

Thomas J. Rogers · Gerard J. Criner
William D. Cornwell *Editors*

Smoking and Lung Inflammation

Basic, Pre-Clinical and
Clinical Research Advances

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Springer

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Introduction

It is clear that chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease state which is the result of multiple insults to the lung, and the resulting inflammatory response is highly complex. Studies over the last 25 years have shown that many elements of the innate and adaptive immune systems are involved, and both local and systemic inflammatory reactions are critical participants. A full understanding of the regulation of the immune system in this disease will require a much more extensive analysis of the development of this disease at both a cellular and molecular level. It is also apparent that patients with this disease should be considered as individuals who have unique clinical and immunological features. This book consists of chapters that cover important elements of the disease process, with an emphasis on the critical components of the innate and acquired immune systems that play a role in the disease.

A common theme in the chapters presented here is the recurring story about the immune response functioning as a “double-edged sword.” Indeed, it is likely that there is an autoimmune component in COPD in at least some COPD patients, and this is an excellent example of how the “hyperactivity” of the immune system can promote this disease. However, our understanding of the role of the immune system in COPD is still evolving, and it is quite apparent that the immune response plays both a beneficial and detrimental role in the evolution of the disease. The implications of this are not trivial, particularly when one considers the best choice of treatments for the disease.

Recent research on COPD immunopathogenesis indicates that two populations of leukocytes substantially participate in the evolution of the disease. These leukocyte populations, the macrophages and neutrophils, make up the majority of the diseased lungs’ inflammatory cell response. Other cells of the immune system (including T cells and dendritic cells) are also important in this disease, and their function in this disease appears to be mediated (at least in part) through the participation of macrophages and neutrophils. Nevertheless, both of these leukocyte populations are typical components of the inflammatory milieu at sites of acute and chronic inflammation, and it is not surprising that they make up a substantial

component of the cells recruited to the lung. Very importantly, recent studies suggest that both of these populations are present in the lung in an “altered” or “heightened” activation state. Moreover, it is possible that the macrophages are derived from circulating monocytes that are already undergoing an “alternative” activation program. These cells then migrate into the lung where they enter into an environment that further promotes this alternative activation status, leading to the production of mediators that result in fibrosis, mucus production, and eventually in inappropriate tissue remodeling.

An important component of this disease is the fact that the stimulus of the inflammatory response is persistent, leading to a chronic overactivation of the immune system. The consequence of this persistence is that the normal “switch” from a pro-inflammatory immunological program to an anti-inflammatory program, which normally results in resolution of the inflammatory response, is not permitted to occur. The normal functional responses which might be made by neutrophils, macrophages, and structural cells of the lung to promote healing are not able to transition away from the initial inflammatory profile. In patients who develop COPD, there is also activation of structural cells, most notably the epithelial cells, and these cells contribute substantially to the production of inflammatory mediators. The hyperactivation of these cells leads to elevated mucus production, and mucus hypersecretion promotes airway obstruction, and a resulting decline in lung function. It also results in greater susceptibility to exacerbation and reduced resistance to respiratory tract infections.

These immunological processes, combined with the toxic mixture of thousands of active substances in tobacco smoke, lead to a more intense, and potentially much more pathogenic, stress response in the lungs. We are only now beginning to understand the biochemical basis for this stress response, and as more of the essential elements of this response are identified, we may have the ability to target this response so that more effective therapeutics may be developed. Of course, this type of treatment effort may be most effective if employed early in the disease process, before the tissue damage has reached an advanced stage. For this reason, early diagnosis and identification of susceptible patients is critical for successful treatment. Therapeutic strategies will almost certainly change as advances are made in identifying susceptibility genes that correlate with disease severity. It is now clear that COPD is a heterogeneous syndrome, and efforts are being made to identify subsets of patients with more selective disease characteristics. As a greater appreciation for clinical phenotypes is gained, it should be possible to unite this information with biomarker and related immunological parameters in order to individualize the therapeutic approach.

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Immunopathology of COPD

Laimute Taraseviciene-Stewart and Norbert F. Voelkel

This review focuses on new aspects of the pathogenic autoimmune mechanisms of COPD unveiled in recent years. We emphasize the importance of lung inflammation, innate and adaptive immune responses, as well as an autoimmune component in lung tissue destruction and repair.

Anatomical Evidence

The umbrella term chronic obstructive pulmonary disease (COPD) covers three conditions (Fig. 1): emphysema, chronic bronchitis with a reactive airway component, and chronic bronchitis without a reactive airway component. COPD affects 24 million people and is the fourth leading cause of death in the United States [1, 2]. Although chronic cigarette smoking is the primary risk factor [3], many other environmental exposures such as biofuel smoke, diesel exhaust, ozone, and microparticles are contributing risk factors for COPD [4]. One in five smokers develops COPD, and 80–90 % of COPD patients have a smoking history.

Lung tissue destruction is frequently progressive, in spite of smoking cessation [5, 6]. The clinical syndrome of COPD includes both pulmonary and extrapulmonary manifestations, which are thought to be driven by chronic inflammation [7]. Emphysema has been described and defined as “airspace enlargement” of the adult

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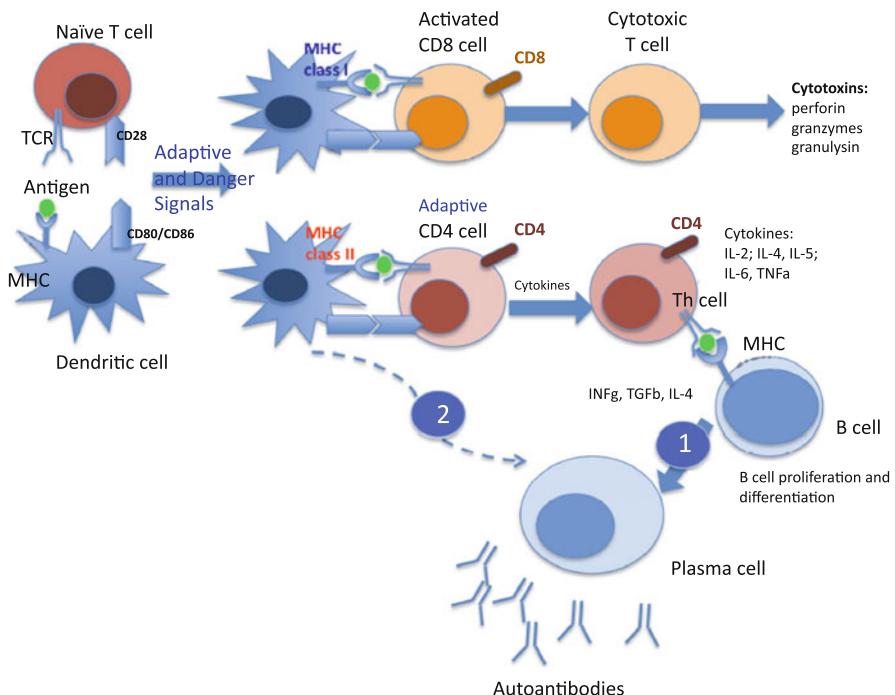


Fig. 1 Dendritic cells link innate and adaptive immunity. MHC class I-restricted DCs promote CD8+ T cell differentiation into cytotoxic effector cells. MHC class II-restricted DCs depending on the local cytokine milieu advance CD4+ T helper (Th) cell differentiation into Th1, Th2, Treg, or Th17 phenotype. Th cells are essential in determining B-cell antibody class switching. Cytokines secreted by T cells help B cells to multiply and mature into antibody-producing plasma cells. The synthesis of autoantibodies might follow conventional T-cell-dependent (1) or T-cell-independent (2) pathways

lung [8] in contrast to the developmentally impaired alveolarization of the neonatal lung. Human emphysema was originally described by the French physician Laennec in the nineteenth century [9]. The landmark study by the British epidemiologists Fletcher and Peto supported the concept that chronic cigarette smoking is indeed the major cause of chronic bronchitis and emphysema [10]. More recently a consensus is building that the lung structure is being attacked and destroyed by inhaled noxious agents contained in cigarette smoke or in polluted air, and the effects of exposure to diesel exhaust, ozone, and microparticles are being examined [11–13].

There is also agreement that not every smoker develops emphysema after smoking two packs of cigarettes per day for 30 years – the painter Pablo Picasso comes to mind who started smoking as a teenager but remained active and productive beyond his ninetieth year – and that there are genetically determined susceptibility factors (recently discussed in a number of reviews), which may increase the risk of developing emphysema at age 50 or 55 [14–19].

In the past, COPD was more prevalent among men. This was attributed to the difference in smoking rates in men versus women. With an increased prevalence of smoking among women, the difference has narrowed. Some studies have suggested

that women may be more susceptible to COPD [20]. Anatomical changes in emphysema occur in distinct morphological types [21]: centriacinar, panacinar, and paraseptal. The common centriacinar (or centrilobular) form of emphysema observed in long-term smokers predominantly involves the upper half of the lungs and begins in the respiratory bronchioles near the thickened and narrowed small bronchioles that become the major site of obstruction in COPD [4]. Panacinar emphysema destroys the entire alveolus uniformly and is predominant in the lower half of the lungs. It is generally observed in patients with homozygous alpha1-antitrypsin deficiency. Paraseptal emphysema (or distal acinar emphysema) preferentially involves the distal airway structures, alveolar ducts, and alveolar sacs. It is localized around the septae of the lungs or pleura. Although airflow is frequently preserved, the apical bullae may lead to spontaneous pneumothorax.

Emphysema commonly presents with chronic bronchitis. Chronic bronchitis leads to obstruction by causing narrowing of both the large and small (<2 mm) airways. In the large airways, an increase in goblet cells, squamous metaplasia of ciliary epithelial cells, and loss of serous acini can be seen. In the small airways, goblet cell metaplasia, smooth muscle hyperplasia, and subepithelial fibrosis can occur. In healthy individuals, small airways contribute little to airway resistance; however, in COPD patients, these become the main site of airflow limitation. The normal inflammatory response to cigarette smoke (or other noxious stimuli) is amplified in persons prone to COPD development. Genetic factors are believed to play a role in this response because not all smokers develop the disease.

Attempts are being made to phenotype COPD patients and identify specific pharmacological therapies for these phenotypes (see the recent review by Anderson and McNee [22]).

An unresolved question remains why many patients continue to worsen and demonstrate deteriorating lung functions several years after smoking cessation. This aspect of the clinical COPD spectrum and an “asthmatic,” reactive airway component in patients with COPD has led to the hypothesis of immune or autoimmune mechanisms of emphysema [23].

Functional Evidence

The presently existing pathogenetic concepts of emphysema, enzymatic breakdown of pulmonary tissue due to protease/antiprotease imbalance, uncontrolled oxidative stress, and increased apoptosis [24, 25], leave room for an immunological contribution to the lung tissue destruction [26–28]. In an early landmark paper [29], Benjamin Burrows reported that smokers have higher serum IgE concentrations than nonsmokers and suggested that smoking might be immunologically mediated. Much later Hogg et al. examined the evolution of the pathological effects of airway obstruction in patients with COPD and related the findings to the disease severity, as characterized by the GOLD classification [30]. Progression of COPD is associated with the accumulation of inflammatory mucous exudates in the lumen and infiltration of the wall by innate and adaptive inflammatory immune cells that form

lymphoid follicles. These changes are coupled to a repair or remodeling process that thickens the walls of the airways and also to areas of fibrosis.

The hyperreactive state of the airways has been historically associated with the asthmatic phenotype [31]. The so-called Dutch hypothesis postulates that asthma and COPD are two different aspects of the same disease and that airway hyperresponsiveness (AHR) predisposes to the development of both clinical conditions [32]. In this scenario, the increased airway responsiveness could be envisaged as a contributing factor to the development of COPD rather than the consequence of the disease [33], as proposed by the international guidelines for COPD [34]. There seems to be epidemiologic evidence that AHR and smoking predispose patients toward the development of asthma and COPD. Subjects with the “host factor” AHR are more susceptible to environmental stimuli that increase the risk of obstructive airway disease. In utero smoke exposure is an established risk factor for asthma, whereas active smoking has been shown to be a risk factor for COPD in a subset of the general population and in a subset of asthma patients (all of whom were hyperresponsive). Furthermore, there is an interaction between AHR and smoking, leading to a greater lung function loss. However, it has not been established whether the underlying mechanisms of the AHR that predisposes a person to asthma and the AHR that predisposes a person to COPD are the same; this will be a challenge for future research in the genetics of asthma and COPD [35].

In recent years, new aspects of the pathogenic mechanisms of COPD were unveiled, emphasizing the importance of lung inflammation and innate and adaptive immune responses as key players in the onset and progression of emphysema. The presence of lymphoid aggregates, CD4+, CD8+ T cells and B lymphocytes, mast cells, dendritic cells (DC), and macrophages, was documented in COPD lung tissue samples [4, 7, 36–38]. In addition to the cells of the immune system, antibodies have also received attention [39–42].

Dendritic Cells

Dendritic cells (DCs) are professional antigen-presenting cells (APC) derived from hematopoietic bone marrow progenitor cells. They play a central role in the initiation of immune responses [43, 44]. The general paradigm of DC function states that DCs reside in an immature state in the periphery of the lung, where they sample the inhaled air for incoming antigens. On triggering the antigen-recognition receptors such as Toll-like receptors, the DCs (Fig. 1) migrate through the afferent lymphatics to a lymph node (LN) where they select and activate naive T cells differentiating them into either cytokine secreting T effector cells or tolerizing Treg cells [45]. Two distinct lineages of DC, myeloid (m) and plasmacytoid (p), have been identified in the circulation [46, 47] and the lungs [48–51].

Myeloid DCs comprise two subtypes: type 1 mDCs, the main APCs that drive cell-mediated immune responses [43], and still poorly characterized type 2 mDCs. The pDCs exert potent antiviral activity due to the ability to produce type I

interferons (IFNs) [52, 53], and they also induce antigen-specific antitumor immune responses by enhancing the ability of mDC to present Ag to T cells [54]. pDCs are critically involved in immune tolerance through their cooperation with regulatory T cells [55]. In mouse lungs, DCs are found in most tissue compartments, including the large extrathoracic- and intrathoracic-conducting airways, the lung parenchyma accessible by lung tissue digestion, the alveolar compartment accessible by bronchoalveolar lavage, the pleura, and the perivascular space; they are also marginating the pulmonary lung vessels [56, 57], where they may respond to blood-borne antigens. A recent study from Jakubzick's laboratory [58] shows that in mice both CD11b^{hi} and the CD103+ lung DCs were able to ingest and traffic latex beads or soluble antigen; however, only CD103+ DCs were able to acquire and transport apoptotic cells to the draining LNs and cross present apoptotic cell-associated antigen to CD8 T cells. The CD103+ DCs selectively exhibited high expression of TLR3, and ligation of this receptor led to enhanced in vivo cytotoxic T cell responses to apoptotic cell-associated antigen [58].

The role of DC in the pathogenesis of COPD is not yet well understood [59]. Masten et al. [60] attempted to characterize DCs from human lungs: They found twice as many mDCs (identified as CD1c(+)CD11c(+)CD14(−)HLA-DR(+) cells) and pDCs (CD123(+)CD11c(−)CD14(−)HLA-DR(+) cells) in lung tissues from COPD patients. The mDC expressed higher levels of costimulatory molecules than pDCs suggesting that both lung DC subsets exert distinct immune modulatory functions. Tsoumakidou and colleagues [61] reported increased amounts of pulmonary immature DCs and decreased amounts of mature DC in the sputum of COPD patients. An imbalance of blood DCs in COPD also has been reported [45]. Demedts and others [62] evaluated DC infiltration in small airways by immunohistochemistry in patients with COPD (stages I–IV), never-smokers, and smokers without COPD and found a significant increase in the DC number in the epithelium and around small airways of patients with COPD compared with never-smokers and smokers without COPD. Moreover, the DC number in the epithelium and around the airways increased with disease severity. The levels of CCL20, the most potent chemoattractant for DC, were also significantly higher in induced sputum in patients with COPD compared with never-smokers and smokers without COPD [62].

Recently Freeman and colleagues [63] characterize different subsets of lung DCs concomitant with the expression of DC costimulatory markers (CD80, CD83, CD86) and a marker of activated CD4 T cells (CD69) present in whole lung tissue obtained from smokers with or without COPD and healthy never-smokers. Interestingly, mDCs were predominant in lungs of all patients, regardless of the smoking history. However, as determined by flow cytometry, progressively more severe disease, as assessed by GOLD criteria, correlated strongly with both DC costimulatory molecule expression and CD4 T cell activation. These findings contribute to a growing number of studies that have begun to assign a place for adaptive immune T cells in the chronic inflammatory pathobiology that leads to the lung tissue destruction in COPD [64]. Important future studies would determine the role of various lung DC subsets in T cell activation, as well as to examine the relative importance of alternative APC such as macrophages [65].

Mast Cells

Mast cells were first described by Paul Ehrlich in his 1878 thesis doctoral on the basis of their unique staining characteristics and large granules that are rich in histamine and heparin [66]. The role of mast cells and their mediators in the pathogenesis of COPD is not well understood, and their relative contribution to airway injury and remodeling in response to exposure to cigarette smoke is poorly documented [67]. In the normal human bronchus, lung mast cells are found in close proximity to epithelia, blood vessels, nerves, smooth muscle cells, and mucus-producing glands [68]. Activation of mast cells can lead to production of a wide array of effector molecules including prestored mediators (serotonin, histamine, proteases) and actively synthesized mediators released within minutes (prostaglandins, leukotrienes, and endothelin) and a large variety of cytokines and chemokines released within hours after activation. The IL-4 and IL-13 released by mast cells can modulate T cell responses, mucus gland hyperplasia, and smooth muscle hypertrophy/hyperplasia [69–71]. In asthma and COPD, mast cells contribute to hypoxia-induced angiogenesis by producing vascular endothelial growth factor (VEGF). VEGF exocytosis requires free radical formation and the activation of Src kinases [72, 73]. Mast cell-derived mediators such as histamine and cysteinyl leukotrienes can also activate lung macrophages to generate nitric oxide, lysosomal enzymes, and proinflammatory cytokines [74].

A study performed in monkeys in 1982 before and after acute exposure to cigarette smoke reported that the total counts of mast cells in the lungs were lower, while the percentage of degranulated cells was higher [75], implying that cigarette smoke induces degranulation of lung mast cells. Because degranulation is accompanied by local release of histamine, which could act on the smooth muscle of the airways, it was suggested that histamine mediates smoking-induced *acute* bronchoconstriction. In the lungs of human smokers, mast cells increase in absolute numbers, and smoking may be associated with increased levels of histamine and tryptase in the bronchoalveolar lavage fluid [76]. Cigarette smoke (CS) exposure *in vitro* stimulated the release of chemokines in a noncytotoxic manner but did not induce mast cell degranulation. CS induced in mast cells induced phosphorylation of Erk1/2, p38, and CREB and increased translocation of p65 without degradation of I κ B- α NF- κ B. These observations suggest that mast cells may contribute to the pathogenesis of emphysema through a direct effect of CS on the production of proinflammatory chemokines [77].

Recently, it has been reported that the number of mast cells were significantly increased in the sputum of smokers compared to ex-smokers [78]. The authors compared sputum, bronchoalveolar lavage (BAL) fluid, and airway wall inflammatory profiles in current versus ex-smokers and related the findings to smoking intensity and lung function in 17 current and 17 ex-smokers with mild to moderate COPD. Current smokers had more sputum mast cells, whereas ex-smokers had increased sputum neutrophils. There was a significant increase in eosinophils in the BAL fluid of current smokers, but ex-smokers had significantly increased numbers of lymphocytes and epithelial cells. There were no cell profile differences observed in airway

biopsies between current and ex-smokers, and there were no correlations between the individual inflammatory cell populations in any of the airway compartments. Only in current smokers smoking intensity was negatively correlated with lung function and associated with a reduction in the overall cellularity of both sputum and BALF. This study showed that airway inflammation persists in ex-smokers with COPD and differs from current smokers. The impact of smoking appears to vary in different airway compartments, and any direct relationships between mast cell cellularity and lung function tended to be negative, i.e., worse lung function indicated the presence of fewer cells [78].

Mast cells were also analyzed in lung tissues from patients with mild to very severe COPD [79]. In all compartments of COPD lungs, especially at the severe stages, the connective tissue MC population was found to be increased in density, whereas the mucosal mast cell population decreased; however, total mast cell density was decreased. This phenomenon was paralleled by increased numbers of luminal mast cells, whereas the numbers of apoptotic mast cells remained unchanged. In COPD lungs, the mucosal and connective tissue mast cell populations showed alterations in morphology and expression of CD88 (C5a-R), transforming growth factor (TGF)- β , and renin. Interestingly, as COPD progresses to its severe stages, the mast cell populations in the lung undergo changes in density, distribution, and molecular expression. These alterations are correlated to lung function; thus, the alterations in mast cell biology may have clinical consequences. Clinical data also show increased levels of mast cell-secreted tryptase and increased numbers of degranulated mast cells in the BALF and bronchial tissue of smokers. Tryptase is known as a potent activator of proteinase-activated receptor (PAR)-2 which, in turn, can induce secretion of IL-8 by mast cells and contribute to the progression of inflammation [80]. Furthermore, activation of calcium-independent phospholipase A2 (iPLA2) via mast cell tryptase could lead to arachidonic acid liberation, PAF production, cell surface P-selectin expression, and increased neutrophil adherence [81]. Activation of iPLA2 also could lead to activation of inflammasome signaling and production of IL-1 β and IL-18 such amplifying lung inflammation and injury [67].

Taken together, these studies provide unequivocal evidence that mast cells contribute to chronic inflammation in the lung. Whether mast cells are central to or only supportive in the pathogenesis of COPD still remains to be clarified, and the mechanisms by which mast cells can be activated in the lungs of COPD patients need further investigation.

Eosinophils

Early studies by Marina Saetta et al. showed elevated numbers of eosinophils in airway wall tissue samples [82] from patients with COPD, and Riise et al. found an increased production of eosinophilic cationic proteins in the BALF [83]. High levels of eosinophils were found in sputum of COPD patients [84] and in the induced sputum and BALF of nonsmoking COPD patients compared to nonsmoking controls [85].

Furthermore, Lams et al. demonstrated increased levels of activated eosinophils in the submucosa of resected lungs from smokers with COPD and current smokers compared to nonsmoking controls [86]. Recently D'Armiento et al. [87] found a significant increase in BALF eotaxin-1 levels in COPD patients compared to normal controls. Eotaxin-1 is a CC chemokine (CCL11) that binds to the CC chemokine receptor 3 (CCR3) on the surface of eosinophils, thereby inducing eosinophil activation and migration. Lung eosinophilia has been linked to bronchial hyperreactivity in COPD patients [88], and the expression of both eotaxin-1 and CCR3 was found to be upregulated during exacerbations of chronic bronchitis [89]. Patients whose lung function subsequently declined over the ensuing 6 months had significantly higher BAL eotaxin-1 levels than those patients with stable lung function over the same time period. In addition, disease stability was associated with decreased plasma eotaxin-1 levels. These data indicate that eotaxin-1-mediated lung eosinophilia may be a critical factor in the progression of COPD [87]. Whether or not eotaxin-1 can be a biomarker for COPD progression still needs to be validated.

Neutrophils and Macrophages

It is widely accepted that the proteolytic potential of neutrophils and macrophages can be important for the destruction of the extracellular matrix in emphysema. Increased numbers of neutrophils and macrophages were found in both airways and parenchyma of patients with COPD [24, 65, 90, 91]. Moreover, animal studies have demonstrated that macrophages and their proteolytic activity are a prerequisite for the development of cigarette smoke-induced emphysema [92–94] and that neutrophil elastase is essential for cigarette smoke-induced emphysema in mice and human [91, 95].

While neutrophils are present in the conducting airways, macrophages are the major cell type in secretions from the small airways and parenchyma [94, 96]. Macrophages are increased throughout the respiratory tract airway lumen and epithelium in COPD and are positively related to the severity of the disease, airway obstruction, and degree of alveolar wall damage in emphysema [97].

Macrophages have remarkable plasticity that allows them to efficiently respond to environmental signals; they change their phenotype, and their physiology can be markedly altered by both innate and adaptive immune responses [98]. In the past macrophages have been classified into classically activated M1 macrophages and alternatively activated M2 macrophages. Recently, the M2 designation has been expanded. The current classification divides macrophages into five different functional phenotypes based on their activation state: (1) panactivated, (2) classically activated, (3) alternatively activated, (4) immune complex-activated, and (5) suppressor-activated macrophages. An immunologically naïve macrophage phenotype (M0) has been also postulated [65]. Macrophages from patients with COPD, when cultured *in vitro*, showed increased expression of inflammatory cytokines TNF-a, IL-8, and matrix metalloproteinase (MMP)-9 [99, 100]. Concurrently the progressive reduction in expression and activity of histone deacetylase-2 (HDAC2)

has been observed. HDAC2 modulates the expression of the inflammatory genes and is associated with increased activation of NF-κB. The decrease in HDAC2 activity correlated with the severity of COPD [101].

Inhibition of HDAC with trichostatin A caused emphysema in rats, and gene silencing of HDAC2 in human pulmonary microvascular endothelial cells resulted in suppression of HIF-1a, VEGF, and lysyl oxidase and an increased expression of p53 [102].

Recently Vandivier's group [103] has demonstrated that acute and subacute cigarette smoke exposure suppressed efferocytosis (removal of apoptotic cells) by alveolar macrophages in a dose-dependent, reversible, and cell type-independent manner, whereas more intense CS exposure had an irreversible effect. CS inhibited efferocytosis through oxidant-dependent activation of the RhoA-Rho kinase pathway. The inhibitors of the RhoA-Rho kinase pathway reversed the suppressive effect of CS on apoptotic cell clearance in vivo and ex vivo. Data also suggest the involvement of sphingolipids in the inhibition of apoptotic cell clearance by alveolar macrophages [104]. These findings provide evidence that impaired efferocytosis may contribute to prolonged inflammation and the pathogenesis of COPD [103].

T Cells

The interpretation of data regarding the number and type of T cells in human disease and animal models of COPD remains controversial. Generally it is accepted that in COPD there is either an increase in the CD8/CD4 T cell ratio or an increase in the total numbers of CD8 and CD4 T cells in the lung tissue [105, 106]. Oligoclonal CD4 T cells have been demonstrated in lung tissues from patients with severe COPD [107] as well as an antigen-specific Th1 response against lung elastin [40], indicating an antigen-specific T cell response in COPD. In a murine model of cigarette smoke-induced emphysema, Maeno and coworkers described a critical role for CD8 T cells in inflammatory cell recruitment and lung destruction by releasing cytotoxic perforins and granzyme B, which cause cell death and apoptosis [108]. A study by Mortaz et al. showed increased CD8 T cell proliferation in the presence of cigarette smoke extract (CSE)-primed conventional dendritic cells [109]. Cigarette smoke affects both suppressor T cells and T helper cells [110], but its final effect on allergic sensitization is not well understood. T cells recruited to the lung in response to irritants such as cigarette smoke might express a restricted TCR repertoire, suggesting their recruitment to the lung in response to a conventional antigenic stimulus. We did not find significantly expanded TCR-Vbeta subsets in ex vivo human COPD lung samples [107]; however, when we cultured CD4+ T cells from five of the patients with emphysema in vitro, we found expansion of seven major TCR-Vbeta subsets. These data suggest that severe emphysema is associated with inflammation involving T lymphocytes that are composed of oligoclonal CD4+ T cells. In a rat model of autoimmune emphysema where we induced antibodies against endothelial cells and against the VEGFR2, we also found that adoptive transfer of selected T cells was sufficient to transfer the disease from HUVEC-immunized rat to the

naïve animals [111]. Recently Michael Borchers' group reported that chronic CS exposure leads to the generation of pathogenic T cells capable of inducing a COPD-like disease in Rag2(–/–) mice. There were increased numbers of circulating Treg cells in Sprague Dawley rats exposed for 2 and 4 months to secondhand smoke (SHS); however, the function of these cells was impaired since they were not able to suppress the proliferation of effector T cells. In addition, athymic nude rats that lack functional T cells, exposed to secondhand smoke for 2 months, did not develop emphysema (Taraseviciene-Stewart, Adelheid Kratzer, 2013). Together these findings support the concept that absence of the main partners for activation of macrophages (Th2 T cells) and B cells (Th1 T cells) protects against cigarette smoke-induced emphysema and that pathogenic T cells play a pivotal role in the early onset of emphysema pathogenesis.

Lymphoid Follicles and B Cells

Hogg and colleagues [7] described the presence of lymphoid aggregates and lymphoid follicles in the lungs from patients with GOLD [30] stages 3 and 4. Moreover, compared with nonsmokers, T lymphocytes were significantly increased in smokers with preserved lung function, while this response was blunted in patients with COPD [112]. Higher numbers of B-cell numbers were found in large airway biopsies from patients with COPD versus controls; moreover, B-cell numbers were higher in patients with GOLD severity stage 3 than stage 2 [113].

The lymphoid follicles consisting of B cells and follicular dendritic cells with adjacent T cells were described in the parenchyma and in the bronchial walls of patients with emphysema [37, 114]. Clonality was observed in all follicles and somatic mutations were observed in 75 % of the follicles, indicating oligoclonal, antigen-specific proliferation. These authors also detected similar lymphoid follicles in mice that had developed pulmonary inflammation and progressive alveolar airspace enlargement after nose-only cigarette smoke exposure. The increase in the number of B-cell follicles was progressive with time and correlated with the increase in the mean linear intercept. The authors concluded that B cells contribute to the inflammatory process and/or the development and perpetuation of emphysema by producing antibodies (Fig. 1) against either tobacco smoke residues or extracellular matrix components [37].

Cytokines

It is well recognized that cytokines are important players during chronic inflammation [64]. The regulation of cytokine production depends on many factors and is tightly regulated during inflammation [115]. Cytokines can affect endothelial permeability and expression of adhesion molecules [116, 117] inhibit angiogenesis [118],

Table 1 Cytokines, chemokines, and growth factors contributing to the pathogenesis of COPD in animal models

Gene	Result	(Ref.)
Overexpression		
IL-1 β	Inflammation, fibrosis	[120, 121]
IL-4	Eosinophilic infiltration, fibrosis	[122]
IL-6	Emphysema-like remodeling	[123]
IL-11	Emphysema-like remodeling	[123]
IL-13	Inflammation	[124]
IL-18	Severe emphysema, ↑IFN-gamma, ↑IL-5, and ↑IL-13	[125]
IFN- γ	Inflammation	[126]
TNF- α	Chronic inflammation	[127]
Knockout (KO)		
VEGF (conditional KO)	Absence of inflammation	[128]
Adiponectin	Progressive COPD-like phenotype; systemic inflammation	[129]
SP-D	Foamy macrophages	[130]
TLR-4	↑Oxidant generation; ↑elastolytic activity	[131]
TIMP-3	Spontaneous progressive air space enlargement	[132]
FGF-23	Premature aging-like phenotype	[133]
β 6-subunit of av β 6 integrin	Age-dependent emphysema	[134]
Smad a 3	Age-related emphysema	[135, 136]
Klotho b	Imbalance of MMP-9 and TIMP-1	[137, 138]
TNF- α R	Protected against emphysema	[139, 140]
IL-18R- α	↓Inflammation; ↓emphysema	[141]
CCR6	↓Cigarette smoke-induced emphysema	[142]
CCR5	↓Cigarette smoke-induced inflammation but not emphysema	[143]
STAT-3	High susceptibility to viral infection	[144]

^aThe SMAD proteins are homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the *C. elegans* protein SMA. The name is a combination of the two. During *Drosophila* research, it was found that a mutation in the gene, *MAD*, in the mother, repressed the gene, *decapentaplegic*, in the embryo. The phrase “Mothers against” was added since mothers often form organizations opposing various issues, e.g., Mothers Against Drunk Driving

^bThe Klotho gene encodes for a transmembrane protein, named Klotho, which in addition to other effects provides some control over the sensitivity of the organism to insulin and appears to be involved in aging. The Klotho protein is a novel β -glucuronidase (EC number 3.2.1.21) capable of hydrolyzing steroid β -glucuronides

lipid metabolism, cell proliferation, and migration; and release microparticles [119]. Cytokines also influence extracellular matrix composition through the alteration of the expression of matrix metalloproteases (MMPs) and their inhibitors TIMPs.

Innate immunity relies on pattern recognition receptors that detect molecular structures common to many microorganisms: LPS and endogenous ligands such as heat shock proteins. Activation of TLR4 by LPS triggers production of both cytokines and reactive oxidant species. Alterations of lung cytokines, oxidant stress, and TLR signaling have all been associated with experimental emphysema. Data from animal models summarized in Table 1 demonstrate the involvement of cytokines in the development of COPD. However, translating results from mice to humans is not trivial. For instance, in patients with tobacco smoke-induced severe emphysema,

lung tissue IL-13 levels were reduced and there was no difference in IFN- γ levels in patients with or without emphysema [145]. Contrary to the results in humans, over-expression of IFN- γ and IL-13 in mice generated an emphysema-like phenotype [122, 146]. Interestingly in rats, exposed to chronic secondhand smoke, IFN- γ levels were not increased (Taraseviciene-Stewart, Kratzer, unpublished observation).

Recently D'Armiento's group [87] examined 25 human plasma cytokine levels (IL-1 β , IL-1Ra, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , GM-CSF, MIP-1 α , MIP-1 β , IP-10, MIG, Eotaxin-1, RANTES, MCP-1) and found a statistically significant ($p < 0.05$) elevation for nine of these cytokines (IL-4, IL-5, IL-7, IL-8, IFN- α , GM-CSF, MIP-1 α , MIP-1 β , and IP-10) when compared to normal controls – of interest, only eight (IL-1Ra, IL-2, IL-6, IL-8, IP-10, RANTES, MCP-1, and eotaxin-1) were detectable in the BALF. Plasma IL-2 was significantly increased in stable COPD patients compared to patients with rapidly progressive disease, while plasma eotaxin-1 levels were significantly lower in stable COPD subjects compared to controls. Interestingly, only one cytokine, IL-8, overlapped in both plasma and BALF samples implying that the microenvironment matters and that BALF cytokine levels might be better indicators of lung inflammatory processes [87]. Two groups of investigators reported elevated serum levels of IL-18 in COPD patients with GOLD stage III and IV COPD when compared to healthy smokers and nonsmokers [141, 147]. We recently found increased IL-18 levels in BAL fluid of GOLD stage II and III COPD patients [117].

Antibodies

With chronic yearlong exposure, approximately 40 % of active smokers develop significant obstructive lung physiology, and, despite smoking cessation, another 10 % also develop lung disease [148]. This does not come as a surprise because for years pathologists had described large numbers of innate and adaptive immune cells embedded within the lung tissues of former smokers with chronic obstructive pulmonary disease (COPD) and emphysema [7, 149]. However, recently these intriguing clinical and pathological observations opened the door to a new concept: in susceptible individuals, cigarette smoke exposure could trigger long-lasting inflammatory memory responses that persist beyond the immediate period of exposure to cigarette smoke. Whereas human and experimental studies have provided unequivocal evidence for the ability of cigarette smoke to activate innate immune cells, new findings are now emerging that support a critical role for adaptive immunity (i.e., T and B cells) and antigen recall years after the smoke exposure has ceased [38].

In 1974 Michaeli and Fudenberg reported antibodies to collagen in patients with emphysema [150]. Seventy percent of 422 patients with emphysema were found to have antibodies to denatured collagen, whereas only 9 % of normal controls matched for age, sex, and ethnic origin had these antibodies. The titers ranged up to 1:16 and

higher, compared to titers of 1:2 and 1:4 in the normal sera. The antibody activity was found exclusively in the IgG fraction. No correlation between titers of antibodies to collagen and levels of $\alpha 1$ antitrypsin could be demonstrated, nor were any of the seropositive patients deficient in $\alpha 1$ antitrypsin. Authors concluded that antibodies to collagen in patients with emphysema probably reflect destruction of pulmonary connective tissue and that the demonstration of their presence may serve as a basis for the development of an assay for early detection of this disease. Four decades later, Brandsma et al. [151] could not reproduce these findings. Authors found no difference in levels of circulating autoantibodies against elastin, collagen, and decorin in COPD patients and healthy control individuals. However, they did find an increased anti-decorin autoantibody response in COPD ex-smokers compared with COPD smokers [151]. The same group of investigators also reported that antinuclear autoantibodies were more prevalent in COPD patients with low body mass index and that there was no association with smoking history [152].

Tobacco anti-idiotypic antibodies have been identified in serum from smokers [153], and recently the prevalence of IgG autoantibodies against pulmonary epithelium in patients with COPD has been described [39]. In 2007 Lee et al. [40] reported the presence of anti-elastin antibodies in serum samples from patients with emphysema and showed that elastin peptides can induce proliferation of peripheral blood CD4+ T cells isolated from COPD patients, but not control individuals or asthma patients. However, later we and other investigators [151, 154, 155] challenged the validity of these observations as we failed to detect significantly elevated titers of anti-elastin antibodies in the serum from COPD patients. Recently Wood et al. [156] reported higher levels of anti-elastin antibodies in smoking individuals without emphysema relative to subjects with alpha-antitrypsin deficiency and COPD; however, there was no relationship between antibody levels and clinical phenotype after adjustment for smoking.

Emerging evidence from human and animal studies suggests that the progression of COPD results from T- and B-cell-mediated inflammation and that T cell activation by endogenous or exogenous antigens released during smoking-induced lung injury can induce autoimmune responses [38, 64, 157] (Fig. 1). Autoreactive adaptive immune responses that involve anti-endothelial cell antibodies (AECA) production may be important in the etiology of this disease. Since autoimmunity constitutes a breakdown of central and/or peripheral tolerance, it is feasible that interventions, classically employed to induce T cell tolerance or activate innate immune responses, can prevent emphysema development. Our recent work has demonstrated in an animal model that anti-endothelial cell antibodies (AECA) can cause emphysema [111] and that serum from emphysematous animals was sufficient to passively transfer disease to naïve animals, suggesting that AECA cause emphysema. We also found that chronic exposure of rats to secondhand smoke (SHS) resulted in AECA formation and increased levels of proinflammatory cytokine IL-18 in BALF fluid.

Recently we have found autoantibodies against endothelial cells (AECA) in a subgroup of patients with COPD. The AECA titers in serum from emphysema patients correlated with the severity of the disease [41]. As shown in Fig. 2, there

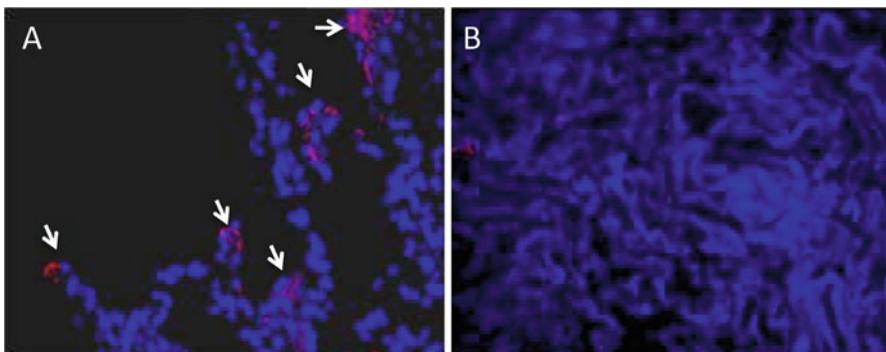


Fig. 2 Antibody deposition (*arrows*) in the lung tissue from emphysema patient (a) and healthy control (b). Immunofluorescence using goat antihuman Alexa 546-labeled IgG (red). Blue – DAPI staining for cell nuclei. Magnification x400

is antibody deposition in the lungs from COPD patients. These findings support a role for autoimmune mechanisms in the pathogenesis of COPD [27, 38] perhaps in a subgroup of patients (approximately 25 % of our cohort have AECA). These results are in agreement with observations from human studies by other investigators [7, 36, 37, 107, 158]. It is important to emphasize that autoantibodies may or may not cause tissue damage [159, 160]. It is generally accepted that those antibodies that have specific targets on the cell surface (such as VEGFR2) or are specific for extracellular or circulating molecules (such as CD146) can cause clinical manifestations suggesting that AECA can be the pathogenic agents in COPD. Recently Kirkham et al. discovered and reported that some patients with COPD had detectable levels of autoantibodies to carbonyl-modified proteins [161] and suggested that carbonyl adducts formed as a result of cigarette smoke-induced oxidative stress may be immunogenic. The antibody levels inversely correlated with the GOLD-staged disease severity; the authors also found deposits of activated complement in the lung blood vessels from smokers and patients with COPD. This study also confirmed our earlier findings of circulating anti-endothelial cell antibodies [41, 161]. Some of the antibodies detected by Kirkham et al. [161] were against acrolein-modified proteins; in this context, it is important to note that acroleinated proteins decorate endothelial cells and smooth muscle cells of the small vessels from the lungs of COPD patients (Fig. 3); moreover, acrolein causes endothelial cell apoptosis and loss of expression of the vascular-homeostatic prostacyclin synthase gene [162].

Apoptotic cell death, now accepted as a cause of emphysema, may occur in the lungs in a variety of scenarios when stress pathways have been activated beyond a point of no return [163]. Pulmonary vascular endothelial cell apoptosis [164] is a characteristic pathological finding of COPD at different stages of the disease. As the most important risk factor of COPD, cigarette smoking may initiate lung structural cell death and inflammation; increased levels of active proinflammatory cytokines

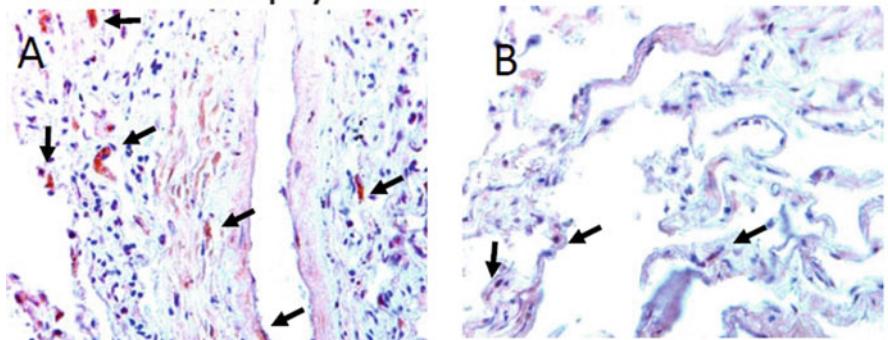
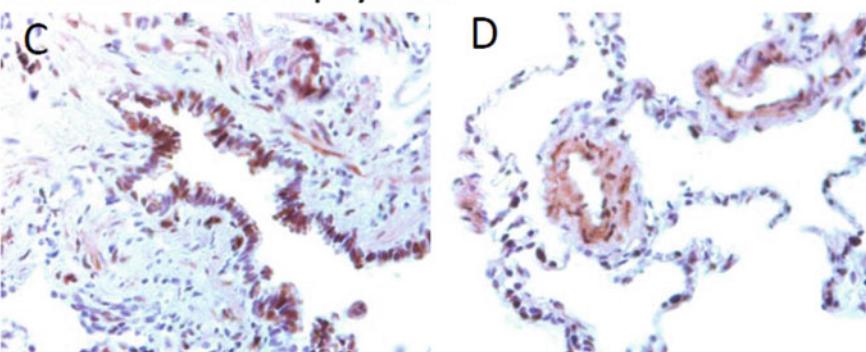
Smoker – no emphysema**Ex-Smoker with emphysema**

Fig. 3 In susceptible individuals, cigarette smoke exposure could trigger long-lasting inflammatory memory responses that persist beyond the immediate period of exposure to cigarette smoke, and chronic cigarette smoke imprints on the cells and acrolein-protein adducts seem to persist. While there are some acrolein-protein adducts in the healthy lungs (**a, b**), acroleinated proteins decorate bronchoepithelial (**c**), endothelial, and smooth muscle cells (**d**) of the small vessels from the lungs of COPD patients. Immunohistochemistry was performed on paraffin-embedded lung sections using rabbit polyclonal anti-acrolein antibody (Novus Biologicals)

such as IL-18 can lead to the induction of endothelial cell death initiating autoimmune processes. Moreover, we also showed that immunomodulatory strategies used to treat classical autoimmune diseases can be successfully used to prevent experimental autoimmune emphysema development [165].

Gordon and coworkers recently reported that smokers exhibit pulmonary endothelial cell apoptosis even in the absence of clinical manifestation of emphysema or airflow obstruction [166] based on the findings that smokers have increased levels of circulating apoptotic endothelial microparticles that appear to originate from the pulmonary vasculature. Based on this study, Chandra et al. [167] raised the question whether endothelial apoptosis occurs in a sub-phenotype of COPD patients or is a prequel to COPD. The study by Gordon et al. adds to the earlier findings by Kasahara

and colleagues that pulmonary endothelial cell apoptosis may be an early event in emphysema development and occurs prior to radiographic manifestation of emphysema. Many investigators find the attack of inhaled cigarette smoke in lung vascular cells counterintuitive: after all, the epithelial cells are the most important barrier. This bias negated the fact that compounds inhaled with cigarette smoke enter the circulation. One example of an inhaled and extremely aggressive compound is the aldehyde acrolein, which can be measured in the blood. Furthermore, the assumption that the alveolar-capillary barrier is impermeable for cigarette smoke – in particular volatile components – may be incorrect.

A recent study by Jack Elias' group [168] highlights the importance of innate immunity and of VEGF receptor signaling in the lung. This study is based on the use of poly(I:C) (a molecular mimic of viral pathogen-associated molecular patterns [PAMP]) and respiratory viruses as inducers of pulmonary inflammation in mice transgenic for the human VEGF₁₆₅ gene. VEGF is a critically important lung maintenance factor [169–171], which plays a critical role during lung development and confers protection against injury and oxidative stress. Poly(I:C) dramatically and dose-dependently inhibited the VEGF-triggered pulmonary edema, inflammation, angiogenesis, and mucin 5AC (Muc5AC) secretion in transgenic mice. The effect of poly(I:C) that selectively regulates VEGFR1 expression and inhibits VEGF-induced activation of ERK, FAK, Akt, and eNOS appears to be mediated by a TLR3-independent, retinoic acid-inducible gene-I like RNA helicase (RIG-like helicase)-dependent pathway. It is possible that the mechanism, whereby poly(I:C) contributes to the pathogenesis of virus-induced COPD exacerbations and lung function deterioration, can also be a contributing factor in the autoimmune mechanisms of emphysema.

Adaptive Immunity in Emphysema

Finally there may be additional mechanisms at play in the smoldering but progressive destruction of the lung parenchyma, which can continue to be active for decades after smoking cessation. What sustains the chronic inflammation for years after smoking cessation continues to puzzle investigators. Chronic, poorly controlled asthma in humans is associated with lung remodeling and a transition toward fixed airway obstruction. Whether or how this fixed airway destruction relates to alveolar destruction is unclear. Whereas allergic asthma (which is understood as an immune disorder wherein the antigen is sometimes known) and animal models of asthma – developed, using a variety of immunization strategies – can be dissected to uncover multiple layers of the adaptive immune response, emphysema until recently has been seen as a problem of proteolysis, not of adaptive immunity. This situation is somewhat perplexing because the burning cigarette can indeed be seen as an antigen delivery device. Recognizing this, most recently, a concept of emphysema pathogenesis based on immune mechanisms has begun to emerge. A comprehensive gene

expression profiling of rat lungs performed by Stevenson and colleagues [172] has demonstrated a sustained, increased expression of a number of genes implicated in the innate and adaptive immune responses. Chronic lung cell damage, and in particular apoptosis, when combined with ineffective phagocytic removal of apoptosed cell bodies [173, 174] may result in the generation of neoantigens and autoantibodies.

Synopsis

It appears – with hindsight – that investigators of the pathobiology of COPD/emphysema have largely ignored the seminal studies by Burrows and perhaps found the Dutch hypothesis inconvenient. When Agusti et al. [23] in their 2003 Thorax paper asked the question: “Does COPD have an autoimmune component?” many years of relative inactivity of research in the area of COPD had gone by. Since then the concept of an immune system participation in COPD has been noticed by many investigators and embraced by few. Lung tissue samples have been examined and the cells of the immune system – suggesting some kind of an immune response – are there. Antibodies have been found in plasma samples, yet immune complex studies are still lacking. We believe that it is important to find out whether immune responses play a role in many or most patients with COPD/emphysema or whether such patients are found in a subgroup. Conceptually immune system participation could amplify the smoldering inflammation and contribute to tissue destruction [175]. If this disease component is not understood and not therapeutically targeted – and steroids may or may not be effective as a treatment of these immune mechanisms – the disease may continue to progress. Recent studies of lung tissue samples from patients with COPD/emphysema have demonstrated chromosomal abnormalities [176] and decreased VEGF [170] and HIF-1a [177] expression. These new findings still need to be integrated into the model of altered (hyperactive) innate and adaptive immunity. For starters, T and B lymphocytes and dendritic cells express VEGF receptors, and how decreased VEGF signaling in COPD affects immune cell function is unclear. Figure 4 attempts to illustrate the multicellular and multimediator interactions of the innate and adaptive immune system and how they influence lung tissue homeostasis – the adult lung tissue maintenance program [178]. Our model has as a starting point lung alveolar epithelial and endothelial cell injury and death caused by inhaled cigarette smoke or activated macrophage and neutrophil products. Ineffective efferocytosis perpetuates inflammation and may promote autoimmunity [179]. Cell damage may expose membrane epitopes which elicit a local T cell response and eventually become an antigen. Many elements of this hypothetical model still need to be investigated and many questions remain unanswered. One important question is whether or to what degree the smoldering fire of inflammation and tissue destruction is being fed by the bone marrow [180]; after all, dendritic cells, mast cells, and eosinophils originate from the bone marrow.

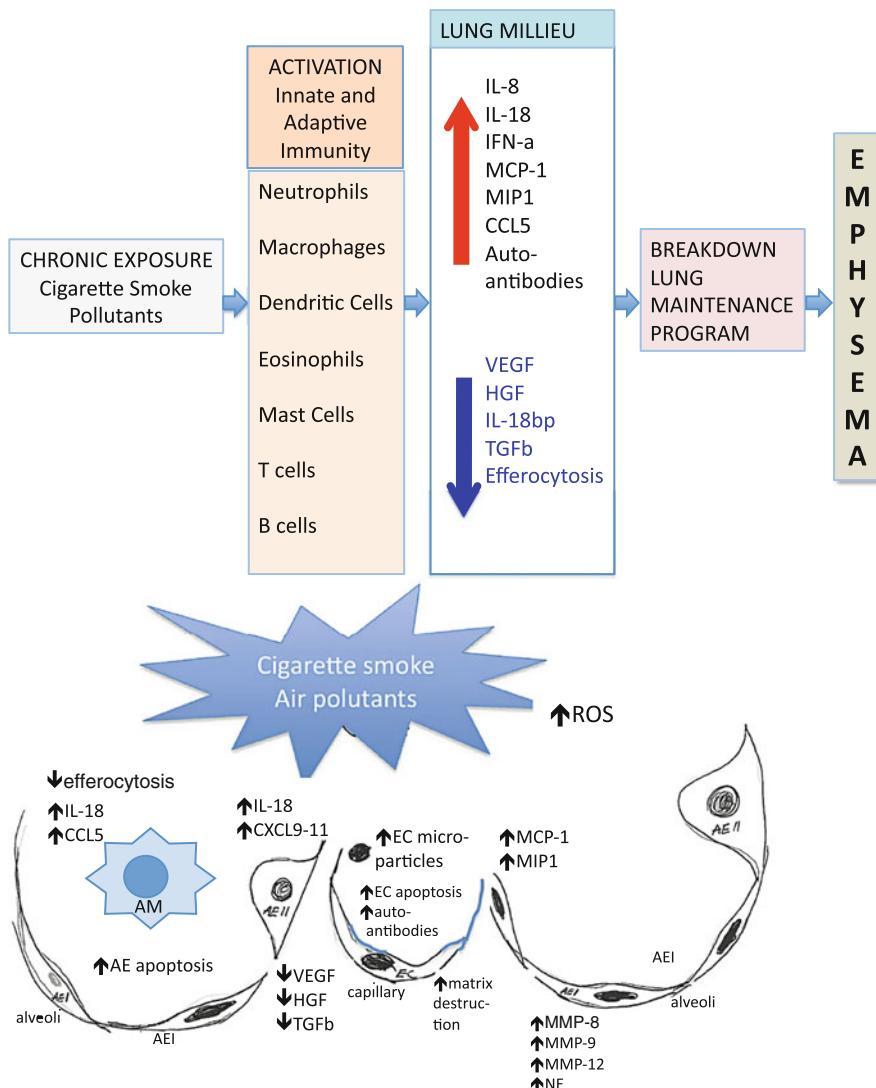


Fig. 4 Destruction of the lung maintenance program in emphysema. Chronic exposure to cigarette smoke leads to a change in the local milieu: increased release of proinflammatory mediators (IL-8, IL-18, CCL5, IL-8, IFNgama, MIP1) that contribute to paracrine and autocrine signaling, impairment in efferocytosis, decreased levels of growth and survival factors (most notably VEGF and HGF), and extracellular matrix degradation (increased levels of matrix metalloproteases MMP-9 and MMP-12). Increased apoptosis of endothelial and epithelial cells might contribute to autoantibody formation. AM – alveolar macrophage; AEI – alveoli type I cell; AEII – alveoli type II cell; EC – capillary endothelial cell; ROS – reactive oxygen species

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Monocyte Populations Which Participate in Chronic Lung Inflammation

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Introduction

Monocytes are a member of the family of mononuclear phagocytes, which are composed of monocytes, macrophages, and dendritic cells. These cells play a crucial role in the innate immune response but are also essential for adaptive immunity. Very importantly, these cells also exert a major influence on both acute and chronic inflammatory reactions. Each of the members of this family is made up of multiple subtypes, and our understanding of the regulation of their function is still not fully developed. In this chapter we will review current knowledge regarding the function of monocytes and macrophages, and in particular we will examine the role of these cells in chronic inflammatory responses in organ systems such as the lung.

Monocyte Development

Monocytes develop from precursor cells in the bone marrow, and the precise nature of this maturation process is still not fully understood. It is possible to identify cell populations that give rise to mature monocytes and distinguish them based on the expression of certain cell surface markers. Monocytes develop from

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hematopoietic stem cells (HSCs) that are renewable, and these cells reside in the bone marrow for long periods of time [1–4]. These HSCs are pluripotent and have the capacity to develop into common lymphoid precursors and common myeloid precursor cell populations which, in turn, develop into mature lymphocytes, dendritic cells, monocytes, and granulocytes (Fig. 1). The development of the common myeloid progenitor (CMP) and common lymphoid progenitor represents the first branch point in the differentiation of the HSC, and the CMP express a phenotype which can be detected based on the expression of cell surface markers CD34 and CD117/cKit, together with the loss of the stem cell antigen (Sca1) (Fig. 1). These cells do not express the lineage-, monocyte-, dendritic-, or granulocyte-specific markers (i.e., Lin-) and at this stage are still capable of development into any of these hematopoietic cell types [2, 5]. In the bone marrow the CMP population gives rise to the granulocyte/macrophage progenitor (GMP), a population that acquires the expression of both CD16 and the receptor for colony-stimulating factor (CSF-1R).

The GMP population appears to be programmed to preferentially develop into erythrocyte progenitors, and this bias is only interrupted in the presence of certain myeloid colony-stimulating factors. Perhaps the most significant of these factors is CSF-1 (alternatively termed macrophage colony-stimulating factor (M-CSF)) [2, 5, 6], a cytokine which influences hematopoietic cell development only after the transition of the CMP population to GMP. A deficiency in CSF-1R in mice results in deficiency of macrophages in a number of organs and tissues, including the lung [7–9]. The expression of CSF-1R is preserved through the development of mature monocytes, and CSF-1 regulates the functional activity of mature monocytes and macrophages [10–12]. Of note is the ability of CSF-1 to induce the expression of CD16 by monocytes, and CD16+ monocytes express higher levels of MHC class II, express higher levels of the proinflammatory cytokines TNF- α and IL-1 β , but exhibit reduced phagocytic activity [13–16]. Recently it has become clear that IL-34 shares many of the activities of CSF-1, and like CSF-1, this cytokine also appears to work through the CSF-1R [7].

The GMP stage of development is followed by the emergence of cells which have committed to the development of monocytes or dendritic cells (Fig. 1), and these cells are designated monocyte/dendritic cell progenitors (MDP). These cells can be identified by a reduced expression of CD117/cKit, combined with expression of the CX3CR1 (the fractalkine or CX3CL1 receptor). These cells are capable of proliferation in response to a variety of signals, including CSF-1 under noninflammatory conditions. In contrast, during inflammatory conditions the expansion and further maturation of these cells can be strongly induced through the activation of Toll receptors TLR2 and TLR4 [17]. The proliferation of these cells in the bone marrow in response to Toll receptor ligands serves to expand the reservoir of monocyte progenitors during the initial period of inflammation and/or infection. This selective proliferation of monocyte precursors is coincident with a substantial loss of overall cellularity in the bone marrow following infection [17, 18].

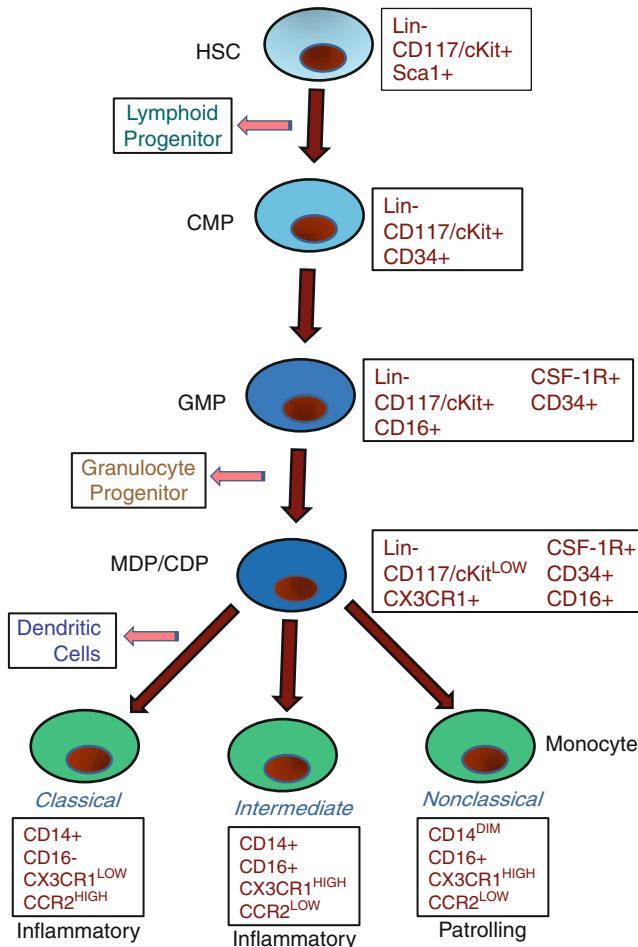


Fig. 1 Development of monocytes and dendritic cells from hematopoietic stem cells. Hematopoietic stem cells (HSC) reside in the bone marrow and give rise to common myeloid progenitors (CMP). These cells further develop into the granulocyte/macrophage progenitor (GMP) population and then into myeloid/dendritic progenitor cells (MDP). At this stage, these cells can further differentiate into either dendritic cells or monocytes before exit from the bone marrow

In humans, it is estimated that 3×10^{11} monocytes are released from the bone marrow per day [2]. However, the emigration of monocytes from the bone marrow is altered during periods of inflammation or in response to infection. Research carried out with mice suggests that the major stimulus of monocyte emigration from the marrow is mediated by CCL2 and its receptor CCR2. Moreover, during periods of inflammation or infection, blood levels of CCL2 increase, and CCR2, CCR5, and CX3CR1 are major regulators of greater monocyte traffic [19–25].

These studies have been confirmed by work showing that CCR2 knockout mice exhibit substantial monocytopenia, and this duplicated in genetically normal mice treated with inhibitors of CCR2 [26]. Current evidence using experimental animals suggests that the influence of CCL2 is greater for the Ly6c⁺monocyte subset (roughly equivalent to the human CD14++CD16- population) [19, 20, 24, 27], and this is likely due to the greater expression by these cells of CCR2 (Fig. 1). There is also evidence that monocyte emigration is promoted by chemokines which activate the chemokine CX3CR1 and CCR5, although the role of these receptors appears to be less significant [20, 25].

There are also retention mechanisms in the marrow which act to resist the emigration of monocytes. The most notable of these is mediated by the chemokine receptor CXCR4. The ligand for CXCR4 (CXCL12) is produced by marrow stromal cells, and the activation of CXCR4 on monocytes in the marrow appears to lead to the arrest of marrow monocytes. Moreover, in certain circumstances, the production of CXCL12 can lead to the loss of monocytes from the blood and into the bone marrow reservoir [5]. These results are consistent with studies which show that pharmacological inhibition of CXCR4 leads to a rapid emigration of bone marrow monocytes into the blood [26].

Monocyte Subpopulations

Monocytes comprise about 5–10 % of the circulating leukocytes in humans, and this is typically a stable frequency in the absence of inflammation or infection. Three populations of monocytes can be identified in humans based on the expression of cell surface markers CD14 and CD16, and these subpopulations exhibit the following phenotype: CD14++CD16-; CD14+CD16+, and CD14^{DIM}CD16+ (Table 1) [2, 5]. The CD14++CD16- “classical” population is the major monocyte subset (80–90 % of circulating monocytes) and expresses high levels of the chemokine receptor CCR2 and low levels of CX3CR1 [29]. The differential expression of these receptors directs these cells to migrate prominently toward sites of inflammation, by virtue of the elevated level of CCL2 expression at these sites. These cells exhibit a weak TNF- α and IL-1 β response, but a strong IL-6, IL-8, CCL2, CCL3, and IL-10 response to bacterial LPS, a TLR4 agonist, and are typically considered “proinflammatory” [14, 30].

The CD14++CD16+ “intermediate” monocyte subpopulation represents 5–7 % of the total blood monocytes, and these cells express low levels of CCR2, but high levels of both CX3CR1 and CCR5. While these cells are unlikely to migrate strongly toward tissue sources of CCL2, the expression of CCR5 presumably allows these cells to migrate to sites of inflammation where the CCR5 agonists CCL3, CCL4, and CCL5 are typically produced at elevated levels. These cells are responsive to bacterial LPS and in general produce an intermediate to high level of proinflammatory cytokines (Table 1). These cells are proinflammatory, although the precise function of these cells *in vivo* remains somewhat uncertain.

Table 1 Characteristics of human monocyte subpopulations

Feature	“Classical” CD14++CD16-	“Intermediate” CD14++CD16+	“Nonclassical” CD14 ^{DIM} CD16+
Function	Inflammatory	Inflammatory	Patrolling
Frequency (%)	80–90	5–7	6–8
Receptor expression	CCR2 ^{HIGH} CX3CR1 ^{LOW} CCR5 ^{HIGH} CD163+	CCR2 ^{LOW} CX3CR1 ^{HIGH} CCR5 ^{HIGH} CD163+	CCR2 ^{LOW} CX3CR1 ^{HIGH} CCR5 ^{LOW} CD163-
Size	Large	Large	Small
LPS-induced:			
Cytokine expression	TNF- α ^{LOW} IL-1 β ^{LOW} IL-6 ^{HIGH} IL-10 ^{HIGH}	TNF- α ^{HIGH} IL-1 β ^{HIGH} IL-6 ^{HIGH} IL-10 ^{MEDIUM}	TNF- α ^{LOW} IL-1 β ^{LOW} IL-6 ^{LOW} IL-10 ^{LOW}
Chemokine expression	CCL2 ^{HIGH} CCL3 ^{HIGH}	CCL2 ^{LOW} CCL3 ^{MEDIUM}	CCL2 ^{LOW} CCL3 ^{LOW}
TLR7/8-induced:			
Cytokine expression	TNF- α ^{LOW} IL-1 β ^{LOW}	TNF- α ^{MEDIUM} IL-1 β ^{HIGH}	TNF- α ^{HIGH} IL-1 β ^{HIGH}
Chemokine expression	CCL3 ^{LOW}	CCL3 ^{LOW}	CCL3 ^{HIGH}

Information derived from Cros et al. [28]

Finally, the CD14^{DIM}CD16+ “nonclassical” monocytes contribute approximately 5–8 % to the total circulating monocytes and express low levels of both CCR2 and CCR5 and high levels of CX3CR1. The absence of both CCR2 and CCR5 significantly restricts the ability of these cells to traffic into the extravascular tissue in response to inflammatory stimuli, since these receptors provide strong signals for extravasation. However, recent work has demonstrated that the expression of CX3CR1 provides these cells with the ability to interact with the chemokine CX3CL1 (fractalkine) expressed on the luminal surface of vascular endothelial cells [28]. This interaction results in prolonged “crawling” of these cells along the blood vessel surfaces. This prolonged crawling activity is not exhibited as prominently by either classical or intermediate monocytes. However, these cells do traffic into extravascular tissues during inflammatory reactions, so their complement of chemoattractant receptors is sufficient to permit mobilization to sites of tissue inflammation. Finally, studies conducted in mice suggest that once these cells leave the blood, they differentiate into the type of macrophage which is designated “M2” or “alternatively activated” [5]. The features of the M2 macrophages will be discussed in a section below.

The nonclassical monocytes express low levels of CD14 (which is a coreceptor with TLR4 for bacterial LPS), and this results in a much weaker cytokine and chemokine response to bacterial LPS (Table 1). However, analysis of the nonclassical monocytes shows that they are very responsive to stimulation by viral pattern Toll receptors TLR7 and TLR8 [28]. The implication of these findings is that the

nonclassical monocyte subpopulation is relatively unresponsive via bacterial pattern receptors (i.e., TLR4) but highly responsive to viral pattern receptors (i.e., TLR7 and TLR8). This suggests that these cells are very likely responsible for “patrolling” in search of anatomical sites where there might be a viral infection, but unlike the other monocyte subpopulations, these cells would be hyporesponsive to bacterial stimulation (Table 1) [28].

The capacity of the classical and intermediate monocyte subpopulations to respond via bacterial pattern receptors is consistent with the functional activity of these cell types. For example, both of these populations are highly phagocytic, and the classical subtype exhibits strong reactive oxygen and myeloperoxidase activities [28]. This is in contrast to the nonclassical subtype which is poorly phagocytic and exhibits a very weak reactive oxygen and myeloperoxidase response. These populations appear to have specialized their abilities to participate in an effective response to either bacterial or viral pathogens.

Monocyte Traffic to the Lung

Studies on the capacity of monocytes to move to sites of inflammation have led to the conclusion that monocytes have at least two distinct phenotypes. The first phenotype has previously been referred to as “patrolling” monocytes, and the second has been designated “inflammatory” [30]. The patrolling monocytes were shown to circulate in the blood and populate tissues in the absence of an inflammatory response, while the inflammatory cells traffic preferentially to sites of inflammation. These cell types can be distinguished on the basis of the expression of cell surface marker, and the major difference was found to be the expression of the two chemo-kine receptors CCR2 and CX3CR1 [30]. In humans, these phenotypes are now designated classical (inflammatory) and nonclassical (patrolling), and the trafficking properties of these cells now appears to be much more complex. For example, there is evidence from studies in mice that classical monocytes can convert to nonclassical monocytes *in vivo* [31–33]. In addition, classical monocytes can differentiate to dendritic cells *in vivo* under conditions of inflammation [34]. Furthermore, unlike nonclassical monocytes, the classical monocyte population is capable of transport from the blood to the marrow and back unless exposed to an inflammatory signal which mobilizes these cells to inflamed tissue [30, 31]. The influences which regulate emigration of monocytes from the marrow to the blood must be sustained in order to maintain a normal level of circulating classical monocytes.

Recent work has shown that nonclassical, but not classical, monocytes traffic to the normal lung, and these cells undergo extravasation and differentiate into lung macrophages [34]. These cells express low levels of CCR2 and CCR5, but a high level of CX3CR1. The tissue signals responsible for the transport of these cells out of the blood vessel into the lung are not understood. Classical monocytes do not appear to have the capacity to develop into lung macrophages in normal (noninflamed) tissue, but these cells do differentiate into lung dendritic cells. In contrast, classical

monocytes are strongly mobilized to the lung tissue during lung inflammation, and these cells differentiate into lung macrophages. Evidence in mice suggests that the major signal for traffic of these cells into inflamed tissue is provided by CCL2 [35].

Macrophages

Macrophages and their phagocytic capacity for microorganisms and other cellular debris were described in detail by Metchnikoff in 1905 [36]. Since that time, two key events propelled our understanding of macrophages and their functions. The first was the discovery of interferon (IFN- γ) in the early 1980s [37]. IFN- γ induces macrophages to produce proinflammatory cytokines and other mediators as well as enhancing phagocytic activity and increased antigen presentation capacity. The second was the elucidation of Toll-like receptors (TLRs) and their role in innate immunity [38, 39]. Signaling through TLRs once the macrophage has been primed with IFN- γ results in a potent antimicrobial response with the most obvious phenotype being phagocytosis and destruction of the invading pathogen. Together these two discoveries rapidly advanced our understanding of the molecular mechanisms of innate immunity to a wide variety of organisms.

Macrophages are found in all tissues and organs of the body in the absence of inflammation or tissue injury, and they serve homeostatic roles and as sentinels of infection or injury. These macrophages are considered “resident” within the tissue and are thought to be derived and replenished from the blood by CD14^{DIM}CD16+CX3CR1^{HIGH} monocytes [28, 30]. Examples of these macrophages include alveolar and interstitial macrophages in the lung, Langerhans cells in the epidermis, Kupffer cells in the liver, microglial cells and perivascular macrophages in the CNS, renal interstitial macrophages in the kidney, and osteoclasts in the bone. Macrophages can also be recruited to sites of inflammation in a number of tissues, or where tissue damage/injury has occurred. In these situations, monocytes in the blood with a CD14++CD16- phenotype are typically recruited to the site infection/inflammation in a CCR2-dependent manner [30]. Upon transit from the blood into the tissue, these monocytes differentiate into macrophages and this differentiation is highly dependent on the tissue microenvironment.

Microbial Pattern Recognition Receptors

Our understanding of innate immunity has greatly advanced with the sequencing of the human, murine, and *Drosophila* genomes. Comparative analysis of these genomes resulted in the identification of a family of ten Toll orthologues (in human), termed Toll-like receptors. These receptors play a critical role in the innate immune response, and the TLRs are expressed in several cell types of the immune system, including monocytes, macrophages, fibroblasts, and endothelial cells. The TLRs are

Table 2 General characteristics of human TLRs

Receptor	Cellular location	Microbial ligand	Organism
TLR1	Cell surface	Triacyl lipoprotein	Bacteria
TLR2	Cell surface	Peptidoglycan	Bacteria
TLR3	Intracellular	dsRNA	Viruses
TLR4	Cell surface	LPS	Bacteria
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	Diacyl lipoprotein	Bacteria, viruses
TLR7/TLR8	Intracellular	ssRNA	Viruses
TLR9	Intracellular	CpG DNA	Bacteria, viruses, protozoa
TLR10	Unknown	Unknown	Unknown
TLR11	Cell surface	Profilin-like molecule	Protozoa

Information derived from several sources [39–41]

“pattern recognition” receptors, and together with other microbial recognition receptors, these proteins play a role in the inflammatory response [39–43].

Toll receptors have the capacity to recognize conserved components and products of a wide range of microbial organisms (Table 2). These receptors have some common structural characteristics including leucine-rich repeats (LRR) that are arranged in tandem domains. This structure confers the ability of these receptors to recognize a wide range of molecules. For example, TLR4 has the capacity to recognize lipopolysaccharide (LPS), as well as the fusion protein of respiratory syncytial virus (RSV), paclitaxel, and fibronectin [42]. Furthermore, some of the TLRs can form heterodimers, and this creates additional structural diversity and expands the capacity to recognize microbial products. Examples of this are TLR1/TLR2, TLR1/TLR6, and TLR6/TLR2. Ligation of TLRs initiates a complex set of signaling cascades that are predominantly mediated through MyD88 and ultimately via the transcription factor nuclear factor- κ B (NF- κ B). The specific outcome of these signaling events varies depending on the cell type but can include cytokine and chemokine production, enhanced phagocytic activity, and production of reactive oxygen species (ROS) and nitric oxide (NO). Macrophage function and activity depends on sensing cues from the environment which are delivered primarily via cytokine/chemokine receptors in combination with TLRs.

Classically Activated Macrophages

Traditionally, activation of macrophages has been studied through the examination of events/functions of macrophages following stimulation with IFN- γ in combination with LPS. These are the macrophages described above which are designated “classically activated” or more recently “M1” cells. Upon sensing a pathogen via opsonic (antibody-mediated) or non-opsonic (pattern recognition receptors) mechanisms, these resident macrophages differentiate (or “polarize”) to the M1 phenotype.

Table 3 Genes transcriptionally upregulated in M1 macrophages

Gene name	Gene symbol
Chemokine (C-X-C motif) ligand 11	CXCL11
Chemokine (C-C motif) ligand 19	CCL19
Chemokine (C-C motif) receptor 7	CCR7
Chemokine (C-X-C motif) ligand 10	CXCL10
Chemokine (C-X-C motif) ligand 9	CXCL9
Chemokine (C-C motif) ligand 5	CCL5
Interleukin 2 receptor, alpha	IL-2RA
Complement component 2	C2
Interleukin 15 receptor, alpha	IL-15RA
Fas (TNF receptor superfamily, member 6)	FAS
Interleukin 15	IL-15
Complement component 1, s subcomponent	C1S
Complement component 1, r subcomponent	C1R
CD40 molecule, TNF receptor superfamily member 5	CD40
Interleukin 32	IL-32
Interleukin 7 receptor	IL-7R
Chitinase 3-like 2	CHI3L2
Superoxide dismutase 2, mitochondrial	SOD2
Mucin 1, cell surface associated	MUC1
Interleukin 12B	IL-12B
CD80 molecule	CD80
Poliovirus receptor	PVR
Interleukin 6	IL-6
Tumor necrosis factor (TNF superfamily, member 2)	TNF- α
Chemokine (C-X-C motif) ligand 13	CXCL13
Chemokine (C-C motif) ligand 20	CCL20
Major histocompatibility complex, class II, DO beta	HLA-DOB
Fc fragment of IgG, high affinity Ib, receptor (CD64)	FCGR1B

Information derived from Martinez et al. [44]. M1 cells are induced by treatment of macrophages with the combination of IFN- γ and LPS

These cells produce several proinflammatory cytokines including TNF- α , IL-6, IL-1 β , and IL-12, express MHC class II, and other costimulatory molecules then drive the inflammatory process for both innate and acquired immunity. In addition, a number of chemokines are produced, including CXCL8, 9, 10, and 11 and CCL2, 3, and 5, which can call into the site of infection additional macrophages, neutrophils, and other granulocytes to assist in the elimination of the invading pathogen. At the same time, M1 cells develop enhanced phagocytic activity which is specifically designed to engulf the pathogen. Once phagocytized, the pathogen resides in a compartment called a phagosome which merges with another intracellular compartment called the lysosome. It is within the lysosome that inducible nitric oxide (iNOS), ROS, and superoxide dismutase (SOD) are present, and these agents are important elements of the antimicrobial mechanism of the macrophage. Table 3 shows a partial list of genes reported to be upregulated in expression in human M1 macrophages.

Alternatively Activated Macrophages

It has recently become apparent that macrophages can be activated in ways that do not necessarily require the presence of a pathogen, IFN- γ , or stimulation via TLRs. These macrophages are collectively referred to as “alternatively activated” or M2 macrophages. This nomenclature of M1 versus M2 cells parallels that of Th1 versus Th2 designations, but it should not be assumed that M2 polarization is tied exclusively to Th2 responses. Although Th2 cells are the major source of IL-4 and IL-13 production, there are other cells of both the innate and acquired immune systems that also produce IL-4 and IL-13 [2, 45].

There are currently three subpopulations of M2 cells that have been identified. First, M2a cells are induced by either IL-4 alone or in combination with IL-13. These cells exhibit increased expression of mannose receptor (CD206), scavenger receptors, DC-SIGN (CD209), fibronectin, complement component 5a receptor (C5aR), and IL-10 [2, 44–46]. Genes that are upregulated by IL-4 (Table 4) and are characteristic of M2 cells include the mannose receptor (CD206) and other scavenger receptors (important for pathogen sensing and lipoprotein clearance) and CD209 (involved

Table 4 Genes transcriptionally upregulated in M2 macrophages

Gene name	Gene symbol
Mannose receptor, C type 1	MRC1
CD36 molecule (thrombospondin receptor)	CD36
Insulin-like growth factor 1 (somatomedin C)	IGF1
Chemokine (C-C motif) ligand 13	CCL13
Fibronectin 1	FN1
CD209 molecule	CD209
Interleukin 1 receptor, type I	IL-1R1
Chemokine (C-C motif) ligand 18 (pulmonary and activation regulated)	CCL18
Scavenger receptor class B, member 1	SCARB1
CD14 molecule	CD14
Macrophage scavenger receptor 1	MSR1
Chemokine (C-X-C motif) receptor 4	CXCR4
Chemokine (C-C motif) ligand 23	CCL23
Toll-like receptor 5	TLR5
Complement component 5a receptor 1	C5AR1
Transforming growth factor, beta receptor II (70/80 kDa)	TGFBR2
Major histocompatibility complex, class II, DM beta	HLA-DMB
Chemokine (C-C motif) ligand 17	CCL17
Fc fragment of IgG, low-affinity III receptor	CD16
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12
Transforming growth factor, alpha	TGFA
Major histocompatibility complex, class II, DMA/B	HLA-DMA/B
CD4 molecule	CD4
Interleukin 17 receptor B	IL-17RB

Information derived from Martinez et al. [44]

in antigen presentation and costimulation) [47]. Fibronectin expression is particularly significant because it is involved with tissue repair and is also important in the development of fibrosis. Dysregulated overexpression of fibronectin may be an important factor in the lung fibrosis observed in idiopathic pulmonary fibrosis (IPF) [48].

M2b macrophages are induced by treatment with the combination of immune complexes (IC) and either LPS or IL-1 β . M2b cells exhibit an anti-inflammatory cytokine expression profile but do produce low levels of IL-1, IL-6, and TNF- α . In addition, Th2 responses and immunoglobulin class switching in B cells are promoted by these cells [2].

Finally, M2c macrophages are induced by stimulation with either glucocorticoids, IL-10, or TGF- β [2, 45]. These cells produce elevated levels of the anti-inflammatory cytokine, IL-10, and these cells can promote tissue repair, remodeling, and wound healing [2, 49]. The relationship of M2b and M2c remains uncertain, and it is not clear that the M2b and M2c phenotypes are truly distinct.

Tumor-associated macrophages (TAMs) appear to represent a hybrid of macrophage subset phenotypes. These cells reside within many solid tumors and appear to regulate angiogenesis, lymphogenesis, and tissue repair functions which promote tumor progression [50–53]. These cells share many of the characteristics of M2 cells such as expression of CD206, scavenger receptors, and TGF- β and can inhibit proinflammatory processes [54]. However, TAMs can also express certain M1 cytokines including TNF- α , IL-1 β , and IL-6 but have little capacity to produce NO or ROS [46, 55, 56]. This is an active area of research understanding the relationship between tumor cells and TAMs.

We would hypothesize that the induction and resolution of immune responses by macrophages are dependent upon local environmental status [5]. For example, in a situation where tissue has been damaged, the resident macrophages would be directed toward an M2 program of tissue remodeling, wound repair, and angiogenesis in an effort to repair the physical damage that has occurred. During an infection, one might expect an M2 macrophage response to occur near the termination of an M1 macrophage response, which would normally have resolved the infection. At the same time, the M2 macrophages would be involved in tissue remodeling and wound repair as needed. However, aberrant or chronic activation of M2 cells would likely result in inappropriate tissue remodeling, and potentially fibrosis which is observed in chronic inflammatory diseases such as idiopathic pulmonary fibrosis (IPF), or scleroderma.

Monocyte Subtype and Bacterial Infection

Macrophages are dynamic and heterogeneous cells; they undergo activation in response to various inflammatory and immune stimuli, and the mode of their activation will determine the success or failure of the host response to the infection [57]. Benoit et al. [58] compared microarray data from 12 studies on the response of human and mouse macrophages to several bacteria and bacterial components and found that the common response of macrophages to bacterial infections mainly

involves upregulation of genes involved in M1 polarization. For example, during the acute infection with *Salmonella typhi*, control of the infection is associated with an upregulation of M1 genes [58]. However, during the convalescence period, M1 macrophages are replaced by M2 macrophages. Convalescent patients who retain the M2 phenotype for prolonged periods may be more susceptible to reinfection, relapse, or the establishment of a carrier state [59].

As stated before in this chapter, the CD14^{DIM} CD16+ monocytes represent about 5–10 % of monocytes in healthy adults, they produce high levels of TNF- α [23], and they are considered to represent an activated or more mature subset than the CD14++CD16- monocytes [60]. These proinflammatory monocytes have been shown to be elevated in bacterial sepsis in adults [61–63], in neonates, and small children [64] compared to normal control subjects. In a case of self-induced urinary tract infection and sepsis with gram-negative bacteria, there was an increase in the level of TNF- α , IL-6, and monocyte colony-stimulating factor (M-CSF) followed by a rise in the number of circulating blood CD14^{DIM} CD16+ monocytes 24 h after the infection [65]. These data suggest that the accumulation of peripheral blood nonclassical monocytes is induced as a part of the response to bacterial infection.

Herra et al. [66] found an increase number of circulating CD16+ monocytes in patients with evidence of systemic or localized bacterial infection when compared to healthy controls. Sixteen patients had positive blood cultures, four patients had gram-negative pneumonia, one patient had peritonitis due to *E. coli*, and another patient had *Clostridium difficile*-associated diarrhea. In this study, infection with gram-negative bacilli appeared to promote a higher level of circulating nonclassical monocytes than gram-positive bacteria. Similar results have been reported which show an elevation of peripheral blood CD14^{DIM} CD16+ monocytes during bacterial infections including patients with erysipelas [67], hemolytic uremic syndrome (HUS) [68], and pulmonary tuberculosis [69].

Macrophage Subtypes and Bacterial Infection

In the acute phase of *Mycobacterium tuberculosis* infection, macrophages exhibit an M1 phenotype [70]. However, there is growing evidence that macrophages infected with *M. tuberculosis* can also induce an M2 polarization. There is a subset of patients who present upregulation of genes involved in Th2 immune responses who can be reversed to a Th1 response after successful treatment [71–73]. Rajaram et al. [74] demonstrated that Mycobacteria upregulates the expression and activity of peroxisome proliferator-activated receptor (PPAR)- γ in macrophages. The upregulation of PPAR- γ induces an M2 phenotype which in turn increases the intracellular survival of mycobacteria [74].

Leprosy is a disease that presents as a clinical and immunological spectrum. Patients develop a clinically progressive or disseminated lepromatous form versus a self-limited or tuberculoid form of the disease depending on the type of immune response that results from the infection of macrophages by the *Mycobacterium leprae* [75]. The M2 phenotype predominates in lepromatous lesions whereas the tuberculoid lesions are

dominated by M1 macrophages [76]. There is also a dynamic change in macrophage functional programs in which a shift from an M2 to an M1 phenotype occurs in patients who convert from the disseminated to the tuberculoid form of the disease.

Whipple's disease is a chronic bacterial infection caused by *Tropheryma whipplei*. In this disease, there are elevated numbers of CD163+ monocytes in the peripheral blood, and M2 cells are the predominant macrophage phenotype in the infected duodenum [77]. Moreover, M2-type chemokines and cytokines (CCL2 and IL-10) are elevated in the duodenum and in peripheral blood of patients infected with *T. whipplei* compared to healthy controls. Therefore, in Whipple's disease, there is polarization toward an alternative macrophage activation which is apparent both locally in the duodenum and also systemically in the circulation. In addition, these macrophages and monocytes produce low levels of nitrite and have a decreased capacity to exhibit an oxidative burst compared to healthy individuals. The overall anti-inflammatory milieu may facilitate the invasion of the intestinal mucosa by macrophages with impaired function, carrying *T. whipplei* which can sustain of the infection in the tissue [77].

Chronic rhinosinusitis (CRS) with nasal polyps is characterized by Th2 inflammation, and CRS without polyps by a Th1 immune response [78]. Krysko et al. [79] demonstrated that the mucosa of CRS patients with nasal polyps had an increased number of M2 macrophages when compared to CRS without polyps and controls, while the number of M1 macrophages among the patient groups was not different. This shift may be caused by IL-33, which is increased in patients with CRS with polyps, and it is known to switch the phenotype of alveolar macrophages to an M2 phenotype [80]. In addition, evidence suggests that the macrophages from patients with CRS with polyps exhibit deficient phagocytosis of *Staphylococcus aureus* [79]. This finding could explain the higher rate of colonization by *S. aureus* seen in this condition compared to controls [81]. The M2 signature combined with the deficient phagocytosis could work to prolong the inflammation in chronic rhinosinusitis with nasal polyps.

Monocyte and Macrophage Subtypes in Viral Infection

Monocytes and macrophages play a crucial role in HIV-1 persistence and contribute to the reservoir of cell-associated virus. A number of investigators have described an increase in the percentage of CD14^{DIM}CD16+ monocytes in patients infected with HIV-1 compared to seronegative controls [82–86]. In normal individuals, this subset of monocytes represents less than 10 % of the monocyte population [1]. However, Thieblemont et al. [85] found that in patients with AIDS the percentage of CD14^{DIM}CD16+ monocytes may represent up to 40 % of the total circulating monocyte cell population, and Pulliam et al. [86] found this percentage to be the highest in patients with HIV-associated dementia.

Monocytes are nearly refractory to HIV-1 infection but become susceptible to infection when they differentiate into macrophages in culture [87, 88]. As stated above, CD14^{DIM}CD16+ monocytes are a more mature subpopulation of monocytes,

and data shows that they are more susceptible to HIV-1 entry, are more permissive for replication, and harbor the virus long term [89, 90]. Furthermore, the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3G) is associated with resistance to HIV infection. CD14+ monocytes express elevated levels of APOBEC3G whereas this protein is inactive in CD16+ monocytes [89] which may explain the increased susceptibility to infection of nonclassical monocytes. Finally, the chemokine receptor CCR5 serves as a coreceptor for M-tropic HIV-1 entry into CD4 expressing cells, and the increased susceptibility may also be explained by the enhanced CCR5 expression following monocyte differentiation [91, 92]. However, little is known regarding the effects of macrophage polarization on susceptibility to HIV infection. Results suggest that polarization into an M1 phenotype is associated with resistance to CCR5-dependent HIV-1 infection [93].

While the immune response to viruses is often largely Th1 mediated, in the case of dengue virus (DV), there is evidence to suggest that a Th2-mediated response is also involved [94]. Miller et al. [94] showed that the dengue virus binds to the macrophage mannose receptor (MR), a protein preferentially expressed by M2 macrophages, and that the type 2 cytokines IL-4 and IL-13 increase the susceptibility of macrophages to DV infection. These cytokines induce M2 macrophage polarization and MR expression. Additionally, these investigators showed that DV infection can be blocked by using an anti-MR antibody.

Merkel cell polyomavirus (MCPyV) is a new member of the two previously known human polyomaviruses JCV and BKV. MCPyV has been implicated in the oncogenesis of Merkel cell carcinoma (MCC) [95], an uncommon but aggressive neuroendocrine skin neoplasia [96]. The transmission route and the reservoir of this virus have not been fully established. There is evidence that monocytes can harbor viruses and establish a long-term reservoir. For example, the nonclassical monocytes CD14^{DIM}CD16+ have been reported to harbor HIV [89], while Mertz et al. [97] identified two patients in which the classical inflammatory monocytes CD14++CD16- were the only reservoir of the MCPyV virus in the blood.

Human cytomegalovirus (HCMV) is an opportunistic pathogen that poses a significant health risk in immunocompromised individuals. It has the ability to evade the immune response and establish a chronic infection, and monocytes are the primary target [98]. HCMV induces an accelerated rate of differentiation of blood monocytes into macrophages, promoting migration, viral replication, and dissemination to host organ tissues [99]. Furthermore, HCMV infection induces simultaneous upregulation of genes and chemokines implicated in M1 and M2 macrophage activation with a predominance of the M1 phenotype [100].

Monocyte and Macrophage Subtypes in Parasitic Infection

Our current understanding of alternatively activated macrophages has been driven in part because of extensive investigation of these cells in parasitic infections. While most of the data comes from animal studies, there are interesting studies conducted in humans, some of which will be reviewed in this section.

Helminthic Infections

Nematodes

Intraperitoneal infection of mice with *Brugia malayi* (a nematode responsible for filarial infection) induces an IL-4-mediated Th2-type response and the recruitment of M2 macrophages. Furthermore, these alternatively activated macrophages can suppress T cell proliferation [101]. Similar results were found in studies with *Litomosoides sigmodontis*, another nematode that causes filariasis [102]. However, the role of M2 macrophages in filariasis is uncertain.

Nippostrongylus brasiliensis is an intestinal nematode that infects through the skin and migrates to the lung where it induces a strong Th2 cell-mediated immune response and activates M2 macrophages [103]. The larvae are then coughed up and swallowed. Even though *N. brasiliensis* is not retained in the lungs, there is evidence that lungs from mice infected with the parasite develop damage to the alveoli and severe airway hyperresponsiveness resembling emphysema and chronic obstructive pulmonary disease (COPD) [104].

Heligmosomoides polygyrus is a gastrointestinal nematode that triggers a Th2-type response and can induce chronic infections. An interesting fact about this parasite is that alternatively activated macrophages in this case protect the host against reinfection [105]. In this infection as in *B malayi*, *L. sigmodontis*, and *N. brasiliensis* infection, M2 macrophages were only detected at the site of infection [102].

Trematodes

Schistosoma mansoni causes schistosomiasis, a disease that affects millions of people worldwide. Alternatively activated macrophages isolated from liver granulomas of a mouse model infected with this parasite were able to anergize T cell (CD4 and CD8) responses [106]. During this infection, a Th2 immune response dominates over a Th1 response and is thought to be protective. This is consistent with the observation that infected IL-4^{-/-} mice are unable to mount a Th2 immune response and die from acute schistosomiasis [107].

Cestodes

Taenia crassiceps infection represents a good model to study the immunopathological mechanisms involved in the parasite-host interactions that occur in cysticercosis [108, 109]. The initial response to *T. crassiceps* infection is a brief Th1 response, followed by a mixed Th1/Th2 response, and finally a predominant Th2 response [110, 111]. Alternatively activated macrophages induced by this parasite have suppressive activity and low proinflammatory properties which could be a defense mechanism to prevent a dangerous inflammatory burst [102]. In human neurocysticercosis (caused by

Taenia solium), a Th2 response promotes a silent resolution of the infection and was associated with asymptomatic neurocysticercosis [112, 113], whereas a high inflammatory profile was associated with severe disease symptoms [114].

Protozoan Infections

During the acute phase of infection with protozoan parasites such as *Leishmania*, *Toxoplasma*, *Plasmodium*, and *Trypanosoma*, a type 1 immune response with classically activated macrophages predominates [115]. However, during the course of the infection, Th2-type anti-inflammatory immune responses can also occur and attenuate exaggerated type 1 responses to avoid damage to host tissue. In human malaria, excessive production of TGF- β and IL-10 inhibit type 1 immune responses and facilitate parasite replication. However, failure to produce these cytokines is associated with severe malaria. Walther et al. [116] found significant interindividual variability in innate responses from 18 healthy subjects with recent *Plasmodium falciparum* exposure and divided them into groups depending on their proinflammatory responses. The group that mounted the highest proinflammatory response had a more rapid control of parasite growth but at the expense of developing clinical symptoms. The group that had an undetectable inflammatory response were less likely to control parasite growth but had no symptoms [116]. A balance between proinflammatory and anti-inflammatory responses is crucial for host survival. In *Plasmodium* infection, early elevated levels of IL-10 and TGF- β resulted in death from overwhelming infection from inhibition of the proinflammatory cytokines IFN- γ and TNF- α [117]. Conversely, *Toxoplasma gondii* infection of IL-10 $^{-/-}$ mice induced an uncontrolled immune response with lethal overproduction of IFN- γ and TNF- α [118].

Blood monocytes from patients with human malaria also exhibit interesting changes. In healthy malaria-exposed individuals, the majority of monocytes are classical monocytes, and in patients with acute uncomplicated malaria, the percentage of intermediate and proinflammatory monocyte subsets increases [119]. Additionally, patients with elevated levels of a unique phenotype of the CD14++ monocyte subset that expresses CCR2 and CX3CR1 had low levels of parasitemia, suggesting that activation of this particular monocyte subset is associated with effective infection control [119].

Trypanosoma cruzi is an intracellular parasite that resides in macrophages and B cells and causes Chagas disease. As with other protozoan infections, there is an initial Th1 immune response with a subsequent switch to a Th2 response and induction of alternatively activated macrophages. These M2 macrophages downregulate the inducible nitric oxide synthase (iNOS) and hence are responsible for persistent *T. cruzi* intracellular growth and might favor chronic infection [101].

It is evident that each of the monocyte subtypes can play an important role in the innate immune response to certain parasitic infections. Excessive activation of M1 or M2 macrophages can be either protective or destructive. Hence, modulation of macrophage activation is paramount since the outcome is dependent on a careful balance between proinflammatory and anti-inflammatory responses.

Monocyte and Macrophage Subtypes in Fungal Infections

Aspergillus fumigatus is a mold that causes severe infections in the immunocompromised host. It is the leading cause of infection-related death in patients undergoing stem cell transplantation and in patients with acute leukemia [120]. Following inhalation of the conidia, the process of germination takes place within the alveolar space of individuals who are unable to mount an appropriate innate immune response. This leads to the formation of hyphae which have invasive and pathogenic properties [121]. During *Aspergillus fumigatus* infections in mice, M2 cells are the predominant alveolar macrophages present in the lungs, and they appear to have a protective effect for effective conidial phagocytosis [122]. While alveolar macrophages are the first line of defense against inhaled conidia [121], there is also evidence that circulating blood monocytes contribute to the innate immune response against these fungal elements. Serbina et al. [123] demonstrated that both human monocyte subtypes have effective phagocytosis capabilities against conidia but, interestingly, only CD14++CD16- monocytes inhibit germination. These results are surprising because CD14^{DM}CD16+ nonclassical monocytes are thought to be superior to the CD14++CD16- classical monocytes in terms of their inflammatory properties [23]. Consistent with these findings are the results seen in the response of human monocytes to *Candida albicans* [124]. Even though both monocyte subsets inhibited the germination of *C. albicans* and did not differ in their phagocytosis capabilities, only CD14++CD16- cells were able to induce a strong Th17 response to effectively kill the fungus. Th17 responses induce IL-17A, an important cytokine that protects the host against systemic, mucosal, and chronic mucocutaneous candidiasis [124].

Both monocyte and macrophage subpopulations play important but different roles in the innate immune response to infection with bacteria, viruses, fungus, and parasites. Understanding the different immune mechanisms involved in various infectious diseases could lead to the development of novel biomarkers or provide targets for therapeutic intervention.

Interstitial Lung Diseases

Interstitial lung diseases (ILD) are a heterogeneous group of rare diseases that are classified together because of similar clinical, pathological, or radiographic manifestations. In general, ILD is characterized by lung parenchyma injury resulting in inflammation and/or fibrosis [125]. There is increasing evidence that suggests a profibrotic role of alternatively activated alveolar macrophages in the pathogenesis of a variety of ILD. Recent studies have shown that bronchoalveolar lavage (BAL) cells from patients with sarcoidosis, scleroderma, and idiopathic pulmonary fibrosis (IPF) exhibit elevated levels of IL1-RA, CCL17, CCL18, and CCL22 compared to healthy controls [48]. These cytokines are M2 markers, indicating an alternatively activated phenotype of alveolar macrophages in patients with these fibrotic lung diseases. Moreover, in patients with IPF, there was an enhanced expression of CD206 and additional marker of M2 macrophages. Additional results showed that while stage I

and II sarcoidosis patients had little radiological evidence of lung fibrosis, stage III and IV had much greater radiological evidence of fibrosis. Interestingly in this study, the stage I patients had similar levels of M2-associated cytokine production compared with controls, but stage III and IV patients had a substantial increase in the production of CCL17, CCL18, and CCL22. These studies also showed that stimulation of alveolar macrophages and monocytes from normal individuals with Th2 cytokines IL-10 or IL-4 induced an M2 phenotype in vitro, and this induction was even more pronounced in patients with fibrotic lung disease. It appears that production of CCL18 by alternatively activated macrophages induces collagen production by fibroblasts, and fibroblast production of collagen enhances CCL18 production by M2 macrophages which sustain the fibrotic process [126]. In line with these findings, several other authors have reported an upregulation of M2-associated cytokines in IPF, sarcoidosis, scleroderma, idiopathic interstitial pneumonias, asbestos-induced lung disease, and hypersensitivity pneumonitis [126–131].

CCL18 has been suggested as a biomarker of disease progression in patients with pulmonary fibrosis [127]. Studies by Prasse et al. [127] showed that there was spontaneous expression of CCL18 by normal macrophages but its production was significantly higher in BAL of patients with pulmonary fibrosis (more than 100-fold). Additionally, there was an inverse correlation between the CCL18 levels in BAL and serum, with total lung capacity (TLC) and diffuse lung capacity for carbon monoxide (DLCO). Furthermore, patients whose TLC improved over the following 6 months had a decrease in CCL18 serum concentration and patients with progressive disease had an increase in CCL18 levels [127]. A more recent study confirmed the latter results showed that patients with serum CCL18 levels above 150 ng/ml exhibited a higher mortality rate and a higher risk of disease progression [132].

As stated before in this chapter, peripheral blood monocytes are the precursors to several cell lineages including macrophages and fibrocytes. There is evidence that peripheral blood monocytes from patients with scleroderma-associated ILD have a fibrogenic potential [133]. LPS stimulation of CD14+ monocytes caused an increased upregulation of the scavenger receptor CD163 (M2 marker) and the profibrotic chemokines CCL18 and IL-10 compared to age matched controls [133]. These are interesting findings because LPS is traditionally associated with classical activation (M1), and in this case, LPS stimulation of peripheral blood monocytes of patients with scleroderma led to an M2 phenotype. Moreover, this phenotype was found on blood monocytes before their transformation into alveolar macrophages and entry into the lungs. These data suggest that monocyte phenotypes may be preprogrammed to mature into M2 macrophages while still in the bloodstream [133, 134].

Chronic Obstructive Pulmonary Disease

Alveolar macrophages play a major role in the pathogenesis of chronic obstructive pulmonary disease (COPD). The number of alveolar macrophages is increased in the lungs of patients with COPD compared to smokers without COPD even after

partial matching for cigarette exposure [135]. Macrophages from COPD patients have been reported to have impaired phagocytic activity compared with cells from healthy smokers or nonsmokers [136]. In line with these findings are the results from another study that found impaired phagocytosis to non-typeable *Haemophilus influenzae* of alveolar macrophages from COPD patients [137]. The defective phagocytic activity may promote bacterial colonization of patients with COPD and increase their susceptibility to exacerbations. Moreover, there is evidence that both healthy smokers and patients with COPD acquire an M2-polarized phenotype with a concomitant downregulation of M1-related genes [138]. These data suggest that cigarette smoke alters the polarization state of alveolar macrophages, and this would be expected to alter the status of the inflammatory response. This downregulation of M1 activity may potentially decrease host defense, leading to higher susceptibility to respiratory infections and the increased airway bacterial colonization in smokers and COPD smokers [138, 139]. The finding that cigarette smoke suppresses the expression of macrophage inflammatory genes in COPD is consistent with these results [140].

Environmental factors in the lungs of COPD patients can influence macrophage activation and polarization. Macrophages incubated with sputum from patients with acute exacerbation of COPD (AECOPD) induced arginase and mannose receptor but no TNF- α induction which is suggestive of a shift to the M2 phenotype [141]. The cytokine IL-4, a known inducer of alternative activation of macrophages, is also increased in BAL from patients with AECOPD [142]. Taken together, these data suggest that there may be a shift to the M2-like phenotype in the lungs during AECOPD. Moreover, even though it was initially thought that COPD was predominantly a Th1-mediated disease, there is now evidence that macrophages in COPD exhibit the alternative activation phenotype [138, 143]. The role of these M2 macrophages in COPD and AECOPD is still unclear. Further research is necessary to elucidate whether they play a beneficial role by suppressing the inflammatory response or whether they are harmful either by promoting the development of destructive tissue remodeling or by increasing susceptibility to opportunistic infections.

Asthma

There is evidence that the innate immune response of children with poorly controlled asthma is impaired. This was suggested by the finding that the phagocytic activity of alveolar macrophages of children with poorly controlled asthma was decreased by 50 % when compared with that seen in adults or pediatric control subjects [144]. Current guidelines for the treatment of persistent asthma include steroid therapy [145]. However, there is a subset of patients with asthma who are corticosteroid resistant. In an effort to characterize this subset of patients, Goleva et al. [145] performed gene microarray analysis of BAL cells from patients with corticosteroid-resistant asthma and found significantly higher levels of M1-related genes and decreased levels of M2-related genes when compared to patients with

corticosteroid-sensitive asthma. These results were further confirmed by results obtained from RT-PCR and analysis of protein expression. Of note, corticoid-resistant asthma patients had elevated levels of LPS and in vitro exposure of monocytes to LPS induced cellular steroid resistance. These findings suggest that endotoxin exposure may contribute to corticoid resistance in these patients [145]. Of course, it is well established that there is upregulation of Th2-type cells and cytokines in asthma [146, 147]. Furthermore, flow cytometric analysis of peripheral blood monocytes from patients with untreated asthma show that there is an increase in the proportion of the CD14^{DIM}CD16⁺ nonclassical monocyte subset in these patients [148]. Finally, the number of macrophages is increased in the lungs of asthmatic patients but, in contrast to COPD, the role of these cells in pathogenesis of asthma remains uncertain [149].

Cystic Fibrosis

Cystic fibrosis (CF) is characterized by chronic airway inflammation. Patients with CF experience recurrent cycles of pulmonary infections that are responsible for the increased mortality and morbidity of this disease. By age eighteen, 80 % of the patients are chronically colonized with *Pseudomonas aeruginosa* [150, 151]. Initial studies on peripheral blood monocytes of patients with CF showed that the patients infected with *P. aeruginosa* produce more IL-4 and less IFN- γ compared to the noninfected patients, upon exposure to *P. aeruginosa* outer membrane proteins [150]. Studies evaluating BAL alveolar macrophages from patients with CF infected with *P. aeruginosa* reported increased levels of IL-4 and IL-13 expression and decreased levels of IFN- γ , and IL-4 and IL-13 levels are inversely correlated with lung function [152]. These findings are consistent with a Th2-type immune response. Murphy et al. [153] evaluated markers of alternative and classical macrophage activation in the lungs of patients with CF with and without *P. aeruginosa* infection. These investigators found that patients infected with *P. aeruginosa* exhibited increased expression of the M2 marker, mannose receptor, and higher arginase activity, consistent with M2 activation of alveolar macrophages. Furthermore, both markers inversely correlated with the forced expiratory volume in one second (FEV1). Of note, more than half of the patients were receiving treatment with azithromycin, especially the patients who had *P. aeruginosa* infection. Murphy et al. [154] have demonstrated that azithromycin can polarize mouse macrophages to the M2 phenotype. However, in a subgroup analysis, these investigators found no difference in expression of macrophage activation markers (arginase and mannose receptor) between the CF patients treated with or without azithromycin. It appears that CF patients infected with *P. aeruginosa* exhibit an enhanced expression of M2 macrophages that is not dependent on azithromycin treatment. However, the long-term consequences of an accumulation of alternatively activated macrophages in CF are still uncertain.

Atherosclerosis

Monocytes and macrophages play a crucial role in vascular plaque formation, and the available data suggests that differences in monocyte subtype populations contribute to the pathogenesis of atherosclerosis in humans. In a group of hypercholesterolemic patients, the number of CD14^{DIM}CD16+ monocytes correlated negatively with high-density lipoprotein (HDL) cholesterol levels and was associated with an increased expression of charged apo E4, which is related to higher plasma cholesterol levels [155]. Moreover, in a large group of 247 patients with angiographic evidence of coronary artery disease (CAD), the numbers of CD14^{DIM}CD16+ monocytes were elevated compared to the control group and correlated with serum levels of TNF- α [156]. Altogether, these data suggest that the proinflammatory CD14^{DIM}CD16+ monocytes may have a potential role in the development of atherosclerotic lesions.

There is evidence that classical monocytes also play a role in the pathogenesis of this disease. Patients with acute myocardial infarction (AMI) have increased levels of both CD14++ CD16- and CD14^{DIM}CD16+ monocyte subsets. In addition, peak levels of CD14++CD16- monocytes correlate negatively with the extent of myocardial salvage 7 days after AMI as well as with the recovery of left ventricular function 6 months after AMI [157]. This study suggests that CD14++CD16- monocytes may promote cardiac injury and remodeling; however, the precise mechanism remains unclear.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects multiple synovial joints. Monocytes and macrophages contribute to the perpetuation of chronic inflammation as well as for the joint damage in RA [158]. The nonclassical monocytes have been implicated in several inflammatory diseases including RA. The percentage of CD14^{DIM}CD16+ monocytes is significantly increased in patients with RA when compared to healthy subjects [159, 160]. Furthermore, patients with an increased frequency of CD14^{DIM}CD16+ monocytes have more active disease indicated by tender and swollen joints, elevated erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF). In addition, patients who respond to therapy have a decrease in the frequency of nonclassical monocytes, while the nonresponders experience an increase in frequency [159]. Baeten et al. [161] found that CD14^{DIM}CD16+ monocytes express human cartilage gp-39 (HC gp-39), a glycoprotein that has been proposed as an autoantigen in RA. These investigators also found that this monocyte subtype is increased in the peripheral blood as well as in the synovium of RA patients with active disease, and the presence of HC gp-39 in the synovial tissue correlates with radiological joint destruction [161]. Furthermore, the expression of the chemokine receptors CCR1, CCR5, and ICAM-1

is stronger in CD14^{DIM}CD16+ monocytes of patients with active RA [159]. Altogether, these findings suggest that nonclassical monocytes have an enhanced ability to infiltrate the inflamed joint and may contribute to joint destruction and persistence of inflammation in RA.

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Neutrophil Inflammation in COPD

Paul A. Kennedy and Laurie E. Kilpatrick

Introduction: Role of Neutrophils in COPD

COPD is a disease of chronic inflammation which persists in patients after smoking cessation and intensifies as the disease progresses [1–3]. Patients with COPD, particularly those at risk for more frequent or severe exacerbations, exhibit an exaggerated innate immune response associated with elevated levels of inflammatory mediators and evidence of oxidative stress [1, 4–7]. Lung inflammation plays a key role in the pathophysiology of COPD and affects the airways, lung parenchyma, and pulmonary vasculature. Inflammation is not limited to the lung compartment. Systemic inflammation is an established feature of COPD, often accompanied by inflammation in the heart, blood vessels, and skeletal muscle which contribute significantly to disease morbidity and mortality [6, 8–10].

Neutrophils are key cells of the innate immune system. While neutrophils are critical to host defense against pathogens, neutrophil dysregulation can contribute to the tissue damage of inflammation. Neutrophilic inflammation is a well described characteristic of COPD and neutrophil infiltration of the lungs is increased in smokers and COPD patients, particularly those with chronic bronchitis [10–14]. There are increased numbers of neutrophils throughout the respiratory tract with the highest numbers found in the airway lumen suggesting rapid directed migration through the airways [6, 10–12, 15–17]. However, neutrophils are also found in lung parenchyma and airway smooth muscle. Neutrophil numbers, particularly those in the sputum, correlate with disease severity in COPD and are associated with the extent of airway obstruction and rate of decline in FEV₁ [2, 6, 10, 11, 17–20]. Exacerbations in COPD are often associated with respiratory infections, which further increase the number of neutrophils migrating into the lung [4, 15, 21]. The elevated levels of

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neutrophils correlate with severity of exacerbation and are independent of the origin of infection (i.e. viral or bacterial) [22].

The sustained recruitment of neutrophils to the lung plays a pivotal role in the pathophysiology associated with COPD [4, 6, 12, 15, 23, 24]. This excessive accumulation of activated neutrophils is the result of persistent inflammation stimulating neutrophil influx, increased cell survival through inhibition of neutrophil apoptosis, and impaired macrophage clearance. Host tissue damage and lung destruction result through the inappropriate release of neutrophil-derived oxygen radicals, proteases, matrix metalloproteinases (MMPs), lipid mediators, and cytokines; which eventually results in extracellular matrix degradation and the development of emphysema [4, 10, 14, 23, 25].

Neutrophil Basics

Neutrophils are produced in the bone marrow where they develop from CD34+ pluripotent stem cells in response to the colony stimulating factors IL-3, G-CSF, and GM-CSF [26]. Neutrophils are released from the bone marrow as terminally differentiated end-stage cells which do not proliferate, rather these cells undergo constitutive apoptosis or programmed cell death [27, 28]. Neutrophils are found in two pools, a circulating pool as well as a marginated pool where neutrophils are sequestered in the microvasculature of various organs including the lung [26]. In the circulation, neutrophils have a relatively short half life of 8–20 h when they are either recruited to sites of inflammation or cleared by macrophages in the lung, spleen, or liver [28, 29]. Cigarette smoke and other particulate pollutants stimulate production and release of mature and immature neutrophils from the bone marrow as well as rapid demargination resulting in increased levels of circulating neutrophils [30–32]. This stimulation of the bone marrow is thought to be the result of alveolar macrophage activation through phagocytosis of inhaled particles and subsequent release of soluble mediators.

Neutrophils are members of the granulocyte family that also includes eosinophils and basophils. Granulocytes contain different types of granules in their cytoplasm, which are released in a hierarchical fashion upon stimulation. Neutrophils contain secretory, primary, secondary and tertiary granules which include over 300 proteins involved in cell adhesion, migration and bactericidal activities [26]. The rate of granule production and maturation time may be influenced by growth factors and cytokines in response to inflammation and infection [27]. Degranulation follows a hierarchy with secretory granules the first to be mobilized in response to chemoattractants such as CXCL8 (IL-8) or the bacterial peptide fMetLeuPhe [26, 33, 34]. These vesicles contain membrane proteins such as β -integrins critical for adhesion and migration, and can be mobilized without releasing cytotoxic mediators [34]. Specific and tertiary granules are then released and have a higher threshold for exocytosis compared to secretory vesicles. These granules contain lactoferrin, lysozyme and matrix metalloproteinases (MMP8, MMP9, MMP25) which degrade collagen, fibronectin and proteoglycans, and facilitate neutrophil migration to sites of inflammation and injury [28].

These proteolytic enzymes can also alter cytokine/chemokine functions, cleave cell receptors, and increase neutrophil migration [35, 36]. The last granules to be released are primary or azurophilic granules which require a stronger stimulus and contain potent enzymes including myeloperoxidase, neutrophil elastase and cathepsin G [34]. These enzymes are capable of degrading the extracellular matrix and their release is tightly regulated to minimize host tissue damage.

Several neutrophil proteases are implicated in the pathophysiology of COPD including neutrophil elastase, proteinase-3 (PR3), cathepsins, and MMP8 and MMP9 [1, 3, 37–39]. Elevated levels of these proteases have been reported in lung samples from smokers and COPD patients as compared to healthy control subjects [36, 38–40]. These proteases can contribute to lung injury and airspace enlargement in COPD through extracellular matrix (ECM) degradation and alveolar wall destruction [38]. These proteases also enhance lung inflammation, promote excessive mucus secretion, and impair mucociliary clearance mechanisms [1, 37, 40]. Degranulation and excessive release of these proteases activate macrophages to release proinflammatory mediators such as LTB₄, cathepsin G, reactive oxygen species (ROS), and proteinases [10]. Neutrophil elastase can also cleave macrophage phosphatidylserine receptor resulting in impaired clearance of apoptotic cells and enhanced inflammation [41]. ECM degradation causes the release of pro-inflammatory fragments, which further attract inflammatory cells to the site of inflammation [42, 43]. MMPs also contribute to ECM destruction, and MMP9 released from neutrophils can degrade the elastase inhibitor serine protease inhibitor α 1-antiproteinase, thus enhancing elastase activity [44]. MMP9 increases CXCL8 activity, a potent neutrophil chemoattractant which further stimulates neutrophil migration [36, 44]. Neutrophil elastase, proteinase-3 (PR3), and cathepsins also stimulate mucus production and secretion from submucosal glands and goblet cells in the epithelium. This mucus hypersecretion is thought to contribute to airway obstruction in COPD [1].

Neutrophil Activation

Upon infection or tissue injury, neutrophils are rapidly recruited to the sites of inflammation. In the lung, the innate immune response is activated by the detection of pathogens, tissue injury or environmental exposures such as cigarette smoke or air pollution [12, 45]. Resident macrophages, neutrophils, dendritic cells, endothelial, and epithelial cells detect pathogens or tissue injury by pattern recognition receptors (PRRs) [46]. Pathogens are recognized by pathogen-associated molecular patterns or PAMPs, while damaged or necrotic cells release intracellular contents that act as damage associated molecular patterns (DAMPS). Several molecules can act as DAMPs including High-Mobility Group Box-1 (HMGB1), hyaluronan, heparan sulfate, heat shock proteins, and ATP [47]. This defense mechanism is critical in the lung where the innate immune system is in constant contact with inhaled pathogens and pollutants [24]. Elevated levels of different DAMPS have been identified in bronchoalveolar lavage fluid (BALF) of COPD patients including uric acid, extracellular ATP, and HMGB1 [1, 48, 49].

PAMPs and DAMPs are recognized by PRRs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and purinergic receptors [47, 50, 51]. PRRs activate multiple proinflammatory signaling pathways including NLRP3 (NLR family, pyrin domain-containing) inflammasome and transcription factors NF κ B, AP-1, and C/EBP- β triggering the release of ROS, proteolytic enzymes (i.e. MMPs and neutrophil elastase), proinflammatory cytokines, lipid mediators (LTB₄), and neutrophil chemoattractants such as CXCL8 [1, 46, 52–54]. Inflammasome activation leads to increased release of IL-1 β and related IL-1 family cytokines. In COPD, IL-1 β levels are elevated and correlate with disease severity [55]. The accumulation of proinflammatory mediators contributes to lung injury and serves as a self-perpetuating stimulus for further immune activation by stimulating increased influx of activated neutrophils, macrophages, and lymphocytes that contribute to lung tissue damage [6].

TLRs are an important PRR family expressed on neutrophils and recognize a wide range of PAMPs and DAMPs [12, 50]. Human neutrophils express all TLRs except for TLR3. TLR2 and TLR4 have critical roles in modulation of neutrophil life span and TLR5 in regulating neutrophil antibacterial responses [12]. In vitro studies demonstrated that cigarette smoke extract (CSE) activates TLR4-mediated signaling, a receptor thought to have a critical role in the development of lung emphysema [56]. Moreover, CSE-induced production of the chemokine CXCL8 is mediated through TLR9 signaling in neutrophils [56]. Studies in smokers with COPD who were in acute respiratory failure, had increased expression of TLR4, but not TLR2, on BALF neutrophils suggesting a role for TLR4 in promoting airway neutrophilia in COPD [57].

Neutrophil Chemoattractants in COPD

Lung inflammation in COPD is associated with increased levels of neutrophil chemoattractants. Neutrophil chemokines CXCL8 (IL-8), CXCL1 (GRO α) and CXCL5 (ENA78) are elevated in the sputum or BAL fluid of COPD patients [10, 20, 21, 58–60]. Other chemoattractants found to be elevated and may contribute to neutrophil migration in COPD include leukotriene B₄ (LTB₄), C5a, and elastase- α_1 -antitrypsin complexes [20]. Two CXCL8 receptors, CXCR1 and CXCR2, are expressed on neutrophils. CXCR1 is specific for CXCL8 and CXCL6 (GCP-2) while CXCR2 interacts with multiple chemokines, including CXCL8, CXCL1, CXCL5, and CXCL7. Both receptors bind CXCL8 with similar affinity yet may mediate distinct functions [61]. Neutrophil activation through CXCR1 leads to degranulation, superoxide anion (O₂ $^-$) generation and upregulation of CD11b. Signaling through this receptor is required for bactericidal activity, and loss of CXCR1 surface expression reduces neutrophil-mediated killing [9, 61, 62]. CXCR2 is functionally different than CXCR1, and does not lead to O₂ $^-$ production in response to ligand interactions [62]. CXCR2 appears to mediate the initial recruitment of neutrophils from the circulation after which the receptor loses signaling capacity as its expression is rapidly downregulated [63]. CXCR1 is generally more

resistant to desensitization and downregulation, and may facilitate directed neutrophil migration to sites of high CXCL8 concentrations [63].

The tripeptide, proline-glycine-proline (PGP), has recently been implicated in the chronic lung inflammation associated with COPD. PGP, an extracellular matrix collagen breakdown product, is a selective neutrophil chemoattractant [43, 64, 65]. PGP is elevated in the lungs of COPD patients and is associated with increased neutrophil numbers [43, 66]. PGP is normally degraded in the lungs by leukotriene A4 hydrolase (LTA4H). This hydrolase converts leukotriene A₄ into the neutrophil chemoattractant LTB₄ [65]. This enzyme is also an aminopeptidase that degrades PGP. Cigarette smoke selectively inhibits LTA4H aminopeptidase activity but not hydrolase activity resulting in increased levels of both LTB₄ and PGP in the lung promoting neutrophil influx and chronic inflammation [43, 65, 67]. PGP-mediated neutrophil chemotaxis is associated with CXCR1 and CXCR2. A recent study suggests that PGP itself does not interact directly with CXCR2 but is dependent on CXCR2-mediated neutrophil recruitment, and PGP may activate an alternate pathway leading to the enhancement of neutrophil chemokines in the lung [64]. PGP is also regulated through degradation by extracellular peptidases. However, an acylated derivative of this tripeptide N-acyl-PGP, also a neutrophil chemoattractant, is resistant to degradation by extracellular peptidases. Increased oxidative stress produced by smoking, or increased oxidative burdens, positively correlate to acylation of PGP further promoting lung inflammation [68].

Recruitment of Neutrophils to the Lung

A key step in neutrophil-mediated lung damage is the migration of neutrophils across the pulmonary endothelium. Recruitment of neutrophils is a dynamic process which requires interactions between neutrophils and vascular endothelium. The endothelium has an active role in the recruitment and activation of neutrophils through the production of cytokines/chemokines and expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [69]. The movement of neutrophils out of the pulmonary circulation is different from other organ systems and the lung architecture makes it an organ that is particularly susceptible to neutrophil accumulation [70–72]. In most organs neutrophil accumulation is mediated through post-capillary venules, while in the lung, neutrophils move out of the pulmonary circulation across capillary endothelium [73–75]. Due to the small diameter of the pulmonary capillary vessels (often less than the size of a neutrophil), neutrophil transit time is prolonged in the lung as the neutrophils must stop and change shape in order to traverse the small vessels. Increased transit time results in a significant accumulation of neutrophils in the lung (marginated pool) [74]. During inflammation, cytokines and proinflammatory mediators activate neutrophils and alter their biomechanical properties. Neutrophil activation results in increased integrin expression and actin polymerization producing neutrophil stiffening. This activation results in increased transit time and neutrophil adherence to activated endothelium thereby trapping neutrophils in

the pulmonary capillaries and precludes the need for selectins normally required to facilitate rolling and tethering to the endothelium [31, 73–79]. Increased neutrophil adhesion to endothelial cells and subsequent transendothelial migration can occur via at least two different pathways; a β 2-integrin dependent and a β 2-integrin-independent pathway. Key adhesion molecules involved include members of the β 2-integrin family (CD11a/CD18, CD11b/CD18, CD11c/CD18), as well as the CD29 integrin $\alpha_4\beta_1$ (CD49d/CD29) on neutrophils, and the adhesion molecules ICAM-1, ICAM-2, VCAM-1, and PECAM-1 on endothelial cells [75, 80–82]. Increased expression of the β 2-integrin CD11b/CD18 (MAC-1) has been reported on peripheral blood neutrophils as well as sputum neutrophils obtained from COPD patients [9, 20, 83–85]. In contrast, neutrophil CD11a/CD18 (LFA-1) expression is not increased in these patients suggesting a selective response.

Transendothelial migration is via a transcellular or paracellular route. The majority of neutrophils migrate using the paracellular route at endothelial junctions at bi-cellular or tri-cellular corners [34, 73, 86]. Neutrophil adherence induces remodeling of tight junctions involving PECAM-1, JAM-A, CD11b/CD18 and CD11a/CD18. This transient remodeling permits paracellular neutrophil migration at endothelial tight junctions. In contrast, during transcellular migration, neutrophils move through the body of the endothelial cell via transmigratory cups that contain clusters of ICAM-1 and VCAM-1. Movement through the endothelial cell may be facilitated by pore formation [34, 78].

Following migration through the endothelium, neutrophils traverse the basement membrane to reach the airway epithelial layer and airspace. Transmigrating neutrophils release proteases, cationic peptides, and oxygen radicals that breakdown basal membrane collagens and lamins increasing tissue permeability [34]. Epithelial passage requires neutrophil adherence, migration, and post-migration cell interactions. Neutrophil adherence to the basolateral surface is via neutrophil CD11b/CD18 ligation with fucosylated glycoproteins [87, 88]. Neutrophil transepithelial migration is only via the paracellular route and neutrophils traverse tight junctions through interactions neutrophil JAM-like protein and CAR (coxsackie and adenovirus receptor). Upon arrival on the apical side of the epithelium, neutrophils are retained on the surface though ICAM-1 and Fc interactions [87, 88]. Thus, neutrophil recruitment to the lung requires a series of steps entailing activation, adherence and migration which starts with sequestration in the pulmonary capillaries, transendothelial migration from the blood to the interstitial space, and finally transepithelial migration from the interstitial space into the airspace.

Neutrophil Killing

At the site of inflammation, neutrophils rapidly clear invading pathogens through phagocytosis, production of toxic oxygen radicals, and the release of proteases and antimicrobial proteins through degranulation. Oxygen radical production is tightly regulated and requires the assembly of an active NADPH oxidase complex.

The NADPH oxidase uses cytosolic NADPH to reduce molecular oxygen to produce superoxide anion (O_2^-). Assembly of the NADPH oxidase and generation of O_2^- requires the translocation of two cytoplasmic components to the plasma membrane: the p67phox:p47phox:p40phox complex and rac2 which exchanges GDP for GTP [34, 89–91]. Upon neutrophil activation, these complexes translocate to the plasma membrane where they interact with integral membrane components p22phox and Nox2 (gp91phox) that form the cytochrome b₅₅₈. The O_2^- formed is then converted to hydrogen peroxide and other reactive oxygen intermediates such as hydroxyl radicals. Myeloperoxidase released from azurophilic granules can also convert hydrogen peroxide to hypochlorous acid (the active ingredient in bleach) [91]. Smoking elevates neutrophil MPO levels and MPO activity demonstrates a negative correlation with FEV₁ in COPD patients [92]. Neutrophils from COPD patients also produce increased levels of reactive oxygen species as compared with nonsmokers and healthy smokers [10, 85, 92, 93]. The increased production of oxygen radicals in COPD patients may be the result of “priming”. Priming does not activate neutrophils directly but affects subsequent activation through inhibition of apoptosis, upregulation of adhesion molecules and assembly of the NADPH oxidase enzyme complex [12]. Neutrophil transmigration through activated pulmonary endothelium leads to a proinflammatory phenotype, increased expression of the $\beta 2$ -integrins and neutrophil priming for enhanced oxygen radical production [94, 95]. This enhanced production of superoxide anion further enhances the oxidative stress produced by inhalation of cigarette smoke and other pollutants. This increased oxidative burden in COPD patients is also reflected by decreased levels of GSH and increased lipid peroxidation in BALF samples [96, 97].

Neutrophils can also kill pathogens by the release of neutrophil extracellular traps or NETs [34, 67, 91]. NETs are composed of extruded strands of decondensed DNA studded with granule contents (i.e. neutrophil elastase, myeloperoxidase, and cathepsin). Calprotectin is also an important component of NETs and possesses potent antifungal properties. NETs serve to trap pathogens, which in turn facilitates their interaction with neutrophil-derived effector molecules [67]. While NETs are important components of the neutrophil arsenal against pathogens, aberrant concentrations of NETs can also produce host tissue damage during inflammation. The precise role of NETs in COPD has yet to be fully elucidated [98, 99].

Neutrophil Apoptosis

Apoptosis or programmed cell death is a means to negatively regulate neutrophil activity and decrease neutrophil-mediated host tissue damage [34]. Neutrophils undergoing apoptosis have decreased oxygen radical production, degranulation, and phagocytosis in response to stimuli [100]. In addition, transcriptional processes are shut down limiting production of proinflammatory mediators [101]. During apoptotic cell death, as opposed to cell necrosis, membrane integrity is maintained which serves to limit the release of toxic neutrophil contents.

Apoptotic neutrophils express specific markers on their cell surface such as phosphatidylserine (PS) residues, which are recognized by macrophages as “eat me” signals leading to their removal by a process termed efferocytosis. Efferocytosis prevents the release of cytotoxic neutrophil contents and also modifies the transcriptional profile of the macrophage stimulating the production of IL-10 and TGF- β , important anti-inflammatory cytokines associated with inflammation resolution and initiation of tissue repair [26, 67, 102, 103]. If neutrophils are not cleared appropriately, apoptotic neutrophils can undergo secondary necrosis and release DAMPS and other proinflammatory mediators which serve to enhance the inflammatory response [104]. Defective neutrophil clearance is linked to the pathogenesis of many inflammatory diseases including COPD [26]. Neutrophil apoptosis can be delayed by proinflammatory cytokines, DAMPS, and PAMPS. In isolated neutrophils, pro-inflammatory cytokines and TLR agonists can delay neutrophil apoptosis through mechanisms that require protein synthesis, the transcription factor NF κ B, and signals transmitted through the PI 3-kinase and MAP kinases pathway [20, 105–108]. The MAP kinase ERK is an important regulator of TNF α , GM-CSF, LPS and CXCL8 anti-apoptotic signaling [109, 110]. Neutrophil survival is also increased following adherence, transmigration, or β 2 integrin cross-linking [111–113]. Delayed apoptosis is associated with increased expression of anti-apoptotic molecules Mcl-1 and A1 [28]. The extended survival of neutrophils at sites of inflammation permits more efficient removal of damaging agents as well as increased interaction with other cells [67]. However, while enhanced neutrophil survival at sites of inflammation promotes increased bactericidal activity, it may also play a role in inflammatory damage via excessive release of oxygen radicals, proteases, lipid mediators and cytokines.

It has been proposed that neutrophil apoptosis in patients with COPD is dysregulated. However, the evidence is controversial. Sputum neutrophil apoptosis has been shown to be decreased, or unchanged as compared to healthy smokers or nonsmokers [15, 23, 114]. Disparate results on the rate of apoptosis in peripheral blood neutrophils from COPD patients has also been reported [23, 84, 115]. It is not clear why there are conflicting results in these studies but may be related to different methodology used to quantify apoptotic neutrophils, or the variable phenotypes in COPD patient populations.

Neutrophil Phenotypes

Neutrophils demonstrate plasticity and can change their phenotypes from a pro-inflammatory phenotype and the production of pro-inflammatory mediators such as PAF and LTB₄ to a more anti-inflammatory state where they release resolvins such as lipoxins [116]. Recent studies suggest that neutrophils can express phenotypes similar to macrophages such as N1 phenotype (classically activated M1) or N2 (Alternatively activated M2) [117, 118]. The neutrophil phenotype is dependent on

microenvironments and environmental factors such as pro-inflammatory or anti-inflammatory mediators. These mediators induce a specific cell phenotype characterized by a distinctive cell surface proteome and a unique pattern of gene expression during cell activation. Although many responses of activated neutrophils do not require protein synthesis, neutrophils can synthesize a variety of cytokines and chemokines [28]. The different neutrophil subpopulations have distinct cytokine/chemokine profiles, expression of specific Toll-like receptors, and differential responses to stimuli. For example, N1 neutrophils express TLR2, TLR4, TLR5, and TLR8 and produce IL-12, TNF, and CCL3 while N2 neutrophils express TLR2, TLR4, TLR7 and TLR9 and produce CCL2, CCL5, IL-10 and IL-4. TLR5 is upregulated to the cell surface in airway neutrophils in patients with chronic lung disease [24]. While neutrophils produce less cytokines than macrophages, the massive number of neutrophils at inflammatory sites contribute significantly to the local concentrations of cytokines and chemokines such as CXCL8, CCL3, CXCL2, and IL-1 β , which serve to amplify the inflammatory response by recruiting more neutrophils and inflammatory cells to the sites of inflammation [27, 34]. Chemokine receptor expression can also be altered in response to DAMPS and PAMPs present in the neutrophil microenvironment. Neutrophils typically only express CXCR1 and CXCR2 receptors however recruitment to sites of inflammation triggers expression of a unique chemokine receptor profile which includes CCR5, CCR2, CXCR3 and CXCR4 [119]. Circulating neutrophils are also heterogeneous cell populations and express different phenotypes dependent on environmental conditions.

Systemic Inflammation and Circulating Neutrophil Phenotypes

Systemic inflammation is an established feature of COPD, is predictive of disease severity, and is increased during exacerbations [6, 8, 10, 120–123]. Systemic inflammation is characterized by increased levels of circulating proinflammatory cytokines, chemokines, and acute phase proteins [124]. The presence of inflammatory mediators such as CXCL8, IL-1 β , TNF α , IL-6, CRP, and GM-CSF triggers neutrophil priming and initiates a positive feedback loop by activating circulating immune cells. Alterations in function have been identified in peripheral blood neutrophils from COPD patients but it is not clear whether these neutrophils are intrinsically different, or are merely altered in response to the proinflammatory environment. Several abnormalities in peripheral blood neutrophils have been reported including alterations in (a) cell surface expression of receptors and adhesion molecules, (b) gene expression, and (c) cell function including degranulation, superoxide anion generation, and chemotaxis.

Gene expression profiling of peripheral blood neutrophils in COPD patients demonstrated systemic inflammation as evidenced by increased expression of

proinflammatory cytokines, chemokines and adhesion molecules. Disease severity, as measured by FEV₁, correlated with expression of multiple inflammatory transcripts including IL-1 β , IL-1R2, IL-1RA, and MIP-1 β [8]. Cell surface expression of adhesion molecules and chemokine receptors are also altered in peripheral blood neutrophils from COPD patients. CD11b/CD18 (MAC-1) is increased with no alterations in CD11a/CD18 (LFA-1) expression [84, 85, 122]. In contrast L-selectin expression is decreased indicating specificity of response. However, during exacerbation both CD11b and CD11a surface expression are decreased as compared to control groups [84]. The authors of this study suggest that during exacerbations there is increased sequestration of neutrophils in the lung and that those mobilized have enhanced expression of adhesion molecules. Thus, the circulating neutrophils may be a subpopulation that do not exhibit increased adhesion molecule expression. Modulated expression of the neutrophil chemokine receptors CXCR1 and CXCR2 are also reported in COPD patients. However, conflicting studies have been reported where CXCR2 expression, but not CXCR1, is decreased on circulating neutrophils [83]. Conversely, CXCR1 was reported to be elevated in COPD with no change in CXCR2, while others report no difference in either CXCR1 or CXCR2 expression [9, 125, 126]. Interestingly, CXCR1 expression correlated with the severity of the airflow limitation suggesting a role in increased neutrophil migration to the lung [9]. In gene profiling studies, peripheral blood neutrophil CXCR1 mRNA expression was characterized in patients with moderate to severe COPD. Only neutrophils from GOLD stage I-II (mild-moderate COPD) patients had a significantly decreased level of CXCR1 expression [8]. The reasons for the reported discrepancies in neutrophil chemokine receptor expression is not apparent, but may be related to the use of isolated neutrophils versus whole blood studies, different control groups, and the severity or GOLD stage of the COPD patients.

Important functional alterations have been reported in peripheral blood neutrophils obtained from COPD patients. Key alterations reported include increased degranulation as evidenced by increased circulating levels of MPO and neutrophil elastase, as well as enhanced production of superoxide anion [10, 84, 85, 122]. Neutrophil migration is also altered and neutrophils from COPD patients show enhanced chemotaxis [127]. Further studies demonstrate that COPD neutrophils move with increased speed but with decreased accuracy as compared to healthy smokers and nonsmoker controls [126]. Moreover, COPD neutrophils formed fewer pseudopods during migration suggesting functional differences. This abnormal neutrophil migration was associated with altered PI3 kinase activity indicating signaling alterations in COPD neutrophils. Similarly, expression of the G-protein subunit G_{as}, but not G_{a11/2} was decreased in circulating neutrophils from patients with COPD irrespective of disease severity [84]. Thus in COPD, the phenotypes of systemic neutrophils show significant modulation of integral components as a result of chronic activation. Neutrophils from these subjects appear to be functionally primed and demonstrate enhanced responsiveness. These findings support the hypothesis that chronic neutrophil activation increases neutrophil effector responses and systemic inflammatory/oxidative burden, both major contributors to the pathophysiology of COPD.

Neutrophils in Disease Progression and Acute Exacerbations in COPD

Neutrophils and neutrophil-derived mediators have been implicated in disease progression in COPD. In BAL samples from subjects with COPD, neutrophil numbers are significantly elevated and these numbers positively correlate to GOLD stage criteria (disease severity and symptoms). Neutrophil numbers and neutrophil-derived proteins (neutrophil elastase, MPO) not only increase with GOLD stage criteria, but also with age, smoking history, and inflammatory mediator levels (CXCL8, IL-6, CRP, GM-CSF, IL-1 β , TNF α , LTB₄) [45, 128–131]. Sputum neutrophil numbers also correlated with GOLD stage, residual volume/total lung capacity (RV/TLC, gas trapping), mean lung density expiratory/inspiratory ratio (pulmonary compliance), and negatively correlated to FEV₁ [19]. In a large cohort study of patients with moderate to severe COPD over 7 years (97 % on steroids), sputum IL-6, plasma fibrinogen levels, and sputum neutrophil numbers increased over time, and these factors were consistent with rapid lung function decline (FEV₁); however, macrophage and lymphocyte numbers showed no correlation [123]. Thus, neutrophil numbers correlate, not only with disease progression by the GOLD stage criteria, but also to many other pulmonary parameters associated with increased morbidity and mortality in these patients.

Acute Exacerbations of COPD (AECOPD) are defined as a sudden worsening of symptoms and is the number one cause of hospitalizations in this patient population [132–134]. AECOPD is also the leading contributor to morbidity and mortality in these patients and is often a common occurrence in patients with advanced COPD [6, 135–137]. The underlying pathophysiology which triggers AECOPD is not well understood but is thought to be an amplification of airway inflammation in response to infectious (bacterial and/or viral) or sterile inflammation, such as particulate matter or DAMPs (HMGB-1) [48, 138–141]. A hallmark of AECOPD is increased levels of sputum neutrophils irrespective of the origin of infection (bacterial vs. viral) or inflammation [1, 140]. Another key feature of AECOPD is the increased presence of neutrophils in the bronchial tree as compared to stable COPD [21]. The increased concentration of neutrophils during AECOPD is reflected by increased sputum purulence. Purulence (green in color) is directly correlated to neutrophil numbers and MPO content, which also correlates with frequency of exacerbation as well as bacterial load [142–144]. The increased numbers of neutrophils during AECOPD suggests increased neutrophil chemoattractant signals or decreased clearance of neutrophils from the airways [115]. COPD patients who suffer from frequent exacerbations have elevated levels of TNF α and IL-6 in induced sputum compared with patients who experience fewer exacerbations [139, 141, 145]. In addition, local concentrations of important neutrophil chemotactic mediators such as CXCL8, CXCL5 and LTB₄ are also transiently elevated during exacerbations, mediators which would enhance neutrophil trafficking into the lung and further amplify pulmonary inflammation. Neutrophil apoptosis is attenuated in systemic neutrophils during AECOPD resulting in increased neutrophil numbers, but it is not known whether airway neutrophils also exhibit decreased spontaneous apoptosis during AECOPD [115].

Potential Therapeutics in the Treatment of COPD

There are limited therapeutic options available for the treatment of COPD, particularly those that target inflammatory cells and pro-inflammatory mediators [1, 146, 147]. Corticosteroid therapy does not effectively modulate the neutrophilic inflammation in COPD and this resistance to steroids is a common feature of neutrophil-mediated inflammatory diseases. In studies with COPD and asthma patients, no significant differences were observed in CXCL8, MPO or neutrophil numbers following corticosteroid treatment [148, 149]. The reason for this lack of responsiveness involves multiple factors. Glucocorticoids can promote neutrophil survival by altering apoptotic signaling through modulations of Bcl-2 apoptosis regulatory proteins, and decreasing Fas expression and caspase-9 activation [15, 150]. In addition, smoking is known to impair the function of histone deacetylase-2, an enzyme recruited by glucocorticoids receptors to suppress proinflammatory gene expression [15, 147, 150]. New therapeutic strategies have been directed towards modulating neutrophil migration and activation to suppress neutrophilic inflammation [151]. Of particular interest are those which target neutrophil chemotactic factors, adhesion molecules, chemokine receptors, and protease inhibitors.

Monoclonal antibodies directed against CXCL8 and TNF have also undergone testing as potential therapeutics in COPD. Administration of monoclonal CXCL8 antibodies over a 3 month time period was found to be safe in a phase two clinical trial with COPD patients [152]. However, only severity of dyspnea was decreased in this treatment group and no significant differences were found in health status, pulmonary function, or frequency of bronchodilator usage [152]. Further clinical trials are needed to ascertain the therapeutic dosing levels, treatment regimens (duration of therapy), and optimal route of administration. A monoclonal antibody directed against TNF α was used in a small trial of patients with asthma and resulted in a reduced presence of sputum inflammatory mediators [25]. However, in a multi-center randomized double-blind placebo controlled trial, the anti-TNF antibody (infliximab) failed to demonstrate clinical benefit in patients with moderate to severe COPD [153]. Of particular concern, it was found that treatment with this antibody increased the development of respiratory cancers in COPD patients. LTB₄ is an important neutrophil chemoattractant and activator by binding to LTB₄ (BLT1) receptors on neutrophils. In vitro BLT1 antagonists limited neutrophil chemotaxis in response to sputum from COPD patients but showed little efficacy in clinical trials [150, 154].

Chemokine receptor antagonists, particularly those directed against neutrophil chemokine receptors are another attractive therapeutic target to attenuate neutrophil inflammation in COPD. CXCR1 and CXCR2 bind CXCL8, while CXCR2 binds CXCL8, CXCL1, and CXCL5, chemokines which are elevated in both stable COPD and AECOPD [1, 21]. A potent CXCR2 allosteric inhibitor with some affinity towards CXCR1, Sch527123, reduced in vitro neutrophil migration and MPO release in response to CXCL8 [155]. While Sch527123 was described as being CXCR2 specific, neutrophil activation and degranulation is CXCR1-dependent indicating that Sch527123 also affects CXCR1. Similarly, another CXCR1/2

inhibitor CXCL8_(3–74)K11R/G31P reduced LPS induced neutrophil migration to rodent lungs. This reduction in neutrophil numbers was accompanied by decreased BALF IL-1 β , IL-6, and TNF α levels, and decreased pyrexia [156]. Ozone is known to cause neutrophil influx into the lung. In a small double-blind proof of principle study Sch527123 was assessed on its ability to reduce ozone-mediated neutrophil recruitment to the lung. Subjects in this study were grouped as placebo, steroids (prednisolone), or Sch527123. Sch527123 treatment was significantly better than both placebo and steroid controls [157]. No adverse effects were observed in this study suggesting this inhibitor may have therapeutic applications. However, caution must be taken to ensure that immunosuppressive phenotypes are not generated by receptor antagonism in neutrophils, as CXCR1 is important in neutrophil bactericidal activity. A CXCR2-specific inhibitor that does not alter superoxide anion generation and degranulation may be more efficacious [61].

Statins and Macrolides are emerging therapeutic options for the treatment of inflammatory diseases. Statins are potent cholesterol-lowering agents that also exhibit pleiotropic anti-inflammatory properties. In contrast to other anti-inflammatory therapeutics, statins do not appear to produce significant immunosuppression [158]. Statins can reduce neutrophil migration, cytokine/chemokine release, and oxidative stress [25, 159, 160]. In retrospective studies, statin use in COPD patients was associated with a reduced rate of lung function decline, decreased exacerbations, and reduced mortality [160–162]. A large Prospective Randomized Placebo-Controlled Trial of SimvaSTATin in the Prevention of COPD Exacerbations (STATCOPE) is currently underway to determine the effect of daily administration of simvastatin taken for at least 12 months on the frequency of exacerbations in patients with moderate to severe COPD who are prone to exacerbations and do not have other indications for statin treatment. Macrolide antibiotics, also display potent anti-inflammatory properties [25, 163]. A study of COPD patients receiving erythromycin for 6 months demonstrated decreased sputum neutrophilia, neutrophil elastase, and frequency of exacerbation; all of which have been previously shown to correlate to COPD GOLD stage severity score [164]. In another study, azithromycin treatment resulted in increased macrophage efferocytosis in patients with COPD in a mannose receptor (MR)-dependent manner, decreased plasma CRP, decreased BALF IL-1 β , and decreased total leukocyte counts [165]. These data suggest that the modulating properties of macrolides may not only reduce the inflammatory response, but enhance the clearance of early apoptotic cells in a MR-phosphatidylserine-dependent manner. This enhancement in macrophage activity may account for decreased lung neutrophil numbers and frequency of exacerbation.

Conclusion

While neutrophils are key components of the innate immune response, inappropriate or persistent neutrophil activation can promote an inflammatory response that contributes to the pathophysiology of COPD. Neutrophil inflammation is not

limited to the lungs and systemic neutrophils display a proinflammatory phenotype and enhanced responsiveness. The presence of continual neutrophilic inflammation results in excessive release of neutrophil-derived mediators such as proteases, MMPs, ROS and proinflammatory cytokines that further contributes to the inflammatory/oxidative burden characteristic of COPD. Understanding the cellular and molecular mechanisms involved in neutrophil activation in COPD is key to the development of effective anti-inflammatory therapies. Therapeutics targeting neutrophil migration, activation, and apoptosis may decrease the frequency of exacerbation and neutrophil-induced host tissue damage observed in COPD.

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Role of Epithelial Cells in Chronic Inflammatory Lung Disease

Victor Kim, Kosuke Kato, K. Chul Kim, and Erik P. Lillehoj

Introduction

Airborne pathogens entering the lungs first encounter the mucus layer overlaying epithelial cells as a first line of host defense [1, 2]. In addition to serving as the physical barrier to these toxic agents, intact epithelia also are major sources of various macromolecules including antimicrobial agents, antioxidants and antiproteases [3, 4] as well as proinflammatory cytokines and chemokines that initiate and amplify host defensive responses to these toxic agents [5]. Airway epithelial cells can be categorized as either ciliated or secretory [6]. Secretory cells, such as goblet cells and Clara cells, are responsible for the production and secretion of mucus along the apical epithelial surface and, in conjunction with ciliated cells, for the regulation of airway surface liquid viscosity. In addition, submucosal mucus glands connect to the airway lumen through a ciliated duct that propels mucins outward. These glands are present in the larger airways between bands of smooth muscle and cartilage. See Fig. 1.

Initially, inhaled toxic agents encounter a mucus layer overlying the respiratory epithelium, become trapped, and are subsequently neutralized by macromolecules. Elimination of these toxic agents depends on mucociliary clearance and cough.

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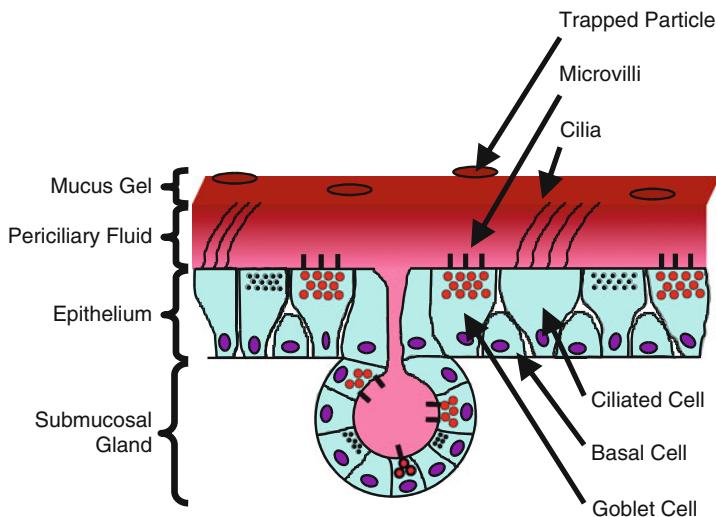


Fig. 1 The airway epithelium and mucus gel

Continuous ciliary movement propels secretions proximally at about 1 mm per minute [7], and this mucosal velocity is modified by hydration of the mucus layer [7, 8] and adrenergic and cholinergic stimuli [7, 9–11]. Efficacy of cough in the elimination of mucus depends on inspiratory muscle strength and expiratory flow velocity, which must detach the mucus from the airway surface and expel the secretions proximally.

A second layer of defense is provided by cell surface receptors (e.g. Toll-like receptors, TLRs) on epithelial cells and resident leukocytes. They bind to various components of the harmful agents and stimulate the production of proinflammatory cytokines (e.g. tumor necrosis factor- α , TNF- α) and chemokines (e.g. interleukin-8). Finally, a third layer of protection is mediated by leukocytes recruited to the lumen of the airways by chemotactic molecules that attack pathogens by direct phagocytosis, as well as through the release anti-microbial proteases (e.g. neutrophil elastase) and oxygen radicals. Details of the role of airway epithelium in the host immune responses are described in a recent review [12]. This review is focused on the role of airway mucus, particularly MUC1 mucin, in the context of chronic inflammatory lung diseases.

Clinical Consequences and Airway Pathology

Mucus is overproduced in several inflammatory lung diseases, a process detrimental to the normal lung defense against environmental toxins. To make matters worse, difficulty in secretion clearance secondary to ineffective cough or poor mucociliary transport leads to mucostasis and paradoxically predisposes to bacterial colonization and infection [13, 14]. Dyspnea, cough and sputum production result from the

Table 1 Airway structural changes in airway diseases^a

Variable	Asthma	COPD	Bronchiectasis
Mucus gland hyperplasia	++	++++	+++
Subepithelial collagen deposition	+++	+	+
Angiogenesis	+++	+	+
Increased smooth muscle	+++	+	+
Increased proteoglycan depositin	+++	+	+
Increased elastin	++	?	+
Epithelial damage	+++	++	+

^aOverall estimate of the significance of these changes in the conditions mentioned. Scores are as follows: + = mild, ++ = moderate, +++ = significant, ++++ = marked, ? = uncertain (Adapted with permission from the American College of Chest Physicians. Bergeron C, Boulet LP. Structural Changes in Airway Diseases: Characteristics, Mechanisms, Consequences, and Pharmacologic Modulation. *Chest* 2006; 129: 1068–1087)

physical obstruction of the airways by mucus and stimulation of intrapulmonary vagal afferent nerves [15, 16]. Lung diseases such as COPD, bronchiectasis, and cystic fibrosis are characterized by mucus hypersecretion, chronic bacterial colonization, and repeated lower respiratory tract infections [17–21].

Airway disease is a crucial pathologic component of multiple inflammatory lung diseases (See Table 1). Pathologic changes in airway epithelium of COPD patients include squamous metaplasia, inflammatory cell infiltration, goblet cell hyperplasia, and mucus metaplasia, a process in which mucus is overproduced in response to inflammatory stimuli (Figs. 2 and 3) [22]. These abnormalities are seen in both the larger central airways as well as smaller respiratory bronchioles [23–26]. Airway inflammation from smoking begins early in the course of the disease, and leads to persistent and progressive airway remodeling, even after smoking cessation [27]. Niewoehner et al. discovered inflammatory changes in the peripheral airways of young smokers who died suddenly, suggesting that airway disease developed before the diagnosis of COPD could be established [28]. As further evidence of this concept, epithelial layer thickness and mucous metaplasia increase incrementally with disease severity [26, 29]. These alterations in the epithelium increase airflow obstruction by several mechanisms: (1) excess mucus occludes the airway lumen [30]; (2) epithelial layer thickening encroaches on the airway lumen, thereby reducing inner diameter [31]; and (3) increased mucus alters surface tension of the airway, predisposing it to collapse during expiration [32]. Hogg et al. found inverse relationships between inflammatory cell infiltration and luminal occlusion of the small airways and lung function [29], strongly supporting the notion that small airway pathology is responsible for severity of illness. Airway disease also has prognostic significance in COPD. Mucus metaplasia in COPD has been associated with worse physiologic response to lung volume reduction surgery [33] as well as greater mortality [34].

Although it is established that quantity of emphysema correlates well with clinical disease staging in COPD, the relationship between airway pathology, physiology and symptom severity is weak at best. Chronic bronchitis exists in 26–45 % of all smokers, but COPD develops in only 15–20 % [35, 36]. Large airway mucous metaplasia correlates poorly with the degree of airflow obstruction [37] and amount

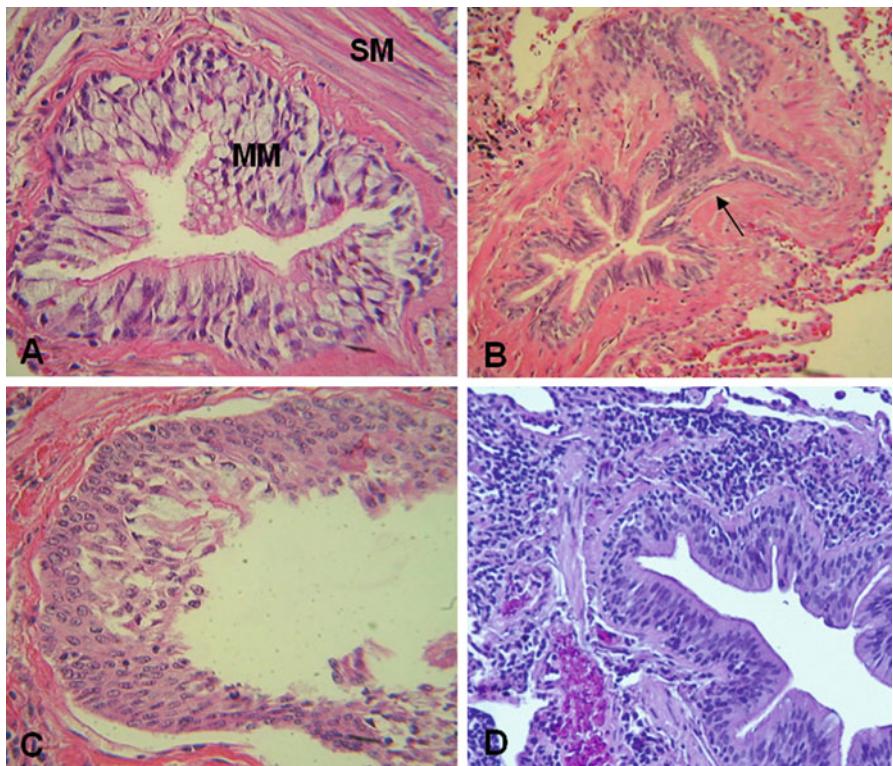


Fig. 2 Examples of airway remodeling in COPD. *A* represents mucous metaplasia (*MM*) of the epithelium and smooth muscle hypertrophy (*SM*). *B* represents peribronchial fibrosis (black arrow). *C* shows squamous metaplasia. *D* shows an inflammatory infiltrate of lymphocytes in the adventitia of a bronchiole (Reprinted with permission from the American Thoracic Society. Copyright© American Thoracic Society. Kim V, Rogers TJ, Criner GJ. New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc Am Thor Soc* 2008; 5: 478–485. Official Journal of the American Thoracic Society)

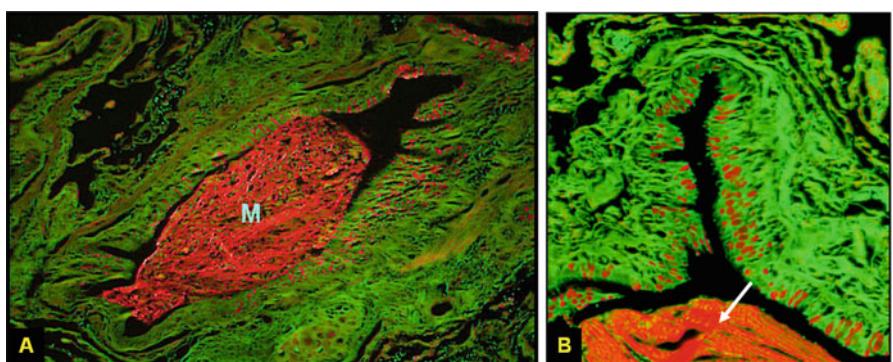


Fig. 3 Periodic Acid Fluorescent Schiff stain of a small airway from a patient with advanced emphysema. The entire airway is seen in *A* and a quadrant of the airway in *B*. Mucin granules are shown in red along the apical border of the epithelium. Note the large intraluminal mucin plug (*M*) in *A*, also noted in *B* (white arrow) (Reprinted with permission from the American Thoracic Society. Copyright© American Thoracic Society. Kim V, Rogers TJ, Criner GJ. New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc Am Thor Soc* 2008; 5: 478–485. Official Journal of the American Thoracic Society)

of mucus expectoration [38]. Small airway disease has been found in surgical lung specimens from those with advanced emphysema, with no clinical or radiographic evidence to suggest its presence preoperatively [29, 33, 39]. More importantly, the degree of small airway mucous metaplasia is difficult to detect clinically by burden of cough or sputum [40].

Despite the disconnect between symptoms and airway pathology, chronic cough and sputum production in COPD have multiple consequences, including an accelerated decline in lung function, [41, 42] increased exacerbation frequency [43–47], greater respiratory symptoms [43, 48], worse health related quality of life [43], and higher mortality [35, 49]. These phenomena are without a doubt a result of increased airway inflammation and worsened airflow obstruction, in addition to the aforementioned mechanisms. In a long term study of over 9,000 adults, an excess yearly rate of FEV₁ decline of 12.6–22.8 mL was attributed to chronic mucus hypersecretion [21]. We have found chronic cough and sputum production in patients with severe COPD were associated with higher dyspnea scores and more upper airway symptoms [43, 48]. In multiple studies, patients with chronic bronchitis and COPD were found to be at a 1.20–1.92-fold increased risk for COPD exacerbation compared to those without chronic bronchitis [43–47]. The cause of the observed increase in all-cause mortality, however, is still a matter of debate. It is hypothesized that the increased lung inflammation associated with chronic bronchitis causes greater systemic inflammation, resulting in numerous downstream consequences, including coronary artery disease, dyslipidemia, osteoporosis, and skeletal muscle weakness [50]. In the Tucson Epidemiological Survey of Airway Obstructive Disease, chronic bronchitis was associated with a 2.2-fold greater risk of all-cause mortality in those under the age of 50, and was also associated with higher serum levels of IL-8 and C-reactive protein at enrollment [49].

In asthma, chronic inflammation and thickening of the small airway epithelium, submucosal space, and smooth muscle has been noted in several pathologic studies [51–53]. In addition, shedding of the epithelial layer has been noted in postmortem studies, bronchoalveolar lavage fluid, and sputum samples [54], most likely as a result of weakened attachment of epithelial cells to the basement membrane. Large airway goblet cell hyperplasia and smooth muscle hypertrophy are prominent pathologic features of asthma. Goblet cell hyperplasia is more consistently seen in asthma compared to COPD, where clinical and pathologic phenotype is a highly variable combination of airway disease and emphysema. In asthma, mucus hypersecretion leads to obstruction of the majority of distal airways and ultimately respiratory failure during fatal asthma exacerbations [55]. Diffuse occlusion of the small and medium sized airways by mucus and cellular debris has been demonstrated in multiple autopsy studies of patients who died from asthma [56, 57]. Goblet cell hyperplasia is also seen in less severe cases as well; *Ordonez et al.* found a greater number of goblet cells and secreted mucins in subjects with mild to moderate asthma compared to control subjects [58].

Similar to asthma, mucus hypersecretion in cystic fibrosis leads to airflow obstruction and small airway occlusion [59]. However, cystic fibrosis is caused by dysfunction of an epithelial chloride channel, which results in sodium and water influx to the epithelial layer and therefore depletion of the airway surface liquid [19].

The increased viscosity and tenacity of secretions makes detachment from the epithelium and propulsion outward during cough exceedingly difficult. Excess mucus production combined with airway surface liquid dessication results in mucostasis, causing colonization by pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia* [18–20].

Airway Mucus and Mucins

Mucus, or the airway surface liquid, is a complex mixture of ions, salts, peptides, proteins, glycoconjugates and water. Strict regulation of mucus production is indispensable for normal lung function. The protective function of mucus depends on its proper composition of constituent components, particularly mucin glycoproteins. Mucins are high molecular weight proteins with O-glycosidic linkages between the first GalNAc residue of the oligosaccharide side chains and serine and threonine amino acids of the polypeptide backbone. Over 20 mucin (MUC in human, Muc in animals) genes have been cloned, 12 of which are expressed in the lung [2, 60]. The airway mucin include secreted gene products (MUC2, 5AC, 5B, 7, 8, and 19) and membrane-tethered mucins (MUC1, 4, 11, 13, 16, and 20). MUC5AC and MUC5B are the two major secretory mucins in the respiratory tract. The levels of these mucins in mucus have been shown to significantly increase, and to directly correlate with, the number of goblet cells under the pathological conditions of goblet cell metaplasia or goblet cell hyperplasia. Although the exact roles of MUC5AC and MUC5B in the airways remain to be fully elucidated, it has been suggested that MUC5AC expression is inducible during airway inflammation, whereas MUC5B expression is constitutive [61]. A recent report supports this notion by demonstrating that MUC5AC levels correlated with the degree of airway obstruction in COPD patients [62]. Cystic fibrosis, in contrast, is characterized by greater MUC5B levels compared to MUC5AC [63, 64], suggesting that impaired mucociliary clearance is the principal mechanism responsible for the overwhelming burden of mucus in these patients.

Epithelial TLRs as Mediators of Airway Inflammation

TLRs, and related molecules, on airway epithelial cells comprise a second line of defense against inhaled microbial pathogens [65]. These pattern recognition receptors (PRRs) constitute an evolutionary conserved family of gene products that interact with pathogen-associated molecular patterns (PAMPs) to initiate downstream signal transduction and innate inflammatory responses. In general, all TLRs possess a leucine-rich repeat region in their ectodomains and an intracellular Toll/interleukin-1 receptor (TIR) domain. TLR signaling is activated by agonist-induced receptor homodimerization, recruitment of cytoplasmic adaptor proteins (MyD88, TIRAP, TRIF) to the TIR domain, and activation of protein kinases (IRAKs,

TRAF6) [66]. Although all of the 10 known human TLRs are expressed by airway epithelial cells, TLR2 and TLR5 are the predominant respiratory PRRs [67, 68]. TLR5 engages flagellin, the major protein component of the bacterial flagellum, while TLR2 recognizes a diverse array of components from Gram-positive and Gram-negative bacteria, including lipoproteins and peptidoglycan. It remains unclear how a single receptor (TLR2) can recognize such a broad diversity of stimuli, but a possible explanation is the ability of TLR2 to form heterodimers with TLR1 and TLR6. For example, bacterial peptidoglycan interacted with the TLR2/6 co-receptor complex on airway epithelial cells to activate NF- κ B and stimulate production of TNF- α [69]. The magnitude of the response generated by the TLR2/6 heterodimer was greater than that produced by TLR2 alone. While TLR5 homodimers are clearly capable of binding to flagellin, Mizel et al. [70] reported that nitric oxide production by airway epithelial cells in response to flagellin was dependent on interaction of TLR5 with TLR4. TLR2 also was shown to be involved in signaling induced by flagellin in human airway epithelial cells, suggesting a possible TLR2/TLR5 heterodimer interaction [67]. Biotinylation of surface proteins of airway epithelial cells followed by co-immunoprecipitation experiments demonstrated that both TLR2 and TLR5 were associated with the ganglioside, asialoGM1, in the plasma membrane [71]. IRAK1 and TRAF6 were also found in the co-receptor complex, whereas TLR4 was not. Furthermore, treatment of airway epithelial cells with *Pseudomonas aeruginosa* pili or flagella mobilized asialoGM1, TLR2, and TLR5 to the apical surface of the cells leading to Ca⁺²-associated activation of mitogen-activated protein kinases (MAPKs), nuclear translocation of NF- κ B, and production of IL-8 [67]. These combined results indicate that TLRs link the asialoGM1 glycoconjugate to intracellular signal transduction leading to a proinflammatory host response following interaction with bacterial components. Other groups, however, have questioned the role of asialoGM1 as a cell surface receptor for *P. aeruginosa*, particularly clinical isolates of the bacterium [72].

Neutrophil Elastase in the Airways

Neutrophils and macrophages constitute a third layer of defense in the clearance of bacteria from the lungs. The anti-microbial function of these immune cells is directly mediated through phagocytosis, and indirectly by the release of anti-microbial agents [73]. Among the soluble mediators released by neutrophils is the serine protease, neutrophil elastase (NE). Studies using NE knockout mice showed that this protease is required for host defense against experimental infection by Gram-negative bacteria [74]. However, the role of NE in the normal lung response to spontaneous bacterial infection needs to be more firmly established. Some evidence suggests that NE promotes neutrophil migration into the lung by degradation of the extracellular matrix, but this remains controversial [75]. In general, NE is considered as a proinflammatory molecule, and NE chemical inhibitors decrease inflammation and lung edema in animal models [76]. Part of the mechanism through

which NE mediates its anti-microbial effects is up-regulation of mucin secretion by goblet cells [77]. Using a co-culture system containing neutrophils and primary tracheal epithelial cells, Kim et al. [78] demonstrated that activation by fMLP/cytchalasin B resulted not only in increased NE production by neutrophils, but also greater mucin release from the epithelial cells. Both effects were blocked in a dose-dependent fashion by pretreatment with α 1-protease inhibitor, implicating a proteolytic effect of NE on the epithelial cells. Kohri et al. [79] reported that NE treatment of NCI-H292 airway epithelial cells stimulated the production of MUC5AC mucin through transforming growth factor- α (TGF- α)-dependent activation of the epidermal growth factor receptor (EGFR). Park et al. [80] showed that NE treatment of well-differentiated primary normal human bronchial epithelial (NHBE) cells cultured at an air-liquid interface (ALI) increased the release of MUC5AC and MUC5B mucins via an intracellular signaling pathway involving protein kinase C δ (PKC δ). To date, NE is the most potent mucin secretagogue described.

Control of Airway Inflammation

Given the intricate and diverse host airway inflammatory mechanisms, a critical balance between these processes and the counter-regulating anti-inflammatory pathways is absolutely required to maintain a homeostatic environment in the airways. This balance ensures that harmful environmental insults are effectively neutralized without excessive bystander tissue damage. Although a large body of literature has characterized the microbial-stimulated pro-inflammatory pathways summarized above, relatively less is known about the compensatory anti-inflammatory responses. Nevertheless, it is hypothesized that failure to down-regulate airway inflammation results in the development of acute or chronic respiratory diseases, including COPD, CF, ARDS, and asthma [25]. A number of anti-inflammatory molecules have been shown to play an important role in controlling the normal inflammatory response in the lung, including IL-10, transforming growth factor- β (TGF- β), peroxisome proliferator activating receptor (PPAR)- γ , and Mucin-1 (MUC1) [25, 81, 82]. However, what is less clear is whether defective expression and/or structure/function of these, or related, anti-inflammatory mediators is responsible for the etiopathogenesis of inflammatory lung diseases. The following sections briefly describe each of these key anti-inflammatory mediators with the goal of stimulating further basic and clinical research on their role in airway inflammatory diseases.

Interleukin-10

IL-10 down-regulates the expression of proinflammatory cytokines, including interferon- γ (IFN- γ), IL-2, and TNF- α , major histocompatibility complex (MHC) class II antigens, and leukocyte co-stimulatory molecules [83]. IL-10 also enhances

B cell survival, proliferation, and antibody production. These pleiotropic effects are mediated through interaction of the IL-10 homodimer with its cognate IL-10 receptor α subunit (IL-10R α), and subsequent binding of this ligand-receptor complex to the IL-10R2 co-receptor. An accumulating body of evidence points toward a role for IL-10 in chronic inflammation during COPD and asthma [84, 85], although a direct causal effect for IL-10 in the pathogenesis of these disorders is unclear. In the case of CF, airway secretions from afflicted patients, as well as CFTR $^{-/-}$ mice, have decreased IL-10 levels compared with secretions from normal individuals or CFTR $^{+/+}$ mice [82].

Transforming Growth Factor- β

TGF- β is an anti-inflammatory cytokine that exists in three isoforms, TGF- β 1, - β 2 and - β 3. TGF- β knockout mice are embryonic lethal as a result of profound multi-organ inflammation. TGF- β $^{+/-}$ heterozygous mice have reduced levels of the cytokine and exhibit exacerbated airway inflammation compared with wild type animals, suggesting a role for endogenous TGF- β in suppressing the development of allergic airway disease [86]. Additional evidence supporting an anti-inflammatory role for TGF- β comes from the observation that intratracheal delivery of TGF- β suppressed allergen-induced airway inflammation in a murine model of asthma [87]. Increased airway inflammation also was evident upon inhibition of TGF- β -dependent intracellular signaling [88]. Genetic studies have demonstrated an association between gene polymorphisms of the TGF- β locus and COPD [89]. Finally, a possible role for TGF- β in CF comes from the report that CF human cell lines and cells from CFTR $^{-/-}$ mice have decreased Smad3 levels and decreased responses to TGF- β [90].

Peroxisome Proliferator Activating Receptor- γ

PPAR- α , - β , and - γ are members of the steroid hormone receptor family of ligand-activated transcription factors [82]. PPARs form heterodimers with retinoid X receptors that regulate gene transcription. PPAR γ is expressed as two isoforms, PPAR γ 1 and PPAR γ 2, that differ by the presence of a unique 30 amino acid segment in the latter [91]. PPAR γ 2 is primarily expressed in adipose tissue, while PPAR γ 1 is expressed in the lung, heart, skeletal muscle, intestine, kidney, pancreas, spleen, breast, and lymphoid tissues [92]. Both PPAR γ molecules are activated by prostaglandins, a subclass of eicosanoids consisting of prostaglandins, thromboxanes, and prostacyclins. Synthetic PPAR γ ligands, such as the thiazolidinediones [93], have been developed that suppress inflammation both in vitro and in vivo [94, 95], including in response to lung infection with *Pseudomonas aeruginosa* [96], the major bacterial species that is responsible for the morbidity and mortality of CF. In the case of CF, at least three lines of evidence have been reported for an anti-inflammatory

role for PPAR- γ . First, PPAR- γ inhibits airway inflammation by competitively inhibiting NF- κ B binding to gene promoters, thereby blocking the activation of pro-inflammatory cytokines [97]. Second, PPAR- γ expression is decreased in lung of CFTR $^{-/-}$ mice compared with CFTR $^{+/+}$ mice [98]. Finally, CF airway epithelial cell lines have reduced PPAR- γ levels compared with normal cells [99]. Thus, decreased PPAR- γ expression likely contributes to defective NF- κ B signaling that favors increased airway inflammation in CF, and possibly other inflammatory airway diseases. However, the exact mechanisms by which PPAR γ down-regulates inflammatory responses in CF and other lung diseases remain to be clarified.

MUC1 Mucin

Of the 20 known mucin genes, MUC1 was the first to be cloned [100, 101]. MUC1 is a single pass, transmembrane glycoprotein located on the apical surface of airway epithelial cells and is composed of two polypeptide chains, a large molecular weight (>250 kDa) subunit containing glycosylated variable number of tandem repeats (VNTR) and a SEA (sea urchin sperm protein, enterokinase, agrin) domain, and a 25 kDa subunit comprised of the transmembrane and intracellular COOH-terminus (CT) regions of the molecule [25]. The two polypeptide structure of MUC1 arises as a consequence of proteolysis within the SEA domain [102]. MUC1 is unique among the membrane-bound mucins because its CT region constitutes a signal transduction domain. The CT contains multiple amino acid sequence motifs predicted as binding sites for Shc, c-Src, Grb-2, β -catenin, and phosphoinositide 3-kinase (PI3K) [25]. These motifs are evolutionarily conserved and undergo tyrosine phosphorylation. The presence of CT phosphorylation sites associated with signaling cascades that have been characterized for other membrane receptors has suggested that MUC1 is functionally analogous to cytokine and growth factor receptors [103].

Anti-Inflammatory Role of MUC1 in Airway Epithelia

Identification of a functional role for MUC1 in the airways was made possible by the generation of Muc1 knockout ($Muc1^{-/-}$) mice [104]. Early experiments demonstrated that $Muc1^{-/-}$ mice were predisposed to developing spontaneous eye inflammation due to infections by *Staphylococcus*, *Streptococcus*, or *Corynebacterium* compared with wild type animals with an intact Muc1 gene [105]. Subsequent studies by Lu et al. [106] using an experimental model of bacterial lung infection showed that $Muc1^{-/-}$ mice exhibited reduced lung colonization by *P. aeruginosa*, greater recruitment of leukocytes and higher levels of TNF- α and KC (mouse IL-8) in BALF compared with their wild type littermates. In vitro and in vivo mechanistic studies have indicated that MUC1/Muc1 plays an anti-inflammatory role during *P. aeruginosa* airway infection by suppressing TLR5 signaling [107–110].

More interestingly, the anti-inflammatory effect of MUC1/Muc1 was not limited to TLR5, but also included TLR2, 3, 4, 7 and 9, suggesting that this cell surface mucin may be a universal, negative regulator of TLR signaling [110]. Given that the host responses to lung pathogens involves the expression of multiple PAMPs, which must be activated and regulated in response to infection, this finding suggests a crucial role for MUC1/Muc1 in the resolution of inflammation, and perhaps in the genesis of chronic inflammatory disorders, such as COPD, CF and asthma.

Regulation of MUC1 Expression by TNF- α

Given the anti-inflammatory role of MUC1 in the airways, it is crucial to understand the mechanisms by which MUC1 gene expression is regulated. Several proinflammatory cytokines have been shown to up-regulate MUC1 expression. Noteworthy in this regard is TNF- α . Skerrett et al. [111] reported that TNFR1 $^{-/-}$ mice treated intranasally with *P. aeruginosa* showed significantly increased airway inflammation compared with wild type mice, as measured by enhanced bacterial clearance from the lungs, increased numbers of neutrophils in BALF, and higher levels of TNF- α in BALF. Subsequently, TNF- α was demonstrated to stimulate MUC1 expression in A549 lung epithelial cells [107, 112]. The molecular mechanism of TNF- α -induced MUC1 up-regulation has been described in detail using a combination of biochemical, pharmacological, and molecular biological approaches [107]. The requirement for TNF- α in increased MUC1 expression has also been observed in A549 cells infected with respiratory syncytial virus (RSV) [109], as well as in mice infected with *P. aeruginosa* [113]. Thus, these results suggest that TNF- α may play a key role in controlling inflammation during airway infection, from the initiation phase of bacterial exposure to the final resolution of inflammation, the latter likely by inducing the expression of key anti-inflammatory molecules, such as MUC1, with possible assistance by IL-10 and/or PPAR- γ .

A Proposed Model for the Anti-Inflammatory Role of MUC1 in the Airways

Based on the accumulated published literature, we propose the following model to account for MUC1, TLRs, and TNF- α in the airway epithelial response to respiratory infection [25]. Normally, transiently inspired pathogens are quickly removed by mucociliary clearance and phagocytosis by resident leukocytes in the airway lumen. With abnormally high pathogen load, for example due to a predisposing condition such as CF, microbial PAMPs activate TLRs resulting in the production of pro-inflammatory mediators (IL-8 and TNF- α), thereby promoting leukocyte influx into the airways. During the early stage of infection, MUC1 expression is sufficiently low and TLR signaling is not antagonized. However, after invading pathogens have been

cleared, increased levels of inflammatory products such as neutrophil elastase and TNF- α up-regulate MUC1 expression which, in turn, suppresses the release of TNF- α , thus inhibiting TLR-dependent airway inflammation. The net effect facilitates pathogen removal and returns the lungs to homeostasis. Future experiments are needed to provide additional support for this proposed negative feed-back loop model system.

MUC1 Mucin and Chronic Inflammatory Lung Disease

TNF is the major pro-inflammatory molecule during airway infection. Ulich et al. [114] demonstrated that intratracheal LPS-induced neutrophilic inflammation in rats can be inhibited by intratracheal administration of soluble TNFR, suggesting that TNF/TNFR interaction plays a key role in LPS-induced airway neutrophilic inflammation. Interestingly, TNFR1 deficient mice not only failed to control either LPS or *Pseudomonas aeruginosa*-induced neutrophilic inflammation [111] but showed greater neutrophilic inflammation to the contrary. Recently Choi et al. [113] showed that Muc1^{-/-} mice behaves exactly the similar way as TNFR deficient mice in response to airway *Pseudomonas aeruginosa* infection, i.e., an increased neutrophilic inflammation, compared with their WT Muc1^{+/+} mice. The relationship between TNFR and Muc1 can be explained by Koga et al. [107] who demonstrated that the levels of MUC1 are controlled by the TNF/TNFR signaling pathway. Thus, MUC1/Muc1 seems to be controlled mainly by TNF both in vivo [113] and in vitro [107]. This timely regulation of inflammation and its resolution has also been demonstrated *in vivo* between TNF and IL-10, in which the former induces the latter, one of the major anti-inflammatory molecule [115]. Thus, failure to induce sufficient levels of anti-inflammatory molecules in a timely manner during the course of lung inflammation will result in lung tissue damage, and the subsequent repair processes will result in lung remodeling, a major characteristic of inflammatory lung diseases such as COPD and CF. Whether other anti-inflammatory molecules are also controlled through the similar mechanism remains to be elucidated.

One of the interesting questions that arise from this study is why there are multiple anti-inflammatory molecules and how they interact with each other during airway infection. For example, it has been shown that MUC1 induces IL-10, an anti-inflammatory cytokine, in dendritic cells [116] and that the levels of IL-10 in the BALF of Muc1 KO mice were significantly greater following *Pseudomonas aeruginosa* infection as compared with those of Muc1^{+/+} mice (unpublished data), suggesting the possible collaboration between the two during inflammation. The same question may be applied to other anti-inflammatory molecules in the lung that have been reported recently, including CD44 [117], aryl hydrocarbon receptor [118] and various lipid mediators [119, 120]. Further studies are required to understand the functional relationships between the known anti-inflammatory molecules during the resolution of airway inflammation.

Summary and Conclusions

In summary, airway epithelial cells play a critical role in the pathogenesis of chronic inflammatory lung disease. Their primary role in the process of host defense becomes dysregulated, and the excess inflammation causes increased mucus production and hypersecretion, resulting in mucostasis, airway obstruction, and tissue remodeling from several downstream events. Clinical consequences include an accelerated decline in lung function, greater respiratory symptoms, exacerbations of underlying lung disease, recurrent lower respiratory tract infection, and higher mortality. Multiple complex interactions between inflammatory cytokines and epithelial cells exist, and the precise roles of each in the generation of mucins and the amplification of lung inflammation remain unclear. There is, however, emerging evidence that the role of MUC1 mucin is essential to the airway epithelium's response to environmental toxic agents, and therefore essential to the development of chronic and persistent inflammation. Further studies are required to better understand the roles of this mucin as well as others in the pathogenesis of inflammatory lung disease.

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The Relationship Between Oxidative Stress Responses and Lung Inflammation with Cigarette Smoking

Changcheng Song and Steven G. Kelsen

Introduction

Cigarette smoking represents a major world health hazard. In fact, chronic cigarette smoking is the leading risk factor for the development of chronic obstructive pulmonary disease (COPD), the world's third leading cause of death and accounts for 90 % of lung cancers [1, 2]. Cigarette smoking produces adverse respiratory effects by exposing the airways and lung parenchyma to a variety of reactive oxygen species (ROS) and other toxic compounds. Although the molecular mechanisms underlying lung and airway damage in response to cigarette smoke remain incompletely understood, ROS are believed to produce tissue injury by affecting the function and gene expression profiles of lung structural cells and inflammatory cells. In fact, cigarette smoke exposure alters the expression of >600 genes in human monocytes [3]. Specifically, ROS exert direct deleterious effects on cell structure and function by damaging protein, lipid and DNA macromolecules which impair cellular function, induce apoptosis, and stimulate dysfunctional matrix remodeling in the lung and in the respiratory tract. Furthermore, cigarette smoke-induced cell damage causes the release of

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alarmins, cytokines, chemokines and up-regulation of adhesion molecules by epithelial cells in the airway and lung which collectively serve to attract an inflammatory cell infiltrate. In addition, bacterial constituents present in cigarette smoke further shape the intensity and inflammatory response by activating PAMPs [pathogen associated molecular patterns] expressed by lung cells which, in turn, interact with the cell's oxidant defense mechanisms [4, 5]. Moreover, the inflammatory process generated by the innate immune system, in turn, increases oxidant stress in the lung through the production of the superoxide ion by infiltrating neutrophils. Accordingly, the inflammatory reaction in the lung induced by oxidants in cigarette smoke has the potential to act as a positive feedback loop or self-amplifying process which exacerbates both conditions.

This chapter will describe the composition of cigarette smoke and the mechanism by which its major constituents induce oxidant stress imposed in the respiratory tract. Furthermore, we will discuss the molecular mechanisms in the lung which deal with oxidant stress. Finally, we will discuss the manner in which antioxidant defense mechanisms in the respiratory tract interact with inflammatory signaling pathways to shape the intensity and nature of both responses to cigarette smoke.

Cigarette Smoke Composition

Cigarette smoke contains more than 4,700 separate compounds many of which are highly toxic and xenobiotic materials [6–9]. In particular, cigarette smoke contains a variety of aromatic and non-aromatic hydrocarbons (dioxin, benzopyrene); alpha and beta aldehydes (acrolein); heavy metals (cadmium, zinc, iron); toxic gases (nitrogen dioxide, nitric oxide, carbon monoxide); and bacterial-derived substances (lipopolysaccharides [LPS]) which induce important biological effects on the innate and adaptive immune systems.

The effects of many of these compounds on oxidant defenses and the immune system have been well characterized (see below). Of considerable importance, the effects of individual compounds of smoke produce effects which in some cases are opposite in sign to those produced by cigarette smoke per se suggesting that the effects of cigarette smoke cannot be predicted from the study of its individual components but are best assessed using cigarette smoke itself.

Reactive Oxygen Species (ROS)

Smoke from a burning cigarette contains approximately 10^{15} ROS per puff [6]. It has been estimated that the concentration of total reactive oxygen species contained in the average cigarette ranges from 16 to 55 nmol H₂O₂/L [10]. Chemical studies of cigarette smoke performed in the 1980s and 1990s have characterized the nature of the ROS generated by both the particulate phase which is retained on a filter (termed cigarette "tar") and the gas or vapor phase which passes through the filter. In fact,

the differing materials in these two phases of cigarette smoke exert different biological effects. The majority of the toxic chemicals generated in the particulate phase are semiquinones, phenol, catechol and nicotine [11]. The tar phase also retains several heavy metal ions including cadmium, nickel, and zinc. The major chemicals in gaseous phase are carbon monoxide, nitrogen oxides, ammonia, formaldehyde, acrolein (aldehyde), toluene, benzene, hydrogen cyanide, and the short-chained amides, acrylamide, acetamide [11, 12].

Cigarette smokers deposit up to 20 mg of tar in their lungs per cigarette smoked and the levels of ROS in cigarette smoke, which correlate closely with the level of tar phase materials [10]. Semiquinones cause sustained production of superoxide (O_2^-), hydroxyl radical (OH $^\cdot$), singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide (H_2O_2) [9]. In the aqueous phase of the surface lining fluid of the respiratory tract, superoxide radicals react quickly to oxidize proteins, lipids or nucleic acid macromolecules. Superoxide also can be enzymatically dismuted to form the more stable oxidant, hydrogen peroxide, by superoxide dismutase. Hydrogen peroxide can also form the very highly reactive hydroxy radical via the Fenton reaction via the iron present in cellular fluids and cigarette tar.

Although free radicals in the gas phase are short-lived and affect primarily the upper respiratory tract, radical concentrations are maintained at high levels in gas phase cigarette smoke for more than 10 min and seem to increase in concentration as smoke ages. In fact, radicals are continuously formed and destroyed. Based on nitrogen oxide chemistry, nitric oxide is slowly oxidized to form the more reactive nitrogen dioxide which reacts with unsaturated compounds such as isoprene to form carbon radicals which then rapidly react with oxygen to form peroxy radicals. These in turn react with nitric oxide to produce more nitrogen dioxide. In general, the gas phase is believed to be less harmful to the lung than the tar phase [13].

CS also promotes the generation of ROS and reactive nitrogen species (RNS) in resident lung structural and inflammatory cells by activation of endogenous NADPH oxidase (NOX). NOX isoforms transport electrons from cytoplasmic high energy electron donor NADPH to generate O_2^- and hydrogen peroxide (H_2O_2) [14]. Of the family of NADPH oxidase (NOX) isoforms, NOX1, 2, 4, 5 and Duox 1 and 2 are expressed in lung epithelial and other cell types [14–19]. Moreover, NADPH oxidase (NOX) isoforms are activated by cigarette smoke in a variety of cell types including alveolar macrophages, airway smooth muscle and pulmonary artery endothelial cells and are believed to contribute importantly to oxidant stress in the lung [14, 20, 21]. LPS in cigarette smoke acting on TLR4 [21] and cytokines secreted from airway and alveolar epithelial cells and inflammatory cells in the lung such as TNF- α and IL-1 β , activate NADPH oxidase. Of considerable importance, NADPH oxidase-derived ROS can induce mitochondrial ROS production indicating the possibility of a positive feedback loop [22, 23]. Of interest, tar- and nicotine-free cigarette smoke is capable of activating NADPH oxidase in PKC-dependent fashion indicating that gaseous phase ROS in addition to cigarette tar may contribute to NADPH-induced oxidant stress in some cell types [24]. In fact, acrolein, a gas phase constituent of cigarette smoke (see below), activates NADPH oxidase and superoxide production in human pulmonary endothelial cells [25].

An additional mechanism can contribute to endogenous ROS production in the lung. Nitric oxide (NO) in cigarette smoke can be transformed to NO by nitric oxide synthase which is expressed by a variety of respiratory cell types. In turn, ·NO can be oxidized to the more potent peroxynitrite (ONOO⁻) by superoxide anion. NO synthase expression can be up-regulated by TNF-α and IL-1β.

Acrolein

Acrolein, a highly water-soluble gas which is highly irritating to eyes, nose and respiratory passages, is deposited mainly in the aqueous lining fluid of the lower respiratory tract when cigarette smoke is inhaled. Acrolein contains a highly reactive alpha carbon which forms carbonylated macromolecules and depletes reduced glutathione and antioxidants in the respiratory tract [26–29]. As such acrolein and other α, β unsaturated aldehydes are major contributors to the oxidative damage induced by gas phase cigarette smoke [30, 31].

Of interest, the concentration of acrolein in sidestream smoke is actually 17-fold greater than in mainstream smoke due to altered combustion chemistry and lower temperatures [32, 33]. Acrolein is also formed endogenously in the lung during inflammation via three separate pathways: (1) by myeloperoxidase oxidation of the amino acid, threonine; by the oxidation of membrane fatty acids; and by thiamine oxidase mediated catabolism of the polyamines, spermine and spermidine. In fact, acrolein concentrations in expired breath condensate and in induced sputum are higher in smokers and ex-smoking subjects with COPD than in healthy nonsmokers [34, 35].

Acrolein induces a variety of deleterious effects on the respiratory tract which mimic the effects of chronic cigarette smoke exposure. On balance, acrolein has a proinflammatory effect but its effects on individual components of the inflammatory response are complex. In rodents, chronic exposure to acrolein increases infiltration of macrophages, neutrophils, and CD8+ T cell into the lungs [36]. Moreover, acrolein exposure increases IFN-γ, IP-10 (CXCL10), IL-12, MCP-1 (CCL2), RANTES (CCL5) and metalloproteinase (MMP)-12 in BAL [36]. Of interest, macrophage accumulation, production of these cytokines and increased MMP12 in response to acrolein does not occur in CD8 deficient mice suggesting that the pro-inflammatory effect of acrolein is initiated by effects on CD8 cells. Acrolein inhibits neutrophil apoptosis [36] but augments alveolar macrophage apoptosis [37]. Acrolein also has novel pro-inflammatory effects by generating chemoattractant peptides from breakdown of lung connective tissue. For example, acrolein acetylates the proline-glycine-protein (PGP) tri-peptide degradation product of collagen breakdown rendering it resistant to breakdown by the aminopeptidase activity of LTA4 hydroxylase [38]. PGP is chemoattractive for neutrophils by acting as a ligand for CXCL1 and CXCL2 [38]. Acrolein does not affect the hydrolyase activity of LTA4 hydroxylase which converts LTA4 to the powerful neutrophil chemoattractant, LTB4 [38].

Acrolein also has direct effects on the master inflammatory gene transcription factor, NF-κB which appear to be anti-inflammatory [39]. For example, acrolein induces alkalization of the p50 subunit of NF-κB which inhibits its

binding to NF-κB consensus DNA sequences. This effect appears to account for acrolein-mediated inhibition of cytokine gene expression induced by endotoxin and TNF-α treatment [40].

Acrolein also has complex effects on lung oxidant defense. It irreversibly inhibits its important antioxidant proteins including thioredoxin reductase, thioredoxin 1 and thioredoxin 2 but increases heme oxygenase-1 (HO-1) expression in human pneumocytes [41, 42].

Cadmium

Cadmium (Cd^{2+}) is abundant in tobacco and, depending on tobacco growing conditions, may be present in μg amounts in each cigarette [43, 44]. In general, the higher binding affinity of heavy metal ions leads to replacement of physiological divalent ions like Zn^{2+} in native proteins thereby altering their structure and function [45, 46]. Cd^{2+} is highly toxic to the lung when inhaled as a vapor or fume or directly instilled [47–50]. In human subjects [51–53] and animal models [54–56], acute exposure induces lung inflammation; chronic exposure induces centrilobular emphysema [47, 48, 57] and pulmonary fibrosis [58]. Cd^{2+} is retained in the body for long periods with a half-life of >10 years [59]. Cd^{2+} binding to metallothioneins in lung cells mitigates its toxicity [60, 61].

It has been suggested that Cd^{2+} present in cigarettes contributes to the development of lung dysfunction and COPD in chronic smokers [62, 63]. In fact, Cd^{2+} concentrations are greater in the emphysematous lung (fourfold) compared to smokers without emphysema and never smokers [64] and in alveolar macrophages from smokers compared to non-smokers [65]. Epidemiological studies indicate that urinary Cd^{2+} levels are greater in smokers and ex-smokers than in never smokers [63, 66]. Moreover, FEV1 and FVC are inversely related urinary Cd^{2+} levels and correlate better with Cd^{2+} concentrations than pack years of smoking [63]. Studies of the effects of Cd^{2+} in cultured pneumocytes, airway epithelial cells and lung fibroblasts indicate that Cd^{2+} causes protein misfolding and may induce an unfolded protein response (UPR) response [see below] [67]. Specifically, Cd^{2+} induces heat shock 70 chaperone expression in rat pneumocytes and human airway epithelial cells [68, 69]. Moreover, Cd^{2+} dose-dependently decreases total protein synthesis in rat type II pneumocytes and a human pneumocyte cell line and procollagen and proteoglycan mRNA and protein expression in human lung fibroblasts [69].

Polycyclic Aromatic Hydrocarbons

Cigarette smoke contains a variety of biologically active, poly-aromatic hydrocarbons which act as ligands for an endogenous receptor, the aryl hydrocarbon receptor (AhR). The AhR is a member of the basic helix-loop-helix family of transcription factors which mediates the biologic and toxic effects of its xenobiotic ligands [70].

The AhR induces expression of a variety of genes including phase I and II enzymes which detoxify toxins contained in cigarette smoke, cytochrome P450 and other monooxygenase activities [71]. The AhR also regulates cell apoptosis and transition through the cell cycle [71].

When bound to its polycyclic aromatic hydrocarbon ligands such as dioxin (tetrachloro-dibenzo-dioxin [TCDD]) or benzopyrene, the AhR translocates from the cytoplasm to the nucleus, heterodimerizes with the AhR nuclear translocator (ARNT) and activates transcription through the xenobiotic response element (XRE). The XRE consists of a canonic motif of 5'-TNGCGTG-3'. After nuclear export, the AhR is degraded via the proteasome. Of interest, the lung contains the highest concentration of AhRs of any other organ in the body [72–74]. In fact, secretory proteins such as surfactant protein A (SP-A) and clara cell secretory protein (CC10) are highly regulated by the AhR [75].

TCDD the best characterized AHR ligand induces a variety of effects in cultured respiratory cells and the lungs of rodents. For example, TCDD induces expression of MUC5AC, COX-2, IL-1 β and MCP-1 mRNA in cultured clara cells [75]. When injected intraperitoneally, TCDD produced similar effects in the whole lung lysate of mice along with increases in TNF- α and reductions in SP-A mRNA. Of interest, the results obtained using AhR knock-out mice exposed to cigarette smoke suggest that the AhR exerts an anti-inflammatory effect. For example, AhR $^{-/-}$ mice demonstrate increased numbers of total cells, neutrophils and lymphocytes in BAL in response to acute cigarette smoke inhalation [76]. Similar results were obtained with LPS treatment, which does not contain AhR ligands. Moreover, AhR $^{-/-}$ fibroblasts demonstrate heightened COX-2 expression and prostaglandin production in response to cigarette smoke which could be rescued by transient expression of AhR [77]. These data suggest that AhR deficient animals are inflammation prone and that the inflammatory responses to TCDD may be a manifest of non-AhR mediated effects.

Nicotine

Nicotine is present in milligram amounts in cigarette tar and, like acetylcholine, is a cholinergic agonist that binds to and activates nicotinic acetylcholine receptors [78]. In cell types which express the $\alpha 7$ nicotinic acetylcholine receptor, nicotine exerts diverse, cell type specific effects on immune cells [79].

In general, nicotine induces anti-inflammatory effects *in vivo* [79–82]. For example, nicotine inhibits LPS-induced elevation of serum TNF- α in mice, an effect which is eliminated in $\alpha 7$ knockout mice [79]. Similar effects were produced in alveolar macrophages *in vitro* [79]. Nicotine treatment also reduced LPS-induced leukocyte infiltration and myeloperoxidase activity in the mouse lungs, and reduced lung MIP-1 α , MIP-2, eotaxin, IL-1, IL-6, and TNF- α [81]. In contrast, nicotine augments dendritic cell capacity to stimulate T-cell proliferation and release TH1 cytokines like interleukin-12 by increasing expression of the co-stimulatory molecules, CD86, CD40, MHC class II receptor, and the adhesion molecules, LFA-1 and

CD54 [83]. These latter results suggest that in contrast to its effects on innate immune responses activated by toll-like receptor stimulation, nicotine may augment adaptive immune responses. The mechanisms by which nicotine exerts these cell type specific effects and the signaling processes involved are unknown.

Antioxidant Defense Systems Involved in the Response to Cigarette Smoke

The respiratory system contains a variety of non-enzymatic and enzymatic antioxidant systems which protect against the injurious effects of oxidants. These systems function to affect electron transfer, enzymatically degrade the chemical compound, as well as scavenge and sequester transition metal ions. The non-enzymatic system scavenges free radicals via electron transfer to electrophilic thiol or carbon groups. This system is comprised of low molecular weight molecules and proteins including glutathione, thioredoxin, peroxiredoxin, α -tocopherol (vitamin E), uric acid, and vitamin C in the extracellular compartment of the lung. Cigarette smoke alters the concentrations of anti-oxidants in lung and other tissues [6, 30, 84 and reviewed by 85]. For example, smokers have lower concentrations of vitamin C in their blood plasma and vitamin E in lung lavage than nonsmokers [86–88].

The enzymatic system comprises the superoxide dismutase (SOD) family, catalase, glutathione peroxidase and heme oxygenase-1 and is also altered by chronic cigarette smoke exposure. SOD which transforms superoxide into hydrogen peroxide includes copper/zinc SOD (Cu^{2+}/Zn^{2+})-SOD in the cytoplasm, manganese (Mn^{2+})-SOD in mitochondria, and extracellular SOD in the interstitial space of the lung [89, 90]. Catalase and glutathione peroxidase catalyze hydrogen peroxide to oxygen and water. Catalase is primarily located in peroxisomes, and glutathione peroxidase is distributed in cytoplasm and extra-cellularly [91]. Heme oxygenase-1 (HO-1) inactivates redox generating heme groups by converting heme to biliverdin-IX α , carbon monoxide (CO) and iron in the presence of O_2 and an electron donor, NADPH/cytochrome p450 reductase [92]. Heme cleavage by HO-1 prevents hydroxyl radical formation through iron. In fact, the importance of the enzymatic systems involved in anti-oxidant stress in the prevention of cigarette smoke induced lung inflammation and injury has been demonstrated repeatedly. The expression of SOD, catalase, glutathione peroxidase, and HO-1 is inducible by chronic oxidative stress including cigarette smoke exposure [93–95] and proinflammatory cytokines [96, 97]. Finally, the phase I enzymes which decarbonylate endogenous proteins and detoxify reactive aldehydes such as acetaldehyde and acrolein and other xeno-biotics, such as aldehyde dehydrogenase, aldo-keto reductase, and NQO1 reductase, are also up-regulated in response to chronic cigarette smoke exposure [98]. Up-regulation of these enzymes in response to cigarette smoke-induced oxidant stress is largely accomplished via the transcriptional activity of the master anti-oxidant transcription factor, Nrf2 (nuclear factor-erythroid 2-related factor-2) [99].

The importance of the enzymatic anti-oxidant enzymes in protecting the respiratory system from cigarette smoke-induced injury has been demonstrated repeatedly. For example, exogenous expression of Cu²⁺-Zn²⁺ – SOD protects CS, elastase and ceramide-induced emphysema in mice [100, 101]. Conditional knockout of extracellular SOD results in the elevation of lung superoxide levels, infiltration of inflammatory cells, and histological changes similar to those observed in adult respiratory distress syndrome [102]. In contrast, increased expression of extracellular SOD attenuates CS-mediated lung inflammation and emphysema in mice [103]. Administration of the SOD mimetic (i.e., MnTBAP) and intranasal administration of SOD-containing microparticles which act to increase lung superoxide levels, reduces mortality and prevents histological alterations [102].

The relevance of antioxidant enzymes in the prevention of cigarette smoke-induced chronic obstructive pulmonary disease in man is strongly suggested by epidemiologic data as well as observations that levels of antioxidant enzymes such as HO-1 are reduced in COPD [104]. Moreover, COPD patients demonstrate a high frequency of mutation of several genes of antioxidant enzymes such as extracellular SOD [105, 106], glutathione S-transferase M1 (GSTM1), GSTT1, GSTP1 and glutamate cysteine ligase (GCL) [107–112].

Nrf2/Keap

Nrf2, a transcription factor that mediates a broad-based set of adaptive responses to intrinsic and extrinsic cellular stresses, regulates expression of enzymes that inactivate oxidants; increase NADPH synthesis; and enhance toxin degradation and export [113, 114]. Nrf2 also enhances the recognition, repair and removal of damaged proteins; augments nucleotide repair; regulates expression of other transcription factors, growth factors, receptors and molecular chaperones; and inhibits cytokine-mediated inflammation [113, 114]. Of particular interest in the setting of cigarette smoke exposure, Nrf2 binds to anti-oxidant response elements in the promoter region of a variety of genes coding for important anti-oxidant enzymes (e.g., heme oxygenase-1 [HO-1], glutathione-S-transferase [GST], glutathione peroxidase [GP], superoxide dismutase [SOD], etc.) [99, 115–117]. In fact, Nrf2 regulates two major redox systems, the glutathione and thioredoxin systems, by promoting expression of enzymes involved in glutathione synthesis, transfer and reduction and thioredoxin synthesis and reduction [99, 115–117]. In addition, Nrf2 regulates several glutathione-dependent (e.g., UDP-glucuronosyl transferase) and glutathione independent enzymes (e.g., NAD(P)H:quinone oxidoreductase1[NQO1]), which are important in the detoxification of tobacco smoke products [116, 117].

Nrf2 and its actin-tethered redox-sensitive inhibitor, Keap1 (Kelch like-ECH-associated protein 1), are widely expressed in bodily tissues [118]. When present in the cytoplasm attached Keap1, Nrf2 has a short half-life as a result of its susceptibility to ubiquitination and proteasomal degradation [118]. Oxidation of Keap1 allows

Nrf2 to dissociate and migrate to the nucleus where it binds to a specific DNA consensus sequence found in the antioxidant response element [5'- NTGAG/CNNNGC-3'] [119]. Nrf2 activity is also regulated by the cytosolic protein, DJ-1, and the nuclear protein, Bach1 [120]. DJ-1 enhances Nrf2 expression by preventing its degradation by the proteasome thereby acting to stabilize Nrf2 in the cytoplasm [121]. The transcriptional inhibitor, Bach-1, on the other hand, inhibits Nrf2 transcriptional activity [122] by competing with Nrf2 for available transcriptional co-factors in the nucleus such as Maf K [119, 120, 123].

Of importance, post-translational modifications of Nrf2 (i.e., phosphorylation and acetylation) affect its functional activity in terms of binding to its inhibitors, its nuclear import and export, and its DNA binding affinity and transcriptional activity [124–127]. For example, phosphorylation of Nrf2 facilitates its dissociation from KEAP1 and its translocation to the nucleus [124–126]. Kinases which phosphorylate Nrf2 include PKC, PI3K and PERK [PKR-like ER resident kinase] [124–127]. At present, however, the Nrf2 phosphorylation sites targeted by kinases which may be activated by cigarette smoke e.g., PERK, PKC, PI3K, etc. and their functional consequences are completely unstudied.

Nrf2 is also acetylated by histone acetyltransferase (HAT) and deacetylated by histone deacetylase (HDAC) 2 [128]. Acetylation of Nrf2 diminishes its transcriptional activity and enhances its export from the nucleus [128]. Accordingly, increases in the level of acetylated Nrf2 are associated with decreases in Nrf2 activity. Specifically, reductions in HDAC2 expression or activity which occur in the setting of cigarette smoke exposure, reduce Nrf2-regulated HO-1 expression and increase sensitivity to oxidative stress in BEAS2B cells and mice [128]. Moreover, HDAC2 knock-down by RNA interference reduces H₂O₂-induced Nrf2 protein stability and activity in cells.

Nrf2 also interacts with the NF-κB family of transcription factors which regulate the innate and adaptive inflammatory response and cell apoptosis (see below). For example, Nrf2 knockout mice demonstrate increased NF-κB activity after treatment with TNF-α, LPS and respiratory syncytial virus [129, 130]. Nrf2 attenuates IκB phosphorylation and increases IKK activity in response to TNF-α or LPS [130]. It is not clear if greater expression of NF-κB and its targets in Nrf2 deficient animals is a result of diminished ability to scavenge ROS or to a direct interaction between the two transcription factors.

Direct evidence of the importance of Nrf2 in the pathogenesis of cigarette smoke-induced lung inflammation and emphysema has been provided in animal models and in cultured lung cells and human subjects [99, 131]. For example, Nrf2 knockout mice are more susceptible to CS-induced emphysema and inflammation while transcriptional induction of Nrf2 by CDDO (2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oicacid) reduces oxidative stress and alveolar destruction in wild-type mouse but not in Nrf2 knockout mice [15, 99, 132, 133]. Mice deficient in Nrf2 demonstrate increased numbers of macrophages in BAL and lung tissue following cigarette smoke exposure [119]. Nrf2 knockout mice demonstrate increased lung infiltration with macrophages, lymphocytes, eosinophils, and neutrophils after

ovalbumin inhalation [134]. Moreover, type II pneumocytes from Nrf2 knockout mice demonstrate impaired growth and increased sensitivity to oxidant-induced cell death [99, 131]. In addition, deletion of KEAP1 in Clara cells in the airways of mice attenuates CS-induced inflammation and oxidative stress [135]. On the other hand, knockdown of DJ-1 in mouse lungs, mouse embryonic fibroblasts and human airway epithelial cells (BEAS2B) impairs antioxidant induction in response to CS [133]. Of considerable interest, expression of Nrf2 and several Nrf2-regulated anti-oxidant enzymes e.g. NQO1, HO-1 and glutamate cysteine ligase modifier subunit, is reduced in subjects with advanced COPD [133]. The potential importance of Nrf2 in cigarette smoke-induced lung inflammation and tissue injury have recently prompted trials of substances which increase anti-oxidant gene expression in respiratory cells in subjects with COPD. For example, sulforaphane, a derivative of broccoli sprouts, and resveratrol, a polyphenolic phytoalexin in grapes, regulate Nrf2 expression [136, 137].

Heat Shock Proteins (HSP)

The HSP family of proteins (e.g., HSP27, 60, 70, 90 and 100) participate in protein homeostasis in the cytoplasm and mitochondria and interact closely the ER chaperones in protein folding and transport [138]. For example, HSP90 interacts with and stabilizes IRE1 and PERK kinases [139].

Hsps (e.g., Hsp27, 60, 70, 90 and 100) are induced in response to cigarette smoke and are highly expressed in the lungs of chronic smokers and subjects with COPD [140]. In addition, serum levels of Hsp27 [141], Hsp70 and Hsp90 are elevated in COPD [142]. In vitro, ROS induce HSP expression in lung structural cells. For example, H₂O₂ increases the levels of Hsp60 in bronchial epithelial cells through a pathway involving NF-κB-p65 [143]. Of interest, however, in animal models, the effects of cigarette smoke exposure on the expression of Hsps are complex and appear to be related to the duration of CS exposure. For example, 1-month exposure of rats to CS increases expression of Hsp70 in airway smooth muscle while a 3-month exposure dramatically reduced it [144]. Hsp expression is also controlled by corticosteroids. For example, dexamethasone increases Hsp72 mRNA and protein expression in the presence of cigarette smoke extract resulting in increased survival of alveolar epithelial cells [145].

Release of HSPs into the extracellular milieu may promote inflammation. For example, HSP60 released by epithelial cells in the setting of oxidative stress stimulates neutrophil activity in COPD patients [143]. Hsp60 also is a key target of T cell responses in chronic inflammation and induces expression of TNF-α and Th1-promoting cytokines, IL-12 and IL-15 in macrophages [146]. In addition, oxidative stress inducers such as CS induce the secretion of Hsp70 from lung structural cells and promote IL-8 release [147, 148], probably through acting as ligand for TLR4 [149].

Molecular Mechanisms of Oxidant Stress Induced Inflammation

Mitogen Activated Protein Kinases (MAPK)

The major redox sensitive signaling system presented in the lung is the MAPK system. MAPKs affect molecular targets which ultimately alter gene transcription in response to environmental stress. MAPK kinases include extracellular signal-regulated kinases (ERK), c-Jun-terminal kinases (JNKs), and p38 kinases. These kinases target a variety of immune effector molecules. For example, MAPK signaling pathways affect T-cell activation and differentiation [150, 151]. MAPK signaling regulates the influx of inflammatory cells into the respiratory tract. Specifically, p38 activation enhances lung inflammation by increasing the expression of inter-cellular adhesion molecule-1, tumor necrosis factor (TNF)- α , and MIP-2. P38 MAPK inhibitors decrease the expression of these pro-inflammatory cytokines and inhibit neutrophil influx in animal models of COPD [152].

Reactive oxygen species in cigarette smoke such as superoxide, hydrogen peroxide and peroxynitrite induce phosphorylation and activation of ERK [153], P38 [154], and JNK [155]. In part, MAPK activation is mediated by activation of the epidermal growth factor receptor and its tyrosine kinase activity [156]. Oxidants can also enhance MAPK signaling by inactivating tyrosine protein tyrosine phosphatase such as PP2a which inactivate MAPK such as JNK and p38 [157]. The importance of ROS-induced activation of the protein tyrosine phosphatases (PTPS) has been demonstrated recently in studies in which PP2a knockdown increases the intensity of cigarette smoke-induced inflammation in the lungs of mice. PTPS may be activated as a result of oxidation of cysteine residues within their catalytic domains. In addition MAPK phosphatases (MKPs) which inactivate MAPK are also inactivated by ROS [158].

Nuclear Factor Kappa B (NF- κ B)

A redox sensitive transcriptional factor NF- κ B, is an important regulator of the inflammatory and cell stress responses [159, 160]. Specifically, NF- κ B regulates expression of a variety of cytokines, chemokines, immunoreceptors, cell-adhesion molecules, stress response genes, regulators of apoptosis, growth factors, and transcription factors. NF- κ B is a family of homo- or heterodimers which contain a conserved Rel homology domain responsible for dimerization and binding to the consensus sequence [5'-GGGRNNYYCC-3'] [159, 160]. The NF- κ B family of proteins can be divided into two distinct families based on the presence of a transactivation domain. RelA (p65), RelB and c-Rel all contain transactivation domains while p50 and p52 do not and require heterodimerization with the Rel proteins for

this function. In the absence of stimulation, NF- κ B is inhibited in the cytosol by association with I κ B [161–163]. In response to appropriate stimuli, I κ B is phosphorylated by I κ B kinases (IKKs) at two separate serine residues which leads its ubiquitination and subsequent proteasomal degradation. Release of NF- κ B from I κ B allows its translocation to the nucleus and subsequent binding to the promoter region of over 100 target genes [164]. In particular, NF- κ B regulates the expression of over 30 cytokines and chemokines, immune recognition receptors and cell adhesion molecules required for neutrophil migration including TNF- α , inducible NOS (iNOS), interleukin-1 (IL-1), intra-cellular adhesion molecule-1 (ICAM-1), and cyclooxygenase (COX-2) [165]. A wide range of agents involved in oxidant stress, immune system activation and bacterial infection stimulate IKK to activate NF- κ B including H₂O₂, TNF- α , IL-1, phorbol esters, microbial infection or PAMPs [165].

The importance of NF- κ B in the inflammatory response of the lung is demonstrated by the fact that NF- κ B knockout mice manifest less lung inflammation and cytokine levels in the BAL compared to wild-type animals in response to inhaled toxic substances. For example, NF- κ B knockout mice demonstrate less neutrophil infiltration and cytokine expression in the lung in response to LPS [166]. Moreover, ROS in cigarette smoke such as hydrogen peroxide activate NF- κ B in several cell lines in vitro [167–169]. In fact, H₂O₂ treatment leads to phosphorylation and activation of IKK. Oxidants may also directly phosphorylate the p65 subunit of NF- κ B.

Of interest, NF- κ B is also regulated by Nrf2. For example, NF- κ B activity is increased in Nrf2 knockout mice after treatment with TNF- α , LPS and respiratory syncytial virus [129, 130]. In fact, Nrf2 attenuates I κ B phosphorylation and increases IKK activity in response to TNF- α or LPS [130]. In addition, Nrf2 appears to regulate expression of at least subsets of the NF- κ B family directly. For example, p50 and p65 are reduced in Nrf2^{-/-} fibroblasts while c-Rel is increased in Nrf2^{-/-} fibroblasts [170]. Greater expression of NF- κ B and its targets in Nrf2 deficient animals may be a result of diminished ability to scavenge ROS and, hence, to greater oxidant stress or to more direct interactions between the two transcription factors. Of note, since a variety of stimuli such as ROS and LPS induce both Nrf2 and NF- κ B activity, an entirely antagonistic relationship between the two transcription factors under all circumstances is unlikely [171–174].

Of note, NF- κ B appears to be negatively regulated by the AhR. For example, AhR^{-/-} mice demonstrate increased NF- κ B DNA binding activity in whole lung lysates [76]. Moreover, heightened prostaglandin responses to cigarette smoke in AhR^{-/-} fibroblasts appear to be explained in part by loss of RelB protein. These data suggest that the AhR represses the NF- κ B complex by interacting with RelB.

AP-1

AP-1, another redox sensitive transcription factor, exerts a pro-inflammatory effect by inducing the expression of a variety of chemokines, in particular, C-X-C chemokines [175] in alveolar macrophages [176] and lung epithelial cells [177, 178].

AP-1 is a heterodimer composed of Fos, Jun and activating transcription factor (ATF) subunits interacted with c-Jun, the most potent activator of the group. Fos stabilizes Jun thereby enhancing its binding to promoter region in target genes.

MAPK signaling is an important pathway for AP-1 activation by phosphorylation of Fos, JUN, or ATF subunits [179, 180]. PERK (protein kinase R-like ER resident kinase) activation also induces Fos expression [181]. ROS can activate AP-1. For example, hydrogen peroxide induces phosphorylation of FOS and JUN and increases the expression of Fos, an effect which is attenuated by the use of ERK or JNK inhibitors indicating the importance of the MAPK signaling pathway [182]. Cigarette smoke induces phosphorylation of c-Jun which in turn promotes the expression of CXCL8.

Histone Deacetylases (HDAC)

The HDAC enzymes deacetylate lysine groups on histones thereby interfering with the binding of transcriptional activators. As such, the HDAC family of enzymes generally inhibits immune responses in the lung [183]. In fact, corticosteroids act by recruiting HDACs to transcriptional co-activators such as p65-CBP thereby inhibiting their activity by inducing deacetylation of the histone complex. HDACs also attenuate inflammation by deacetylating and, hence, inactivating the RelA subunit of NF-κB [184]. Acetylation of RelA inhibits IκB- α binding [184] and augments binding to IKK α causing export of the NF-κB complex from the nucleus [184]. The effect of HDAC activity, therefore, is to attenuate NF-κB transcriptional activity.

Of considerable interest, HDAC activity is affected by the redox state of the cell and is inhibited under conditions of oxidative stress. For example, cigarette smoke and H₂O₂ augment histone acetylation by decreasing the expression and activity of HDAC in human bronchial epithelial cells [185–187]. In the rodent model, cigarette smoke exposure increases histone acetylation, decreases HDAC activity and enhances NF-κB mediated signaling [188, 189]. In contrast, cigarette smoke increases HAT activity contributing to increased acetylation of histone proteins [186]. Of interest, reduced HDAC2 activity in COPD may contribute to increases in Nrf2 acetylation, reduced Nrf2 stability and impaired anti-oxidant defense [128].

The Unfolded Protein Response (UPR)

The UPR alters the activity of signaling pathways which control protein synthesis, transport and degradation [125, 126, 190]. Moreover, the UPR up-regulates expression of a wide array of genes vital for cell survival including genes which promote oxidant defense (e.g., Nrf2, ATF4, HO-1). The UPR is activated in response to ROS and protects against oxidant-induced cell injury and death while defective function of UPR activity impairs the response to oxidant stress, increases ROS burden and diminishes cell survival [125, 126, 190–195]. Of considerable importance, signaling

pathways activated by the UPR also enhances the activity of pro-inflammatory pathways which regulate the immune response and thus have the potential to augment the innate inflammatory response to cigarette smoke [196].

Oxidant stress in general and cigarette smoke exposure, in particular, cause protein oxidation and misfolding in the lungs [197, 198] and cultured respiratory cells [197, 199]. The effects of cigarette smoke on protein oxidation appear to be largely due to the action of acrolein, superoxide and H₂O₂. In addition, nicotine induces a UPR response in several cell types presumably by increasing cytosolic calcium [200]. Protein misfolding induced by cigarette smoke can be attenuated by ROS scavengers and by pre-treatment with an anti-oxidant, the glutathione precursor, n-acetyl cysteine [190, 194, 201–204].

Misfolded proteins are non-functional and potentially cytotoxic when present in sufficient amount. Accordingly, cells have evolved mechanisms to refold misfolded proteins using a variety of chaperones, protein disulfide isomerases and oxidoreductases to isomerize, oxidize and reduce thiol groups on target proteins [196]. The processes involved in protein refolding are energy dependent and require oxidation of thiol groups and the formation of intramolecular and intermolecular disulfide bonds [205]. Electron transport during disulfide bond formation involves two ER-resident enzymes: protein disulfide isomerase [PDI] and ER oxidoreductase 1 [ERO1] [206]. PDI accepts electrons resulting in cysteine oxidation and disulfide bond formation. Electrons are then transferred by ERO1 to reduce molecular oxygen (O₂) and form H₂O₂, thereby increasing the oxidant burden of the cell.

The presence of misfolded proteins is sensed by a triad of ER resident proteins [i.e., PERK (protein kinase R like-ER resident kinase); ATF6 (activating transcription factor 6); and IRE1 (inositol requiring enzyme-1)] [191, 192, 207–209]. Although the precise mechanism by which an increase in the load of misfolded proteins is sensed is uncertain, dissociation of an inhibitor protein, the chaperone, GRP78, from the luminal surface of the sensors increases their activity and triggers a UPR. IRE1 α and IRE1 β (which is present in the lung and gut only), are transmembrane kinases with RNase activity, which splice XBP1 mRNA into a transcription factor (sXBP1) which also up-regulates the above ER resident chaperones, as well as genes involved in protein ubiquitination and degradation, lipid biosynthesis and expansion of ER mass. Activation of IRE1 induces a conformational change which leads to its formation of a complex with the adapter protein, TRAF2 [TNF- α receptor associated factor-2] which recruits IKK leading to the phosphorylation and degradation of I κ B [210, 211]. The IRE1-TRAF2 complex can also recruit JNK which phosphorylates and activates AP-1 [212].

PERK is a transmembrane kinase which phosphorylates and thereby inhibits eIF2 α , the eukaryotic translation initiation factor-2 α . Phosphorylation of eIF2 α is a crucial feature of the UPR since it inhibits protein translation globally, but facilitates translation of selected mRNAs containing appropriate open reading frames. In fact, inhibition of eIF2 α up-regulates translation of Nrf2 and ATF4, a basic zipper transcription factor which enhances ER chaperone expression, and which up-regulates expression of HO-1 and NQO1metabolizing enzymes [116, 117]. Of interest, phosphorylation of eIF2 α and attenuated translation increases expression of NF- κ B [213]. Since I κ B, which has a much shorter half-life than NF- κ B, attenuating expression of

I κ B increases the ratio of NF- κ B to I κ B thereby freeing NF- κ B to translocate to the nucleus. PERK also directly phosphorylates Nrf2, which facilitates its dissociation from the cytoplasmic inhibitor, KEAP1, and its translocation to the nucleus [124–126]. ATF4 also induces expression of the pro-apoptotic transcription factor, CHOP (CCAAT/enhancer protein-homologous protein) which contributes to a UPR driven apoptosis pathway of cell death in lung structural and inflammatory cells [214, 215].

ATF6 is a proto-transcription factor, which upon proteolytic cleavage of its N-terminal transcriptionally active form in the Golgi apparatus, traffics to the nucleus where in conjunction with sXBP1, it activates genes encoding GRP78, calreticulin, calnexin, and PDI (protein disulfide isomerase).

Cigarette smoke induces a UPR response in the lungs of chronic cigarette smokers and in cultured human airway epithelial cells as reflected by up-regulation of expression of the hallmark UPR effector proteins, GRP78, calreticulin, calnexin and PDI [197]. Of interest, the UPR response to cigarette smoke appears to be partially reversible with smoking cessation since expression of these proteins is significantly less in ex-smokers than in active smokers. Cigarette smoke exposure also increases the expression of genes involved in protein folding and the ubiquitin-proteosome pathway in human monocytes suggesting impaired protein folding in this cell type [3]. Moreover, *in vitro* studies in human airway epithelial cells [202, 203, 215, 216] indicate that the cigarette smoke induced UPR is rapid in onset (within hours) and dose-dependent [197, 202, 203, 215, 216]. Furthermore, PERK activity is increased since phospho-eIF2 α , ATF4 and Nrf2 are up-regulated [197, 203]. In contrast, the IRE1 signaling pathway is not activated by cigarette smoke since XBP1 mRNA splicing is unchanged [202, 203, 215]. Activation of the PERK pathway without increase in activity of the IRE1 pathway in the setting of cigarette smoke exposure appears to be explained by active suppression of XBP1 splicing by cigarette smoke [203].

Of considerable interest, cross-talk between components of the UPR and components of both the MAPK pathway (i.e., JNK) and Toll-like receptor pathways (i.e., TLR4) affect the intensity of the inflammatory response and inflammatory cell survival [4, 5, 217]. These interactions may augment the intensity of the inflammatory process in the lung. For example, activation of the kinase activity of the IRE-1 arm of the UPR by misfolded proteins activates both JNK and NF- κ B and increases IL-8 mRNA and protein in human alveolar pneumocytes and airway epithelial cells [218–220]. Moreover, the IRE1 arm of the UPR can be activated by PAMPs to amplify the intensity of the innate immune response to pathogens. For example, LPS-induced activation of TLR 2 or 4 acting through the TRAF6 adapter protein activates the endonuclease activity of IRE1 with resultant increases in sXBP1 [5]. In turn, sXBP1 augmented production of IL-6 thereby magnifying the innate immune response to microbial infection in human monocytes [221]. Prior activation of the UPR by inducing protein misfolding using the canonical stimulus, thapsigargin, potentiated IRE activation of potentiated the IL-6 response to LPS. Of interest, the PERK and ATF6 arms of the UPR were inhibited. These data suggest that the UPR may potentiate the innate inflammatory response to PAMPs. Specifically, the combination of cigarette smoke exposure and TLR activation may act cooperatively to increase lung inflammation. In fact, mice treated with a combination of cigarette smoke and the viral PAMP and TLR3 ligand, poly (I:C),

demonstrate synergistic augmentation of lung inflammation and emphysema compared to either treatment alone [222].

Of interest, TLR4 activation by LPS also inhibits translation of ATF4 through a TRIF-dependent pathway in mouse monocytes [4, 217]. Inhibition of ATF4 expression diminishes expression of its downstream target, the pro-apoptosis transcription factor, CHOP, thereby promoting cell survival. Of interest, CHOP also appears to induce IL-8 expression at the transcriptional level by binding to the IL-8 promoter in human airway epithelial cells [218]. Inhibition of CHOP expression by LPS may therefore reduce IL-8 expression. Nonetheless, enhanced survival of TLR4-activated monocytes in the lung is likely to augment the inflammatory response to LPS present in cigarette smoke. Moreover, the combination of cigarette smoke exposure and activation of a TLR may act cooperatively to increase lung inflammation. In fact, mice treated with a combination of cigarette and the TLR3 ligand, poly (I:C), demonstrated synergistic augmentation of lung inflammation and emphysema compared to either treatment alone [222].

Oxidant stress is heightened in subjects with COPD and persists for prolonged periods even after subjects have stopped smoking [104, 133, 223]. In part, oxidant stress is heightened because Nrf2 expression is reduced in subjects with COPD [15, 122, 133, 223]. Reductions in Nrf2 in lung tissue and in alveolar macrophages appears to explain reductions in both glutathione dependent and glutathione-independent anti-oxidant defense, in particular, HO-1, which is transcriptionally regulated by Nrf2 and ATF4 [92, 224]. Nrf2 up-regulates the expression of the components of the 26 S proteasome [223]. Accordingly, decreased Nrf2 expression decreases proteasomal activity, impairs protein degradation and leads to accumulation of misfolded proteins in the lung of subjects with COPD [223]. Accumulation of misfolded proteins in the lungs of subjects with COPD may be expected to enhance UPR activity and contribute to the NF- κ B-induced inflammatory process. However, UPR activity in the lungs of subjects with COPD is unstudied.

Variations in Antioxidant Gene Expression and Susceptibility to Lung Inflammation

Of considerable importance, the propensity to develop lung disease varies widely across cigarette smokers and correlates only weakly with the smoking history as reflected in the number of cigarette pack years [225, 226]. In fact, it is estimated that only a minority (i.e., 15–35 %) of chronic, continuous cigarette smokers develop COPD [226, 227]. That the majority of long-term smokers do not develop lung damage or COPD suggests that compensatory mechanisms protect the lung from RONS or xenobiotic materials. In this regard, the magnitude of up-regulation of mRNA for several anti-oxidant genes e.g., glutathione peroxidase, glutathione synthase, HO-1, etc., varies considerably across individual cigarette smokers [228, 229]. The mechanism(s) underlying this inter-individual variability in important anti-oxidant gene expression is unknown.

In this regard, the expression of UPR related genes in response to pharmacological stimuli like thapsigargin or tunicamycin also varies widely in healthy human subjects but is concordant in monozygotic twins [230]. Moreover, polymorphisms in the PERK promoter affect PERK function and expression [231]. These findings suggest that UPR responses are genetically determined and that inter-individual differences in UPR function may affect the response to cigarette smoke and the development of lung inflammation in chronic smokers. This issue is unstudied, however.

Conclusion

The complex mix of compounds present in cigarette smoke exposes the respiratory tract to oxidant stress. Many of these compounds induce an inflammatory response by activating redox sensitive, pro-inflammatory pathways including NF- κ B, AP-1 and MAPKs and by inhibiting redox sensitive anti-inflammatory pathways such as HDACs. Conversely, an elaborate network of protein and small molecule anti-oxidants exist to scavenge ROS in the respiratory tract and maintain redox balance in the cell. The regulation of anti-oxidant defense is largely under the control of the redox sensitive transcription factor, Nrf2. Moreover, the UPR which is activated when proteins are oxidized and misfolded in the ER, regulates anti-oxidant defense per se by both Nrf2 dependent and Nrf2-independent mechanisms. Of considerable importance, the NF- κ B mediated pro-inflammatory and Nrf2 mediated anti-oxidant pathways interact to shape the intensity of the inflammatory response to cigarette smoke. Moreover, the UPR acting through its IRE1 arm appears to paradoxically have a pro-inflammatory aspect as well by affecting cytokine expression directly and indirectly via the NF- κ B and AP-1 signaling pathways. Of considerable importance, genetically determined inter-individual responses to oxidant stress and UPR activation vary considerably and are likely to contribute to differences in susceptibility to cigarette smoke-induced lung inflammation and lung damage. Further studies will be required to characterize the effects of cigarette smoke on the Nrf2 and UPR systems in the lung and their role in the development of cigarette smoke-induced lung inflammation and tissue damage.

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Autoimmune Mechanisms Contributing to Chronic Obstructive Pulmonary Disease

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Summary

The pathophysiology of smoking-related chronic obstructive pulmonary disease (COPD) and lung parenchymal destruction (emphysema) has evolved over time from simplistic concepts involving only neutrophils and macrophages to more comprehensive models that further include adaptive immune cells such as T cells and B cells in addition to antigen presenting cells (APC). Evidence from human studies specifically point to a role for the recruitment and activation of pathogenic lymphocytes and lung APC in emphysema; similarly, animal models have confirmed a complex role for the immune response in progressive smoke-induced emphysema. Increased numbers of activated APCs, T helper 1 (Th1), and Th17 cells are now clearly associated with smoke induced lung inflammation and the canonical cytokines produced by these cells, including IFN- γ and IL-17A, constitute critical effectors of disease through their ability to promulgate the pro-elastolytic lung environment that leads to emphysema. T and B cells with autoimmune specificity directed toward lung matrix proteins, especially elastin and potentially vascular endothelium and airway epithelium, specifically appear to distinguish COPD patients with emphysema from those without this devastating complication. These and further discoveries will permit the development of improved diagnostic, prognostic and therapeutic strategies in COPD.

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Distinguishing COPD Subsets: Emphysema Versus Chronic Bronchitis

As most often applied clinically, the term chronic obstructive pulmonary disease (COPD) broadly refers to two clinical syndromes, chronic bronchitis and emphysema. COPD is currently the fourth but is expected to become the third leading cause of death in the world by 2020 [1, 2]. Whereas tobacco smoking in Western societies is considered the main causative factor, in developing nations exposure to toxins (e.g. coal dust, biomass fuels) and chronic respiratory infections, together with a rapidly rising prevalence of tobacco smoking, all contribute significantly to the expression of COPD. The most common clinical presentation of COPD includes symptoms of chronic bronchitis that consist of a daily cough productive of sputum for 3 months out of the year for two consecutive years. Emphysema, on the other hand, represents the loss of lung tissue, often with a predominant upper lobe distribution, but disease may be seen in a variety of patterns, including diffuse involvement. By definition, chronic bronchitis is a symptomatic condition and diagnosis is readily made with a thorough patient history. In contrast, the lack of specific symptoms in smokers with milder forms of emphysema contributes to the long delay in diagnosis that is typical of these patients. Compounding this issue, whereas patients with chronic bronchitis often manifest pulmonary function test (PFT) abnormalities, especially reductions in the forced expiratory volume in one second (FEV1), PFTs and conventional chest roentgenograms are insensitive means of detecting the early stages of emphysema [3, 4]. Consequently, smokers all too often present at their initial clinical evaluation with severe physical limitation and end-stage emphysema.

Computed tomography (CT) scanning of the chest, the most reliable non-invasive method of detecting emphysema, is not obtained as a routine part of evaluating symptomatic smokers and most radiology reports do not commonly describe the presence or quantify the extent of emphysema (Fig. 1). Consequently, a complete phenotypic characterization of lung disease in smokers is often delayed until the onset of self-reported clinical symptoms and findings of airflow obstruction on PFTs, which further obscures the true prevalence and extent of tissue damage in the smoking population. Further, epidemiological studies have shown that lung cancer risk is strongly associated with radiographic emphysema, independent of airflow obstruction and chronic bronchitis [5, 6]. Thus, although they share a final common etiology, smoke exposure, clinical evidence strongly argues that chronic bronchitis and emphysema represent distinct entities with likely different pathophysiolgies.

Innate Immunity in Emphysema

Several classes of enzymes display elastolytic properties and when given intratracheally to rodents, can induce lung parenchymal lesions that resemble emphysema [7]. The most frequently associated and well-studied of these enzymes are the matrix

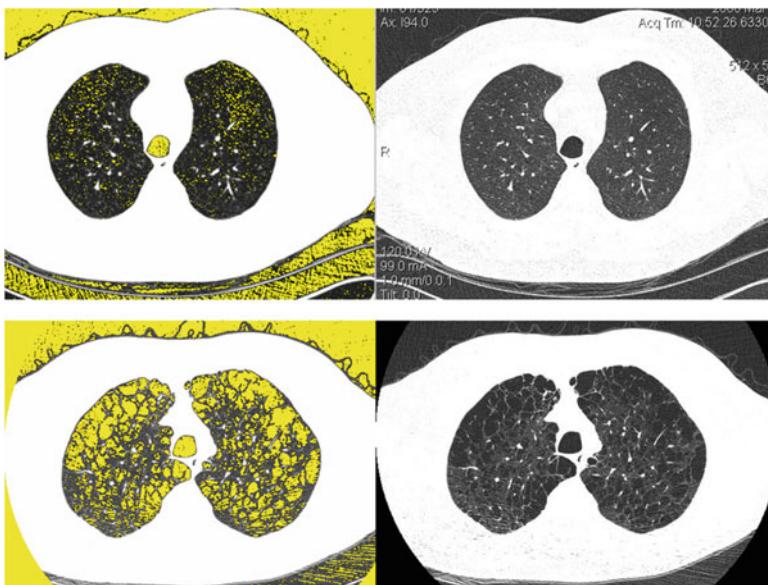


Fig. 1 Quantitation of emphysema by chest CT scan. Chest CT scan images from a patient without (top) and with (bottom) emphysema are shown. The transverse plane images are from approximately the same mid-thoracic level and include conventional (right) and background-enhanced (left) images. Emphysematous spaces are revealed as *yellow* areas with this technique and the percent of emphysematous (*yellow*) lung in each image is computed as a fraction of total imaged lung. Emphysema is defined as >7 % emphysema

metalloproteinases (MMPs) and the serine proteinase neutrophil elastase (NE) that are primarily produced by macrophages and neutrophils, respectively [8]. In particular MMP9, MMP12, and NE are among the most abundantly expressed enzymes reported in the lungs of smokers and are required for induction of emphysema [9, 10]. The activity of most of these enzymes is controlled by endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and A1AT, the principal antagonist of elastases. Besides their potent elastin degrading property, MMP12 neutralizes the action of A1AT, re-enforcing the potent elastolytic function of NE, while cigarette smoke exposure is sufficient to enhance elastase activity [11]. These biochemical events are critically important to the expression specifically of emphysema because elastin is an important matrix protein that is in part responsible for both the structural integrity of the lung and its elastance (ability to stretch). Emphysema, therefore, is the clinical expression of the innate immune-based loss of lung integrity due to enhanced expression of elastases [12].

In parallel to the possible role of smoke in inducing the recruitment of innate inflammatory cells to the lungs, proteolytic cleavage of other matrix molecules has been shown to provide additional pro-inflammatory stimuli in COPD [13, 14]. In particular, proteolytic degradation of collagen elaborates N-acetyl Pro-Gly-Pro (PGP) tripeptides that act as potent neutrophil chemoattractants *in vivo*, a process that is mediated through activation of the chemokine receptor CXCR2 [15].

Emphysema and Autoimmune Inflammation

A role for autoimmune inflammation in COPD was first suspected when histology-based studies of human lung tissue revealed a preponderance of CD8 T cells in the small and large airway biopsies of ever-smokers with COPD [16]. The Lung Health Study, a multi-center longitudinal study of ever-smokers in the United States and Canada revealed a subset of smokers who developed a more rapid decline in lung function despite long abstinence from smoking [17]. The progressive inflammatory nature of emphysema that is seen even when exposure to the provocative agent has ceased provided an especially strong rationale for searching for evidence of systemic autoimmune inflammation in smokers with emphysema [18].

Although human autoimmune diseases all differ greatly in terms of their clinical manifestations, where studied in detail, these same syndromes are also remarkably similar with respect to fundamental immune characteristics. In addition to unremitting inflammation without apparent cause, many autoimmune syndromes are characterized by the enhanced expression of a unique pattern of pro-inflammatory cytokines that includes interferon gamma (IFN- γ), tumor necrosis factor (TNF) and, more recently described, IL-17A [19]. Importantly, IFN- γ and IL-17A are the canonical cytokines secreted by major subsets of effector T helper cells, T helper type 1 (Th1) and Th17 cells, respectively. Experimental models of human autoimmune diseases have confirmed that both Th1 and Th17 cells and cytokines are essential mediators of disease [20, 21]. The importance of TNF is further underscored by the remarkable efficacy of inhibitors of the TNF signaling pathway that are now widely used to suppress debilitating pain and inflammation in diverse autoimmune disorders [22].

One of the first human emphysema studies that supported a potential role for autoimmunity showed that former smokers with emphysema who were free of active infection yet harbor lung Th1 cells [23]. The same study further revealed that while IFN- γ does not affect the production of MMP12, indirectly IFN- γ inducible protein of 10 kD (IP-10; CXCL10) induces the expression of this MMP in lung tissue macrophages [23]. Analysis of human bronchoalveolar cells has further shown that Th1-specific chemokine receptors (e.g., CXCL10) are expressed in the lung of humans with COPD but not other inflammatory disorders such as asthma [24].

Additional evidence of autoimmunity in COPD came from the discovery of a specific memory T cell recall response to elastin fragments in human smokers with emphysema [25]. This matrix molecule is a logical autoantigen to investigate because emphysema has long been associated with elastin breakdown and the loss of elastin structural support is thought to be the final common mediator of lung destruction in emphysema [26, 27]. T cells isolated from peripheral blood of ever-smokers showed a significant increase in secretion of IFN- γ , while no changes were noted in Th2 cytokines such as interleukin 4 (IL-4) and IL-13 after elastin peptide restimulation [25]. Further analysis of T cells extracted from the peripheral lung of smokers with stable, uncomplicated emphysema confirmed that these cells spontaneously secrete IFN- γ and IL-17A in the absence of IL-4 and IL-13 [28].

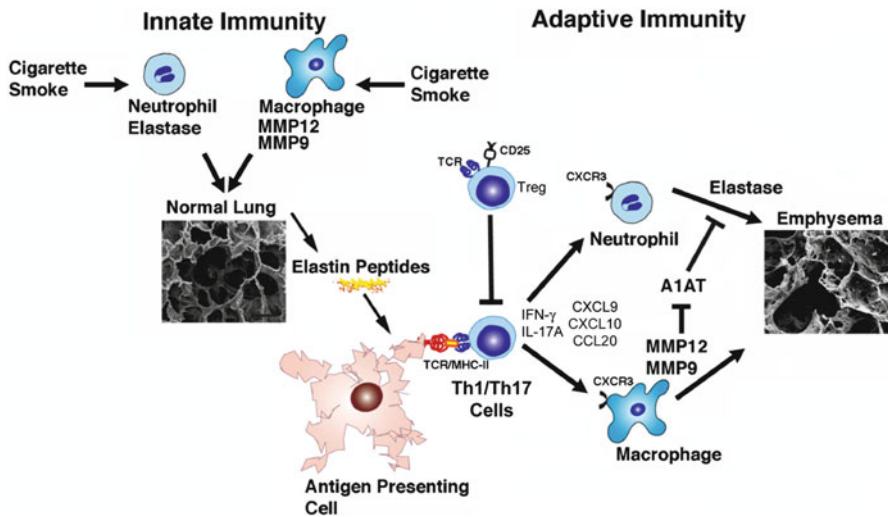


Fig. 2 Schematic view of autoimmune inflammation in emphysema. According to this immune-based model of emphysema pathogenesis, innate and adaptive immune processes contribute to disease. Cigarette smoke likely directly activates macrophages and neutrophils to initiate elastolysis through induction of elastase production. Elastin peptides are then processed by lung antigen presenting cells such as dendritic cells (DC) and presented to T helper cells. In genetically susceptible hosts, Th1 and Th17 cells reactive to elastin and potentially other endogenous antigens develop under these conditions and expand. The cytokines (IFN- γ , IL-17A) and chemokines (CCL20, CXCL9, CXCL10) produced by Th1 and Th17 cells act cooperatively to further promote the recruitment and activation of lung DC and secretion of elastases such as neutrophil elastase, MMP9, and MMP12, which degrades A1AT. Th1 and Th17 cells therefore establish a vicious cycle of ongoing T cell activation and accelerated elastolysis, which if sufficiently prolonged leads to emphysema (Modified from Lee et. al. [25])

Thus, these studies were the first to confirm that human emphysema is characterized immunologically by the expression of the key autoimmune signature consisting of Th1 and Th17 cells and their canonical cytokines and related chemokine receptors and identify the first potential autoantigen in emphysema.

The discovery of Th1 and Th17 cytokines in emphysematous lung further proved to be the key to unlocking the immunological basis of how lung destruction results from smoking. IL-17A promotes secretion of neutrophil chemokines, in part accounting for the chronic neutrophilia of COPD [29]. IL-17A and IFN- γ further act coordinately to promote a pro-elastolytic lung environment. Chemokines induced by IFN- γ such as CXCL10 and IL-17A act directly to stimulate MMP12 secretion by macrophages. IL-17A further stimulates macrophage MMP12 secretion by promoting production of the chemokine CCL20, which simulates macrophage MMP12 release. Thus, tobacco smoke acutely elicits elastase secretion from innate immune cells, but ultimately induces a Th1/Th17-predominant adaptive immune response that underlies chronic inflammation and progressive emphysema even in persons who have stopped smoking (Fig. 2).

Whereas elastin was the first T cell-reactive autoantigen identified in emphysema, autoantibodies have also been found in human COPD. Three major classes of such antibodies have been defined, including anti-epithelial, anti-endothelial, and anti-elastin antibodies. Anti-epithelial antibodies are present in most smokers with air-flow obstruction relative to smokers without obstruction and normal control subjects and such antibodies may have cytotoxic potential, at least when tested in vitro [12]. The antigen recognized by anti-epithelial antibodies and their precise role in disease causation are currently unknown.

Anti-endothelial antibodies have also been described in COPD [30]. Such antibodies, when generated in the context of immunization against xenogeneic endothelial cells, are associated with the development of emphysema in rodents, although again the nature of the antigen(s) recognized and the precise role of the antibodies in disease are uncertain. Anti-elastin antibodies have further been detected in some [25, 31, 32], but not all [33, 34], subjects with COPD. The varying ability of anti-elastin antibodies to distinguish patients with emphysema from other manifestations of COPD and even those entirely free of disease likely reflects differences in the methods used. Moreover, few, if any, autoimmune inflammatory diseases are driven exclusively by pathogenic antibodies and antibodies to self-proteins have been reported to exist in disease-free subjects [35, 36]. Ultimately, whereas autoreactive Th1 and Th17 cells have clear pathophysiological significance, autoantibodies in COPD may not participate in disease, but have potential for development as part of novel diagnostic and prognostic strategies.

Antigen Presenting Cells in Emphysema

CD4 and CD8 T cells are activated by professional antigen presenting cells (APCs) such as the dendritic cell (DC), which include myeloid DC (mDC) and plasmacytoid (pDC) subtypes [37]. By virtue of their long dendritic processes, mDCs have the unique capacity to sample antigens in the airway via DEC205 (CD205), while macrophage mannose receptors (MMR) and other cell surface molecules can mediate receptor-mediated endocytosis and antigen processing in deep lysosomes or peripheral endosomes, respectively [38]. The digested foreign- or self-peptides are then presented at the cell surface in the context of major histocompatibility (MHC) molecules, and only then is antigen capable of being recognized by T cells. Additional studies to further characterize specific APC subsets in normal and diseased lung and their specific costimulatory molecules (CD80, CD86, CD83, CD40) showed a functional role for T cell activation in human emphysema [28]. How DCs undergo this unique maturation process remains an intense area of investigation, but lack of robust costimulatory molecule expression on APC promotes induction of tolerance against foreign antigens (e.g. ovalbumin), which is marked by the production of antigen-specific immunosuppressive or regulatory T cells termed Tregs [37, 39]. Although difficult to assess rigorously, under normal conditions, human lung DC are thought to lack co-stimulatory molecules and therefore favor immunosuppressive Treg

development. Unfortunately for smokers, the tolerogenic mission of the lung is usurped by the overwhelming presence of mature mDC replete with co-stimulatory molecules, apparently induced by tobacco smoke exposure, that induce and perpetuate T cell-dependent inflammation [28].

The immune phenotype of lung APCs in current and never smokers has recently been evaluated in detail [40, 41]. Clinical data derived from lung function studies and chest CT scans have shown positive correlations between disease severity and APC activation (assessed by relative expression of the costimulatory markers CD80, CD83, and CD86) concomitant with a marker of activated CD4 T cells (CD69) [42]. Mature APCs expressing co-stimulatory molecules could activate CD4 T cells and promote their differentiation into Th1 and Th17 cells merely through physical contact. Notably, however, members of the B7 family of co-stimulatory molecules (CD80 and CD86) which bind to CD28 to activate T cells, can also efficiently bind to cytotoxic T lymphocyte antigen-4 (CTLA-4), which is also expressed on CD4 T cells, and deliver an inhibitory signal to activation. Therefore, the complex interplay between T cells and DCs requires better understanding of the activation status of T cells and DC maturation under normal and pathogenic conditions. These findings indicate that T cells become activated through signals from lung APCs, implying that a process more fundamental than activated T cells most likely is the ultimate endogenous governor of the pathobiology of smoke-induced emphysema. Future studies are needed to define the genes and environmental factors (i.e., substances in tobacco smoke) regulating activation of lung APCs that are critical for T cell activation.

Respiratory Infections, Autoimmunity and Emphysema

The otherwise stable deterioration in lung function that characterizes COPD is punctuated in many patients by bouts of disease exacerbation marked by sudden, but transient worsening of lung function in the setting of an acute and febrile pneumonic or bronchitic syndrome. These episodes are thought to represent bouts of airway infection with viruses, especially human rhinovirus (HRV), or bacteria such as *Streptococcus pneumoniae* [43, 44]. These exacerbations are ameliorated through the use of anti-inflammatory agents and antibiotics, but lung function may not return to baseline status and patients often require temporary mechanical ventilatory support. Activation of co-stimulatory signals by infectious pathogens through pathogen recognition receptors (PRRs) such as Toll like receptors (TLRs) on APCs can activate this critical cell population in the lung and initiate or worsen baseline Th1 and Th17-predominant inflammation in smokers, thereby potentially exacerbating the baseline pro-elastolytic lung environment that exists in susceptible smokers [45, 46]. Thus, emphysema appears to be a Th1/Th17-driven lung inflammatory process that is initiated by cigarette smoke and possibly recurrent infections in which both the innate and autoimmune components promote elastolysis, loss of lung integrity and impaired lung function [12].

Genetic Correlates of Emphysema and Autoimmunity

The genetic basis of emphysema is consistent with a heritable multi-factorial trait that is reminiscent of a number of autoimmune genetic disorders [47]. Although cigarette smoke can provoke activation and recruitment of inflammatory cells to the lung, not all smokers develop lung disease. The exact prevalence of emphysema is currently unknown and to date, the only known gene linked to increased risk of early onset emphysema is mutations in the alpha-1-antitrypsin (A1AT) protein encoded by the SERPINA1 gene. This genetic association is of particular interest because A1AT has potent immunomodulatory effects, having the ability to suppress the rejection of organ transplants and attenuate autoimmune inflammation and disease afflicting diverse organs in experimental systems [48–54]. A1AT does not appear to influence T cells directly, but rather indirectly promotes the development and recruitment of inflammation-suppressing FoxP3+ T regulatory cells (Treg) to sites of inflammation. Additional studies further suggest that partial deficiency in A1AT may be linked to autoimmune diseases such as ulcerative colitis [55]. Thus, A1AT replacement therapy not only helps to restore a proper elastase balance, it may suppress autoimmune inflammation by promoting Treg responses.

Allelic variance in the SERPINA1 accounts for only 1–2 % of all diagnosed cases of emphysema, suggesting that many additional genes, as yet undiscovered, influence the expression of disease. Furthermore, even among carriers of different forms of A1AT proteins, there is large phenotypic variability suggesting that together modifier genes and environmental exposures exert a combined effect. Many other susceptibility loci for emphysema and COPD have been considered, most recently the transcription factor regulating chondrogenesis, SOX5 [56–58]. However, none of these proposed susceptibility loci are clearly linked to inflammation and autoimmunity.

Recent technological advances in high throughput genetic analyses have promoted collection of new information about the genetics of COPD and emphysema. The three basic approaches include: (I) candidate gene association studies, (II) linkage analysis in multiple families, and (III) genome-wide association studies (GWAS). To date only a few genetic studies have explicitly examined emphysema as the main diagnostic criterion to determine susceptibility loci in human smokers. One such study attempted to identify genetic determinants of emphysema using GWAS in a large cohort using CT based diagnosis of emphysema. This effort described a single nucleotide polymorphism (SNPs) in the BICD1 locus encoding a protein critical for dynein-dynactin interactions that was significantly associated with emphysema, and verified using distinct cohorts [59]. Most other reports, however, have examined the genetics of COPD without distinguishing the individual component of emphysema as a risk factor [60]. In particular, scanning the entire genome for SNPs associated with COPD (irrespective of the presence or absence of emphysema), has led to the identification of several new risk-related genes such as the α -nicotinic acetylcholine receptor (CHRNA 3/5), and hedgehog interacting protein (HHIP) [61]. It should be noted that some of the identified risk loci (e.g. CHRNA) have also been linked to

smoking behavior and lung cancer [62, 63], further underlining the complexity of genetic association studies in humans.

With these new insights into the genetics of smoke induced lung disease, a predictably complex genetic basis is emerging in which multiple genes appear to contribute to the increased disease risk, although most such genes (with the exception of A1AT) generally confer a very modest independent risk [57]. Such heterogeneity among different phenotypes within smokers is reminiscent of the common genes that underlie multiple autoimmune disorders because much like genetic associations in COPD, such gene associations are typically extremely modest. These findings further suggest that the more important utility of discovering arrays of new genes may be the generation of new hypothesis about the pathogenesis of autoimmune diseases [47].

Summary and Future Directions

Clinical and pathological observations made over the past few years have opened the door to a new concept: in susceptible individuals, cigarette-smoke exposure may trigger long-lasting inflammatory memory T cell responses that can persist beyond the immediate period of exposure to cigarette smoke. Given that not all smokers develop lung disease, we propose that the perpetual inflammation seen in ever smokers with emphysema is driven by multiple insults that include: (I) T cell activation against specific lung matrix and cell derived autoantigens (e.g. elastin, collagen, endothelial and epithelial antigens); (II) presentation of lung-specific antigens that results in the accumulation of activated, auto-reactive T cells; (III) transient production of the cytokines that induce proliferation of autoreactive T cells (i.e. IL-6, IL-1, and IL-17A); and (IV) a positive feedback loop in part governed by genetic factors that shapes the host response and may increase the clonal T cell bias toward Th1/Th17 differentiation in the lung. Most importantly, the continuous recruitment of activated lung APCs could further ensure propagation of autoinflammatory T cell responses and the development of chronic progressive lung destruction (Fig. 2). Although activation and clonal expansion of T cells is an exceedingly important part of the chronic inflammation of emphysema, individual genetic susceptibility factors that govern how the immune system responds to antigen most likely contribute importantly to the degree of autoimmune inflammation that results. For example, certain MHC II haplotypes may be incapable of binding and presenting elastin fragments and therefore inducing anti-elastin T and B cell responses. Therefore, during the multi-step activation and expansion of autoreactive T cells, a spectrum of disease severity would be seen in human smokers (Fig. 2). This contingent model of autoimmune inflammation potentially explains why despite smoking cessation, the lung inflammation rages on in only a subset of exposed subjects [64].

Chronic, autoreactive systemic inflammation increasingly appears to be most strong in ever-smokers with emphysema. Unresolved questions regarding the wide spectrum of lung diseases seen in smokers have prompted a global mobilization of

resources to assess different subphenotypes of human smokers. Most likely, differences in response to antigens (self or foreign) by major immune effector cells from peripheral blood and lung APCs will provide the greatest insight into emphysema pathogenesis. Although the fundamental mechanism by which lung inflammation is sustained in human smokers remains unknown, the finding of autoimmunity involving elastin and perhaps other endogenous antigens presents a solid platform to begin this important research [25, 65]. Even more fundamentally, however, we can now postulate that smoking triggers an as yet unknown innate inflammatory response that activates lung APCs. Among many possibilities, this signaling pathway could comprise adjuvant-like ligands generated during smoking that signal through APC-expressed TLRs. The many chemically-reactive molecules identified in cigarette smoke could further sufficiently alter the antigenic character of endogenous molecules such as elastin to render them more recognizable (i.e., less tolerogenic) to T and B cells, providing a mechanism for breaking the lung tolerogenic state that normally precludes such autoreactivity. Obviously, much work remains before our understanding of emphysema pathogenesis begins to impact how we predict, diagnose and treat this devastating malady. However, we now have a roadmap for dissecting the relevant cells and molecular pathways that likely play key roles.

Over the next 5 years, the results of several ongoing multi-center studies will provide much needed insight into the pathobiology of human emphysema. Predictably, characterizing distinct clinical phenotypes and identifying susceptibility genes that correlate with them will offer new means of developing personalized therapy to those with pure emphysema, airway obstruction or mixed types, but also will provide novel means of identifying smokers who are at a higher risk of developing COPD and emphysema. This will in turn aid in implementation and delivery of personalized medicine to those at risk, and may potentially represent a means of identifying smokers at higher risk of lung cancer who could then be efficiently screened using serial CT scans. Firm identification of lung autoantigens linked to emphysema, e.g., elastin, further establishes the rationale for developing screening tools to identify smokers at risk for lung parenchymal damage, and offer therapy aimed at halting the destructive action of the pathogenic lung T cells. In mice, evidence is mounting that many of the early immunological changes occur in response to pathogenic APCs prior to the onset of lung disease; while this could also be true in humans, there is no evidence to support this at the present time. Therefore, longitudinal T cell-based studies in early smokers could help identify whether development of pathogenic T cells take place at an early time point during chronic exposure to smoke.

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Chronic Obstructive Pulmonary Disease (COPD): Local and Systemic Disease

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Introduction

COPD is a common and important disorder that affects more than 200 million people worldwide. Its incidence is rising and by 2020 it is projected to become the fourth leading cause of death worldwide [1]. COPD is characterized by an abnormal inflammatory response in the lung to noxious particles or gases [2], but there is increasing evidence and acceptance that COPD also has important systemic manifestations. The Global Initiative for Obstructive Lung Disease (GOLD) definition includes the statement that COPD has “significant extrapulmonary effects that may contribute to the severity in individual patients” [2]. Indeed, careful analysis of data from the Towards a Revolution in COPD Health (TORCH) study found that only 40 % of deaths in patients with well-characterised moderate to very severe airflow limitation were definitely or probably related to COPD. Cause-specific mortality was: cardiovascular 27 %, respiratory 35 %, cancer 21 % (predominantly lung cancer), other 10 % and unknown 8 % [3]. In this chapter we will highlight the critical role of airway and systemic inflammation in COPD and review the impact of exacerbations on both the local and extrapulmonary manifestations of this important disease.

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Local Disease and Inflammation

Long-term tobacco smoking is the most common precipitant for the development of abnormal airway inflammation in COPD patients. Indeed, cigarette smoke has been shown to trigger an abnormal inflammatory reaction in even in the absence of established airflow obstruction that can involve the entire tracheobronchial tree [4, 5]. Bronchial biopsies of smokers with a history of chronic sputum production demonstrate increased total leukocytes both in the lamina propria and epithelium and an increase number of macrophages and T-lymphocytes in the lamina propria [5] and further inflammatory changes have been demonstrated in patients with COPD. Studies using bronchoalveolar lavage (BAL) and induced sputum in patients with COPD have shown increased numbers of neutrophils, and elevated levels of tumor necrosis factor-alpha (TNF- α) and interleukin-8 (IL-8) in subjects with COPD compared with smoking and non-smoking controls [6].

Excessive local inflammation is thought to lead to fibrosis of small airways and a protease-antiprotease imbalance resulting in destruction of the lung parenchyma, the relative contributions of which are highly heterogenous between subjects [2].

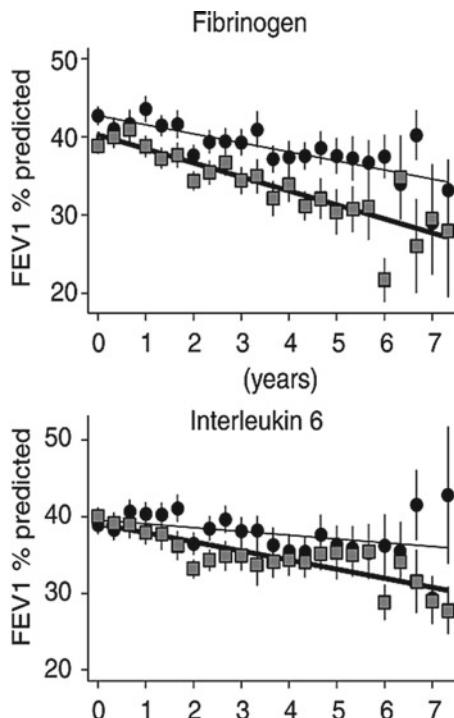
Exacerbations

Episodes of acute deterioration of respiratory symptoms are termed exacerbations and are responsible for much of the morbidity [7] and mortality [8] associated with COPD. These events are usually caused by airway infection [3] and have a critical influence on airway inflammation in COPD. During exacerbations, sputum IL-6 levels are increased compared to the stable state and COPD patients with frequent exacerbations also have higher stable sputum levels of IL-6 and IL-8 compared to those with less frequent exacerbations [9]. Airway inflammation (as reflected by sputum IL-6 and sputum neutrophils) increases over time and frequent exacerbators have a faster rise in sputum IL-6 over time compared to infrequent exacerbators. Furthermore, patients with higher levels of airway and systemic inflammatory markers have a faster decline in lung function (Fig. 1) [10].

Upper Airway Disease

In addition to increased concentrations of sputum inflammatory markers, stable COPD is associated with increased nasal inflammation, as shown by IL-8 concentrations, the severity of which reflects that occurring in the lung [11]. Nasal inflammation is also increased at exacerbation and the degree of upper airway inflammation at exacerbation is correlated with the degree of lower airway inflammation [12].

Fig. 1 Mean FEV1% Predicted in 4-month periods for low plasma fibrinogen and sputum IL-6 group patients (circles and thin line) and for high plasma fibrinogen and sputum IL-6 group patients (squares and thick line) against time from recruitment of each patient. Bars are 2xSE. Error bars increase in size with time, as not all patients participated in the study for 7.33 years. No adjustment was made for covariates. (Reproduced with permission: [10])



Bacteria and Viruses

Bacteria and viruses also play an important role in the determination of airway inflammation in COPD, particularly at exacerbation (Fig. 2 [13]). Rhinovirus is the organism most commonly responsible for the common cold and Seemungal and colleagues found that up to 64 % of exacerbations could be associated with a symptomatic cold that occurred up to 18 days prior to exacerbation onset [14]. The presence of cold symptoms at exacerbation onset has been associated with larger falls in peak expiratory flow rate, prolonged recovery times and also higher levels of sputum IL-6 [9, 15].

Airway bacterial load at exacerbation correlates to the rise seen in sputum IL-8 and a synergistic effect of viral and bacterial infections during exacerbations of COPD has also been demonstrated [16]. Exacerbation symptoms and FEV₁ decline are more severe in the presence of both bacteria and colds than with a cold or bacterial pathogen alone, and exacerbations associated with both human rhinovirus and *Haemophilus influenzae* exhibit greater systemic inflammation (as measured by serum IL-6) than those without both pathogens [16].

In the stable state, total bacterial count is related to airway inflammation, as measured by sputum IL-8 [17]. In longitudinal studies, patients with increasing airway bacterial load over time suffered accelerated decline in FEV₁ compared to patients

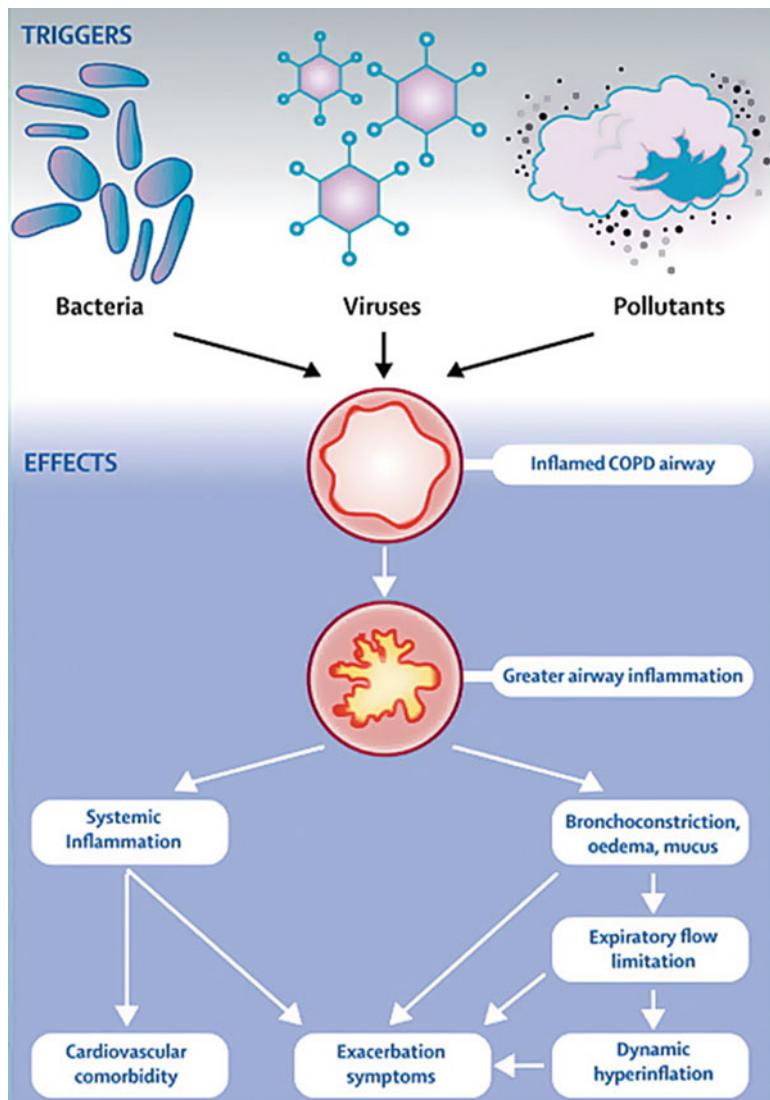


Fig. 2 Triggers of COPD exacerbations and associated pathophysiological changes leading to increased respiratory symptoms (Reproduced with permission: [13])

with stable or decreasing loads and patients with a higher bacterial load had higher sputum IL-8 levels [18]. The relationship between airway inflammation and bacteria has recently been shown to differ according to species, with colonisation by *Haemophilus influenzae* but not *Haemophilus parainfluenzae* relating to higher sputum concentrations of the inflammatory mediators IL-1 β and IL-12 [19].

Macrophages

Colonisation of the lungs in COPD patients may be due to dysfunctional phagocytosis of pathogens by macrophages. Alveolar macrophages from COPD patients phagocytose fewer apoptotic epithelial cells [20] and *Escherichia coli* [21] compared with non-smokers and less *Haemophilus influenzae* compared with smokers without COPD [22]. Furthermore, monocyte-derived macrophages (MDM) from COPD patients demonstrate reduced *in-vitro* phagocytosis for *Streptococcus pneumoniae* and *Haemophilus influenzae* with recent evidence revealing that the lack of pathogen removal is an inherent defect in circulating monocytes from COPD patients that unmasks during maturation into macrophages. Thus defective phagocytosis of bacteria is likely to be an important factor in the development of lower airway colonisation and bacterial exacerbations [23].

Systemic Disease and Inflammation

Exacerbations are associated with increased systemic inflammation [12, 24], and are thought to be a key link between COPD and extrapulmonary manifestations.

However, the source of systemic inflammation in COPD remains unclear. Tobacco smoke alone may contribute significantly to systemic inflammation in COPD and systemic oxidative stress and peripheral vascular dysfunction has been noted in passive smokers and smokers with few pack-years [26, 27]. Hypoxia may also play a role in the generation of systemic inflammation in COPD. Hypoxia *in vitro* leads to increased macrophage-induced cytokine production and circulating TNF- α and soluble TNF-receptor levels are inversely related to hypoxemia (PaO_2) in COPD patients [28].

The Over-Spill Hypothesis

More likely, systemic inflammation in COPD may be the result of a spill-over of airway and lung parenchymal processes and there is considerable evidence to support the movement of proteins from the lung to the systemic circulation [29]. Cigarette smoking is the most important cause of COPD, and bronchial epithelial cells from smokers with COPD show increased trans-epithelial permeability and reduced antioxidant properties, as well as increased released of pro-inflammatory mediators in response to smoke [30].

Strong evidence to support the over-spill hypothesis comes from alveolar macrophages which are responsible for ingesting and clearing inhaled particles. Both human and animal studies have demonstrated that phagocytosis of air pollutants by alveolar macrophages causes pulmonary inflammation [31], (including the release of inflammatory mediators such as TNF- α [32]) which is accompanied by increased concentrations of circulating cytokines, systemic inflammation and microvascular

endothelial dysfunction in the systemic circulation [31, 33]. Furthermore, phagocytosis of small carbon particles by alveolar macrophages stimulates the release of polymorphonuclear leukocytes from bone marrow [34] confirming that alveolar macrophages initiate both a local and systemic inflammatory response in response to lung insults. These experiments have been supported by studies in healthy volunteers exposed to diesel exhaust. Following exposure, neutrophils and B-lymphocytes were found in bronchial lavage fluid and concurrently, significant increases in systemic inflammation (neutrophils and platelets) were observed in peripheral blood [35].

Biomarkers

Further evidence for the role of systemic inflammation in COPD is from acute phase reactant and cytokines, many of which have been proposed as potential biomarkers.

Surfactant Protein D

Surfactant protein D (SP-D) is a large multimeric collagenous glycoprotein produced mainly by type 2 pneumocytes and Clara cells in the lungs, which plays a role in innate defence against micro-organisms [36]. SP-D deficient mice develop chronic lung inflammation and emphysema that can be prevented by administration of truncated recombinant human SP-D [37]. Serum SP-D concentrations are increased in COPD patients and related to disease severity (as measured by FEV₁) and symptoms [38]. Thus, because SP-D is predominantly lung-derived, this biomarker supports the premise that lung inflammation leads to inflammatory changes in the systemic circulation. Data from the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) cohort has also shown that the risk of exacerbations appears to increase with highly elevated baseline serum SP-D concentrations and that serum SP-D may also be a biomarker of anti-inflammatory treatments [39].

PARC (Pulmonary and Activation-Regulated Chemokine)/CCL-18 (CC-Chemokine Ligand-18)

Another biomarker assessed in the ECLIPSE study was PARC/CCL-18, a protein that is secreted predominantly in the lungs. Serum levels are elevated in acute coronary syndromes [40] and in idiopathic pulmonary fibrosis, concentrations of serum PARC/CCL-18 may reflect fibrotic activity and correlate with survival [41]. In COPD, serum PARC/CCL-18 levels are higher than in smokers or never smokers without COPD [42] and plasma PARC/CCL-18 concentrations are further increased during COPD exacerbations [43]. Furthermore, elevated PARC/CCL-18 levels have been associated with increased risk of cardiovascular hospitalization or mortality in the Lung Health Study (LHS) and with total mortality in the ECLIPSE cohort [42].

Fibrinogen

Plasma fibrinogen is an independent risk factor for cardiovascular disease and is elevated in patients with stable COPD and increase further at exacerbation [24]. Viral exacerbations in particular are associated with higher plasma fibrinogen concentrations than nonviral exacerbations [14]. Stable-state plasma fibrinogen levels increase more quickly over time in frequent exacerbators compared with infrequent exacerbators and elevated plasma fibrinogen is also associated with a faster decline in lung function (Fig. 1 [10]).

C-Reactive Protein (CRP)

CRP is a member of the pentraxin protein family and is synthesized in the liver as part of a coordinated cascade known as the acute-phase response. Major stimuli that induce CRP synthesis include the cytokines IL-6 and IL-1, however a number of different agents can alter its expression including corticosteroids [44]. CRP plays an important role in the innate immune system by activating complement, binding to Fc receptors and acting as an opsonin for various pathogens. Interaction of CRP with Fc receptors leads to the generation of proinflammatory cytokines such as IL-6 and IL-8 that enhance the inflammatory response [45]. However, CRP is non-specific and can be elevated in infection, inflammatory and malignant conditions.

Nevertheless, the relationship between COPD and CRP has been a focus of intense scrutiny in part due to the association between raised systemic inflammatory markers and mortality. Dahl and colleagues [46] performed a cohort study with a median of 8 years follow-up of 1,302 individuals with airway obstruction selected from the Copenhagen City Heart Study. Individuals with $\text{CRP} > 3 \text{ mg/l}$ had increased risk of COPD hospitalisation and death than individuals with $\text{CRP} \leq 3 \text{ mg/L}$: after adjusting for sex, age, $\text{FEV}_1\%$ predicted, tobacco consumption, and ischemic heart disease. Hazard ratios for hospitalization and death due to COPD were increased at 1.4 and 2.2 in individuals with $\text{CRP} > 3 \text{ mg/L}$ versus $\leq 3 \text{ mg/L}$. They also identified that these risks were higher with increasing age, $\text{FEV}_1 < 50\%$ predicted, and a higher smoking pack-year history. CRP was also an independent predictor of mortality over several years in 4,803 participants in the LHS with mild to moderate COPD [47]. However, this finding was not replicated in a smaller cohort of patients with moderate to very severe disease [48].

Another large population-based study showed that CRP and fibrinogen levels were incrementally related to airflow limitation [49]. Elevated CRP was also related to health status, exercise capacity and body mass index (BMI) [50] in COPD patients, indicating a clear association with both lung-related and systemic features.

COPD and cardiovascular disease commonly coexist and atherosclerosis may not manifest on electrocardiography or in terms of clinical symptoms. The suggestion that CRP may potentially be raised in COPD patients by occult cardiovascular disease rather than COPD itself was refuted by research that demonstrated that in 88 COPD patients with no evidence of ischemic heart disease on cardiopulmonary exercise

testing, CRP was raised compared with controls (5.03 mg/L vs. 2.02 mg/L), an effect that was independent of cigarette smoking [51]. The same study also reinforced the link between local and systemic inflammation by demonstrating that patients taking inhaled corticosteroids had lower CRP levels. Furthermore, the withdrawal of inhaled corticosteroids led to increases in CRP levels [52] confirming that therapeutic modulation of airway inflammation in stable COPD may affect levels of systemic inflammation.

CRP and Exacerbations

Systemic inflammatory markers (serum CRP and IL-6) are related to lower airway neutrophilic inflammation (leukocyte count and myeloperoxidase) at exacerbation [12] and serum CRP is related to the presence of a potentially pathogenic microorganism in the lower airway [12]. In proteomic studies, CRP has also been demonstrated to have value in the diagnosis of exacerbations. In a comparison of 36 molecules in 90 paired stable state and exacerbation serum samples, nine of these potential biomarkers were significantly higher at exacerbation (defined by well validated symptom criteria and requiring additional prescribed treatment). CRP was clearly the most clinically useful of those tested, with a median value of 4.0 mg/l at baseline compared with 15.6 mg/l at exacerbation. However, when evaluated alone as a diagnostic test, CRP had an area under the receiver operating characteristic curve (AUC) of 0.73, below the threshold of 0.8. However, the combination of CRP with one or more major exacerbation symptoms (increased breathlessness, increased sputum volume, increased sputum purulence) gave an AUC of 0.88, which was significantly greater than for either CRP ($p < 0.0001$) or symptoms alone ($p = 0.004$) [43].

CRP can also inform on exacerbation recovery. Perera and colleagues [53] demonstrated that non-recovery of symptoms at exacerbation is associated with persistently heightened systemic inflammation, as measured by serum CRP. In the same study, patients who developed a recurrent exacerbation within 50 days had a higher CRP overall at 14 days after the index exacerbation compared with those who remained exacerbation-free. Therefore, monitoring of CRP during exacerbation recovery may guide novel anti-inflammatory treatments designed to improve recovery and prevent recurrence.

Cardiovascular Disease and Exacerbations

An increasingly recognised predictor of outcome in COPD exacerbations is the presence of cardiac complications. COPD patients are at increased risk of cardiovascular events [47, 54], with approximately 30 % deaths due to cardiovascular disease [3, 55]. Acute exacerbations may provide a potentially important mechanism to link COPD and cardiovascular disease, through the increased systemic

inflammation at exacerbation. Increased levels of systemic inflammatory markers have been directly or indirectly linked with an increased risk of thrombus formation and cardiovascular events [46, 56, 57] and the upregulation of inflammatory pathways and platelet activation at exacerbation may precipitate an acute cardiovascular event. The formation of platelet-monocyte aggregates is an early process in atherothrombosis [58] and recent data has shown that patients with stable COPD have increased circulating platelet-monocyte aggregates compared with well-matched controls [59]. Platelet activation is further increased in patients with COPD during an acute exacerbation and this mechanism may in part explain the increased cardiovascular risk in COPD [59].

Further evidence to support a link between acute exacerbations and cardiovascular disease has been provided by analysis of data from 25,857 patients with COPD in The Health Improvement Network database [60]. A 2.27-fold increased risk of myocardial infarction (MI) was found 1–5 days after exacerbation (defined by prescription of both steroids and antibiotics) and one in 2,513 exacerbations was associated with an MI within 1–5 days. Additionally, a 1.26-fold increased risk of stroke was seen 1–49 days after exacerbation.

Recent studies have also investigated cardiac involvement in acute COPD exacerbations by analysing cardiac-specific biomarkers at exacerbation [61, 62]. Cardiac troponins are commonly used to diagnose myocardial infarction, being specific markers of myocardial necrosis [63], and N-terminal pro-brain natriuretic peptide (NT-proBNP) is an established marker of left ventricular dysfunction and associated with increased mortality in acute and stable cardiac disease [64]. Chang and colleagues examined [61] NT-proBNP and troponin levels in patients admitted to hospital with exacerbations of COPD, but without clinical evidence of acute cardiac disease. NT-proBNP was elevated ($>220 \text{ pmol/l}$) in 27.5 % of patients and significantly predicted 30-day mortality (OR 9.0, 95 % CI 3.1–26.2, $p < 0.001$). Troponin T was elevated ($>0.03 \mu\text{g/l}$) in 16.6 % of patients and also predicted 30-day mortality (OR 6.3, 95 % CI 2.4–16.5, $p < 0.001$). These associations persisted even after adjusting for other clinical and laboratory predictors of mortality such as BMI and arterial CO₂ pressure, and NT-proBNP and troponin T levels appeared to have additive associations with mortality: 30-day mortality among patients with abnormalities of both NT-proBNP and troponin T being 15-times higher than among patients with normal values. A separate study of 99 patients hospitalised with acute exacerbations of COPD has also recently reported increased levels of high-sensitivity cardiac troponin (hs-cTNT) at exacerbation and found that troponin elevation was independently associated with death (Fig. 3 [62]), particularly amongst tachycardic patients [62].

These studies challenge the way that exacerbations are conventionally treated. Cardioselective β-blockers such as atenolol and bisoprolol have been demonstrated to be safe in COPD and perhaps may be beneficial [65, 66]. Patients receiving β-blockers appear to have a lower risk of COPD exacerbations [67] and a lower mortality from exacerbations [67, 68]. Cardiovascular medications such as statins and angiotensin pathway drugs may benefit COPD patients [69] and randomized trials to investigate this further are ongoing. Therefore in the future, COPD patients, especially those with elevated cardiac biomarkers, may be considered for increased

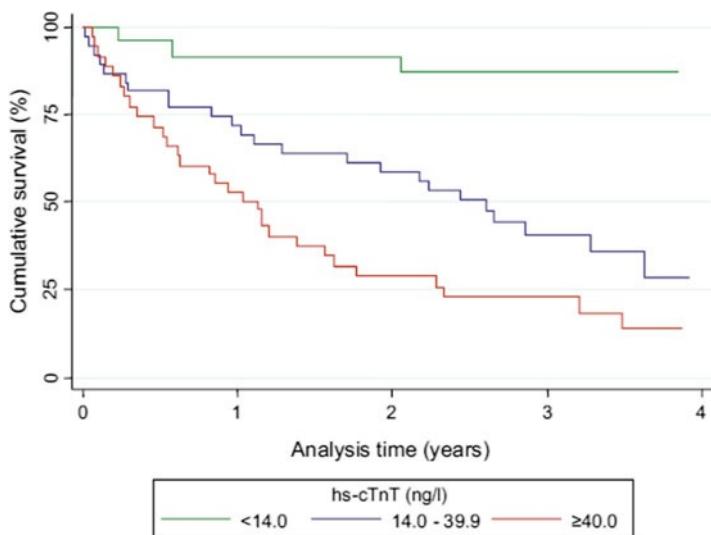


Fig. 3 Survival after admission for acute exacerbations of chronic obstructive pulmonary disease by level of high-sensitivity cardiac troponin T (hs-cTnT), n=99. The figure is based on 219 admissions among 99 patients (Reprinted with Permission: [62])

cardiac treatment at exacerbation. In addition, by reducing the systemic inflammation that is likely to play a key role in the mechanisms underpinning acute cardiac events, the use of novel anti-inflammatories may also reduce the frequency and/or severity of acute cardiac events at exacerbation.

Gastro-Oesophageal Reflux Disease (GORD)

Another comorbidity in COPD patients that may be linked to exacerbations is GORD. Symptoms of GORD are common in COPD patients and are associated with more severe disease [70]. Reflux involves the movement of stomach contents into the oesophagus. This can ascend as high as the larynx and mouth and may be more common in COPD due to coughing, increased use of abdominal muscles during respiration and hyperinflation causing a low lying diaphragm [71].

In asthma, GORD may be accompanied by neutrophilic airway inflammation [72] and is able to elicit nocturnal bronchoconstriction in asthmatics with moderate to severe GORD [73]. Furthermore, bronchoconstriction severity and duration in this group were related to duration of gastro-oesophageal reflux [73].

In COPD, a history of heartburn or reflux was identified as an independent predictor of frequent exacerbator status in the ECLIPSE study [74]. Also, a prospective study of 82 patients with COPD and 40 age matched controls examined the relationship between exacerbations and GORD symptoms using the Frequency Scale for

the Symptoms of GORD (FSSG) questionnaire [75]. Positive GORD symptoms were reported in 22 patients with COPD and in 5 controls, and the frequency of exacerbations was significantly associated with the FSSG score. Multiple regression analysis revealed that GORD symptoms were significantly associated with the occurrence of exacerbations. Therefore, future studies should assess the ability of specific GORD medications to reduce exacerbation frequency.

Conclusions

COPD is characterized by inflammation that is seen both at a local and systemic level. Systemic inflammation is likely to be an important link between the pulmonary and extrapulmonary manifestations of COPD. Extrapulmonary comorbidities are common and important in COPD and significantly impact on morbidity and mortality, especially cardiovascular disease. Systemic inflammation is increased during exacerbations and such events provide a potentially important mechanism to link COPD and cardiovascular disease. COPD patients are at high risk of acute cardiac events at exacerbation and biochemical markers of cardiac dysfunction predict mortality at exacerbation. Future studies should investigate the impact of cardiac treatments and novel anti-inflammatories at exacerbation.

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Infectious Mechanisms Regulating Susceptibility to Acute Exacerbations of COPD

Karin Provost, Himanshu Desai, and Sanjay Sethi

Introduction

Acute exacerbations of COPD (AECOPD) are defined by clinical criteria, outlined in the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [1]. These include an acute increase in one or more of the following cardinal symptoms, beyond day to day variability: dyspnea, increased frequency or severity of cough and increased volume or change in character of sputum, which represent an acute increase in airway inflammation. The role of infection in the pathogenesis of COPD, acute exacerbation and disease progression has been a clinical and research question for many years, and the pendulum has swung from infection as a major cause of acute exacerbation and COPD (British Hypothesis) [2], to infection as an unrelated epiphomenon in acute exacerbation [3–5], and back again to infection as integral in the development of AECOPD and likely an important contributor to COPD progression [6–19]. Upwards of 80 % of AECOPD are driven by infectious stimuli, with 40–50 % associated with bacterial infection and 30–50 % associated with acute viral infection, with some exacerbations having dual bacterial and viral causation [20]. Much of the advancement in our understanding of the role of infection in AECOPD is due to the advancement of clinical and research tools that have allowed researchers to accurately characterize the microbial pathogens, and better understand the host-pathogen interactions (Table 1).

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Table 1 Microbial pathogens in COPD [139]

Microbe	Role in exacerbations	Role in stable disease
Bacteria		
<i>Haemophilus influenzae</i>	20–30 % of exacerbations	Major pathogen
<i>Streptococcus pneumoniae</i>	10–15 % of exacerbations	Minor role
<i>Moraxella catarrhalis</i>	10–15 % of exacerbations	Minor role
<i>Pseudomonas aeruginosa</i>	5–10 % of exacerbations, prevalent in advanced disease	Likely important in advanced disease
<i>Enterobacteriaceae</i>	Isolated in advanced disease, pathogenic significance undefined	Undefined
<i>Haemophilus haemolyticus</i>	Isolated frequently, unlikely cause	Unlikely
<i>Haemophilus parainfluenzae</i>	Isolated frequently, unlikely cause	Unlikely
<i>Staphylococcus aureus</i>	Isolated infrequently, unlikely cause	Unlikely
Viruses		
<i>Rhinovirus</i>	20–25 % of exacerbations	Unlikely
<i>Parainfluenza</i>	5–10 % of exacerbations	Unlikely
<i>Influenza</i>	5–10 % of exacerbations	Unlikely
<i>Respiratory syncytial virus</i>	5–10 % of exacerbations	Controversial
<i>Coronavirus</i>	5–10 % of exacerbations	Unlikely
<i>Adenovirus</i>	3–5 % of exacerbations	Latent infection seen, pathogenic significance undefined
<i>Human metapneumovirus</i>	3–5 % of exacerbations	Unlikely
Atypical Bacteria		
<i>Chlamydophila pneumoniae</i>	3–5 % of exacerbations	Commonly detected, pathogenic significance undefined
<i>Mycoplasma pneumoniae</i>	1–2 %	Unlikely
Fungi		
<i>Pneumocystis jiroveci</i>	Undefined	Commonly detected, pathogenic significance undefined

With the more recent scientific acceptance of infectious organisms, both viral and bacterial, as significant players in AECOPD, the host response in patients with COPD must also be questioned. The airways of patients with COPD have significant infiltration of inflammatory cells (polymorphonuclear cells and CD8+ T lymphocytes) and much higher numbers of alveolar macrophages than are seen in healthy persons [21–27]. The nature of the inflammatory infiltrate suggests and supports the findings of both viral and bacterial infections, with recruitment of immune cells pertinent to both the innate and adaptive immune response. Despite the recruitment of appropriate effector immune cells, in many patients with advanced stage COPD (GOLD III–IV), there is persistent presence of pathogens in the airway, rather than eradication [19, 28], suggesting an impaired host response to infection. Numerous studies have delineated impaired macrophage phagocytosis and cytokine secretion [29–31], impaired humoral immune response to infection [32–37] and impaired T-lymphocyte responses [38]. The mechanisms of this impaired responsiveness of both innate and adaptive immune cells to infection has not been clearly delineated, and remains a target of investigation.

Bacterial Etiology of AECOPD

Although the temporal association of bacterial presence in the airway and COPD exacerbation was first recognized in the early 1950s, there was divergence away from bacterial causation of AECOPD during the 1970s and 1980s related to several observations. There were no differences observed in sputum bacterial isolation rates (at a species level) in between stable state and exacerbations, and studies of immune response studies to bacterial pathogens and placebo controlled antibiotic trials showed inconsistent and contradictory results [4, 39]. Beginning in 1992, multiple investigators began to recognize an effect of bacterial infection and colonization in stable COPD [11, 17, 32, 40–45], but a direct association with AECOPD was not initially recognized. The bacterial isolation in these early studies was done predominantly by sputum culture, and as such, most pathogens isolated can be nasopharyngeal commensals in healthy adults, raising the issue of specimen contamination. In addition, the older studies could not differentiate among strains of a pathogenic species, assuming that all strains isolated from sputum over time were identical. Advancing diagnostic techniques of bronchoscopy with protected specimen brushes and bronchoalveolar lavage, as well as molecular bacterial typing allowed identification of bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* and others at potentially pathogenic concentrations) in the distal airways in stable COPD [9, 17, 28, 40, 41, 46–51], with more severe GOLD stage disease being associated with identification of *Pseudomonas*. Subsequent studies went on to associate colonization with more severe spirometric airflow obstruction [43]. Expanding on the recognition of bacterial colonization during stable COPD, it was recognized that the prevalence of bacteria in the lower respiratory tract increased significantly during AECOPD as compared to stable COPD, when sampled by bronchoscopy [10, 46–48, 52]. Scientific advancement led to the recognition that changes in the overall numbers of potentially pathogenic bacteria in the airways between periods of colonization and acute exacerbation mattered less than the acquisition of a new strain of bacteria [10, 15–17, 28, 47]. Acquisition of a new strain of bacteria (*H. influenzae*, *M. catarrhalis*, *S. pneumoniae* or *P. aeruginosa*) was associated with a greater likelihood of symptoms of an exacerbation, increased inflammatory markers both locally and systemically (TNF- α , IL-8, IL-6, CRP, Neutrophil elastase) and development of a specific host immune response to the infecting pathogen [8, 10, 11, 15, 28, 47]. Although some authors have described a link between the presence of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella* infection and AECOPD [53–55], these studies measured single serologic titers rather than serologic conversion, and an additional respiratory pathogen was often identified. Studies using serologic conversion as a diagnostic criterion or molecular detection to identify the presence of atypical bacterial DNA in sputa [56] during AECOPD indicate only a minor role of these bacteria in exacerbations, often with co-infection with typical bacterial pathogens (Table 1).

The significant role of bacteria in both pathogenesis of COPD and AECOPD, combined with recent data from improved microbiological detection techniques that

the normal lung is not sterile, has led to recent research focused on understanding the microbiome of the lung in stable COPD and during exacerbations. Two groups recently published data using PhyloChip microarray analysis and quantitative PCR and pyrosequencing of the variable regions of the 16S rDNA. The first study identified the bacterial diversity seen during severe AECOPD requiring intubation in eight patients [57], noting significant bacterial richness (as defined by the number of bacterial taxa detected) that waned with prolonged intubation. The common ‘core’ of 75 bacterial taxa representing 27 classified bacterial families was identified in all patients studied. This group included members of the Pseudomonadaceae, Enterobacteriaceae, Campylobacteraceae and Helicobacteraceae families, among others. The majority of the bacteria belonged to the Proteobacteria phylum, with smaller contributions from Firmicutes and Bacteroidetes. The second study addressed the effects of cigarette smoke on the bacterial diversity in comparing healthy smokers to smokers with COPD to non-smokers [58]. The investigators demonstrated lung resident bacteria in all groups, and the dominant phyla were Proteobacteria, Firmicutes and Bacteroidetes, as noted in the first study. There was heterogeneity in the bacterial communities in the non-smoker, healthy smoker and mild COPD patients, which was lost in the patients with moderate and severe COPD. Within each patient, geographic differences in bacterial heterogeneity were also noted, suggesting micro-anatomic differences in bacterial communities within the lung. Whether the micro-anatomic, spatially distinct bacterial communities within the lung or the overall airway bacterial diversity represent mechanisms of disease progression or contribute to AECOPD remains to be determined.

Viral Infections in AECOPD

Respiratory tract viral infections have long been suspected as capable of inciting inflammation sufficient to generate an acute exacerbation of COPD. The diagnosis of viral infection was initially done by cell culture and serologic methods, with more recent studies detecting viral infection by PCR in either sputa, BAL or nasopharyngeal swabs, with the greatest recovery seen in sputa as compared to nasopharyngeal swabs [59, 60]. The most commonly recovered viruses (varying in prevalence in various studies) in AECOPD using the more sensitive PCR detection methods were influenza, rhinovirus, respiratory syncytial virus (RSV), parainfluenza, with the majority of infections due to rhinovirus. These viruses were also found in stable COPD, and it is not clear if those findings were subclinical infection or colonization, as there were no symptoms of active infection in the preceding 30 days [13, 14, 59–62]. Therefore, the presence of viruses in respiratory samples detected by sensitive techniques such as PCR may not always correlate with an acute infection and should be interpreted in its clinical context.

Though there are studies that suggest viral exacerbations are more severe or protracted than non-viral exacerbations [13, 14, 62], these studies did not study bacterial infection concurrently. Virally-induced AECOPD were associated with higher airway levels of IL-6 as compared to AECOPD in which virus was not detected [61,

63, 64]. The presence of eosinophils in sputa samples recovered from AECOPD of viral etiology also differs from the inflammatory cells present during bacterial or non-infectious AECOPD [11]. In studies that have examined viral and bacterial infection simultaneously, presence of dual infections are associated with increased clinical severity of exacerbation [62].

Altered Host Defense to Infectious Challenge in COPD

COPD is now recognized as a state of chronic inflammation, with periods of exacerbation marking acute increases in this inflammation, both locally and systemically. Both pathogenic and host factors determine the outcome of the acquisition of a bacterial strain. Approximately half of the acquisitions of pathogenic bacteria lead to an exacerbation. Pathogen virulence is likely to also play a role in determining which acquisitions lead to acute exacerbations. Strains of *nontypeable H. influenzae* (NTHI) that are associated with exacerbations have more effective adherence to airway epithelium and result in increased IL-6 and IL-8 secretion in vitro and mouse models, as compared to those strains associated with colonization [48, 65, 66], and demonstrated higher levels of neutrophil recruitment to the airway [9, 10, 65].

There are also significant differences in the host response to different pathogens. Patients with COPD are able to eradicate *Moraxella catarrhalis* and *Streptococcus pneumoniae* from the airway quite well following exacerbations, most likely related to an effective immune response [16, 37]. However, though antibody responses develop to NTHI or *Pseudomonas* following exacerbations, effective clearance is often not seen with these pathogens.

Alterations in Innate Immune Responses to Infectious Pathogens in COPD

The innate immune response is the most immediately responsive immune defense to the invading pathogen, and both anatomic and functional barriers (mucociliary clearance, epithelial tight junctions), as well as cellular immunity (recognition of invading pathogens via germline encoded pathogen recognition receptors (TLR, NOD)) and soluble mediators (SLPI, lysozyme, collectins). This initial innate response is multifaceted, including epithelial cells of the upper and lower airways, airway resident macrophages, dendritic cells and recruited polymorphonuclear cells.

Impaired Macrophage Phagocytosis

Multiple authors have demonstrated an association between increased inflammatory cytokines present in the airways of patients with COPD and bacterial infection and

colonization of the lower airway [7, 9, 52, 67]. The dominant cytokines involved, IL-6, IL-8, TNF- α are also seen in AECOPD due to infection, and suggest a common innate immune response at the time of exacerbation. The source of these cytokines may be from either the airway epithelium, after adhesion and invasion by bacterial or viral pathogens, or from the alveolar macrophage after recognition of pathogen associated molecular patterns (PAMP's) and activation of toll like receptors (TLR). Alveolar macrophages are also less able to phagocytose bacteria in patients with COPD [29, 30, 68] and have a less robust response to bacterial proteins, specifically OMP6 and LOS of NTHI [29], and are less able to clear apoptotic cells from the airway [69]. Both disease and cigarette smoke exposure contribute to this relative hypo-responsiveness, as alveolar macrophages from smokers who had ceased smoking have better phagocytic ability than those who continue to smoke [30, 31], and both were reduced relative to healthy controls. The ongoing presence of apoptotic cells in the airway may function as a source of endogenous ligand for inappropriate self-directed immune responses.

Mucociliary Clearance

Normal mucociliary clearance (MCC) maintains the sterility of the tracheobronchial tree by effectively trapping and clearing inhaled and micro-aspirated particles, including infectious pathogens [70, 71]. Smoking disrupts MCC by inducing structural abnormalities in the ciliary apparatus [72]. Other investigators have shown that impairment of MCC is universal, though variable in moderate-to-heavy smokers [73]. Development of chronic bronchitis and airway obstruction in smokers is associated with further deterioration in MCC [71, 73, 74]. Infiltrating neutrophils likely contribute to MCC impairment, probably mediated by increased mucus production (mediated through proteolytic cleavage of TGF α and increased EGF receptor (EGFR) binding), reduced ciliary beating and reduced viscoelastic properties of mucus [75].

Soluble IgA

Tobacco smoke also has effects on airway levels of soluble IgA. The literature has demonstrated conflicting results [76–81], and though these trials were quite heterogeneous, they supported the issue of the importance of secreted airway immunity. The more recent trials support geographic changes in the structural integrity of the lung, demonstrating localized areas of IgA deficiency associated with altered epithelial cell integrity, and reduced pIgR expression (polymeric IgG receptor), which is required for transcytosis of the structural components of the IgA molecule from the basolateral to the apical surface of the epithelial cell. The reduction in

expression of the IgA transport system was supported by findings of reduced total IgA in the BAL of patients with COPD [81, 82]. In contrast, the systemic IgA response elicited in the bronchial mucosa seems preserved in COPD, as demonstrated in COPD patients with *Chlamydia pneumoniae* infection [78].

Antimicrobial Peptides

An increasing number of polypeptides with antimicrobial activity have been identified in the airway surface fluid that may play an important role in host defense in the respiratory tract [83–85]. One major group of these peptides is cationic polypeptides. These include lysozyme, which is lytic to many bacterial membranes; lactoferrin, which excludes iron from bacterial metabolism; defensins, which are released from leukocytes and respiratory epithelial cells; and the cathelicidin family of proteins, (of which only LL-37 is found in humans), which are present in specific granules of neutrophils and airway epithelial cells [86–96]. Deficiency in salivary lysozyme and sputum secretory leukocyte protease inhibitor (SLPI) has been related to more frequent exacerbations [15, 97–99]. In patients with normal baseline SLPI levels, these levels drop significantly at the time of infective exacerbations, which return to normal after resolution of the exacerbation [89]. Lower levels of lysozyme and lactoferrin are noted with both colonization and infective exacerbations with NTHI and *Moraxella catarrhalis*, as compared to pre-acquisition levels [89]. In contrast, LL-37 levels have been shown to increase in the presence of airway bacteria, both in states of colonization as well as infection, with greater increases during infective exacerbation, as compared to colonization [89]. The divergent responses of the airway anti-microbial peptides during infective exacerbation underscores the fundamental importance of host-pathogen interactions. Lower levels may be related to consumption in the face of infection, or a mechanism of bacterial evasion of immune clearance. The role of tobacco smoke specifically on the anti-microbial peptides has not been well studied, though murine models suggest that at least with regards to SLPI, the presence of tobacco smoke was deleterious to the protease inhibitory activity [100].

Another important group of antimicrobial polypeptides are the collectins. Surfactant protein-A (SP-A) and surfactant protein-D (SP-D) are collectins with broad spectrum antimicrobial activity that also promote phagocytosis of particulates by alveolar macrophages [101]. Concentrations of SP-A and SP-D are decreased in smokers, and are further decreased in association with emphysema [102, 103], a finding noted in both human and animal models [104]. Mannose binding lectin (MBL) deficiency has been strongly implicated in infection and COPD exacerbations, with an odds ratio of 4.9 for infective exacerbations, as compared to normal MBL levels [105]. Though considerable progress has been made in understanding the basic biology of these polypeptides, their role in response to respiratory infections and in the pathogenesis of COPD is still poorly understood.

Table 2 Bacterial ligands from COPD pathogens that trigger signal transduction pathways in the human respiratory tract through pattern recognition receptors [139]

Pattern recognition receptor	Bacterial ligand	Bacterial species
TLR-1		<i>S. pneumoniae</i>
TLR-2	P6 P2 porin Lipoproteins Lipoteichoic acid Pneumolysin Lipoooligosaccharide	<i>H. influenzae</i> <i>S. pneumoniae</i>
TLR-4	Pneumolysin Lipoteichoic acid	<i>H. influenzae</i> <i>M. catarrhalis</i> <i>P. aeruginosa</i> <i>S. pneumoniae</i>
CD-14	Lipoooligosaccharide	<i>H. influenzae</i>
LPS binding protein	Lipoooligosaccharide Peptidoglycan	<i>H. influenzae</i> <i>S. pneumoniae</i>
TLR-5	Flagellin	<i>P. aeruginosa</i>
TLR-7		<i>H. influenzae</i>
TLR-9	CpG dinucleotides	<i>S. pneumoniae</i>
Nod1, Nod2	UspA1 Peptidoglycan	<i>M. catarrhalis</i> <i>H. influenzae</i> <i>S. pneumoniae</i>
CEACAM1	UspA1	<i>M. catarrhalis</i>
Platelet activating factor receptor	Pneumolysin Lipoteichoic acid	<i>S. pneumoniae</i>
C-reactive protein	UspA2 Phosphorylcholine	<i>M. catarrhalis</i> <i>S. pneumoniae</i>

TLR toll like receptor

LPS lipopolysaccharide

Nod nucleotide-binding oligomerization domain

UspA Ubiquitous surface protein

CEACAM Carcinoembryonic antigen-related cellular adhesion molecule

Pattern Recognition Receptor Expression

Pattern recognition receptors, of which the transmembrane toll-like receptors (TLR) and cytosolic nucleotide-binding oligomerization domain (NOD) like receptors (NLR) and RIG-I-like receptors predominate, are germline encoded receptors that recognize conserved sequences present on multiple infectious organisms (pathogen associated molecular patterns, PAMP). This non-specific recognition provides an immediate immune response (innate), without the initial requirement for effector memory responses (Table 2).

Recent evidence suggests that TLR signaling is not restricted to microorganism particles but that TLRs recognize a wide variety of signals such as heat shock proteins [106], hyaluronan fragments [107], oxidative stress [108], and neutrophil

elastase [109, 110]. This non-classical activation (or damage-associated molecular pattern (DAMP) activation), may explain the pro-inflammatory state seen in cigarette smoking healthy controls. Recent data defines a direct effect of cigarette smoke induced MMP and CXCL-8 secretion from epithelial cells that is mediated by activation of TLR4 [111–113], and independent of LPS present in the extract. This was neutralized by the addition of anti-oxidants, suggesting an oxidant stress induced activation of TLR4.

The classical pattern-recognition role for toll-like receptors through PAMP recognition, are affected by both tobacco smoke as well as the development of COPD, both on antigen presenting cells (dendritic cells and alveolar macrophages) as well as the airway epithelium. Decreased levels of TLR2 and TLR4 on both airway epithelial cells as well as alveolar macrophages have been demonstrated in the presence of cigarette smoke as well as in patients with established COPD [114–116]. It has been presumed in the past that lower levels of these TLR's contribute to impaired immune responses to pathogenic bacteria, but in the face of newer data demonstrating activation by reactive oxygen species present in cigarette smoke, these may as yet represent downregulation in an attempt to curb detrimental inflammatory processes, though this has yet to be investigated. The presence of soluble forms of both TLR 2 and TLR4, as well as CD14 are an emerging field of study, and how levels of these proteins factor into the lower levels of cell surface expression is not yet defined.

Natural Killer Cells

Natural killer cells are classified as lymphocytes based on their morphology, their lack of antigen specific receptors puts their responses within the realm of the innate immune response [117]. These cells have been found within the airways as well as the lung parenchyma, and act as cytolytic effector lymphocytes, inducing cell death in infected and structural cells in the lung [118, 119] through secretion of perforins and granzyme B. Increased numbers of CD3⁺CD56⁺ NK cells and NKT cells (CD3⁺CD56⁺) have been noted in the induced sputum of patients with COPD as compared to healthy smokers or normal controls [119]. The CD56⁺ cells of patients with COPD expressed greater cytolytic activity, which inversely correlated with FEV1 [119].

Dendritic Cells

Dendritic cells are the canonical antigen presenting cells that link the innate and adaptive immune responses. Dendritic cells act both locally upon antigen recognition (cytokine secretion) and in the lymphoid follicle, after migration from the primary site. Dendritic cells function primarily by promoting differentiation of the CD4⁺ T helper lymphocytes, as well as CD8⁺ cytotoxicity [120].

Tobacco smoke has been associated with an expansion of dendritic cell populations in the lung. Both CD1a Langerhans-like dendritic cells (subepithelial and BAL) and myeloid dendritic cells are selectively recruited into the airways [120–124], and demonstrate increased expression of co-stimulatory markers (CD80, CD86) in smokers as compared to non-smokers [121].

In patients with COPD, all dendritic cell subsets (myeloid and plasmacytoid) demonstrated increased co-stimulatory molecule expression (CD80, CD83 and CD40) that correlated with COPD severity and was not explained by smoking alone [122]. Lung dendritic cells coordinate and co-localize with CD4+ T lymphocytes, and induce Th1 and Th17 responses [122, 125].

The increased number and advanced maturation state of both myeloid and plasmacytoid dendritic cells in the airways of patients with progressive GOLD stage COPD, suggests an enhanced antigen presenting capacity that develops with more severe disease. Whether this develops in response to bacterial colonization and more frequent antigen recognition, leads to self antigen presentation (anti-elastin and anti-endothelin) and progressive auto-immunity or whether these mature dendritic cells are engendering a state of tolerance (via CTLA-4 interaction with T lymphocytes) has not been defined.

Alterations in Adaptive Immune Responses to Infectious Pathogens in COPD

Alterations in Cellular Immunity

Both the innate and adaptive immune responses are altered in COPD and smoking. Antigen specific effector memory responses, of both cell mediated and humoral arms of the adaptive immune response, are required to effectively clear established infection. These effector memory responses respond more slowly than the innate immune response, but are highly specific for the pathogens involved.

The airways of patients with COPD have been noted to retain significant numbers of inflammatory cells of both the innate and adaptive immune responses. Of the adaptive immune response, effector lymphocytes of both CD4+ and CD8+ lineages have been documented in the airways, and subepithelial spaces, with a predominant CD8+ presence [118, 126, 127]. Numbers and cytolytic activity of the CD8+ cells increases substantially in patients with COPD and progressive GOLD stage disease [119, 126]. Elevated levels of interferon gamma have been found in both the BAL and intracellular staining of these lymphocytes, suggesting a Th1 or Tc1 effector phenotype.

Of the CD4+ lymphocytes found in the airways of patients with COPD, there have been two sub-types identified, both Th1 and Th17 cells [128–130]. Lung lymphocytes of the Th1 CD4+ lineage increase with severity of COPD and emphysema and secrete more interferon gamma than control smokers [131].

Th17 cells are a newly recognized CD4+ lymphocyte subset and regulate tissue inflammation by producing IL-17A and IL-17F [132]. Th17 cells regulate immunity to extracellular pathogens, but have also been associated with auto-immunity [132]. IL-17A and IL-17F act on the airway epithelial cells to induce anti-microbial peptides, G-CSF, GM-CSF and chemokine secretion. Recent data may support a geographically restricted role for the two forms of IL-17, with IL-17A localized to the inflammatory hematopoietic cells in the sub-epithelium of small airways, and IL-17F localized to the lymphoid follicles and epithelial cells [129].

Alterations in Humoral Immunity

B cells have been shown to be present in increased numbers in the large and small airways of patients with COPD, organized into lymphoid follicles around the airways and in the parenchyma of patients with COPD with advanced GOLD stage disease [133–137]. These follicles contain memory and naïve B cells, T cells, dendritic cells and follicular dendritic cells, which allow for T and B cell priming and clonal expansion [135, 136]. The B and T cells in the BALT are oligoclonal suggesting antigen specific immunity [134, 138]. The antigens involved in this response have not been identified, but leading candidates include microbial antigens, cigarette smoke-derived antigens, damage-associated antigens from apoptosis or extracellular matrix degradation and auto-antigens.

Despite recruitment of the appropriate effector cell populations, patients with advanced COPD are not able to effectively clear bacterial infection and bacterial colonization results. Whether the breakdown is solely at the level of the innate immune response, at the interface between the innate and adaptive immune response or due to derangements in the adaptive immune response alone, is not clear. It is likely that with disease progression, there is progressive dysregulation of the immune response to invading pathogens, and downward spiral of inflammation, infection, altered immune response, inflammation and tissue injury, that is amplified during periods of AECOPD.

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COPD: Biomarkers and Phenotypes

Kartik V. Shenoy and Gerard J. Criner

Chronic obstructive pulmonary disease (COPD) is a syndrome with deterioration in lung function and quality of life. It is also the fourth leading cause of death in the United States. The disease is characterized by airflow obstruction that is not reversible, which deteriorates over time. Through the course of the disease, there are acute exacerbations of COPD (AECOPD) which cause decline in lung function, worsened quality of life, and adds considerable cost to the healthcare system. COPD classically has been characterized and treated based on the degree of airflow limitation. However, with recent advancements, it has been shown that COPD is indeed a heterogeneous disease with many different presentations, radiographic appearances, responses to treatment, and variable decline in lung function. With this heterogeneity, it is no longer realistic to base assessment and treatment of this disease solely on the degree of airflow limitation. Researchers and clinicians have proposed phenotyping to better characterize the heterogeneous nature of COPD. A phenotype has been defined as “a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression, or death)” [1]. With phenotyping, we are able to group patients into distinct groups with similar prognosis and therapeutic options.

Biomarkers are also an area of interest in COPD. A biomarker is defined by a “characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or a pharmacologic response to therapeutic intervention” [2]. They are clinically useful when they have a well-defined

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relationship to disease development, are easily accessible, and are cost-effective. Biomarkers are even more valuable when they are simple to use, validated, and represent sentinel clinical outcomes and pathogenesis of disease. Clinicians want biomarkers that enable them to quantify disease severity, predict disease progression, predict response to treatment, and are capable of characterizing unique patient clinical phenotypes.

It is clear that both biomarkers and phenotyping are areas that will advance the care of COPD patients. Defining distinct groups of patients that have higher mortality and different response to therapy and those that are prone to exacerbation is important both clinically and from a research standpoint. Finding biomarkers that are then linked to certain phenotypes would be the ultimate goal, thus defining those at-risk patients earlier and developing targeted therapy that will improve disease survival and quality of life and reduce healthcare utilization.

Biomarkers

There are a wide variety of sources available for biomarker testing in COPD. These sources range from easily obtainable to requiring invasive surgical procedures to obtain specimens. There is also increased cost and complexity to more invasively obtained biomarkers (Table 1). Most of the sources are from the lung itself; however, systemic sources of biomarkers are available including blood, serum, and urine. Barriers to COPD biomarker discovery are difficulties accessing suitable lung tissue specimens, poor reproducibility, and lack of standardization of measurements (Table 2). However, further studies should lead to less invasive sources of biomarkers with improved correlation with clinically relevant phenotypes and outcomes.

Table 1 Pulmonary biomarkers

**Pulmonary Biomarkers Vary in Invasiveness,
Costs, Lung Specificity and Complexity**

Invasiveness, Lung Specificity		Costs, Complexity
	Open lung biopsy	↑
	Transbronchial biopsy	
	Bronchial biopsy	
	Bronchoalveolar lavage	
	Exhaled breath condensate	
	Exhaled Gas	
	Sputum	
←	Blood, Serum, Urine	

Table 2 Advantages and disadvantages of pulmonary biomarkers

Lung biomarker source	Advantages	Disadvantages
Blood	Easy to obtain, repeat assessment, measurement of systemic	Variability, sensitivity, and reproducibility? Not lung specific
Induced sputum	Easy to obtain, study entire spectrum of patient severity,	Samples large airways, activates neutrophils, degrades proteins?
Exhaled gas	repeat assessment	reproducibility
Exhaled breath condensate		Variability, sensitivity, and reproducibility?
Bronchoalveolar lavage	Samples cells and mediators	Variable fluid yield, sampling error, supernatant quantification, limited reassessment
Bronchial biopsy	Airway wall structures and different cell subtypes can be isolated and identified Lung tissue, large and small airway can be studied Airway wall structures and different cell subtypes can be isolated and identified; large and small airways can be studied	Difficult to recruit patients, proximal airways only with bronchial biopsy, limited reassessment. Difficult to perform bronchoscopy on severely limited patients

Adapted from Barnes, AJRCCM 2006; 174: 6–14

Bronchial Biopsies

There is an advantage to endobronchial biopsies because it is a direct sample of the airway tissues. These biopsies provide assessment of the structure of the airway wall, epithelium, basement membranes, and other structures that are important in the local and systemic inflammatory process of COPD. Having tissue biopsies also affords study of biomarkers of structural damage such as uncontrolled proliferation and apoptosis.

Biomarker studies of lung tissue show considerable inflammation in the central airways, peripheral airways, and the lung parenchyma itself. In smokers with established COPD, there is airway wall inflammation and fibrosis along with smooth muscle hypertrophy and mucous plugging. There is also increased infiltration of macrophages and activated T lymphocytes. Specifically there are elevated numbers of CD8-positive lymphocytes over CD4-positive lymphocytes in COPD. This relationship becomes stronger as the degree of airflow obstruction worsens [3]. Others have found that loss of lung function in COPD is associated with high percentage of T lymphocytes that expressed chemokine receptors CCR5 and CXCR3 [4]. In AECOPD, there are increased numbers of neutrophils and eosinophils which are associated with upregulation of chemoattractants [5, 6]. Thus, there are increased inflammatory biomarkers in lung tissue in the stable and exacerbating COPD patient.

Given the overall increase of inflammatory biomarkers in COPD and AECOPD, there have been several studies that have looked at the anti-inflammatory effects of treatments on bronchial biopsies. In a double-blind placebo-controlled biopsy study, inhaled corticosteroids had no significant reduction in the amount of CD8-positive T lymphocytes or neutrophils. There was a slight reduction in the CD8/CD4 ratio. Despite the minimal changes in biopsy biomarkers that are germane to COPD, there were significant improvements in symptoms and reduced AECOPD [7]. The combinations of corticosteroid with bronchodilator have more favorable effect on biopsy biomarkers. The use of combination of salmeterol and fluticasone showed decreased amounts of CD8-positive T cells and also reduced cells expressing genes for interferon gamma and tumor necrosis factor alpha (TNF- α) [8]. Phosphodiesterase inhibitors also have favorable reduction of inflammatory biomarkers in lung biopsies. Cilomilast, an oral phosphodiesterase-4 inhibitor, reduced the number of CD8-positive cells on biopsy [9].

There are several limitations to measuring biomarkers with bronchial biopsies. The procedure is invasive so it may be difficult to recruit patients for clinical trials. It may be unsafe for patients with more severe disease and those in exacerbation, given that bronchoscopy can be associated with significant hypoxemia and hypercapnia.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is also an invasive procedure that can be performed to evaluate biomarkers in COPD. It has the advantage of sampling peripheral airways. It can be performed relatively safely. BAL can also be done during bronchial biopsy which adds additional information to biopsy specimens.

Specific cellular makeup and biomarkers of COPD have been studied. From the cellular standpoint, neutrophils, macrophages, and CD8-positive lymphocytes are frequently found in BAL studies with COPD [10]. Neutrophils are found abundantly in BAL fluid both in stable COPD patients and during exacerbation. Neutrophils play a role in the development of mucous metaplasia in chronic bronchitis and are involved in the destruction of lung tissue in emphysema. Macrophages are also found in BAL specimens. Interestingly macrophages account for the majority of inflammatory cells recovered by BAL regardless if the subject is a nonsmoker, healthy smoker, or with COPD [11]. As in bronchial biopsy, there is an increased number of lymphocytes in BAL specimens [12]. In patients with chronic bronchitis, there is an increased number of CD8-positive cells in BAL fluid [13]. Some studies have also found an increased number of eosinophils in COPD patients with chronic bronchitis symptoms, although this is not consistently shown in publications.

Mediators released by the macrophage express a variety of matrix metalloprotease enzymes (MMPs). These enzymes are believed to facilitate leukocyte migration and infiltration into injured tissue and play a large role in the development of emphysema [10]. They also play a major role in tissue remodeling and repair. Increases in

MMP-1, MMP-9, and MMP-12 have all been seen in BAL fluid of COPD. MMP-12 in particular has been a focus of increasing attention in emphysema. MMP-12 in particular is important for macrophage migration through the extracellular matrix, and in mouse models, alveoli destruction is associated with MMP-12. Although this specific MMP is seen in normal subjects, it is considerably increased in BAL fluid of COPD patients [14]. Another biomarker that has been implicated in the pathogenesis of COPD is interleukin-8 (IL-8). It is a chemoattractant that is needed to recruit and activate neutrophils and is increased in BAL specimens of COPD patients. IL-8 has also been found in the BAL fluid of subjects with subclinical emphysema compared with age-matched smokers without emphysema [15]. One can speculate that IL-8 plays a key role in the development and progression of COPD. Other inflammatory markers of such tumor necrosis factor (TNF)-alpha and myeloperoxidase have also been found to be increased in BAL fluid of COPD patients.

BAL does have some disadvantages. For one it still requires bronchoscopy which is an invasive procedure. It can cause transient fever, discomfort, and hypoxia. BAL fluid return in those with more severe emphysema is often reduced, making sample size an issue with these particular patients. There is also no standard for quantification of biomarkers in the supernatant of BAL [2]. This may be a factor that contributes to the variability in biomarker measurements.

Sputum

Many COPD patients produce sputum without difficulty. However, spontaneous sputum may contain a high proportion of dead cells. This often produces misleading cell counts and biomarker measurements. Thus, most research involving sputum collection centers around induced sputum. Sputum induction with hypertonic saline is generally well tolerated by the COPD patient.

Many of the elevated biomarkers in sputum are similar to what is increased in BAL fluid. The cellular makeup of sputum in COPD displays increased inflammation. Most COPD patients have an increased number of neutrophils and some have an increased number of eosinophils. As in biopsy and BAL specimens, there is an increased amount of CD8-positive T cells. Inflammatory biomarkers have been reported to be increased in COPD patients. Sputum IL-8 is one particular biomarker that has been studied extensively. There are increased levels of sputum IL-8 in COPD patients compared to asthma patients and healthy control subjects. IL-8 also has a negative correlation with FEV₁ [16, 17]. Increases in IL-8 are even more pronounced in AECOPD [18]. As in BAL samples, there are also increases in sputum proteases. One specific study showed that there was increased MMP-8 and MMP-9 in patients with mild to moderate COPD compared to smoking controls [19].

Sputum samples are easy to obtain and can reveal much information about the inflammatory biomarkers in the lung. However, there are several limitations. Sputum induction generally involves the large airways. Thus, information from sputum samples may not be telling of peripheral airway inflammation. Also sputum

induction with hypertonic saline causes neutrophilic inflammation itself. This inflammation can persist up to 24 h [20]. By and large the induction process is well tolerated; however, it may cause significant bronchoconstriction despite pre-administration of beta-agonist. This can make it an unsafe procedure for those COPD patients with significant impairment. Processing sputum also requires a solubilization process. This usually takes place with dithiothreitol. This product can disrupt sulfhydryl and alter the composition of protein, making them unrecognizable by antibodies. Both the collection and processing of sputum may affect both the validity and reproducibility of biomarker studies.

Exhaled Gases/Exhaled Breath Condensate

These two methods of biomarker measurement are attractive. They are relatively easy to collect and can be done repeatedly even in patients with significant impairment. However, both techniques have several factors that affect the measurements. Thus, guidelines and recommendations have been set for sample collection. For exhaled breath condensate, it is recommended that collection occurs during tidal breathing using a nose clip and a saliva trap [21]. Both the cooling temperature and collection time should be defined. The condenser used should be an inert material, and a filter between the subject and condenser is not recommended. There are also recommendations for standardized collection of exhaled gases. Most of these recommendations stem from the study of nitrous oxide. Generally expiration needs to be continuously sampled by a gas analyzer. This would result in an exhaled gas profile that allows the administrator to confirm the required flow rate and pressure parameters. There are also other considerations such as the ambient levels of the gas being measured, time of day, and use of nose clips to avoid sampling gas from the upper airways [22].

In exhaled breath condensate of COPD patients, there is an increase in markers of oxidative stress. Hydrogen peroxide is found to be increased in stable COPD patients compared to normal controls [23]. This increase is heightened in those patients with exacerbation. 8-isoprostanate, another marker of oxidative stress, has also been shown to be elevated in COPD. One study found that COPD patients that were either current smokers or ex-smokers had similar levels of 8-isoprostanate [24]. These levels were approximately twofold higher compared with healthy smokers and fourfold higher than healthy nonsmokers. Levels of 8-isoprostanate are further increased in AECOPD. These results show that reactive oxygen species may play a role in the pathogenesis of COPD and AECOPD.

Not only are oxidative stress markers increased in exhaled breath condensate of COPD patients, but there are also elevations of inflammatory mediators [2]. Specific inflammatory mediators that have been studied are leukotriene B4, prostaglandin E2, and IL-6. Though these mediators do play a large role in inflammation, it is unclear how their elevation in exhaled breath condensate relates to COPD.

There are several exhaled gases that have been studied in COPD. However, exhaled nitric oxide (eNO) is most extensively studied. In asthma, eNO not only correlates with sputum eosinophilia but also correlates with the degree of bronchial hyperresponsiveness [25]. Other studies show that treatment of asthma with inhaled corticosteroids reduces eNO levels[26]. This makes eNO an attractive biomarker to follow because it marks the degree of airway inflammation and is reduced when this inflammation is treated. However, in patients with COPD, measuring eNO is less useful. In most studies, eNO is either normal or only slightly increased in the stable COPD patient. Those with AECOPD do have increased eNO levels [27]. More recent studies concentrate on eNO in the peripheral airways in COPD patients. To study these airways, one needs to perform measurements of eNO at different flows. This will allow the investigator to determine nitric oxide levels from flow-dependent and flow-independent airways of the lung. These studies show that there is an increase in eNO in the peripheral airways of COPD patients [28]. Caution should be used in interpreting these studies since there is lack of standardization of measurement of eNO in peripheral airways. Other gases such as volatile hydrocarbons and carbon monoxide have been measured in COPD with varying results.

Though exhaled gases and condensate are attractive because of their relative ease of measurement, there are some disadvantages to consider. The reproducibility of measurement is a considerable problem. Also many of the substances measured can be elevated in healthy smokers. Further work on standardization of measurements needs to take place before these methods have clinical utility.

Blood

Blood is an attractive source for biomarkers. Blood can be measured on multiple occasions and is safe to obtain during AECOPD. Recently researchers have hypothesized that systemic inflammation plays an important role in the pathogenesis of COPD and complicating comorbidities [29, 30]. Several serum biomarkers such as C-reactive protein (CRP), TNF- α , and interleukins support this theory. In COPD, CRP has been studied extensively. CRP is a marker of impaired energy metabolism and has been linked to other diseases, such as pneumonia and chronic inflammatory conditions. Particular interest in this biomarker exists because it may establish a link between systemic inflammation, cardiovascular disease, and identification of infectious related exacerbations in the COPD patient. In stable COPD, increased CRP levels are linked to reduced functional capacity and lower FEV₁ [31]. Elevated CRP is also associated with reduced arterial oxygen tension and lower 6-min walk distance and is controversially associated with increased mortality [32]. Some studies show that increased CRP is linked with mortality in mild to moderate disease [33]. However, in patients with moderate to very severe COPD, CRP is not associated with increased mortality [34]. CRP has also been studied in AECOPD. Increased CRP along with major symptoms (dyspnea, sputum volume, and sputum purulence)

is a good predictor of acute exacerbation, but does not predict severity of exacerbation [35]. CRP also correlates with inflammation present in the lower airways during exacerbation, particularly in the presence of bacterial pathogens in the sputum [36]. Other studies have shown that CRP is normal in approximately half the patients with AECOPD, except when there is purulent sputum [37]. Thus, CRP may be predictive of exacerbations caused by bacterial infections. More studies are needed to confirm this hypothesis, but if true, it could lead to reduced antibiotic usage, less healthcare expenditure, and fewer drug-resistant bacterial pathogens. Not only is CRP predictive of bacterial exacerbation, but it is also elevated in patients that have slower AECOPD recovery. The effect of COPD treatment on CRP levels has also been evaluated. Some studies show that the use of inhaled corticosteroids as well as oral corticosteroids in mild to moderate COPD reduces CRP levels [38]. However, these trials were limited because of study design and small sample sizes. Larger multicenter trials show that inhaled corticosteroids with or without long-acting beta-agonists do not significantly reduce CRP levels [39].

Another Blood Biomarker That Is of Considerable Interest in COPD Is TNF- α . TNF- α is associated with increased metabolism, protein turnover, and systemic inflammation. In animal models, overexpression of TNF- α causes increased collagen, thickened plural septa, and increased lung volume, all of which are seen in emphysema [40]. In humans, polymorphisms of the TNF- α gene have been implicated in the development of COPD. Particularly in East Asian populations, there is increased presence of TNF- α polymorphisms in COPD patients compared with healthy smoking controls [41]. TNF- α is also elevated in those COPD patients with increased unintended weight loss [42]. This is of particular interest given that low body mass index (BMI) is associated with increased mortality in COPD and lower fat-free mass (FFM) affects exercise capacity, peripheral muscle function, and quality of life.

Other blood biomarkers that have been studied are copeptin, sTREM-1, and B-natriuretic peptide (BNP). Copeptin is increased during exacerbation of COPD compared to non-exacerbating controls [43]. A high copeptin level at admission for AECOPD is associated with greater length of stay and higher risk of long-term clinical failure [44]. The relationship between copeptin and long-term clinical failure remains strong even when controlling for confounding variables such as age, baseline lung function, and comorbid conditions. sTREM-1 levels are significantly elevated in patients with COPD compared with controls and is associated with worse lung function [45]. sTREM-1 can also be elevated during acute exacerbation with purulent sputum. Like CRP, sTREM-1 may be useful in predicting exacerbations related to bacterial infection. BNP, a marker of cardiac stress, is significantly elevated in AECOPD, and it is helpful in predicating need for intensive care [46]. However, BNP is not linked to short- or long-term survival in COPD.

Though blood is an attractive biomarker source, there are some limitations to its use. Measurement of blood biomarkers can be variable throughout the day and may be elevated in collagen vascular disease and infections. Newer technologies such as protein microarray platform (PMP) have been used to identify a large number of

serum biomarkers that are associated with clinically useful endpoints in COPD. Though these types of studies are important in the initial evaluation of COPD biomarkers, issues with confounding variables must make us interpret these studies with caution. Overall, larger prospective studies are needed to fully define the sensitivity and specificity of blood biomarkers.

Phenotypes

Phenotypes of COPD have been studied with great interest, attempting to combine patients with comparable disease attributes into groups that have similar outcomes and prognosis. It is clear now that COPD is indeed a heterogeneous disease with many different clinical, radiographic, and systemic manifestations. One of the initial phenotypes of COPD is the differentiation between the “pink puffer” and “blue bloater.” A pink puffer is a COPD patient with predominantly emphysema. Emphysema results from destruction of the airways distal to the terminal bronchioles. This eventually leads to destruction of the capillary bed and difficulty with blood oxygenation. The blue bloater is a COPD patient where the underlying pathology is chronic bronchitis. These patients have mucous hypersecretion with resulting airway obstruction. The increased mucous results from hyperplasia of mucus-producing cells and airway inflammation. Pink puffers are classically described as having pink lips due to their tendency to hyperventilation. Because of the hyperventilation, these patients often suffer from muscle wasting. Blue bloaters often suffer from hypoxemia, polycythemia, and hypercapnea. Because of the significant airflow obstruction, there is increased residual volume in the lung causing the blue bloating. Both emphysema (pink puffers) and chronic bronchitis (blue bloaters) suffer from different clinical and psychological symptoms of COPD [47]. The mainstays of COPD therapy involve treatment of airflow obstruction with bronchodilators, smoking cessation, pulmonary rehabilitation, and oxygen therapy for those who qualify. However, there are some treatments that benefit one group over the other. In general, surgical therapies for COPD apply to those with emphysema (described below) [48]. Newer drugs have been developed that prevent AECOPD in select patients with chronic bronchitis [49].

Spirometry has been a useful tool to help diagnose and characterize COPD. Patients with COPD have irreversible airflow obstruction on spirometry. Stages of COPD have been developed based on spirometry to guide treatment. In North America, the most followed guidelines are of the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The hallmark of these guidelines centers around the forced expiratory volume in 1 s (FEV_1) on spirometry; as the FEV_1 decreases, the severity of COPD increases and therapy escalates. Spirometry has also been used to delineate phenotypes of COPD. One specific phenotype is that of the rapid decliner. Those with rapid decline of FEV_1 have increased morbidity with more frequent hospitalizations and increased mortality [50]. There are plasma biomarkers

that are associated with rapid decline. One small study found and analyzed polymorphism in the antioxidant gene glutathione S transferase M1 in relation to decline of FEV₁. Those patients with a family history of COPD and a specific polymorphism of GST M1 have rapid decline in FEV₁ [51]. This provides an interesting example of the link between phenotypes and biomarkers.

Along with rapid decliners, pulmonary function testing may help delineate another phenotype. The hallmark of COPD is its irreversible airflow obstruction. However, asthma and COPD overlap and produce subsets of patients that have COPD and airway hyperresponsiveness. Spirometric testing often tests bronchodilator responsiveness. Response to bronchodilator has little if any prognostic value in COPD [52]. However, standardized methacholine challenges which test airway hyperresponsiveness (AHR) may be of clinical utility. An increasing proportion of COPD deaths are associated with increasing hyperresponsiveness on methacholine challenge [52].

Assessment of COPD by computer tomography (CT) scanning provides an objective measure of burden of disease. Quantitative measurements of emphysema correlate well with pulmonary function testing [53]. Measurement of proximal airway wall thickness also correlates with pulmonary function testing and rates of exacerbation [54]. Further studies are evaluating airway wall thickness of more distal airways. Though this research is interesting, it may push the limits of accuracy of current CT devices. More structuralized protocols are needed for the study of distal airways in COPD. It is currently unclear if different radiological characteristics of COPD lead to clinical meaningful outcomes. However, the National Emphysema Treatment Trial (NETT) provides some evidence for radiographic-based phenotypes in COPD. In this trial, those with homogenous emphysema on CT, reduced FEV₁, or low diffusion capacity of carbon monoxide undergoing lung volume reduction surgery (LVRS) had increased risk of mortality, whereas those with upper lobe predominant emphysema with decreased exercise capacity developed a functional as well as mortality benefit from surgery [48]. This data supports that radiography along with other clinical parameters helps define clinically useful phenotypes.

Phenotypes of COPD can also be based on clinical symptoms. One clinical parameter that has been used to define a phenotype is exacerbation. Those who have frequent AECOPD may be a separate clinical phenotype of COPD. However, the definition of an exacerbation is somewhat vague and often arbitrarily determined by the treating physician. It is clear that symptoms of increased dyspnea and sputum production are often present without seeking medical attention. Whether these increased symptoms lead to clinically significant outcomes is not known [55]. However, those with frequent AECOPD requiring treatment with hospital admission or increased outpatient therapy have increased morbidity and mortality. Firstly frequent AECOPD has negative effects on longitudinal lung function. Those with frequent exacerbations have lowered FEV₁ that declines at a faster rate compared to non-exacerbators [56]. Frequent AECOPD leads to significant negative effects on short- and long-term quality of life [57]. However, patients with frequent AECOPD tend to be responsive to therapy with inhaled corticosteroids [58]. These patients may also benefit from more novel therapies such as long-term macrolide antibiotics and phosphodiesterase inhibitors [49, 59].

Given the significant heterogeneity of COPD, phenotypes will help us triage patients that have increased risk of worse clinical endpoints. By making phenotypes, we can develop therapies for select groups of patients. Some of this is coming to light with targeted phenotypes benefiting from lung volume reduction surgery and medications such as inhaled corticosteroids and phosphodiesterase inhibitors. More importantly biomarkers could help the process of determining distinct COPD phenotypes. They have the potential to help us predict who will be frequent exacerbators, who are more likely to have rapid decline in lung function, and determine novel therapies to prevent end-stage lung disease.

We have moved considerably from the original diagnosis of COPD being a homogeneous syndrome. There are developed stages of disease, phenotypes that are benefited by targeted therapies, and biomarkers that predict adverse outcomes. With continued study biomarkers and phenotypes can be linked together. Perhaps using biomarkers one day will predict distinct phenotypes and help target disease more precisely.

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Anti-Inflammatory Therapeutics in COPD: Past, Present, and Future

Peter J. Barnes

Introduction

Corticosteroids are highly effective in the therapy of asthma; whereas they have little or no anti-inflammatory effects in most patients with COPD. This has prompted a search for alternative anti-inflammatory treatments, and this has so far proved to be a great challenge. The only effective anti- inflammatory treatment for COPD that is in current use is theophylline, which is widely used in developing countries but little use in Western countries because of its side effects at high doses. There is a pressing need to develop new anti-inflammatory treatments in order to more effectively treat systems, prevent exacerbations, reduce disease progression, and reduce mortality.

Inflammation in COPD

COPD involves chronic inflammation of the airways, particularly peripheral airways and lung parenchyma, which results in progressive small airway fibrosis (chronic obstructive bronchiolitis) and alveolar wall destruction (emphysema) [1]. Recent evidence shows that there is an early loss of peripheral airways that appears to precede the development of emphysema [2].

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Inflammatory Cells and Mediators

There is chronic inflammation predominantly in small airways and the lung parenchyma, with an increase in numbers of macrophages and neutrophils in early stages of the disease indicating an enhanced innate immune response, but in more advanced stages of the disease, there is an increase in lymphocytes, particularly cytotoxic CD8⁺ lymphocytes (Tc1 cells) and CD4⁺ Th1 cells, as well as lymphoid follicles that contain B- and T lymphocytes, indicating acquired immunity [3, 4]. Alveolar macrophages play a critical role in the orchestration of this pulmonary inflammation, since they are activated by inhaled irritants such as cigarette smoke and release chemokines which attract inflammatory cells, such as monocytes, neutrophils and T cells, into the lungs [5]. There is evidence for both innate and adaptive immune response activation, with the involvement of dendritic cells to link these two types of inflammation [6]. The inflammation in COPD persists despite smoking cessation so appears to be driven by endogenous factors, such as chronic bacterial infection (microbial colonization) and/or autoimmune mechanisms [7].

Prominent mediators are those that amplify inflammation, such as tumor necrosis factor- α (TNF- α), interleukin(IL)-1 β (IL-1 β) and IL-6, and chemokines which attract inflammatory cells such as CXCL8 (IL-8), CXCL1 (GRO α), CXCL10 (IP-10), CCL1 (MCP), and CCL5 (RANTES) [8, 9]. Elastolytic enzymes account for the tissue destruction of emphysema and include neutrophil elastase and matrix metalloproteinase-9 (MMP-9). There is an imbalance between increased production of elastases and a deficiency of endogenous antiproteases, such as α_1 -antitrypsin, secretory leukoprotease inhibitor, and tissue inhibitors of MMPs. MMP-9 may be the predominantly elastolytic enzyme causing emphysema and also activates transforming growth factor- β a cytokine that is expressed particularly in small airways that may result in the characteristic peribronchiolar fibrosis. Oxidative stress is a prominent feature of COPD and is due to exogenous oxidants in cigarette smoke and endogenous oxidants release from activated inflammatory cells, such as neutrophils and macrophages [10]. Endogenous antioxidants may also be defective. Oxidative stress enhances inflammation and may lead to corticosteroid resistance.

Systemic Features

Patients with severe COPD also develop systemic features, which may have an adverse effect on prognosis, and this may be due to systemic inflammation which spills over from the lung periphery [11]. A common systemic feature is weight loss due to loss of skeletal muscle bulk, and this is largely explained by reduced physical activity as a result of exertional dyspnea. Cardiovascular diseases (ischemic heart disease, chronic cardiac failure, and hypertension) are most common in COPD than in normal smokers and are the common cause of death amongst COPD patients. Metabolic diseases such as diabetes and metabolic syndrome, osteoporosis, renal disease, and depression are also common. There appears to be a difference in susceptibility to systemic features between patients as they do not always correlate

with disease severity. It is important to manage these comorbidities, and this may involve treating the systemic inflammation in the future.

Exacerbations

Acute exacerbations are an important feature of COPD and may lead to hospitalization, accelerated disease progression, and mortality, accounting for a high proportion of the costs of COPD. Exacerbations are usually due to infections, either due to bacteria (especially *Haemophilus influenzae* or *Streptococcus pneumoniae*) or to upper respiratory tract virus infections (especially rhinovirus or respiratory syncytial virus) [12]. Exacerbations of COPD tend to increase as the disease progresses, but some patients appear to have more frequent exacerbations than others, which may suggest genetic predisposing factors [13]. Exacerbations are due to an increase in inflammation, with increased numbers of inflammatory cells (especially neutrophils) and mediators, so should be prevented and treated by effective anti-inflammatory treatments. Chronic microbial colonization may predispose to bacterial exacerbations, and this in turn may reflect a defect in bacterial phagocytosis by macrophages and neutrophils [14].

Past and Current Anti-Inflammatory Therapy

Current therapies are poorly effective against COPD inflammation but may provide clues to future drug development.

Bronchodilators

Long-acting bronchodilators are the most effective therapies for COPD and work mainly by dilating peripheral airways and thus reducing air trapping and lung hyperinflation. However, it has been suggested that they may have some anti-inflammatory properties since they significantly prevent exacerbations, with reductions of 15–20 %. Muscarinic receptors are expressed on several inflammatory cells, including macrophages, neutrophils, and T cells, and acetylcholine may have some proinflammatory effects [15, 16]. However, tiotropium bromide has no obvious anti-inflammatory effect based on measurements of inflammatory cells and mediators in induced sputum in patients in whom it effectively reduces exacerbations [17]. Long-acting β_2 -agonists (LABA) may theoretically reduce inflammation. For example, formoterol and salmeterol inhibit the release of inflammatory cytokines from human macrophages *in vitro* [18]. However, there is no convincing evidence that LABA therapy alone reduces inflammation in COPD patients. It seems more likely that long-acting bronchodilators reduce exacerbations by stabilizing the airways.

Corticosteroids

Corticosteroids are poorly effective in most patients with COPD, even when high oral or inhaled doses are used [19]. Patients with COPD are commonly treated as if they have asthma, and many patients with a diagnosis of COPD are currently treated with high doses of inhaled corticosteroids (ICS), often in combination with a LABA as a fixed combination (fluticasone/salmeterol or budesonide/formoterol). Over 70 % of patients with diagnosed COPD are now treated with high doses of ICS, yet there is still little evidence for their clinical benefit and increasing evidence that the high doses currently recommended are harmful and they are costly [20, 21]. ICS are recommended in currently used management guidelines for COPD; for example, the GOLD suggests that ICS should be introduced only in patients with severe disease ($FEV_1 < 50\% \text{ predicted normal}$, GOLD3) who have two or more exacerbations a year [22]. It is likely that this would amount to less than 10 % of all patients, rather than the more than 70 % that currently prescribed this therapy, suggesting that ICS are grossly overprescribed. Indeed, the question is whether they should be used at all, unless patients have concomitant asthma.

Many placebo-controlled clinical trials have been conducted with ICS in patients with differing severities of COPD. Four large studies then looked at high doses of ICS compared to a placebo over a 3-year period, and all showed no effect on disease progression, measured by annual decline in FEV_1 [23–26]. However, there was a reduction in the number of exacerbations, a secondary outcome measure, in two of the studies, although there were differences in how exacerbations were defined. An earlier study specifically looked at exacerbations of COPD and showed that high dose ICS had no overall effect on exacerbations, but a post hoc analysis showed a reduction in severe exacerbations [27]. Combining these studies in a meta-analysis failed to show any effect on FEV_1 decline [28]. None of these studies was sufficiently powered to look at mortality, but pooled analysis of several trials comprising over 5,000 patients suggested that there was a reduction in all cause mortality of approximately 25 % [29]. In the large TORCH study including approximately 6,000 patients with COPD studied over 3 years, in which all cause mortality was the primary outcome measure, there was no evidence that high dose fluticasone alone reduced mortality – in fact there was a small but insignificant increase (~6 %) in mortality at the end of the study [30]. A post hoc analysis of the TORCH study showed that ICS has a small but statistically significant effect in reducing the annual rate in FEV_1 decline [31]. Taking all the studies together, a meta-analysis, which included over 13,000 COPD patients, found no significant effect if ICS on rate of FEV_1 decline or on mortality, although exacerbations were reduced by approximately 25 % [32]. Suissa has identified several shortcomings in the randomized controlled studies of ICS in COPD. A major limitation of these studies was the requirement that patients should stop using their prescribed ICS at the time of randomization, so that a large proportion of patients in the placebo or comparator arms were abruptly withdrawn from a high dose of ICS. A detailed reanalysis of one such

trial showed that the effect of ICS on the likelihood of the first exacerbation was significantly protective *only* amongst patients who were previously treated with ICS but had to discontinue [33] Furthermore, it showed no effect of ICS in patients who were naïve to ICS prior to randomization. Thus, trials that have reported a benefit for ICS may have simply shown an effect of abruptly discontinuing high dose ICS therapy, which may lead to side effects, such as relative adrenal insufficiency and other rebound steroid effects, since there are clear systemic effects with high doses of ICS such as fluticasone propionate.

Another problem that was identified was the incomplete follow-up of patients, who were observed only until they discontinued the study drug rather than to the end of planned follow-up. This is a major problem in view of the high and early rates of discontinuation in these studies. This bias was demonstrated in studies with incomplete follow-up that found a ~25 % reduction in all-cause mortality with ICS [29], which was not confirmed in the TORCH trial, in which all patients were followed for 3 years to identify all deaths using a proper intent-to-treat analysis [30]. The OPTIMAL trial, which also avoided this bias by identifying exacerbations, the primary outcome, for the entire 1 year follow-up period also found no benefit of ICS [34]. Measurement of FEV₁ decline was also misinterpreted due to failure of intention-to-treat analysis. In the TORCH study, nearly 18 % of placebo patients did not contribute a single FEV₁ value to the analysis of FEV₁ decline compared with only 9 % of patients allocated to combination therapy [31]. The excluded patients are likely to have had the lowest FEV₁ values at their initial visit, so that the slope of FEV₁ decline in the remaining patients with better FEV₁ initial values at the first visit may have been affected by regression to the mean, thus giving the impression that ICS affect FEV₁ decline [35]. Observational studies that have suggested a reduction in mortality with ICS use were flawed by “immortal time bias,” as there was a survival advantage to the ICS users by defining exposure in such a way that they had to be alive to receive their ICS prescription [36]. Indeed, a correct analysis of the data completely eliminated any apparent protective effect of ICS [37].

COPD is a heterogeneous disease with several different pathological mechanisms, including emphysema, small airway disease, and mucous hypersecretion, so it is possible that corticosteroids might work more effectively on some components of disease compared to others. However, this has so far not been investigated or proven in clinical trials. COPD patients who have some of the clinical features of asthma, with greater reversibility of airways obstruction, may have increased sputum eosinophils and an increase in exhaled nitric oxide concentration, which are characteristics of asthmatic airway inflammation [38]. These COPD patients probably have coexistent asthma. COPD patients with increased sputum eosinophils show a reduction in sputum eosinophils with oral steroids [39] and a management strategy that increased ICS dose or added oral steroids with increased sputum eosinophils reduced exacerbations, as had previously been observed in patients with asthma [40]. A meta-analysis of over 13,000 COPD patients failed to identify any clinical factors that were associated with better responsiveness to ICS [32].

ICS, especially in high doses, may cause oral candidiasis and hoarseness, and these local side effects are increased in COPD patients on ICS [32]. High doses of ICS are well known to have systemic effects due to lung absorption. Elderly patients with COPD who have poor mobility and nutrition, who smoke and have comorbid diseases, such as ischemic heart disease and diabetes, may be at greater risk of developing corticosteroid side effects, but these may take time to develop and this may be longer than the duration of a clinical trial, even over a 3-year period. Even low doses of ICS are associated with increased risk of cataracts in elderly patients [41]. There is a dose-related increase in the risk of fractures with use of ICS amongst elderly patients in the community [42]. Patients with COPD may be at even greater risk as COPD itself is associated with osteoporosis, and cigarette smoking, immobility, and poor nutrition are additional risk factors [11, 43]. In the Lung Health Study, lumbar and hip bone density was reduced in the patients treated with inhaled triamcinolone, but there was no increase in fractures [44]. However, in the TORCH study, although there was a high incidence of osteoporosis amongst COPD patients, there was no detrimental effect of ICS on bone mineral density or on fracture rate [45]. Several large studies have shown that ICS (alone or in combination) are associated with a significant increase in risk of pneumonia, although this has not been well characterized [46–48]. This has been confirmed in a population-based study of over 175,000 COPD patients with hospital-diagnosed pneumonia, where there was a clear dose-related risk [49]. The increased risk of pneumonia with ICS has also been confirmed in meta-analyses, which have also identified an increased risk of death from pneumonia [32, 50, 51]. This increased risk of pneumonia with ICS may reflect the increased susceptibility of COPD patients to bacterial infections as a result of impaired mucosal innate immunity in the lungs. More studies are needed to define the pneumonia, the dose-relationship to ICS, and whether there are differences between different corticosteroids.

The inflammation in patients with COPD is corticosteroid resistant and not suppressed even by high doses of inhaled or oral corticosteroids (Fig. 1) [52–54]. The molecular basis of corticosteroid resistance in COPD as now been elucidated. Oxidative stress is increased in the lungs of patients with COPD and reduces the activity and expression of the nuclear enzyme histone deacetylase-2 (HDAC2) [55, 56]. Corticosteroids bind to glucocorticoid receptors in the cytoplasm which rapidly translocate to the nucleus and bind to activated inflammatory gene complexes, resulting in the binding of HDAC2, which causes deacetylation of hyperacetylated activated inflammatory genes and thus to suppression of inflammation [57]. HDAC2 is also important for deacetylating acetylated glucocorticoid receptors so that they are able to suppress inflammatory genes [58]. A reduction in HDAC2 prevents corticosteroids from switching off activated inflammatory genes and also leads to amplification of inflammation, and inflammatory genes cannot be inactivated. The mechanism whereby oxidative stress reduces HDAC2 involves the activation of phosphoinositide-3-kinase- δ (PI3K δ) leading to phosphorylation of HDAC2 and nitration of critical tyrosine residues on HDAC2 leading to ubiquitination and degradation by the proteasome [59, 60]. Hypoxia also reduces the gene expression of HDAC2 via the activation of the transcription factor HIF-1 α [61].

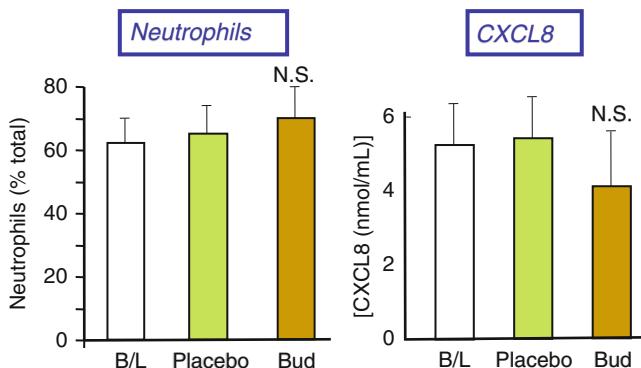


Fig. 1 Lack of anti-inflammatory effect of corticosteroids in COPD. Effect of inhaled budesonide (Bud: 800 µg b.i.d.) on sputum neutrophils and CXCL8 concentrations in patients with moderate to severe COPD, showing no significant (N.S.) effect after 4 weeks of treatment (Adapted from reference [76])

Combination Inhalers

ICS/LABA combination therapy (fluticasone/salmeterol and budesonide/formoterol) inhalers are now widely used in the management of COPD, but these studies are often difficult to interpret as it is difficult to disentangle the effect of the LABA from the effect of the ICS. Several trials have shown beneficial effects of combination inhalers, but the effects of the ICS alone when used as a comparator were less marked or absent [30, 34, 47, 62–65]. Overall, treatment with ICS/LABA combination therapy has little or no additional clinical benefit compared with LABA alone, although there is a risk of corticosteroid side effects [66]. Combination inhalers consistently reduce exacerbations by ~20 %, which is similar in magnitude to the effects of long-acting bronchodilators alone. In the TORCH study, there was a reduction in all cause mortality with fluticasone/salmeterol, which did not quite reach statistical significance ($P=0.052$). A meta-analysis, which included the TORCH data, found no effect of combination inhalers on mortality, however [67]. Taken together, these data provide little evidence that ICS reduce disease progression or mortality, even when combined with LABA. However, there is a consistent reduction in exacerbations and hospitalizations, similar in magnitude to that seen with long-acting bronchodilators such as LABA and tiotropium. Indeed, a direct comparison of fluticasone/salmeterol and tiotropium in COPD patients showed no statistical difference in exacerbation rates over 2 years [48]. A 2×2 factorial study analysis of the TORCH trial data to measure the independent contribution of the LABA and the ICS found that the reduction in mortality was entirely explained by the salmeterol component and none could be attributable to the ICS [33, 68].

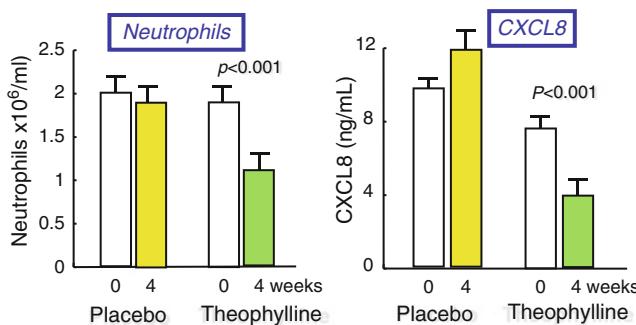


Fig. 2 Effect of theophylline (plasma concentration 9.8 $\mu\text{g}/\text{mL}$) on sputum neutrophils and CXCL8 concentrations in patients with moderate to severe COPD, showing significant inhibitory effects ($P<0.001$) (Adapted from reference [76])

Theophylline

Theophylline in high doses (with plasma concentration 10–20 mg/L) is a useful additional bronchodilator in patients with severe COPD [69], and oral administration has the advantage of treating small airways [70, 71]. Withdrawal of theophylline causes a clinical deterioration in COPD patients [72]. It may have additional properties such as effects on mucociliary clearance and on respiratory muscles that are useful. The major limitation to the use of theophylline is its side effects when high doses are used.

More recently it has been established that lower doses of theophylline (plasma concentration 5–10 mg/L) have anti-inflammatory effects in COPD [73]. Low-dose theophylline significantly reduces exacerbations of COPD (by approximately 50 %) [74] and accelerates the recovery from acute exacerbations of COPD [75]. Low-dose theophylline specifically reduces neutrophilic inflammation in the sputum as well as neutrophil chemotaxis and the concentrations of CXCL8 (Fig. 2) [76, 77]. Low-dose theophylline reduces neutrophilic inflammation in COPD patients, whereas high doses of ICS and a systemic β_2 -agonist (tulobuterol) are without any effect [53, 77, 78].

The bronchodilator effect of theophylline is due to inhibition of phosphodiesterases (PDE), particularly the PDE3 isoenzyme, resulting in increased cyclic AMP concentrations in airway smooth muscle. Theophylline is also a specific antagonist of adenosine receptors, which may be involved in the activation of inflammatory cells. The anti-inflammatory effects of theophylline may be mediated by inhibition of PDE4, although there is relatively little inhibition of this enzyme at low therapeutic concentrations. More recently it has been shown that low concentrations of theophylline are able to reverse corticosteroid resistance in COPD cells in vitro and in COPD patients [75, 77, 79]. This effect of theophylline is through direct inhibition of oxidative stress-activated PI3K δ , which results in increased histone deacetylase-2

(HDAC2) activity [59, 80]. This suggests that low-dose theophylline may be able to restore corticosteroid sensitivity in COPD patients and large clinical trials on long-term low-dose theophylline in combination with ICS are now underway. The major side effects of theophylline include nausea, diarrhea, and headaches, which are largely due to inhibition of PDE4 and cardiac arrhythmias, and seizures are due to adenosine A₁-receptor antagonism, so the low doses needed for reversal of corticosteroid resistance may largely avoid these side effects.

Macrolide Antibiotics

Antibiotics, including macrolides, are used for the treatment of bacterial exacerbations of COPD but have been considered unsuitable for long-term prophylaxis because of the development of corticosteroids resistance. Recently macrolide antibiotics have been shown to be effective in reducing exacerbations of COPD [81, 82]. How this impacts on bacterial resistance and microbial colonization is not yet known. With azithromycin there is an increased incidence of deafness. It has long been known that macrolides have anti-inflammatory effects, although these are not very well defined [83]. These anti-inflammatory effects are distinct from any antibiotic actions [84], and several nonantibiotic macrolides are now in development as anti-inflammatory therapies [85].

Mucolytics

Several mucolytics have been used in the treatment of COPD, although there is little evidence that they provide significant clinical benefit. Some of these mucolytics, based on cysteine molecules, are also antioxidants, and since oxidant damage may be critical in the pathophysiology of COPD, antioxidant therapy is logical. *N-acetylcysteine* (NAC) and carbocisteine were originally developed as mucolytics but have well-documented antioxidant effects [10, 86]. Although a meta-analysis of several small studies demonstrated a beneficial effect of NAC in reducing exacerbations of COPD by approximately 25 % [87, 88], in a large prospective controlled trial, NAC failed to reduce exacerbations, improve health status, or reduce disease progression [89]. However, patients not already treated with ICS obtained some benefit. A large study from China showed that carbocisteine reduced exacerbations by approximately 25 % over a year of therapy in treatment-naïve patients [90]. This suggests that mucolytic/antioxidants may be used in patients who are not already treated with inhaled corticosteroids and may provide benefit in reducing exacerbations, and these effects may be through reduction of oxidative stress-driven inflammation. These cysteine-based antioxidants are inactivated by oxidative stress, so more potent or stable antioxidants need to be developed in the future.

Roflumilast

Roflumilast is the first anti-inflammatory treatment specifically developed for COPD that has been brought to the market. It is an orally active selective PDE4 inhibitor that has been shown to have significant anti-inflammatory effects in COPD patients. PDE4 is the predominant phosphodiesterase expressed in neutrophils, T cells, and macrophages, suggesting that PDE4 inhibitors would be effective in controlling inflammation in COPD [91, 92]. Roflumilast reduces the numbers of neutrophils (by 36 %) and CXCL8 concentrations in sputum of COPD patients [93]. Roflumilast is indicated for COPD patients with severe disease, frequent exacerbations, and chronic bronchitis, the diseased phenotype that showed the most favorable response in the initial clinical trials. In clinical trials in this selected population, roflumilast (500 μ g once daily) given over 12 months improves lung function in COPD patients (FEV₁ increase ~50 ml) and reduces exacerbations (by ~15 %) [94]. It is effective when added to a LABA or tiotropium [95], so may be used as an additional treatment in patients with severe disease who have frequent exacerbations. The limited clinical efficacy reflects the fact that the dose after oral administration is limited by side effects, such as nausea, diarrhea, and headaches, which are also mediated by PDE4 inhibition.

New Anti-Inflammatory Treatments

In view of the poor response to corticosteroids, there is a pressing need to find new anti-inflammatory therapies for COPD, particularly since the inflammation persists after smoking cessation. There are several new approaches to developing new anti-inflammatory treatments for COPD [96].

Blocking Inflammatory Mediators

Many mediators have been implicated in the pathophysiology of COPD [97], but as in asthma, it seems unlikely that these will prove to be very effective therapies as there is considerable redundancy in their effects. Several drugs that block specific mediators, including lipid mediators, proinflammatory cytokines, and chemokines have now been investigated in COPD patients with mainly disappointing results (Table 1). The most promising drugs are CXCR2 antagonists which blocks the chemokine receptor that is important in the recruitment of neutrophils into the lung [98, 99]. A drug that blocks CXCR1 and CXCR2 markedly reduces the increase in sputum neutrophils after ozone challenge [100] and is currently in clinical trials in COPD patients.

Table 1 Mediator antagonists for COPD

Drug class	Clinical development
Leukotriene B ₄ receptor antagonists	Ineffective, development stopped
5'-lipoxygenase inhibitors	Ineffective
Anti-TNF therapy	Ineffective, serious side effects
Anti-IL-1 therapy	In clinical trials
Anti-IL-8	Ineffective
CXCR2 antagonists	Promising against neutrophilic inflammation

Antiproteases

In COPD there is an imbalance between proteases that digest elastin (and other structural proteins) and antiproteases that protect against this. This suggests that either inhibiting these proteolytic enzymes or increasing endogenous antiproteases may be beneficial and should prevent the progression of emphysema. However, several proteases are implicated in COPD so that blocking a single enzyme may not have a major therapeutic effect. Endogenous antiproteases (α_1 -antitrypsin, secretory leukoprotease inhibitor, elafin, tissue inhibitors of MMP) may be given either in recombinant form or by viral vector gene delivery, but these approaches are unlikely to be cost effective as large amounts of protein have to be delivered and gene therapy is unlikely to provide sufficient protein. A more promising approach is to develop small molecule inhibitors of proteases, particularly those that have elastolytic activity. Neutrophil elastase inhibitors have been developed but have so far all failed in clinical trials [101]. Matrix metalloproteinases (MMPs) with elastolytic activity are also a target for drug development, and MMP-9 appears to be the predominant enzyme, which is released from macrophages, neutrophils, and epithelial cells. Nonselective MMP inhibitors, such as marimastat, have major side effects, suggesting that isoenzyme-selective inhibitors or inhaled delivery may be needed. A dual MMP9/MMP12 inhibitor, AZ11557272, has been shown to prevent emphysema, small airway thickening, and inflammation in guinea pigs exposed to cigarette smoke over 6 months [102]. MMP-12 has also been implicated in animal models of COPD, and selective inhibitors have been developed [103].

New PDE4 Inhibitors

In view of the disappointing results with blockage of a single mediator, several broad-spectrum anti-inflammatory treatments are now in development for COPD (Table 2). Several oral PDE4 inhibitors have failed in early clinical development because of the side effects, which has prompted strategies to avoid these systemic

Table 2 Broad-spectrum anti-inflammatory treatments for COPD

Drug class	Clinical development
Phosphodiesterase-4 inhibitors	Roflumilast on market, inhaled drugs in development
p38 MAP kinase inhibitors	Phase II studies but problems with side effects and toxicity
NF- κ B (IKK2) inhibitors	Preclinical but concerns about side effects
PI3 kinase- γ / δ inhibitors	Early clinical development
JAK inhibitors	Inhaled drugs in development
PPAR- γ agonists	Already developed for diabetes, clinical studies in progress

side effects. This problem could be overcome by inhaled delivery, but so far inhaled PDE4 inhibitors have been found to be poorly effective in clinical studies, although well tolerated. GSK256066 is a very potent PDE4 inhibitor that is effective by inhalation in animal models and is currently in clinical trials [104].

Another approach is the development of isoenzyme-selective inhibitors. Based on murine gene knockout models, PDE4D inhibition appears to account for nausea and vomiting, whereas PDE4B inhibition may account for the anti-inflammatory effects, so that PDE4B selective inhibitors may be better tolerated. Selective PDE4B inhibitors are now in clinical development for COPD. PDE7A is also expressed in the same inflammatory cells as PDE4, so inhibition of PDE7 may be beneficial. However, a selective PDE7 inhibitor had only a small anti-inflammatory effect alone, but potentiated the anti-inflammatory effects of a PDE4 inhibitor, suggesting that a combined inhibitor may be useful as it should not increase the side effects [105, 106]. PDE3 inhibitors may produce bronchodilatation so that dual PDE3/4 inhibitors may combine bronchodilatation with anti-inflammatory activity [107]. However, there are concerns about the potential cardiovascular toxicity of PDE3 inhibitors, so these drugs may also have to be given by inhalation.

NF- κ B Inhibitors

The transcription factor nuclear factor- κ B (NF- κ B) regulates the expression of chemokines, many inflammatory cytokines, as well as MMP9. NF- κ B is activated in macrophages and epithelial cells of COPD patients, particularly during exacerbations. Inhibitors of inhibitor of NF- κ B kinase(IKK)2 are effective in some animal models of COPD (LPS exposure) but not in others (neutrophil elastase instillation), indicating that the effects may be complex [108]. Although several IKK2 inhibitors are now in development, so far none have been tested in COPD patients. A major concern about long-term inhibition of NF- κ B is that effective inhibitors may result in immune suppression and impair host defenses, since mice which lack NF- κ B genes may die of septicemia.

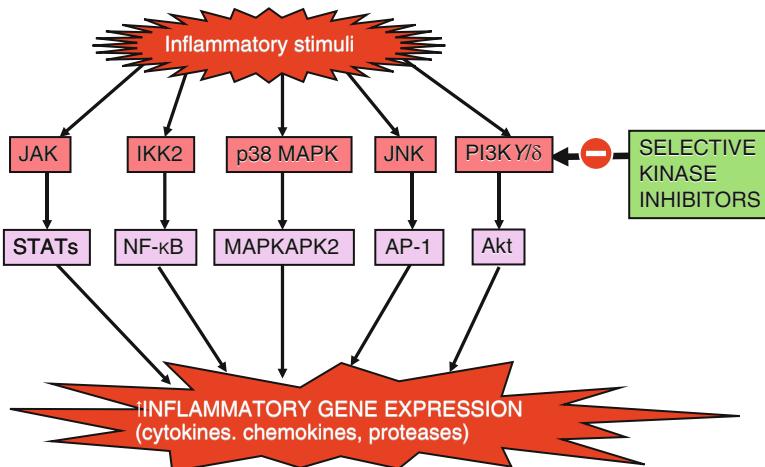


Fig. 3 Several kinases are activated in COPD lungs, resulting in activation of downstream kinases or transcription factors, which switch on multiple inflammatory genes. Selective inhibitors of these kinases are now in clinical development

MAP Kinase Inhibitors

Mitogen-activated protein kinases (MAPK) play a key role in chronic inflammation, and several complex enzyme cascades have been defined (Fig. 3). One of these, the p38 MAPK pathway, is activated by cellular stress and regulates the expression of inflammatory cytokines, including CXCL8, TNF- α , and MMPs. p38 MAPK (measured by phosphorylated p38 MAPK) is activated in alveolar macrophages of COPD lungs [109]. Several small molecule inhibitors of p38 MAPK have now been developed. A potent inhibitor of p38- α isoform, SD-282, is effective in inhibiting TNF- α release from human lung macrophages *in vitro* [110] and in suppressing inflammation in a smoking model of COPD in mice in which corticosteroids are ineffective [111]. Several p38 MAPK inhibitors have entered clinical trials [112], but there have been major problems with side effects and toxicity after oral administration, indicating that it is probably necessary to deliver these drugs by inhalation to reduce systemic exposure. Inhaled p38 MAPK inhibitors are now in clinical development for COPD [113].

Recent studies indicate that other MAPK pathways, particularly extracellular signal-regulated kinase (ERK1/2), may also play an important role in regulating the expression of proinflammatory cytokines in alveolar macrophages, in contrast to its lack of effect in blood monocytes [114].

Phosphoinositide 3-Kinase Inhibitors

PI3Ks are a family of enzymes that lead to the generation of lipid second messengers that regulate a number of cellular events, including innate and adaptive immune

responses. A particular isoform, PI3K γ , is involved in neutrophil recruitment and activation. Knockout of the PI-3K γ gene results in inhibition of neutrophil migration and activation as well as impaired T-lymphocyte and macrophage function, so PI3K γ inhibitors may be potential anti-inflammatory therapy for COPD [115]. PI3K δ is also involved in the expression of inflammatory genes, and several PI3K δ or mixed PI3K γ/δ inhibitors are now in development [116]. Pan-isoform inhibitors of PI3K are likely to be associated with side effects as these enzymes appear to serve a number of key cell function, but the γ and δ isoforms have a distribution more restricted to leukocytes and may therefore be better tolerated, especially if delivered by inhalation. PI3K δ inhibitors also have the potential to reverse corticosteroid resistance in COPD patients, as discussed above [59, 117].

JAK Inhibitors

Janus kinases (JAK) are non-receptor tyrosine kinases that are activated by various cytokine receptors and regulate gene expression through phosphorylation of seven signal transducers and activators of transcription (STAT) proteins. JAKs are good targets for COPD therapy as JAK 1, 2, and 3 regulate the gene expression of several cytokines and chemokines through phosphorylation of several STATs. JAK3 play a key role in T cell signaling, and JAK1/3 heterodimers regulate T cell survival, whereas JAK2 mediates GM-CSF mediated neutrophil survival and is involved in interferon- γ and IL-12/IL-23 signaling. STAT4, which is activated by IL-12 and IL-23, is increased in the airways of COPD patients [118]. STAT3 (and its downstream genes) is also activated in lung parenchyma of COPD patients [119]. JAK inhibitors therefore have potential as anti-inflammatory treatments in COPD, but systemic administration has several adverse effects, including pancytopenia as a result of JAK2 inhibition [120]. This means that inhaled administration is probably necessary.

PPAR Activators

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors belonging to the steroid receptor superfamily, and the three recognized subtypes PPAR- α , PPAR- γ , and PPAR- δ are widely expressed. There is evidence that activation of PPAR- α and PPAR- δ may have anti-inflammatory and immunomodulatory effects. For example, PPAR- γ agonists, such as troglitazone and rosiglitazone, inhibit the release of inflammatory cytokines from monocytes and induce apoptosis of T lymphocytes, suggesting that they may have anti-inflammatory effects in COPD [121]. PPAR- γ agonists also inhibit lung fibrosis and therefore have the potential to prevent progression of small airway fibrosis in COPD [122]. There is a reduction in PPAR- α expression in skeletal muscle of COPD patients that correlates with muscular weakness, indicating that PPAR- α agonists, such as clofibrate, may be useful in treating muscle weakness in severe disease [123].

Table 3 Drugs to reverse corticosteroids resistance in COPD

Drug class	Clinical development
Theophylline (low dose)	Already available
Nortriptyline	Already available
PI3K δ inhibitors	In phase II studies
Nrf2 activators	In clinical development
Nonantibiotic macrolides	In clinical development

Reversal of Corticosteroid Resistance

As discussed above, there is a resistance to the anti-inflammatory actions of corticosteroids in COPD patients, and this appears to be due to a decrease in activity of HDAC2 as a result of oxidative and nitrative stress [56]. This results in increased acetylation of the glucocorticoid receptor which prevents it from inhibiting NF- κ B-driven inflammation [58]. A novel therapeutic strategy is therefore reversal of this corticosteroid resistance by increasing the expression and activity of HDAC2, and this may be achieved in several ways (Table 3, Fig. 4) [124].

As discussed above, low doses of oral theophylline increase HDAC2 expression in alveolar macrophages from COPD patients and thereby restore steroid responsiveness [73]. Theophylline appears to reverse steroid resistance by inhibiting oxidant stress-activated PI3K δ , and selective PI3K δ inhibitors are also effective [59]. The tricyclic antidepressant nortriptyline also increases HDAC2 and reverses corticosteroid resistance by inhibiting PI3K δ [125]. A nonantibiotic macrolide (EM-703) reverses corticosteroid resistance due to oxidative stress by increasing HDAC2 activity [126]. Several nonantibiotic macrolides are now in development as anti-inflammatory therapies.

Antioxidants

Oxidative stress reduces steroid responsiveness via a reduction in HDAC2 activity and expression. This suggests that antioxidants may reverse corticosteroid resistance and also reduce inflammation. Unfortunately currently available antioxidants based on glutathione are relatively weak and are inactivated by oxidative stress, so new more potent and stable antioxidants are needed, such as superoxide dismutase mimics and NADPH oxidase inhibitors. The transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) plays a key role in the regulation of endogenous antioxidant genes and is defective in COPD patients. Several Nrf2 activators, such as sulforaphane (which occurs naturally in broccoli) and the synthetic triterpenoid 1-[2-cyano-3-,12-dioxoleana-1,9-dien-28-oyl]imidazole-methyl ester, have now been identified [127]. The Nrf2 sulforaphane reverses corticosteroid resistance in COPD patients by increasing HDAC2 [128].

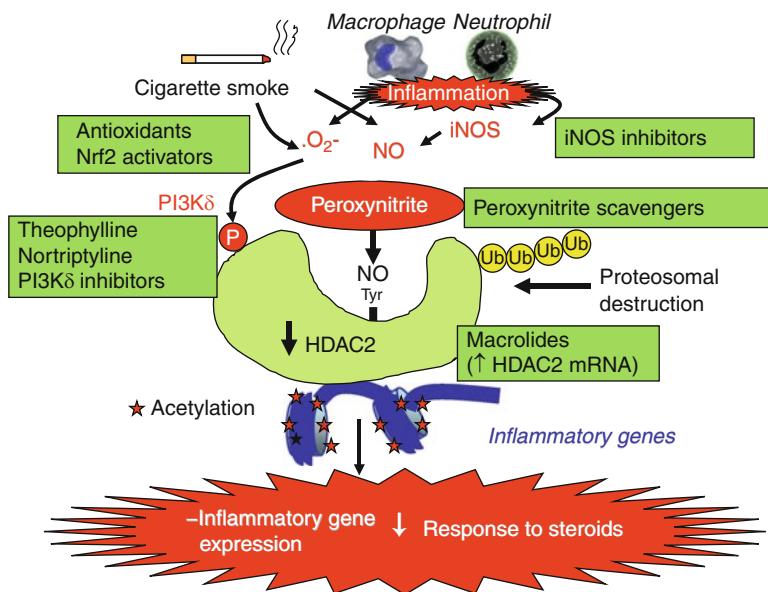


Fig. 4 Mechanisms for decreased histone deacetylase(HDAC)2 in COPD and its reversal. Superoxide anions (O_2^-) and nitric oxide (NO) generated by cigarette smoke and inflammatory cells combine to form peroxynitrite. NO production from inflammatory cells is derived from inducible NO synthase (iNOS) in response to inflammatory stimuli. Peroxynitrite nitrates HDAC2 at a tyrosine (Tyr) residue within the catalytic site, which inactivates HDAC2 and also leads to its ubiquitination (Ub) and proteasomal degradation. Oxidative stress also activates a phosphoinositide-3-kinase(PI3K) δ pathway that phosphorylates (P) and inactivates HDAC2. Loss of HDAC function then results in enhanced inflammatory gene expression and blocks the anti-inflammatory action of corticosteroids. HDAC2 function may be restored by antioxidants (including Nrf2 activators), iNOS inhibitors, or peroxynitrite scavengers, which reduce tyrosine nitration, or by theophylline, nortriptyline, or selective PI3K- δ inhibitors which restore HDAC function to normal through inhibition of PI3K δ . HDAC2 mRNA may also be increased by macrolides

Future Developments

New drugs for the treatment of COPD are greatly needed, and there has been an enormous effort now invested by the pharmaceutical industry to find such treatments. While preventing and quitting smoking is the obvious preferred approach, this has proved to be very difficult in the majority of smokers. Furthermore, it is now recognized that not all COPD is due to cigarette smoking, particularly in developing countries. It is important to identify the genetic factors that determine why only a minority of heavy smokers develop COPD, and identification of genes that predispose to the development of COPD may provide novel therapeutic targets in the future. However, it will be difficult to demonstrate the efficacy of novel treatments on the rate of decline in lung function, since this requires large studies over 3 years.

Hence, there is a need to develop novel outcome measures and surrogate biomarkers, such as analysis of sputum parameters (cells, mediators, enzymes) or exhaled condensates (lipid mediators, reactive oxygen species) [129]. The use of imaging techniques, such as high-resolution computerized tomography (CT), to measure disease progression is another promising approach as scanning resolution increases. It may also be important to more accurately define the presence of emphysema versus small airway obstruction using CT scans, as some drugs may be more useful for preventing emphysema, whereas others may be more effective against the small airway inflammatory-fibrotic process. More research on the basic cellular and molecular mechanisms of COPD and on more useful animal models is urgently needed to aid the logical development of new therapies for this common and important disease, for which no effective preventative drugs currently exist.

Of the drugs currently in development, PDE4 inhibitors and p38 MAP kinase inhibitors appear to be promising, but there are concerns about side effects so that inhaled administration is likely to be needed. CXCR2 antagonists show promise as an anti-neutrophilic and anti-macrophage therapy and should be well tolerated by oral administration. It is likely that effective anti-inflammatory therapies would not only reduce exacerbations but would also improve symptoms and health status. In the long term, these drugs should slow the decline in lung function and prevent the considerable morbidity imposed by this common disease. Perhaps the most promising approach is reversal of corticosteroid resistance, which is the main barrier to effective anti-inflammatory treatments in COPD. Drugs derived from theophylline and PI3K δ inhibitors may be effective through increasing HDAC2 activity and expression and should be relatively well tolerated. More potent antioxidants and nonantibiotic macrolides also deserve further study.

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