and TNF- $\alpha$  receptor knockout mice were protected against elastase-induced emphysema (193). These double transgenic mice showed protection against inflammation and alveolar cell apoptosis caused by elastase instillation. IL-6 knockout mice showed reduced cell proliferation in terminal bronchiolar epithelium and proximal alveolar regions, and maintained Clara cell secretory protein expression after exposure to cigarette smoke and/or ozone (366). However, a role for IL-6 in human emphysema has not been studied in detail.

In airways of COPD patients, T lymphocytes preferentially expressed IFN- $\gamma$  and the chemokine receptor CXCR3 (242). Lymphocytes obtained from advanced emphysematous lungs (from lung volume reduction surgeries) were strongly polarized. These cells secreted high levels of IFN- $\gamma$ , CCR5, and CXCR3 and had increased expression of CXCR3 ligands, MIG and IP10 (97). These findings support a strong polarization of alveolar lymphocytes towards a Th1 phenotype in advanced emphysema. When treated with IP10 or MIG, cultured alveolar macrophages expressed MMP-12 protein, which could also be detected in alveolar macrophages in emphysematous lungs. These studies provided substantial insight into the nature of the parenchymal lymphocytic infiltrate in emphysema, and mechanistically linked the observations of increased chemokines in emphysema and activation of MMP-12.

The evidence of the role of cytokines/chemokines in the pathogenesis of emphysema was better delineated

through the work of Elias and collaborators; these investigators showed that inducible and lung specific overexpression of the prototypic Th1 cytokine IFN- $\gamma$  (344) or the Th2 cytokine IL-13 (371) produced emphysema associated with inflammation, variable degree of fibrosis with MMP, and cathepsin expression. In the IL-13-overexpressing mice, MMP-9 and -12 were responsible for the emphysema phenotype, since the combined MMP-9 and -12 knockout mice were partly protected against emphysema due to lung expression of IL-13 (173). Furthermore, inhibition of cathepsins also contributed to the protection against emphysema (370).

#### **D. Extracellular Matrix Proteolysis: Protease/Antiprotease Imbalance**

Destruction of the elastin framework of the lung has been a leading potential mechanism of alveolar destruction in emphysema (Fig. 2). Neutrophil elastase and MMPs are the most studied candidates that account for the protease/antiprotease imbalance in COPD.

The evidence in support of a significant role of neutrophil elastase in the pathogenesis of emphysema has relied on the finding of early emphysema in patients deficient in  $\alpha$ 1-antitrypsin, the major inhibitor of neutrophil elastase (303). Pallid mice, a strain with reduced  $\alpha$ -1 antitrypsin levels, developed emphysema earlier than C57BL/6 mice after cigarette smoke exposure (39, 311). Emphysema caused by lung instillation of porcine pancreatic elastase has been a disease model widely used in support of the protease/antiprotease imbalance over the past 30 years (80, 109, 124, 132–134, 146, 193, 289, 290). Furthermore, neutrophil elastase-null mice were significantly protected against chronic cigarette smoke-induced emphysema, which was associated with a decrease of MMP-12/TIMP-1 ratio (287). The treatment with a synthetic neutrophil elastase inhibitor ZD0892 reduced BAL neutrophils and BAL desmosine and hydroxyproline to levels similar to controls. This elastase inhibitor also reduced levels of MIP-2, MCP-1, and TNF- $\alpha$  and decreased airspace enlargement (351).

There is substantial evidence of increased expression of several MMPs in emphysematous lungs (10, 324). MMPs comprise at least 20 proteolytic enzymes that play an essential role in tissue remodeling and repair associated with development and inflammation, by degrading collagen, laminin, and elastin. Depending on substrate specificity, amino acid similarity, and identifiable sequence modules, the MMP family can be classified into distinct subclasses as collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), membrane-type MMP (MMP-14 to MMP-25), matrilysin (MMP-7), and macrophage metalloelastase (MMP-12) (169). The major physiological inhibitors of the MMPs *in vivo* are  $\alpha$ -2

macroglobulin and the TIMP family, which are naturally occurring proteins specifically inhibiting these proteases and produced by different cell types. The TIMP family at present comprises four structurally related members, TIMP-1, -2, and -3, and the recently discovered TIMP-4.

Because it degrades elastin and is predominantly produced by alveolar macrophages, MMP-12 activation has been a leading candidate proteinase responsible for pulmonary emphysema. MMP-12 protein was observed in the sputum, BAL, bronchial biopsies (65, 218), and peripheral lung tissue of patients with advanced emphysema (33). Patients with COPD caused by cigarette smoke as well as wood smoke from domestic heating and cooking fuels showed higher expression levels of MMP-2, -9, and -12 transcripts in macrophages from BAL than control patients (219). CSE [prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO)] or cytokine mix (TNF- $\alpha$  and IFN- $\gamma$ ) induced MMP-12 mRNA in HBEC (177, 178). MMP-12 expression was associated with H<sub>2</sub>O<sub>2</sub> production and was dependent on NADPH oxidase, p67phox, and p51 (NOXA1) (177). IL-1 $\beta$  induced the expression of MMP-12 mRNA via activation of JNK and PI3K (357). IL-1 $\beta$ -mediated expression of MMP-9 and -12 was demonstrated in IL-1 $\beta$  overexpressing transgenic mice as well (175).

There are abundant data linking MMP-12 and experimental emphysema. MMP-12 immunoreactivity was evident in alveolar macrophages and septal macrophages after cigarette smoke exposure in male C57BL/6 mice (331). Chronic inhalation of cigarette smoke for 1, 3, and 6 mo caused significant increases of MMP-12 mRNA and protein in the lung and mRNA in BAL cells of male C57BL/6 mice. There were no differences in the expression levels of TIMP-1 and -2, indicating an increase of MMP-12/TIMP ratio (26). Shapiro and colleagues (107) demonstrated that MMP-12-null mice are protected from the development of cigarette smoke-induced emphysema. Infiltrating macrophages in wild-type mice exposed to cigarette smoke might have been attracted by elastin fragments released by MMP-12, as MMP-12 knockout mice did not show evidence of increased alveolar macrophage accumulation due to chronic cigarettes smoke inhalation (124). When exposed to cigarette smoke for 2–3 days, MMP-12-null mice had an attenuated number of lung neutrophils and decreased expression of MIP-2, MIP-1 $\alpha$ , and MMP-9 activity in BAL, suggesting that the MMP-9 response is dependent almost entirely on MMP-12 expression (180). MMP-12 is specifically involved in cigarette smoke-induced inflammation, because there was no difference in the number of neutrophils in BAL between knockout and wild-type mice treated with LPS. MMP-12 also acted as a TACE, promoting the release of active TNF- $\alpha$  for subsequent endothelial activation, neutrophil influx, and elastase release in cigarette smoke-induced acute pulmonary injury (50). These acute lung changes

were not dependent on NF- $\kappa$ B activation, since both MMP-12-null mice and wild-type mice showed a rapid increase of NF- $\kappa$ B DNA binding activity.

There has been increasing evidence that cells other than alveolar macrophages are the source of MMP-12. MMP-12 was expressed by dendritic cells sorted from nonlavaged lung, and bone marrow-derived dendritic cells upregulated MMP-12 expression after treatment by aqueous CSE or LPS (26). Although the role of MMP-12-expressing dendritic cells is unknown, the authors speculated that dendritic cells could harm the surrounding tissues during lung recruitment to orchestrate adaptive immunity responses.

Despite evidence of MMP-9 and -2 (which also degrade elastin) expression in COPD lungs, the evidence that either MMP has a central role in emphysema remains unclear. MMP-9 knockout mice were not protected against emphysema (10). On the other hand, experimental emphysema caused by lung overexpression of IL-13 or deletion of surfactant protein D involved both MMP-9 and MMP-12 (173, 348), as surfactant D-deficient mice expressed high levels of MMP-9 and -12 (108, 364). However, surfactant D-deficient mice lacking MMP-9 or -12 developed air space enlargement similar to surfactant D-deficient mice expressing both MMPs (368). Supporting evidence for the involvement of MMP-9 was provided by the finding of increased MMP-9 expression by tissue samples of COPD patients (280).

Collagenases (i.e., MMP-1) might have a significant role in emphysema. MMP-1 expression was increased in COPD lungs when compared with control lungs (128). Furthermore, there is growing support that human MMP-1 participates in the pathogenesis of emphysema (60, 81). Interestingly, despite the suggested lack of significant fibrosis in emphysema (297), advanced emphysematous lung exhibited a complex pattern of collagen deposition, which eventually replaced the elastin framework (77).

The remodeled extracellular matrix may modulate alveolar inflammation. Elastin fragmented by MMP-12 had chemoattractant activity for monocytes via interaction with the elastin-binding protein (124). In addition, the collagen-derived peptide *N*-acetyl Pro-Gly-Pro (PGP) originated from the breakdown of extracellular matrix, attracted neutrophil infiltration in lungs, and caused pulmonary inflammation dependent on activation of the chemokines receptor CXCR2 (347). Repeated intranasal dosing of PGP for 12 wk resulted in alveolar space enlargement of C57BL/6 mice, an effect putatively attributed to neutrophil-mediated lung destruction. Furthermore, PGP levels in the BAL were significantly increased in COPD patients when compared with control subjects. The authors pointed out that the basis for PGP activity lies in its molecular similarity to the GP motif present in all Glu-Leu-Arg (ELR motif)-CXC chemokines such as IL-8 (CXCL8), GRO- $\alpha$ , - $\beta$ , and - $\gamma$  (CXCL1–3) in humans and KC



(CXCL1) and MIP-2 $\alpha$  (CXCL2) in mice. Thus degraded products from extracellular matrix might represent critical factors in the pathogenesis of destructive air space enlargement.

TIMP-3-null mice developed spontaneous alveolar enlargement, which started at 2 wk of age and subsequently progressed with age (181, 203, 204). The mice exhibited reduced collagen and fibronectin, fragmented collagen in the peribronchiolar space, and disorganization of collagen fibrils in the alveolar walls with no changes of MMP-2, -7, -8, -9, and -12 expression, indicating a shift to MMP/TIMP imbalance to favor MMP-mediated destruction of lung extracellular matrix. Cigarette smoke exposure of male BALB/c mice increased MMP-12/TIMP-2 ratio after a 10-day exposure (59). An increase of MMP-9 and a decrease of TIMP-1 expression in emphysematous lung were reported in the senescent mice lacking the aging-protective protein Klotho (86). In humans, flow cytometry-sorted alveolar macrophages of patients with COPD produced lower levels of TIMP-1 than observed from healthy smokers and nonsmokers, therefore favoring a proteolytic environment in patients with alveolar destruction (251). Two polymorphisms of the TIMP-2 gene involving +853 G/A and -418 G/C nucleotide substitutions were identified in COPD patients (116). It is possible that the former is related to the downregulation of TIMP-2 activity, while the latter, located on the consensus sequence for Sp1 binding site, might downregulate the transcriptional activity of TIMP-2.

### E. Disruption of Alveolar Cell and Molecular Maintenance Program: Role of Growth Factors and Bone Marrow

The finding that decreased VEGF or VEGF signaling caused experimental emphysema (149, 312) and the evidence that COPD lungs have decreased expression of VEGF and VEGF receptor-2 (VEGFR-2) led to the concept that alveolar maintenance (Fig. 3) was required for structural preservation of the lung. Cigarette smoke would then disrupt this maintenance program, causing emphysema (324). In fact, several experimental models underscored a potential disruption of alveolar maintenance via genetic manipulations of genes or alterations of the extracellular matrix (323).

Among the lung cells targeted for destruction in emphysema, endothelial cells are likely affected by a decrease in signaling by VEGF and its receptor VEGFR-2. Kasahara et al. (148) suggested that an increase of endothelial cell apoptosis correlated with reduced expression of these molecules in human tissues, while others showed decreased VEGF levels in induced sputum of patients with COPD (143), and BAL from healthy smokers (162). Although the emphysema caused by VEGFR-2 blockade is

a model independent of inflammation, inhibition of VEGF and VEGFR-2 signaling was mediated by oxidative stress in rodents (144, 326). The overall significance of these studies was further expanded by the findings that cigarette smoke decreased the expression of VEGFR co-receptor neuropilin-1 and heparin sulfate proteoglycan glypican-1, an accessory molecule that potentiates VEGF binding to VEGFR-2 (207).

Ito et al. (136) reported that, when compared with younger mice, older animals had decreased expression of VEGF-A, -B, and -C; VEGF-A isoforms 120, 164, and 188; and VEGFR-1, -2, and -3 mRNA. These molecules were then further downregulated by LPS injection (136). These results suggest that pulmonary expression of VEGF types and isoforms and VEGF receptors decline with age, and additional stimuli such as LPS, oxidative stress, and cigarette smoke further decrease their lung expression. The concept is supported by the finding of downregulation of VEGF protein in the emphysematous lungs of senescence-accelerated prone (SAMP)-1 mice, when compared with age-matched senescence-accelerated resistant (SAMR-1) mice. The expression of VEGF in SAMP-1 mice was further downregulated by cigarette smoke exposure (147).

Evidence of the critical role of apoptosis in emphysema caused by VEGFR blockade begs the question whether maintaining cell homeostasis by growth factors such as VEGF could rescue emphysema. VEGF treatment improved short-term survival in prematurely delivered mice with respiratory distress syndrome (RDS), attributed to an increase in surfactant production (54). Hyperoxia-induced bronchopulmonary dysplasia, which is characterized by air space enlargement and loss of lung capillaries, was associated with decreased lung VEGF and VEGFR-2 expression in newborn rats. Postnatal gene delivery of VEGF improved survival, promoted lung capillary formation, and preserved alveolar enlargement (315). However, no data are available on the potential benefits of VEGF gene therapy in emphysematous lung. VEGF is a tightly regulated gene in the lung, playing specific growth and differentiation roles in different structural and cellular compartments. While VEGF protein and VEGF189 mRNA contents in the lung were reduced in severe emphysema, VEGF gene expression was increased in pulmonary arteries of smokers and patients with moderate COPD and correlated with medial thickening of the pulmonary vascular walls (274). In guinea pigs exposed to cigarette smoke, pulmonary artery pressure and medial thickness were increased and associated with heightened gene and protein expression of VEGF, endothelin-1, and endothelial nitric oxide synthase (353). These findings suggest that, while loss of alveolar maintenance by VEGF might contribute to severe lung disease, the early induction of VEGF during cigarette smoke exposure may contribute to pulmonary vascular remodeling characteristically described in the disease (15).

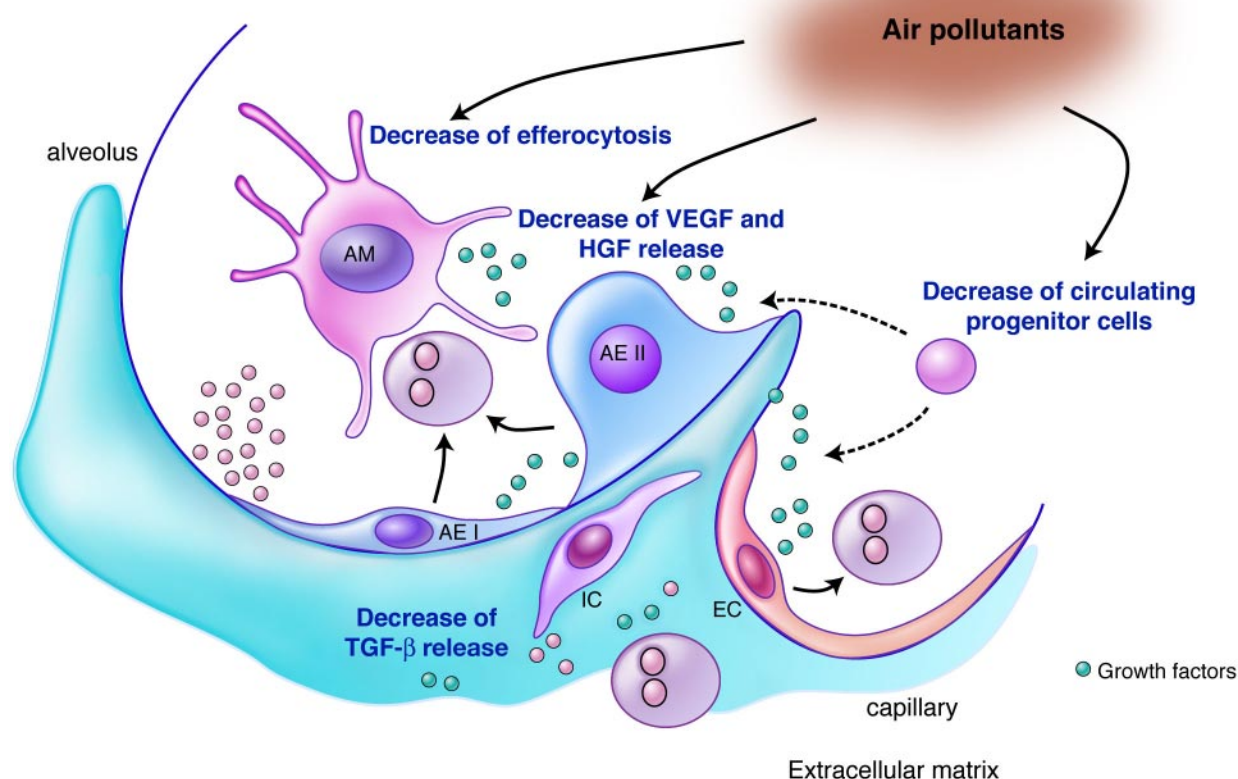


FIG. 3. Breakdown of cellular and molecular maintenance program in emphysema. Air pollutants, including cigarette smoke, might inhibit the production and release of growth and survival factors (most notably VEGF and HGF) from alveolar epithelial cells (type I and II cells), endothelial cells, and interstitial cells (fibroblasts and myofibroblasts), resulting in apoptosis. Impairment of efferocytosis causes decreases of growth factor release from cells involved in apoptotic cell clearance (macrophages as well as epithelial and endothelial cells), and thus might also contribute to persistent inflammation. Decreased circulating progenitor cells might delay replenishment of damaged lung epithelial and endothelial cells. Reduced production and function of TGF- $\beta$  by cells involved in apoptotic cell clearance might delay repair processes and contribute to inflammatory cell activation. Apoptotic and inflammatory cells might be the sources of enzymes involved in extracellular matrix proteolysis.

Primary targeting of type II cells for apoptosis may trigger failure of alveolar maintenance as type II and lung endothelial cells rely on VEGF for growth, survival, and differentiation (336). Tsao et al. (317) proposed a unique mechanism of cell death in alveolar epithelial cells and endothelial cells, because transgenic mice with lung overexpression of placental-like growth factor (PLGF) (a homolog of VEGF, which reacts with VEGFR-1, but not VEGFR-2) showed pulmonary emphysema, starting at 6 mo of age and becoming prominent at 12 mo (317). In accordance with these changes, decreases of numbers of lung endothelial cells and VEGF mRNA expression were observed. In vitro treatment with PLGF promoted death of type II epithelial cells, leading the authors to suggest that overexpressed PLGF induces epithelial cell death via reduction of VEGF expression. A lower availability of VEGF might result in endothelial cell damage and impaired microcirculation, subsequently promoting further epithelial cell death.

Hepatocyte growth factor (HGF), which acts as a potent multifunctional pulmotrophic factor in repairing lung injury and promoting angiogenesis, may represent a promising approach to stimulate lung repair in emphysema (239, 272). Elastase instillation in rat lungs enhanced HGF mRNA and protein expression in plasma and BAL, followed by a significant decline to levels below the baseline. Reduced levels of HGF correlated with progressive emphysematous changes and deterioration in pulmonary physiology. Lung HGF replenishment using a hemagglutinating virus of Japan (HVJ)-mediated transduction in alveolar endothelial and epithelial cells improved pulmonary function by decreasing alveolar cell apoptosis and enhancing alveolar regeneration and angiogenesis (290). HGF likely induced proliferation of bone marrow-derived cells and resident endothelial cells in alveolar walls of mice treated with intratracheal instillation of pancreatic elastase (134). However, a role of HGF in the pathogenesis of COPD is still controversial. A higher

plasma HGF level in COPD patients correlated inversely with the number of bone marrow progenitor cell-derived colonies (241). Intracellular HGF levels in fibroblasts from emphysematous lung were lower than in control fibroblasts both under baseline conditions and after stimulation with IL-1 $\beta$  and PGE<sub>2</sub> (250). The authors pointed out that a decreased secretion of HGF by pulmonary fibroblasts could contribute to the insufficient alveolar repair in pulmonary emphysema. Cohen et al. (52) found that HGF synthesis in human lung fibroblasts was regulated by oncostatin M, a pleiotropic cytokine of the IL-6 family, IL-1 $\beta$ , and MAPK signaling (52).

An elegant demonstration of the interaction among lung structural maintenance, alveolar cell apoptosis, oxidative stress, and protease/antiprotease imbalance was the recently documented emphysema in Toll-like receptor 4 knockout mice. These mice developed age-dependent emphysema due to oxidative stress, generated by enhanced expression of an endothelial cell NADPH-oxidase (NOX-3). The expression of NOX-3 was increased because of loss of Toll-like receptor 4. Furthermore, these mice had increased alveolar cell apoptosis and loss of antielastolytic properties in lung lysates (369).

Inadequate cell repair may contribute to the development of emphysema (262). Aqueous CSE as well as acetaldehyde and acrolein significantly inhibited human airway epithelial cell chemotaxis, proliferation, and contraction of cells plated onto three-dimensional collagen gels, an *in vitro* model of extracellular matrix remodeling (341). This impairment was associated with inhibition of TGF- $\beta$  and fibronectin synthesis. Since TGF- $\beta$  potentially inhibits cellular proliferation and regulates cellular differentiation and extracellular matrix production, reduced activity of TGF- $\beta$  results in destruction of alveolar structure. Latent TGF- $\beta$  binding proteins (LTBPs) modulate the secretion and activation of latent TGF- $\beta$ . LTBP-3- (53) and -4-deficient (302) mice showed air space enlargement at 10 days of age or at birth, respectively. These mice had reduced expression of TGF- $\beta$  and TGF- $\beta$  activation based on decreased expression of phospho-smad 2 and/or 3. Furthermore, LTBP-4-deficient mice also had evidence of activated bone morphogenic protein-4 (BMP-4) signaling, which might have contributed to the lung phenotype (159). Smad3-null mice also developed emphysema at postnatal day 28, associated with increased MMP-9 and -12 expression (22, 41). Transient overexpression of a lung-inducible TGF- $\beta$ 1 transgene caused progressive pulmonary fibrosis with increased levels of extracellular matrix components and matrix proteinases when compared with wild-type mice, while no changes were seen in Smad3-null mice (22). These *in vivo* data suggested that a tightly regulated TGF- $\beta$  signaling is critical in repair processes as well as lung development. Consistent with these findings, a human study showed that the expression of TGF- $\beta$ 1 and TGF- $\beta$  receptor I was significantly lower in

COPD patients (GOLD stage II) than in control patients. The expression of decorin, a key TGF- $\beta$  signaling target, was also lower in COPD stage IV than in control patients (367).

Tissue turnover may be enhanced by apoptosis and activation of cell proliferation by growth factors (Fig. 3). Prosurvival factors VEGF (94) and HGF (223) and the anti-inflammatory factor TGF- $\beta$ 1 (75) were released by macrophages ingesting apoptotic cells, also known as efferocytosis (114, 332). Because macrophages committed to efferocytosis also downregulate the expression of proinflammatory cytokines such as IL-1 $\beta$  and -8 (75), it is conceivable that proper clearance of apoptotic cells might contribute to the maintenance program in the lung. Instillation of apoptotic Jurkat and PLB-985 cells rescued LPS-induced pulmonary injury in a TGF- $\beta$ 1-dependent manner (125). In COPD patients, macrophage efferocytosis of apoptotic 16HBE airway epithelial cells was significantly decreased when compared with control macrophages (119). Cigarette smoke-, acrolein-, and 4HNE-modified extracellular matrix (fibronectin and/or collagen IV) significantly reduced the uptake of apoptotic neutrophils by human monocyte-derived macrophages (154). In addition, IFN- $\gamma$  (a cytokine increased in COPD lungs) suppressed glucocorticoid-mediated augmentation of apoptotic neutrophil clearance (110). Efferocytosis is tightly regulated by ligands (phosphatidylserine, calreticulin) expressed on apoptotic cells and cell surface receptors (phosphatidylserine receptor, CD14, CD31, CD44,  $\alpha$ v $\beta$ 3/5 integrins, surfactant proteins A and D) on cells involved in clearance, such as macrophages, fibroblasts, epithelial, or endothelial cells (114, 332). Because surfactant protein D-deficient mice developed pulmonary emphysema by 3 wk after birth, with progressive air space enlargement in older animals, one alternative explanation for these results is that surfactant protein D is required for apoptosis clearance and lung homeostasis (348). Rho GTPases regulate the ingestion of apoptotic cells. Rac-1 is an activator of efferocytosis, while RhoA inhibited efferocytosis via Rho kinase (114, 224). Of note, simvastatin, a 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitor, blocked the development of cigarette smoke-induced emphysema (183), probably through the inhibition of RhoA (224).

In fact, several developmental models of emphysema may indeed fall within the realm of disruption of structural maintenance of the lung. One of the most dramatic examples of the interaction between alveolar maintenance and inflammation was the description that mice lacking  $\alpha$ v $\beta$ 6, an integrin abundantly expressed in bronchiolar and alveolar epithelial cells, could not activate TGF- $\beta$ , thus losing its anti-inflammatory and antiprotease effects. These mice developed marked emphysema that could be rescued by the forced expression of an active form of  $\alpha$ v $\beta$ 6, or deletion of MMP-12 (226). Models of emphysema based on manipulations targeted at extracel-



lular matrix, surfactant protein, or key cellular receptors involved in alveolar cell function were summarized elsewhere (323). The role of bone marrow-derived cells in lung maintenance is described below.

Cigarette smoke has profound systemic effects on bone marrow and circulating precursor cells. Intrauterine exposure to nicotine during fetal development in mice resulted in a significant decrease in the number of bone marrow hematopoietic progenitor cells and serum level of IL-6 (a regulator of cell proliferation) via nAChR (282). Nicotine may modulate hematopoiesis by affecting the functions of the bone marrow stromal microenvironment because of reduced expression of CD44, required for the interactions between stromal cells and hematopoietic progenitor cells (expression of CD-44 was reduced in both cells) (151). Benzo(a)pyrene reduced the numbers of adherent human endothelial cell progenitor cells (EPCs) and EPC colonies in a dose-dependent manner. The actions of benzo(a)pyrene were probably mediated by aryl hydrocarbon receptor (AhR), which upon binding to polycyclic aromatic hydrocarbons (PAH), heterodimerizes with the AhR nuclear translocator (ARNT or HIF1 $\beta$ ), translocates into the nucleus with subsequent interaction with xenobiotic response element in the promoter of PAH responsive genes, such as the cytochrome *P*-450 CYP1A1 (333).

Michaud et al. (213) isolated circulating EPC from chronic smokers and examined their functional properties. The numbers of EPCs that adhered to cultured umbilical vein endothelial cells in response to TNF- $\alpha$  and induced the formation of vascular tubes were lower in smokers than control subjects. These changes were accompanied by a higher formation of ROS in smoker's EPCs, and lower serum levels of antioxidants and nitrite in smokers. In moderate to severe COPD patients, CD34+ or AC133+ circulating hematopoietic and endothelial cells were reduced in number compared with controls, with a decreased ability to form colonies (241). Exercise capacity and FEV<sub>1</sub> correlated inversely with CD34+ cell counts.

The role of lung homing of circulating progenitor remains unclear. Peinado et al. (245) localized AC133+, CD34+, and/or CD45+ progenitor cells in both endothelial surfaces and the intimal layer of pulmonary arteries in the patients with COPD. These findings were suggestive that circulating cells, which attach to the vascular intima, might contribute to pulmonary vascular remodeling. However, additional experimental data suggested that bone marrow cells might contribute to alveolar maintenance. The suppression of bone marrow cells after sublethal irradiation induced emphysematous changes in lungs of mice instilled with LPS. Furthermore, bone marrow progenitor cells isolated from donor GFP-expressing mice migrated to alveolar walls of recipient mice with emphysema caused by LPS treatment, with evidence of differentiation into (GFP+, CD34+, CD45 negative) donor-

derived endothelial cells and (GFP+, cytokeratin+, CD45 negative) donor-derived epithelial cells (358). A similar bone marrow-derived cell differentiation to lung cells was supported in the elastase-induced emphysema model in mice (134).

Improving the number and function of circulating EPCs might enhance the therapeutic options for COPD. Young healthy smokers who drank green tea (8 g/day) for 2 wk had an amelioration of the numbers of circulating and cultured CD45<sup>low</sup>, CD34+, VEGFR-2+ EPCs as well as endothelial cell function, as measured by flow-mediated endothelium-dependent vasodilation of the brachial artery ultrasound (153). It is interesting that smoking cessation rapidly increased circulating CD45<sup>low</sup>, CD34+, CD133+ progenitor cells and CD45<sup>low</sup>, CD34+, CD133+, VEGFR-2+ EPCs, and their recovery was more evident in light smokers than in heavy smokers (160).

## F. Oxidative Stress

COPD is an abnormal and excessive inflammatory disease of the respiratory system, thought to be caused by oxygen and nitrogen free radical species, such as those generated by exogenous sources, ozone, NO<sub>2</sub>, cigarette smoke, environmental air pollutants, and endogenous sources, particularly of pulmonary inflammatory cells. A compilation of the alterations involving regulators of oxidative stress is shown in Figure 4 and summarized in Table 4. A recent comprehensive review of oxidants and airway diseases has highlighted the link between cellular damage/response (as apoptosis, necrosis, cell proliferation) and transcriptional control of inflammation (such as NF- $\kappa$ B or AP-1 activation) (252). The concept of lung oxidant toxicity relates the formation of reactive and unstable free radicals, superoxide, nitric oxide, peroxy-nitrite, hydroxyl radicals, etc., with subsequent chain reactions resulting in uncontrolled destructive oxidation. Cigarette smoke contains free radicals in both the gas and tar phases. The gas phase has an estimated 10<sup>15</sup> free radicals per puff, including ROS, epoxides, peroxides, nitric oxide, nitrogen dioxide, and peroxy-nitrite, while the tar phase has stable ROS, including organic compounds such as semiquinone and phenol, and other radicals including hydrogen peroxide and hydroxyl ions, with more than 10<sup>18</sup> free radicals per gram (for a comprehensive review, refer to Ref. 253). Indeed, there is solid support for increased lung and systemic oxidative stress based on assessment of markers in peripheral blood cells and BAL of COPD patients. There is recent evidence that the lung tissue of COPD patients also shows evidence of oxidative stress (254). Two broad groups of antioxidant defenses counterbalance the effects of oxidants. The first represents glutathione, vitamin C, urea, and the enzymatic systems such as superoxide dismutase, catalases, and

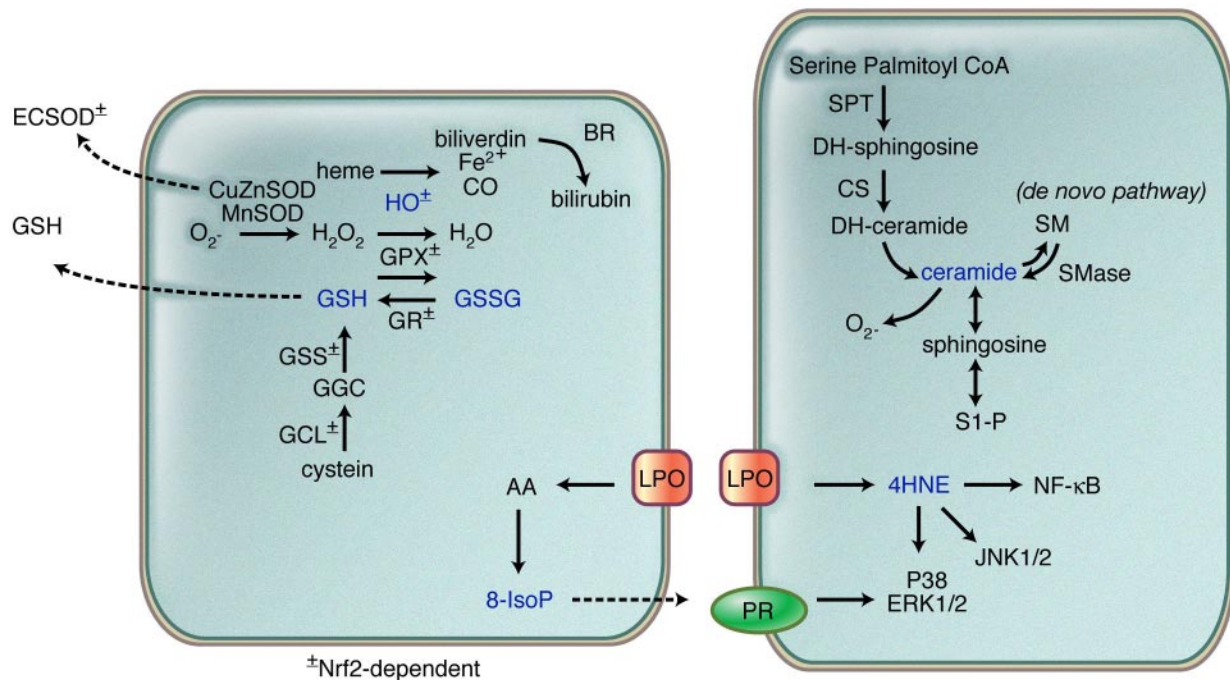


FIG. 4. Oxidative stress responses in chronic obstructive pulmonary disease (COPD). Oxidative stress has a central role in the pathogenesis of COPD as oxidants mediate inflammation, extracellular degradation, and failure of alveolar maintenance. Endogenous signaling molecules and pathways, such as ceramide, may further amplify alveolar destruction despite cigarette smoke cessation. GSH, reduced glutathione; GSSG, oxidized glutathione; GSS, glutathione synthetase; GCL, glutamate cystenyl ligase; GGC,  $\gamma$ -glutamylcysteine; AA, arachidonate; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; 8-IsoP, 8-isoprostane; PR, prostanoid receptor; CuZnSOD, copper-zinc superoxide dismutase; MnSOD, manganese SOD; ECSOD, extracellular SOD; LPO, lipid peroxidation; 4HNE, 4-hydroxynonenal; BR, biliverdin reductase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; ERK1/2, extracellular signal-regulated kinase 1 and 2; JNK1/2, c-Jun NH<sub>2</sub>-terminal kinase 1/2; SPT, serine palmitoyltransferase; CS, ceramide synthetase; DH-, dehydro-; S1-P, sphingosine-1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase.

peroxidases. A second enzymatic group is directly involved in recycling the first level antioxidant defenses. This group includes heme oxygenases and reductases including peroxyredoxins, thioredoxins, and glutaredoxins (256).

Glutathione (GSH), the most abundant non-protein thiol in the BAL, is one of the most important antioxidants against free radicals in its reduced form. Its concentrations in BAL reach  $\sim 500 \mu\text{M}$  in the BAL (253), which would afford protection against oxidative damage at the alveolar epithelial fluid interface. GSH steady-state levels result from the balance of the de novo synthesis by glutamate cystenyl ligase (GCL) (formerly called glutamylcystein synthetase) or recycling derived from reduction of oxidized glutathione by glutathione reductase. Furthermore, GSH overall consumption occurs during the reduction of hydrogen peroxide and lipid peroxides by glutathione peroxidase. The ratio of reduced glutathione (GSH) to oxidized GSH (or GSSG or glutathione disulfide) controls the expression of several critical enzymes involved in free radical detoxification, including superoxide dismutases, glutathione peroxidase, metallothionin, thioredoxin reductase, and GCL (256).

Beeh et al. (17) reported increased levels of total GSH and GSSG in supernatants of processed sputum of patients with moderate and severe COPD, compared with

healthy nonsmokers. These data are supported by Drost et al. (73), who showed that reduced GSH is decreased in BAL obtained from patients with severe exacerbations COPD, compared with healthy nonsmokers. Conversely, reduced GSH was increased in BAL of healthy smokers and stable patients with moderate COPD (73). Collectively, patients having moderate or severe COPD may show an overwhelming oxidative burden from ROS, while healthy smokers and patients having stable COPD may show adaptive responses against oxidative stress, which tend to preserve the ratio of reduced to oxidized GSH. These responses are probably mediated by GCL, the main enzyme regulating GSH synthesis, as the mRNA of GCL heavy chain, the catalytic subunit was increased in the bronchiolar and alveolar epithelial cells of COPD patients with no evidence of infection (255). Another group, however, reported that the immunopositive reaction of GCL heavy chain was diminished in the bronchial epithelial cells and alveolar macrophages of COPD patients (104), suggestive of differential responses of GCL in lung anatomical sites. An important finding was the differential response of GSH with age and history of cigarette smoke exposure (231). Young nonsmokers (22–29 yr old) had higher total GSH in BAL than that in older nonsmokers (37–77 yr old). Furthermore, current smokers of both age

TABLE 4. Increase of antioxidant and detoxification-related gene expression in current microarray data

Samples	Genes	Reference Nos.
Airway epithelial cells, smokers	Glutathione peroxidase 2 and 3, glutathione reductase, glutamate-cysteine ligase (regulatory and catalytic subunits), glutathione-S-transferase A2, NADP <sup>+</sup> mitochondrial isocitrate dehydrogenase 2, alcohol dehydrogenase 7, aldo-keto reductase 1C3 and 1B1, thioredoxin reductase 1	102
Alveolar macrophages, smokers and/or COPD patients	Glutathione reductase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, soluble [nicotinamide adenine dinucleotide phosphate (oxidized form)] isocitrate dehydrogenase 1	112
Lung, SD rats exposed to cigarette smoke for 3 h or 3 wk	Heme oxygenase-1, NAD(P)H:quinine oxidoreductase 1, Nrf2	87
Lung, SKH-1 hairless mice exposed to cigarette smoke for 28 days	Cytochrome P450 CYP1A1, microsomal UDP-glucuronosyl-transferase 1-1 and 1-6 precursors, catalase 1, oxidative stress-induced protein, manganese superoxide dismutase 2 precursor	137
Lung, Nrf2 WT mice exposed to cigarette smoke for 5 h	Heme oxygenase-1; superoxide dismutase 3; glutathione peroxidase 2; glutathione peroxidase 3; glutamate cystenyl ligase (catalytic, regulatory); transcobalamine II; ferritin light chain 1; peroxiredoxin 1; glutathione reductase; thioredoxin reductase 1; glucose-6-phosphate dehydrogenase; phosphogluconate dehydrogenase; glutathione-S-transferases $\alpha 1$ , $\alpha 2$ , $\alpha 3$ , $\theta 1$ , $\pi 2$ , and GT8.7; NAD(P)H:quinone reductase 1; alcohol dehydrogenase 7 (class IV); aldehyde dehydrogenase family 3 subfamily A1; aldo-keto reductase 1 member B8; retinol oxidase/aldehyde oxidase; esterase 10; UDP-glucuronosyl transferase; UDP-glucose dehydrogenase	258
Alveolar macrophages treated with diesel exhaust particles, SD rats	Heme oxygenase-1, thioredoxin reductase 2, glutathione-S-transferase P subunit, NAD(P)H dehydrogenase	158
Swiss 3T3 cell exposed to aqueous cigarette smoke extract	Heme oxygenase-1, methalothionein 1 and 2, heat shock protein 105 and 90, CCAT/enhancer binding protein, JunB, Atf3, Ferritin, Freac-2	24

Nrf2, nuclear factor E2-related factor 2; WT, wild type.

groups showed significantly higher total GSH in BAL than nonsmokers, with GSSG levels significantly increased in older smokers when compared with younger smokers. These findings suggest that the extracellular antioxidant system in the lungs of older individuals might have an impaired ability to resist damage from cigarette smoke, probably through the inactivation of GCL.

Copper-Zinc (Cu-Zn), manganese (Mn), and extracellular (EC) superoxide dismutase (SOD) are three main isoforms of the enzymatic system that catalyzes superoxide to hydrogen peroxide. These enzymes play a central role in the protection against oxidative stress originated from cellular oxygenases and from mitochondrial sources. Cu-Zn SOD is located in airway epithelial cells, alveolar type II cells, and alveolar macrophages. MnSOD is located in mitochondria of airway epithelial cells and alveolar macrophages. ECSOD is expressed ubiquitously in alveolar cells. There is no detailed information about the expression pattern of SODs in the different stages of COPD. However, a single study reported increased expression of MnSOD in airways of patients with COPD (103). There is limited evidence that overexpression of SOD(s) resulted in protection against experimental cigarette smoke-induced emphysema, particularly of Cu-ZnSOD (80). We reported the protective effect of a MnSOD-like small molecule, M40419, in the experimental emphysema model caused by inhibition of VEGFR (326).

Little is known about the role of catalase in COPD. We have recently noted that in the experimental model of emphysema caused by VEGFR blockade, catalase activity was increased as these lungs were undergoing oxidative stress. The expression of catalase was reduced by overexpression of human  $\alpha$ -1 antitrypsin (247). Glutathione peroxidase also metabolizes hydrogen peroxide at the expense of glutathione, playing important roles in free radical scavenging. However, as with catalase, its expression and role in COPD remains undetermined.

Peroxidative degradation of lipids yields the highly reactive aldehyde 4-hydroxy-2-nonenal (4-HNE), a major product of the lipid peroxidation. 4-HNE triggers cell signaling by reacting and forming adducts with sensor proteins and thus initiating or inhibiting cell response pathways (246). Rahman et al. (254) reported enhanced immunohistochemical detection of 4-HNE-modified proteins in airway and alveolar epithelial cells, endothelial cells, and neutrophils in subjects with COPD, when compared with subjects without COPD. Interestingly, these investigators also found a significantly inverse correlation between FEV<sub>1</sub> and the levels of 4-HNE adducts in alveolar epithelium, airway endothelium, and neutrophils. Increased levels of 4-HNE adducts were also observed in bronchiolar epithelial and alveolar epithelial cells, particularly type II cells in male C57BL/6 mice after 1 h of cigarette smoke exposure (5). Exposure of bovine lung



microvascular endothelial cells to 4-HNE decreased endothelial cell permeability via phosphorylation of JNK, ERK, and p38 MAPK, with no evidence of apoptosis (330). However, 4-HNE was reported to induce JNK-mediated apoptosis in a variety of other cell lines (43). Physiologically relevant concentrations of 4-HNE inhibited transcriptional activation of NF- $\kappa$ B by preventing I $\kappa$ B $\alpha$  phosphorylation in cultured Kupffer cells treated with *Chlamydia pneumoniae* (70) and in human monocytic lineage cells treated with LPS (194). Because the increase of 4-HNE may represent a useful oxidative stress marker, and is responsible for signal transduction and apoptosis, further studies are needed to clarify its role in the pathophysiology of COPD.

Isoprostanes are a series of novel prostaglandin-like compounds formed in vitro and in humans from the free radical-catalyzed peroxidation of arachidonate, a reaction that occurs independently of cyclooxygenase (227). Isoprostanes are widely recognized as useful markers of membrane lipid peroxidation, with a growing recognition of their powerful effects on a variety of cell functions as well (139). The concentrations of the oxidative stress marker 8-isoprostane in breath condensate in COPD ex-smokers and current smokers were increased ~1.8-fold compared with healthy smokers, who had 2.2-fold higher 8-isoprostane levels than healthy nonsmokers (220). The levels of 8-isoprostane were higher in patients with more severe COPD than in mild-to-moderate cases (157), and during infective exacerbations of COPD, followed by slow recovery after antibiotic treatment (19). As these effects apply particularly to the lung, the role of 8-isoprostane needs to be examined in COPD. Isoprostanes potentially orchestrate cytokine expression, airway constriction, pulmonary vasoconstriction, inflammation, endothelial permeability, bronchial artery vasoconstriction, epithelial permeability, and mucus secretion (139).

Activation of antioxidant and detoxification gene expression was observed in Swiss 3T3 cells treated with aqueous CSE (24), alveolar macrophages from Sprague-Dawley rats treated with diesel exhaust particles (158), smokers and/or patients with COPD (102, 112, 300), cigarette smoke-exposed Sprague-Dawley rats (87), SKH-1 hairless mice (137), and nuclear erythroid-related factor 2 (Nrf2) wild-type mice (258) assessed by gene microarray (Table 4). The master antioxidant transcription factor Nrf2 was activated by diverse oxidants and chemopreventive agents (45). Nrf2 translocates to the nucleus and binds to an antioxidant response element (ARE) located in the DNA promoter region of several antioxidant genes. Through transcriptional induction of ARE-bearing genes that encode antioxidant-detoxifying proteins such as heme oxygenase-1 (HO-1), glutathione reductase, glutathione peroxidase, GCL, etc., Nrf2 activates critical cellular pathways that protect against oxidative injury, inflammation/immunity, apoptosis, and carcinogenesis. Nrf2-

null mice were highly susceptible to cigarette smoke (126, 258), showing more pronounced inflammation and neutrophilic elastase activity in BAL, enhanced alveolar expression of 8-oxo-7,8-dihydro-2'-deoxyguanosine (a marker of oxidative stress), an increased number of apoptotic alveolar septal cells, predominantly endothelial and type II epithelial cells (258), and decreased secretory leukoprotease inhibitor (126), when compared with wild-type littermates. The marked emphysema seen in Nrf2-null mice treated with intratracheal instillation of elastase also supported an important role for Nrf-2 in maintaining the balance between proteases and antiproteases (132), as well as between oxidants and antioxidants.

Because HO-1 catalyzes heme to generate bilirubin, ferritin, and carbon monoxide, it plays a wide cytoprotective role against inflammation and apoptosis (266). The three enzymatic isoforms consist of the inducible HO-1 and the constitutive HO-2 and HO-3. Human studies showed that HO-1 localized in alveolar macrophages, which expressed the enzyme at higher levels than blood monocytes (171). HO-2 had a more widespread distribution in alveolar wall cells, including type II epithelial cells, adventitial fibroblasts (from pulmonary arteries), bronchiolar cells, and vascular smooth muscle cells (201, 202). Expression of HO-1 was increased in healthy smokers and COPD patients (201), while it was decreased in patients with severe COPD (202). On the other hand, the expression of HO-2 was increased in both healthy smokers and COPD patients (201, 202). Experimental studies confirmed that HO-1 was induced by many factors such as cigarette smoke exposure (84), aqueous CSE (156), acrolein (354), crude diesel exhaust particles (DEP), aromatic and polar DEP fractions, and benzo(a)pyrene quinone (187), ultra fine particulate (PM<sub>0.1</sub>) (186), and 4-HNE (127) in a variety of cell lines. Of note, adenovirus-mediated overexpression of rat HO-1 cDNA attenuated porcine pancreatic elastase-induced pulmonary emphysema (293), as well as LPS- (130) and *Pseudomonas aeruginosa*-induced (318) acute lung injury in mice. The protective effect of HO-1 was associated with concomitant expression of IL-10, an anti-inflammatory cytokine (293), and Bcl-2, an antiapoptotic molecule (318). The administration of biliverdin, a product of HO-1 action, also protected against LPS-induced acute lung injury (275).

## G. Alveolar Cell Apoptosis and Proliferation

The present definition of emphysema emphasizes the unique aspect of the destructive nature of alveolar enlargement without fibrosis set up by a board of experts convened by the National Institutes of Health in 1985 (297). However, the precise mechanisms of alveolar destruction postulated to cause alveolar enlargement remain unclear. Cell death occurs by apoptosis (pro-

grammed cell death) or necrosis. Excessive protease/antiprotease imbalance would result in extracellular matrix destruction, indirectly targeting alveolar cells. Therefore, alveolar cell apoptosis would follow extracellular matrix destruction (149).

Studies in the past 5 years have supported that alveolar cell destruction in emphysematous lungs might occur due to apoptosis, possibly unrelated to preceding matrix protease degradation (Fig. 3). This concept was initially proposed by Aoshiba and Nagai in 1999 (6). The first documentation of lung cell apoptosis in emphysema occurred a year later by studies that showed that COPD lungs exhibited an enhanced number of TUNEL-positive cells, predominantly involving endothelial cells when compared with normal or non-COPD smoker's lungs (280). Furthermore, rat lungs treated with a VEGFR blocker had apoptosis-dependent emphysema (149). A similar outcome was observed when the VEGF gene was deleted in the lung by transduction of the CRE recombinase gene under an adenoassociated virus vector (312). The latter experimental studies were supported by the demonstration that lungs with advanced emphysema show increased apoptosis of septal endothelial and alveolar epithelial cells when compared with lungs of normal individuals, non-COPD smokers, and pulmonary hypertensive patients (148). Alveolar septal cell apoptosis was subsequently confirmed in COPD lungs as well, and shown to correlate with a decrease in alveolar surface area (129). Moreover, alveolar cell apoptosis was linked to cigarette smoke-induced emphysema in strains of mice with low lung levels of VEGF (16). Recently, STAT3-deficient mice were shown to develop emphysema with evidence of apoptosis and caspase-3 expression when infected by adenovirus (209).

The proof of concept that apoptosis could initiate alveolar enlargement was provided by intratracheal instillation of active caspase-3, causing acute apoptosis and alveolar enlargement. These alterations were reversible ~1 wk after the initial caspase-3 instillation (7). Of note, apoptotic cells retrieved by BAL were capable of degrading elastin, a finding suggestive of activation of an elastolytic enzyme, possibly a cathepsin. The first evidence of alveolar cell apoptosis in an experimental model of cigarette smoke-induced emphysema was the study in emphysema-prone Nrf-2 knockout mice (258). The requirement for apoptosis in inflammation-induced emphysema and its interaction with lung elastolysis was highlighted by the demonstration that lung cell apoptosis was required for cigarette smoke-induced mouse emphysema and that cathepsin S was mechanistically upstream of lung cell apoptosis and alveolar enlargement (370). Interestingly, blockade of apoptosis with a broad-spectrum caspase inhibitor or by deletion of the caspase 3 gene also resulted in decreased inflammation, suggesting that apoptosis may act proximally to alveolar cell inflammation. Apoptotic

cells may release intracellular proteases, oxidants, and inflammatory mediators and also express several caspases on their cell surface (332).

The exact apoptotic pathways involved in cigarette smoke-induced emphysema have not been elucidated thus far. Cigarette smoke induces oxidative stress, which might induce apoptosis via the intrinsic or mitochondrial pathways. Evidence of increased expression of Bax by immunohistochemistry in COPD lungs suggested that cigarette smoke-induced emphysema activates the intrinsic pathway of apoptosis (129). The evidence of the role of TNF- $\alpha$  in emphysema (50, 82) supports a participation of the extrinsic pathway as well. Caspase-8, an activator caspase that interacts with the death domain of the TNF receptor and CD95 (Fas) receptor, was activated in experimental emphysema caused by lung overexpression of either IFN- $\gamma$  (370) or IL-13 (196). There is contradictory evidence of increased FasL in COPD lungs (308). Kuo et al. (167) proposed two main mechanisms on cigarette smoke-induced apoptosis in rat models. The first one relies on the activation of p38/JNK-Jun-FasL signaling. The second is mediated by p53 stabilization, increased Bax/Bcl-2 ratio, and release of cytochrome *c* (167). Evidence of p38 MAPK, JNK, and p53 signaling were also reported in ferrets exposed to cigarette smoke, partly mediated by MAPK phosphatase-1 (MKP-1) (189).

The net result of alveolar cell death on alveolar structure depends on the lung's ability to undergo cell proliferation, which is pivotal for the maintenance of normal tissue homeostasis. The data on the balance of apoptosis versus cell proliferation have been discrepant. Patients with emphysema had higher rates of both apoptosis and cell proliferation than healthy smokers and nonsmokers (363). No correlation between apoptosis and cell proliferation was observed (129). On the other hand, Calabrese et al. (31) reported an increase of apoptosis, but not in cell proliferation, in emphysematous lung (31), indicating a higher apoptosis/cell proliferation ratio in patients with emphysema. Because efferocytic macrophages release growth factors as described above, efferocytosis might play a role in controlling the balance of apoptosis and cell proliferation.

## H. Aging and Senescence

Aging is an independent risk factor for cigarette smoke-induced emphysema. At any given smoking history, age plays a critical role in determining an individual's risk for COPD. However, there is little insight into the role of aging in the pathogenesis of COPD. There are several theories underlying aging, including whether aging is genetically determined (i.e., there are genes and gene products that drive the process of aging) or it results from a stochastic interaction between the environment

and the host. It is apparent that natural selection of the gene pool favoring older individuals may ease after the years dedicated to procreation, as aging individuals would not be required for survival of the species. The leading hypothesis of disposable soma predicates that aging occurs due to pay off as consequence of the energy dispensed to survive and cope with environmental hazards before and during procreation. This biological investment would translate in the accumulation of structural and molecular damages over the life of an individual, particularly later in life (155). This damage would eventually lead to organ inflammation, secondary to structural damage accumulated over several years. A similar parallel may underlie the lung damage due to cigarette smoking and emphysema (325).

As the lung ages, there is a progressive air space enlargement, which has been considered as the result of a nondestructive process (versus the destruction that is characteristic of centrilobular emphysema). The air space enlargement parallels a progressive decrease in airflow parameters, which during the normal life span do not cause significant respiratory impairment (140). The non-destructive nature of aging emphysema has been questioned by the finding of elastin fiber fragmentation and loss of elasticity in aged lungs. Lysyl oxidase and the lysyl oxidase-like protein play a critical role in the formation and repair of the extracellular matrix by oxidizing lysine residues in elastin and collagen (142). Lysyl oxidase-like protein 1-deficient mice did not deposit normal elastic fiber and showed early onset of emphysema (190). This protein specifically interacted with the elastic fiber binding protein fibullin-5. Fibullin-5-deficient mice also developed similar early emphysematous lesions (360). Among the fibullin family members, loss of fibullin-4 also has a causal role in the development of emphysema (210).

There are several recent studies demonstrating the presence of molecular signatures of aging in the emphysematous lungs and in COPD patients. Peripheral blood mononuclear cells of patients with COPD have decreased telomere length, a hallmark of senescing cells *in vitro*, when compared with normal individuals (225). This observation has been extended to lung cells, as alveolar epithelial and endothelial cells of emphysema patients exhibited enhanced expression of markers of cell senescence including p16<sup>Ink4a</sup> and p21<sup>CIP1/WAF1/Sd1</sup> and telomere shortening when compared with smokers without emphysema and normal individuals (320). TGF- $\beta$ 1 played a role in the upregulation of p21<sup>CIP1/WAF1/Sd1</sup> in the alveolar type II-like epithelial cell line A549 (205). Lung fibroblasts from emphysema patients showed a senescence marker, senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal), along with upregulation of senescence-associated insulin-like growth factor-binding protein-3 (IGFBP-3) and IGFBP-related protein-1 (IGFBP-rP-1) (229). Senescing cells had increased production of cytokines/chemo-

kines and enhanced matrix protease activity, which can stimulate active tissue destruction (325).

Oxidative stress resulting from mitochondrial free radical generation (the Hartman hypothesis) is the leading pathophysiological hypothesis of aging (13). Indeed, aging tissues and cells had increased expression of markers indicative of oxidative stress. As mentioned previously, cigarette smoke is a rich source of oxidants, and oxidative stress plays a central role in the lung destruction of emphysema. It is apparent that aqueous CSE caused cellular senescence in the human alveolar type II-like epithelial cell line A549, and this change was significantly inhibited by the presence of an antioxidant, *N*-acetyl-cysteine (319). Increased ceramide levels in the smoker's lung may also contribute to aging as ceramide promoted senescence in cultured cells (236). With age, neutrophils and macrophages reduce superoxide production, accompanied by impairment of chemotaxis, cytokine production, and signal transduction (298).

Animal models prone to premature aging have been used to study the link between aging and cigarette smoke-induced emphysema. Senescence accelerated mice (SAM) developed premature aging, including enhanced and global pattern air space enlargement (313). When exposed to cigarette smoke, 6-mo-old SAM mice developed a slight enhancement of alveolar enlargement than those exposed to room air (313). The *klotho* protein is an  $\alpha$ -glycosidase linked to protection against human and rodent aging (8, 168). Although the precise anti-aging functions of the *klotho* protein have not been clarified, it apparently protects the vascular endothelium and thus the vasculature against diseases characterized by endothelial cell dysfunction such as arteriosclerosis (271). *Klotho*-deficient mice developed premature air space enlargement, starting soon after birth (304). FGF23-null mice had a short life span and showed numerous biochemical and morphological features consistent with premature aging-like phenotypes (259). Pulmonary emphysema appeared as early as 3 wk of age with extensive calcifications. There is a lower expression of *klotho* mRNA in FGF23-null mice compared with wild-type mice, implying that part of the premature aging process may be regulated by a common humoral signaling pathway in both strains of mice.

The interaction between cigarette smoke and aging has been recently addressed more thoroughly. The senescence marker protein-30 (SMP-30) was identified based on a differential screen of protein with decreased expression in aging rat livers (83). The functions of SMP-30 were more clearly defined with studies using knockout mice, which developed aging-related changes in the lung, including alveolar enlargement that resembled emphysema (131, 222). The process of senile emphysema in SMP-30 knockout lungs was associated with increased destructive index (i.e., counts of focal ruptures of alveolar septa) and tissue markers of oxidative stress (276). When chron-



ically exposed to cigarette smoke, SMP-30 knockout mice developed marked alveolar enlargement, associated with increased expression of oxidative stress markers and alveolar cell apoptosis (276). This synergistic effect between cigarette smoke and SMP-30 deficiency might be explained by the interaction of shared pathophysiological events, leading to enhanced alveolar destruction. Alternatively, SMP-30 might act itself as a protective mechanism against injury promoted by cigarette smoke inhalation (322).

## V. MEDIATORS AND SIGNALING PATHWAYS LINKING INFLAMMATION, PROTEASE/ANTIPROTEASES, OXIDATIVE STRESS, AND APOPTOSIS

### A. Ceramide

Ceramide is a naturally occurring membrane sphingolipid that functions as a critical second messenger molecule in multiple functional organelle domains within cells. Ceramide has been more specifically linked to transducing stress-related signals and mediating apoptotic cell death (100). The investigation of the role of ceramide and sphingolipids in lung disease has been addressed more recently. The lung level of ceramide, especially the long-chain form, was markedly higher in individuals with emphysema when compared with control patients (248). Ceramide synthesis is controlled by two well-known pathways, the sphingomyelin-dependent pathway involving sphingomyelinase cleavage of ceramide from membrane or intracellular sphingomyelin, and the *de novo* pathway mediated by serine palmitoyl CoA transferase and ceramide synthetase (301). There are three isoforms of sphingomyelinases, one that is active at neutral pH in the endoplasmic reticulum and inner leaflet of the plasma membrane, an acid sphingomyelinase in lysosomal compartments, and a secreted variant of the acid sphingomyelinase with a potential for extracellular enzymatic activity. VEGFR blockade was associated with an increase in lung levels of ceramide, which was mediated by the *de novo* synthesis pathway. Pharmacological inhibition of this pathway by targeting serine palmitoyl CoA transferase with myriocin and ceramide synthase with fumonisins-B attenuated emphysema in rodents reduced oxidative stress and alveolar cell apoptosis (248). Early activation of the *de novo* pathway was followed by subsequent activation of acid sphingomyelinase, potentially leading to further amplification of ceramide synthesis and alveolar destruction. This hypothesis was supported by the beneficial role of intratracheal administration of a ceramide neutralizing antibody against alveolar cell apoptosis caused by VEGFR inhibition. Intratracheal instillation of short-chain ceramide into the lung activated acid

sphingomyelinase, causing accumulation of very-long-chain ceramide and amplification of alveolar cell apoptosis. This feed-forward mechanism of ceramide synthesis may increase the extracellular pool of ceramide and promote lung injuries together with oxidative stress and proteolysis. Because sphingosine 1-phosphate (S1-P) generated from sphingosine promotes cell proliferation, the net imbalance between ceramide (prodeath) and S1-P (prosurvival) may influence critical events involved in emphysema. It is apparent that endogenous mediators of oxidative stress and apoptosis such as ceramide may act as amplification hubs controlling cell signaling and generation of mediators of cell death and alveolar destruction, independently of the presence of the causative agent of emphysema, i.e., cigarette smoke.

### B. Nuclear Factor- $\kappa$ B

Several studies focused on NF- $\kappa$ B as a central inflammatory hub that controls inflammatory processes in COPD. However, NF- $\kappa$ B activation plays disparate roles, some with clear beneficial outcomes as far as lung destruction is concerned. NF- $\kappa$ B is not only a central transcription factor in inflammatory cell networks, but it also stimulates cell survival, by means of enhanced expression of prosurvival Bcl-2 family members and cell proliferation-related genes (c-myc, cyclin D1).

Signaling pathways involved in apoptosis, oxidative stress, and inflammation appear to intersect with NF- $\kappa$ B signaling. There are two distinct NF- $\kappa$ B activation pathways. The classical pathway involves inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (IKK)-I $\kappa$ B-p50/pREL-A (p65), and the alternative pathway involves NF- $\kappa$ B-inducing kinase (NIK)-IKK-p100/p65-p52/REL-B (21). Briefly, the classic pathway is triggered by pathogens and proinflammatory cytokines, leading to activation of the IKK complex. IKK is composed of two catalytic subunits, IKK- $\alpha$  (IKK1) and IKK- $\beta$  (IKK2), and a regulatory subunit, IKK- $\gamma$  (NEMO). The IKK complex, especially IKK- $\beta$ , phosphorylates I $\kappa$ B(s) bound to REL-A (p65)/p50, targeting I $\kappa$ B for proteasomal degradation. The release from the inhibitory I $\kappa$ B renders NF- $\kappa$ B dimers free to translocate to the nucleus and activate gene transcription. The alternative pathway in response to certain members of the TNF family involves the upstream kinase NIK, which activates IKK- $\alpha$  homodimers, leading to the phosphorylation and processing of p100, which is degraded to p52 by the proteasome.

Szulkaowski et al. (307) documented I $\kappa$ B $\alpha$  proteasomal degradation and NF- $\kappa$ B DNA binding in the human lung. I $\kappa$ B $\alpha$  levels were significantly decreased while NF- $\kappa$ B DNA binding was significantly increased in healthy smokers and current smokers with moderate COPD, compared with healthy nonsmokers (307). The degree of NF- $\kappa$ B DNA binding was similar in ex-smokers with

COPD and in healthy smokers. This study suggested that ongoing cigarette smoking may increase NF- $\kappa$ B DNA binding in people with or without COPD. NF- $\kappa$ B was likely expressed in HBEC, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and alveolar macrophages, but not in neutrophils (34, 69). However, another study showed that macrophage expression of NF- $\kappa$ B as well as AP-1 was decreased in BAL samples of smokers and patients with severe exacerbations of COPD, when compared with nonsmokers (73). In support of activation of NF- $\kappa$ B in COPD, NF- $\kappa$ B was clearly responsible for enhancing the levels of IL-1 and TNF- $\alpha$ , via depletion of GSH and generation of ROS in the human macrophage-like cell line MonoMac6 stimulated by aqueous CSE (361).

The mechanism of NF- $\kappa$ B activation due to cigarette smoke is controversial. CSE-induced IL-8 was mediated by IKK and NF- $\kappa$ B signaling in MonoMac6 cell (361). Cigarette smoke induced NF- $\kappa$ B DNA binding in the lung of mice *in vivo* (334), but the authors failed to observe phosphorylation and degradation of I $\kappa$ B. Induction of IL-1 $\beta$  caused by CSE was dependent on ERK1/2 and NF- $\kappa$ B in the human alveolar type II-like epithelial cell line A549 and HBEC. Surprisingly, phosphorylated I $\kappa$ B levels were decreased in a dose-dependent manner in spite of sustained activation of NF- $\kappa$ B (113). Exposure of male Sprague-Dawley rats to cigarette smoke for 3 days and 8 wk increased NF- $\kappa$ B DNA binding in both time points, but these changes were not associated with I $\kappa$ B degradation (206). Therefore, some researchers suggested an I $\kappa$ B-independent NF- $\kappa$ B activation after cigarette smoke treatment. Gebel et al. (88) observed a decreased DNA binding of NF- $\kappa$ B during the first 2 h of treatment with aqueous CSE, which was followed by a more than twofold increase over controls after 4 and 6 h (88). This kinetic change was not regulated by I $\kappa$ B $\alpha$ , as evidenced by a lack of phosphorylation and degradation of I $\kappa$ B $\alpha$ . They proposed a mechanism based on thioredoxin regulation in that immediate loss of thioredoxin, an analog to GSH, together with an oxidized status of Cys-62 of NF- $\kappa$ B, results in decreased NF- $\kappa$ B DNA binding rates, whereas the reappearance of reduced thioredoxin mediated by thioredoxin reductase with the availability of NADPH would reduce the oxidized form of NF- $\kappa$ B and increase DNA binding.

Anto et al. (4) considered that the response of NF- $\kappa$ B DNA binding and I $\kappa$ B degradation were dependent on the nature of CSE. They showed a similar pathway to TNF- $\alpha$ -mediated activation of NF- $\kappa$ B, with an early DNA binding mediated by TRAF-2, NIK IKK, and I $\kappa$ B $\alpha$  after 15 min of treatment using DMSO-dissolved CSE (with lipophilic components of cigarette smoke) when compared with aqueous CSE (smoke-bubbled into PBS). It should be pointed out that the kinetics of I $\kappa$ B $\alpha$  degradation by CSE (dissolved in DMSO)-treated cells is not completely similar to TNF- $\alpha$ -regulated pattern; TNF- $\alpha$  causes degrada-

tion of I $\kappa$ B $\alpha$  after 5 min of treatment followed by recovery at 60 min, while CSE (dissolved in DMSO) begins to degrade I $\kappa$ B $\alpha$  after 15 min and is complete at 60 min, with weak recovery at 120 min. Because cigarette smoke contains thousands of ROS and potent organic oxidants, the kinetics of NF- $\kappa$ B activation may be affected by the nature and preparation method of CSE. Activation of NF- $\kappa$ B without degradation of I $\kappa$ B was also reported in PM<sub>10</sub>-treated alveolar epithelial cell A549 (141).

### C. Histone Acetyltransferases and Histone Deacetylases

Efficiency of gene activation by transcription factors depends on DNA remodeling by the nuclear histone acetylation/deacetylation balance. This balance results from the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (252). DNA supercoiling around a nucleosome core comprising the histones H2A, H2B, H3, and H4 suppresses the accessibility of NF- $\kappa$ B, AP-1, and other transcription factors to their cognate DNA sequences. Acetylation of lysine residues in the NH<sub>2</sub>-terminal tails of the core histones results in uncoiling of the DNA, allowing increased DNA accessibility to transcription factors and RNA polymerase II. Lung tissues from patients with COPD had lower HDAC activity, HDAC(s) 2, 5, and 8 mRNA levels, and expression of HDAC2 protein in a disease severity-dependent manner, while histone-4 acetylation at the IL-8 promoter and IL-8 mRNA were increased in these patients (135). Aqueous CSE reduced HDAC activity and HDAC proteins in MonoMac6 cell and human type II alveolar epithelial cell A549 cell (221, 361). In rats exposed to cigarette smoke, HDAC2 activity and protein expression significantly decreased at 3 days of treatment, while histone-3 phospho-acetylation (acetylated histone 3 was phosphorylated at serine-10) and histone-4 acetylation increased at 8 wk of treatment (206). These alterations were associated with nitrotyrosine, 4-HNE, and aldehyde-modified HDAC proteins, leading to altered deacetylase activity.

Therefore, a higher degree of histone acetylation caused by inhibition of HDACs may be a pivotal event in the activation of transcriptional factors and sustained lung inflammation after cigarette smoking. The evidence in support of this paradigm led to a proposal of enhancing the balance of HDAC vis-à-vis HAT activity to control lung inflammation in COPD (286).

## VI. CONCLUSIONS

After three decades of studies centered on the inflammation/protease-antiprotease hypothesis, the COPD field has experienced a dramatic expansion of paradigms to explain the pathobiology of the disease that resulted from

well-designed human and experimental studies, incorporations of novel experimental techniques, and especially of cellular and molecular processes involved in cellular signaling and cell fate discovered in the recent years. Since no single hypothesis can adequately address the complexity of COPD, novel paradigms will continuously rewrite the pathobiology of the disease. The pathways discussed herein are not unique to COPD as they are also involved in other manifestations of the impact of cigarette smoke on health, namely, cardiovascular disease and cancer.

When one considers the extent to which the present understanding of the disease impacts treatment, identification of biomarkers, or susceptible populations, it becomes clear that we are far from reaching these goals. Nevertheless, we are confident that some of the significant advances outlined herein will inevitably translate into novel therapies and improved outcomes for patients with COPD.

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