Inflammation and remodelling in experimental models of COPD - Mechanisms and therapeutic perspectives

Pera, Tonio

Chapter 1

General Introduction

COPD

Chronic obstructive pulmonary disease (COPD) is defined as “a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (1). Tobacco smoke exposure is the major risk factor for the development of COPD, although other forms of insult to the lungs as well as genetic predisposition may also contribute (1, 2). COPD has been predicted by the Global Burden of Disease Study to become the third leading cause of death worldwide in 2020 (3).

Innate and adaptive immune system in COPD

The inflammatory response in COPD consists of infiltration of various innate immune cells, including macrophages and neutrophils, as well as adaptive immune cells, such as T and B lymphocytes (4-6). Macrophages clear the lung of inhaled particles, bacteria and apoptotic cells by phagocytocis. After phagocytocis, the macrophages are removed by mucociliary clearance, which constitutes an important mechanism of defense against infection. However, the phagocytic capacity of alveolar macrophages may be decreased in COPD (7), whereas the epithelial cilia function is impaired by cigarette smoke, resulting in increased inflammation in the lung (8-10). In addition to the removal of foreign material from the lung, macrophages are also considered important regulators of immune responses in the lung due to their ability to release various pro-inflammatory cytokines and chemokines. Macrophages release tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β), cytokines which induce local and systemic inflammatory responses as well as increased mucus production. Furthermore, the release of chemokines such as interleukin-8 (IL-8) and leukotriene B_4 (LTB_4), which are potent neutrophil chemoattractants, may potentiate neutrophilic inflammation in the lung (4-6, 11, 12). In addition, macrophages are a source of proteolytic matrix metalloproteinases (MMPs) as well as reactive oxygen species which inhibit antiproteases, both of which may contribute to the development of emphysema (4, 6, 11, 12).

Neutrophils in the lung are also a source of proteolytic enzymes, including neutrophil elastase, an elastolytic enzyme which is considered to play a key role in the development of emphysema. In addition to its role in the breakdown of lung tissue, neutrophil elastase has also been implicated in increased mucus production and decreased mucociliary clearance, as well as stimulation of IL-8 and pro-fibrotic transforming growth factor-β (TGF-β) release. Neutrophils also release IL-8 and LTB_4, thereby perpetuating neutrophilic inflammation.

Azurophilic granules in neutrophils contain the enzyme myeloperoxidase (MPO), which participates in the formation of hypochlorous acid, a strong oxidant which may inactivate antiproteases in the lung, thus further augmenting tissue breakdown by elastolytic enzymes. Additionally, released MPO may result in lipid peroxidation and the formation of reactive nitrogen species, leading to tissue injury (4, 6, 13, 14).

Various studies have demonstrated increased numbers of T and B lymphocytes in the lungs of COPD patients, suggesting a role for the adaptive immune system in this disease (6, 13, 15-17). The numbers of T cells in the parenchyma and peripheral airways were shown to correlate with the degree of emphysema and airflow obstruction, respectively, suggesting an important role for these cells in the pathogenesis of COPD (15, 16, 18). Although $CD4^+$ and $CD8^+$ T cells are both increased in the lung, $CD8^+$ cells are the predominant type. $CD8^+$ T cells may contribute to tissue breakdown in COPD by releasing cytotoxic perforins and granzymes (6, 19). $CD8^+$ cells in sputum of COPD patients were shown to be highly activated and express high levels of perforins, associated with increased cytotoxic activity of these cells in vitro (20). In addition, the degree of $CD8^+$ cell activation in peripheral blood of COPD patients correlated with disease severity (21). Due to their strength in numbers, $CD8^+$ cells have received more attention in COPD research than the $CD4^+$ cells. The role of $CD4^+$ cells in COPD is still unclear, but it has been suggested that they contribute to the development of adaptive immune responses by priming and prolonging survival of $CD8^+$ cells as well as by taking part in the activation and differentiation of B cells (19).

B cell follicles have been observed in both the parenchyma and the airways of COPD patients (17, 18, 22, 23). Although the small airway area occupied by these follicles correlates with airflow obstruction in COPD (18), the contribution of B cells to the pathogenesis of this disease is unknown (24). There is evidence that B cells produce immunoglobulins, which are directed against antigens in the lung (25). The nature of these antigens is presently unclear, however. It has been suggested that the immunoglobulins may be directed against microbial antigens, since there is increased colonisation in the lungs of COPD patients (18). Alternatively, the protein content of cigarette smoke may be a source of antigens, as well as protein adducts formed in the lung as a result of cigarette smoke exposure (17). In addition, immunoglobulins directed against elastin peptides have been found in plasma of COPD patients, indicating that the breakdown of extracellular matrix proteins may result in the formation of antigens (26).

Structural changes in the lung

The persistent inflammatory response in the lung may lead to tissue damage and result in structural changes in the lung. The major structural abnormalities which develop in COPD include airway remodelling and emphysema. Emphysema is the destruction of alveolar structures, which leads to impaired gas exchange in the lung. In addition, loss of elastic recoil due to a decreased number of alveolar attachments results in collapse of the airways, contributing to airflow limitation (5, 27). As mentioned above, various inflammatory cells have been implicated in the development of emphysema and a particularly important role is attributed to elastolytic enzymes, such as neutrophil elastase.

Airway remodelling in COPD is characterized by peribronchial fibrosis, mucus gland hypertrophy, goblet cell hyperplasia and increased airway smooth muscle mass (5). These structural abnormalities result in thickening of the airway wall and mucus plugging of the lumen, thereby contributing to airflow obstruction. Although some features of airway remodelling may be observed in the large airways, they are most pronounced in small airways (<2 mm diameter), which are considered the major site of airflow obstruction in COPD (18, 27-29).

The pro-fibrotic growth factor TGF-β and its downstream Smad signalling pathway may be importantly involved in fibrotic processes in COPD (5). Increased TGF-β expression has been found in the airway epithelium of smokers and COPD patients (11, 30, 31). In addition, cigarette smoke exposure was shown to increase TGF-β expression and to induce Smad signalling in rat tracheal explants and in mouse lung (32-34). Furthermore, exposure of latent (recombinant) TGF-β to cigarette smoke in a cell-free system resulted in the release of active TGF-β (35).

Mucus gland hypertrophy and goblet cell hyperplasia in COPD result in mucus hypersecretion, which has been associated with morbidity and mortality in this disease (36-38). In the peripheral airways of COPD patients increased expression of the mucin MUC5B is observed in the lumen, whereas MUC5A/C expression is increased in the epithelium (39). The increased presence of mucus in the airways, combined with a cigarette smoke-induced deficiency of the ciliary apparatus, impairs the mucociliary clearance, leaving the lung susceptible to microbial colonisation (5, 6, 8, 36).

Airway smooth muscle remodelling is discussed separately below.

In addition to the airway remodelling, structural changes in the pulmonary vasculature have also been observed in COPD (5). Pulmonary vascular remodelling in COPD is characterized by thickening of the vessel wall, due to proliferation of the intima and the thickening of the media. In addition, muscularization of microvessels, which lack a smooth muscle layer under healthy condition, has also been observed (5). Remodelling of the pulmonary vasculature may contribute to pulmonary hypertension, a co-morbidity of COPD, which is present in a large proportion of the patients and may manifest itself at rest or during exercise. The mechanisms underlying pulmonary vascular remodelling in COPD are not known, but may involve hypoxia or pulmonary inflammation, leading to endothelial dysfunction and increased expression of growth factors in the vessel wall (5, 40-45). In addition, pulmonary hypertension results in remodelling of the right ventricle, which becomes hypertrophied as a result of the increased afterload (41).

Extrapulmonary manifestations of COPD

COPD may be associated with a wide range of extrapulmonary manifestations, including cardiovascular disease, loss and dysfunction of skeletal muscle, diabetes, osteoporosis, anemia, increased gastro-oesophageal reflux and clinical depression and anxiety (46, 47). These may be the result of a spill-over of lung inflammation into the circulation, causing low grade systemic inflammation or, alternatively, systemic inflammation which does not originate from the lung (46-49).

Pharmacological treatment of COPD

Currently, inhaled β-adrenergic receptor agonists and anticholinergics constitute the main bronchodilator therapy, whereas inhaled corticosteroids are the major anti-inflammatory therapy in COPD (50). Although glucocorticosteroids may be used to reduce exacerbations (51) and some recent studies have indicated that long-term therapy with high doses of inhaled corticosteroids, with or without long-acting bronchodilators, may decrease inflammation and the rate of lung function decline in (subgroups of) COPD patients (52, 53), the effectiveness of inhaled corticosteroids on progression of COPD is still a topic of discussion (54). Thus, it appears that the sensitivity to glucocorticosteroids is reduced in most patients with COPD, which may involve inhibition of histone deacetylase-2 by oxidative stress (54). PDE4 inhibitors, such as roflumilast, are a novel class of anti-inflammatory agents bearing perspectives in (severe) COPD (55).

The role of airway smooth muscle in the pathogenesis of COPD

Airway smooth muscle area may be increased in the small airways of COPD patients (16, 18, 56, 57). This contributes to the increase in airway wall area which leads to airflow obstruction. Indeed, both airway smooth muscle mass (16) and total airway wall area (18) were shown to correlate with a decrease of FEV_1 in COPD. Airway smooth muscle form COPD patients was shown to have an increased capacity to generate force in vitro (58, 59); the generated force negatively correlated with both FEV_1/FVC ratio and FEV_1 in these patients (59). These data suggest that increased area and altered function of airway smooth muscle may contribute to airflow obstruction and hyperresponsiveness in COPD (27).

In addition to their role as contractile cells regulating airway diameter, airway smooth muscle cells may also act as synthetic cells, producing chemokines, growth factors and extracellular matrix proteins in response to various G-protein-coupled receptor agonists, growth factors, pro-inflammatory cytokines or cigarette smoke (28). The increased release of chemokines, such as IL-8, may contribute to airway inflammation, whereas increased deposition of extracellular matrix proteins and the release of growth factors may contribute to fibrosis as well as airway smooth muscle cell proliferation (28, 60-67).

Cell proliferation is a mechanism underlying the increased airway smooth muscle area in obstructive airways diseases (68, 69) and may be induced by the release of growth factors from structural cells of the airway wall, such as epithelial cells, macrophages or even airway smooth muscle cells themselves (60, 70, 71). Growth factors and other mitogens, including various G-protein-coupled receptor agonists, cytokines and extracellular matrix proteins, can induce a proliferative phenotype of airway smooth muscle cells (66, 72-81). This phenotype is characterized by an increased rate of proliferation as well as a decreased expression of contractile proteins and decreased contractile function (74, 76-80). Phenotypic modulation is a reversible process; removal of the proliferative stimulus or altered extracellular matrix expression can induce maturation of the cells to a normo- or even hypercontractile phenotype, resulting in airway smooth muscle tissue with an increased force-generating capacity (74, 77, 82, 83).

Mitogen-activated protein (MAP) kinase pathways have been shown to play a major role in growth factor-induced signalling and the induction of a proliferative, hypocontractile phenotype of airway smooth muscle (72, 75, 77, 78, 84, 85). Even though airway smooth muscle mass may be increased in COPD, the potential contribution of disease-relevant stimuli, such as cigarette smoke or

lipopolysaccharide (LPS), to airway smooth muscle proliferation and phenotype modulation by direct action on the airway smooth muscle cells has not yet been addressed.

In vitro models of COPD

Cigarette smoke

Cigarette smoke extract (CSE) is the cornerstone of in vitro COPD modelling. CSE is made by passing CS through cell culture medium or a physiological buffer solution. There is no standard for preparing CSE. The number of cigarettes, volume of cell culture medium and type of cigarette used vary between different laboratories. Furthermore, cigarettes may be burned with or without filter and the CSE may be used as is or filtered. Therefore, a detailed description of the procedure used to prepare the CSE is required for comparing results obtained in different laboratories.

Various cell types have been exposed to CSE in order to mimic CS exposure in the lung. These include structural cells such as fibroblasts, epithelial and airway smooth muscle cells (62-65, 86-88) as well as inflammatory cell types such as macrophages and neutrophils (89-91). Upon CSE exposure, functional responses and gene expression changes, as well as the underlying intracellular signalling mechanisms, have been evaluated in these cells. Experiments in various cell types, including bronchial epithelial and airway smooth muscle cells as well as macrophages and neutrophils, have indicated that CSE induces increased release of the neutrophil chemokine interleukin-8 (IL-8) (63-65, 86, 89, 90). Some of these studies revealed that oxidative stress and activation of NF-κB and MAP kinase pathways play a role in this process (63, 64). In addition, experiments using CSE exposure of macrophages and neutrophils have indicated that TLR receptors may play a key role in CS-induced IL-8 release (89, 90). Various studies have also indicated that CSE may induce cell death (92-95). However, in the majority of these studies cells are exposed for prolonged periods of time (92, 93), whereas smokers' lungs are usually exposed for several minutes at a time. Effects of short, pulsatile CSE-stimulation of cells are still unknown.

CS instead of CSE has also been used to stimulate epithelial cells (96, 97), since these cells are directly exposed to inhaled air in the lung and this type of exposure may therefore be suitable for studying the effects of CS. Exposure of other airway wall cells, such as fibroblasts and airway smooth muscle cells, may possibly be more suitably modelled using CSE rather than CS, as in vivo components of CS may only reach these cells by diffusion through the airway wall.

Although most in vitro models have focused on CS or CSE exposure of cultured cells, airway or lung tissues have also been exposed to these stimuli (33, 98-100). A particularly elegant model using rat tracheal explants embedded in agar and exposed to CS on the epithelial side, thereby mimicking airway CS exposure in vivo, allows determination of the processes in the airway wall that are activated by CS without the involvement of immune cells (33, 101). Studies using this model have revealed that CS may induce pro-fibrotic changes in the airway wall in the absence of an inflammatory response. These findings were subsequently confirmed using an in vivo mouse model, showing that the pro-fibrotic gene expression in the airways is increased by CS before inflammatory cell numbers are increased (32).

Components of CS

Cigarette smoke is a complex mixture of over 5300 identified components (102). Exposure of cells to known components of CS, such as acrolein, formaldehyde or acetaldehyde, has been used to elucidate the contribution of these components to the release of pro-inflammatory cytokines and mucus production (103-106). This approach is indeed useful for identifying the contribution of individual CS components to the pathophysiology of COPD, but may be less suitable as a model as the contribution of a large number of other components is excluded.

Bacterial components

Stimulation of cells with bacteria (P. aeruginosa, H. infuenzae) or bacterial components, such as LPS, as well as viruses (RSV, rhinovirus) has been used to model exacerbations of COPD. Airway infections have been associated with exacerbations of COPD, which contribute to the progression of the disease (107, 108). Increased levels of LPS have been demonstrated in BAL fluid from COPD patients, indicating that it may be a relevant stimulus for disease progression (109). LPS has been shown to induce IL-8 release from lung fibroblasts as well as from epithelial and airway smooth muscle cells (110-112). LPS has also been shown to induce connective tissue growth factor release or mucin production from bronchial epithelial cells, indicating a possible contribution of LPS to fibrotic processes and mucus hypersecretion in the airways (113, 114).

Elastase

Elastase is a proteolytic enzyme, which is released by activated neutrophils in the lung and is considered to be a major contributor to the breakdown of alveolar tissue, resulting in emphysema (115). However, several studies have shown that elastase may induce pro-inflammatory gene expression and IL-8 release from bronchial epithelial cells (115-119). In addition, neutrophil elastase has also been

shown to induce mucus production by these cells (120-122). Elastase was also found to lower the ciliary beat frequency of epithelial cells, suggesting a role in impaired bacterial clearance in COPD (123). Mechanisms underlying the effects of elastase treatment appear to be complex and may involve activation of protease-activated receptors (PARs) (124, 125) or the proteolytic release of epidermal growth factor (EGF) from lung fibroblasts (126, 127). Interestingly, similar to CS, elastase was found to activate TLRs, supporting the importance of these receptors in the pathophysiology of COPD (128, 129).

Animal models of COPD

Animal models of disease are used for studies, which for ethical reasons cannot be performed in humans. The major disadvantage of these disease models is that they are per definition based on species different from human. However, once this limitation is considered when interpreting results, animal models can be used to gain insight into specific pathophysiological processes and putative therapeutic interventions. Each animal model has its own specific advantages and disadvantages; therefore, the suitability of a particular model will highly depend on the aims of the study.

In the last 30 years, modelling of COPD in animals has largely been performed in small laboratory animals, such as mice, rats and guinea pigs. Mice offer the advantage of a wide range of molecular and genetic tools, such as antibodies and knock-out mice. On the other hand, the lung and airway structures, the distribution of mucus glands and goblet cells, the autonomic innervation of the airways and airway vascularisation in guinea pigs closely resemble that of humans whereas in mice and rats they do not (130) . Despite the fact that several studies have used rats, these animals appear to be relatively resistant to the development of experimental COPD (131).

Cigarette smoke exposure

Cigarette smoke exposure is the major risk factor for the development of COPD and is therefore considered by many to be the ideal choice for modelling COPD (131). The protocols used for the exposure of animals vary greatly between the different studies. Thus, there is large variation in the length, frequency and number of exposures, the type of exposure (nose-only or whole-body exposure) and the type of cigarette used. Exposure of small animals to cigarette smoke has been performed in commercially available or home-made CS exposure apparatuses of varying degrees of complexity (131). The nose-only exposure system involves extensive handling and restraining of the animals, which may be

very stressful. Whole-body exposure results in deposition of CS particles on the pelt, which can be ingested during grooming; however, the animals are unrestrained and the exposure requires less animal handling. It has been demonstrated that animals suffered less weight loss after whole body exposure than after nose-only, suggesting this method may be preferred in order to avoid unwanted effects due to handling and restraining stress (131-133).Nevertheless, the nose-only cigarette smoke exposure has successfully been used for modelling COPD in animals (17, 134, 135) Because animals may change their breathing patterns as an avoidance reaction to inhalation of CS it has been suggested that monitoring of serum cotinine or blood carboxyhaemoglobin should be performed in order to quantify the exposure (131, 132, 136).

Despite these limitations and challenges, CS exposure has been shown to induce various features of COPD in animals, including pulmonary infiltration of macrophages and neutrophils, airway fibrosis and emphysema (32, 131, 134, 137-141). Although emphysema has consistently been reported after chronic CS exposure (131, 137-141), the degree varies between studies. This may be due to variation in the smoking protocol or species and strain differences (131, 142)
In guinea pigs, CS has also been shown to induce pulmonary hypertension (PH), associated with pulmonary vascular remodeling, both of which are observed in patients with COPD (135, 143-145). In addition, CS-induced PH has also been reported in rats but not in mice, although vascular remodeling does occur in the latter species (131, 146).

A major advantage of CS-models may be that the stimulus used is a major contributor to the development of COPD in human subjects. CS-exposure of experimental animals induces several pathological features of COPD. Another drawback is that CS-induced models are costly and time-consuming, as these models require 5 days per week CS-exposure for several hours, during 6 months. The severity of disease in these models is rated as GOLD stage 2, which indicates mild disease (131).

Elastase model of emphysema
This model consists of instillation of elastolytic enzymes in the lung resulting in tissue damage. A single instillation results in the development of emphysema. Early studies by Gross and colleagues (147), using papain instillation were instrumental in the establishment of the protease/anti-protease hypothesis and were a major contribution to the understanding of the role of α1-antitrypsin deficiency in the development of emphysema.

Although intuitively the mechanism of elastase-induced emphysema should be simple, involving destruction of tissue structure by enzymatic digestion, several lines of evidence suggest a much more complex mechanism. The half life of instilled elastases in the lung was found to be as short as 45-50 min (148), whereas the enlargement of alveolar spaces is progressive over a period of days and continues therefore after the exogenously applied elastases are no longer present in the lung (149-151). In accordance, treatment with elastase inhibitors prior to or immediately after instillation of elastase results in inhibition of airspace enlargement, whereas treatment 4 or 8 h post instillation does not (152-155). Elastase instillation induces an inflammatory response in the lung. Thus, increased expression of TNF-α, IL-1β, IL-6 and IL-8 as well as infiltration of macrophages and neutrophils have been reported, suggesting that inflammation might play a role in the development of elastase-induced emphysema (156, 157). Accordingly, mice lacking TNF-α- and/or IL-1β- receptors are strongly protected against the development of elastase-induced emphysema (156, 158).

Major advantages of the elastase model are the technical ease of inducing disease by a single instillation of the enzyme in the lung and the ability to control disease severity by adjusting the amount of enzyme (131). This model is particularly suited to study potential mechanisms of emphysema and regeneration processes in the lung. However, the model is not suitable for studying airway remodeling.

Starvation-induced model of emphysema

Starvation has been shown to induce emphysematous changes in the lung parenchyma. The study which revealed this phenomenon for the first time was conducted in the Warsaw Ghetto during World War II, where autopsy findings showed that a high percentage of people who died from starvation (13.5%) had emphysema (159). Subsequently, a number of studies reproduced this observation in rats, by subjecting the animals to severe caloric restriction for periods of several weeks. These studies confirmed that starvation induces emphysema-like changes as well as changes in lung mechanics. Reduced number of alveoli, increased alveolar volume and decreased alveolar surface area were reported (160-164). More recently, a study in anorexic subjects has shown that long-term caloric restriction is associated with loss of lung tissue and that in anorexic subjects the body mass index correlates with the diffusion capacity of carbon monoxide in the lung (165). However, despite similarities between the emphysematous changes induced by starvation, and emphysema in COPD, there are major differences in the pathology of these two conditions. The abnormal enlargement of airspaces and alveolar wall destruction observed in COPD is permanent, whereas the emphysema-like changes induced by starvation are

being reversed to normal levels after sufficient caloric intake (166, 167). Moreover, in rats with elastase-induced emphysema, calorie restriction induces a further increase in emphysema severity which is reversed by refeeding back to levels observed in animals treated with elastase alone (167). Furthermore, starvation-induced emphysematous changes, unlike emphysema in COPD, are not associated with airflow obstruction (160). In addition, this model lacks inflammatory cell influx in the lungs and structural changes in airways and pulmonary vasculature, which are observed in COPD (131). These data suggest that the starvation-induced emphysematous changes are likely the result of abnormal lung maintenance or growth. Therefore, this animal model is not well suited to study COPD.

LPS-induced model of COPD

LPS is a component of the cell wall of gram-negative bacteria, which is present as a contaminant in cigarette smoke, air pollution and organic dusts (168, 169). Exposure to environmental dusts containing LPS has been shown to induce chronic airflow obstruction and has been associated with the development of COPD in farmers (170, 171). In animals, a single LPS exposure induces pulmonary inflammation, characterized by infiltration of neutrophils and macrophages, as well as airway hyperresponsiveness (172-176). In addition, an increased number of airway mucus cells has also been observed after a single LPS exposure (177-181). Animals chronically exposed to LPS develop pulmonary inflammation as well as structural changes in the lung which are characteristic for COPD. Chronic LPS exposure induces increased numbers of macrophages and neutrophils (180, 182-188). Furthermore, CD8(+) T-cells as well as peribronchial and perivascular lymphocytic aggregates containing both CD8(+) and CD4(+) T-cells and B-cells were found to be increased in mice after chronic LPS exposure (188). Interestingly, 12 weeks of twice-weekly LPS exposure resulted in increased gene expression of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-18 as well as increased numbers of macrophages and lymphocytic aggregates after 1- and 8-week recovery periods (188). This indicates that, similarly to COPD (189, 190), chronic LPS exposure induces inflammatory responses, which persist even after the stimulus is no longer present.

In addition to inflammation, chronic LPS exposure has been shown to induce airway remodelling. Increased numbers of Goblet cells have been observed in airways of both mice and guinea pigs after repeated LPS exposure (180, 188). Chronic LPS exposure also resulted in increased peribronchial deposition of collagen and thickening of the airway wall in mice (182, 184-188, 191). Although 12 weeks of twice weekly LPS exposure resulted in increased airway smooth

muscle mass in mice (188), such changes were not observed in guinea pigs after 3 weeks of 3 times per week LPS exposure (180). The cause of this discrepancy could be the difference in the number of exposures, but species differences might also play a role. Chronic LPS exposure has also been shown to induce emphysema in mice, guinea pigs and hamsters (188, 192-195). Vernooy and colleagues showed that the airway remodelling and emphysematous changes are still present in mice 8 weeks after the LPS exposure had ceased (188). In addition, Brass et al. showed that airways of mice exposed to LPS for 4 or 8 weeks, followed by 4 weeks of recovery, still show submucosal thickening and increased collagen deposition, associated with pro-fibrotic gene expression (186, 191). The numbers of goblet cells in the airways of LPS-exposed mice, although still above control levels, appeared to be decreased after 8 weeks of recovery, indicating this may be a reversible feature of LPS-induced disease (188). Collectively, these data indicate that chronic LPS-exposure induces persistent inflammatory responses in the lung as well as alterations of airway and lung structure, which closely resemble the pathological changes observed in COPD.

Several studies have shown that the LPS-mediated effects are dependent on the Toll-like receptor 4 (TLR4). Thus, features of LPS-induced disease, including AHR, inflammation and airway remodelling, were considerably impaired in mice expressing deficient TLR4 (182) or lacking LPS-binding protein (183) or CD14 (196), which are both required for the activation of TLR4 by LPS. In addition, LPS-induced AHR, inflammation and airway remodelling were inhibited by treatment of mice with a TLR4 antagonist, E5564 (Eritoran) (187).

The complex mechanisms underlying the LPS-induced inflammation and structural changes in the lung are not fully known. LPS induces the release of pro-inflammatory cytokines including TNF-α, IL-1β, IL-8, IL-6 and GM-CSF in the lung (175, 183, 188, 196-198). This leads to the influx and activation of inflammatory cells, which may contribute to the development of LPS-induced disease. In addition, LPS-induced activation of TLR4 in macrophages and/or neutrophils may perpetuate inflammation due to an additional IL-8 and TNF-α release (199, 200).

IL-1 appears to play an important role in LPS-induced pathophysiology. Thus, IL-1 receptor antagonist (IL-1Ra) was found to partially inhibit LPS-induced airway inflammation in rats, whereas IL-1R-knockout mice were protected against development of LPS-induced airway wall thickening and fibrosis, indicating a crucial role for this receptor in chronic LPS-induced airway inflammation and remodelling (184, 201).

Interestingly, TLR4 has also been shown to mediate CS-induced inflammation in vivo. Pulmonary infiltration of inflammatory cells as well as increase of pro-inflammatory cytokines in the BAL fluid after up to 5 weeks of CS exposure was strongly reduced in mice lacking a functional TLR4 (202, 203). In addition, IL-1R-knockout mice were similarly protected against CS-induced pro-inflammatory responses in the lung (202). These data indicate that signalling pathways required for the development of LPS-induced pathological changes also play an important role in the inflammatory responses to CS.

Major advantages of the LPS-induced COPD model are the wide range of pathological features which are induced over a relatively short period of time, requiring only 2-3 LPS exposures per week. Several studies have indicated that TLR4 may play a role in CS-induced inflammatory responses (89, 202, 203) as well as in the development of COPD (170, 171), suggesting that LPS is a relevant stimulus for modeling COPD. This model is also suitable for testing therapeutic interventions and is less labour intensive and less costly than CS-exposure. Like CS-exposure, chronic LPS is considered to induce relatively mild disease, up to now (131). Although LPS and CS may share common mechanisms to induce disease it is important to realize that there still may be differences in the etiology of disease.

Caenorhabditis elegans model of COPD?

Recently, one group has exposed Caenorhabditis elegans (C. elegans) to CS in order to study its effects on gene expression of the innate immune system while eliminating any involvement of the adaptive immune system, which is not present in this nematode (204). The authors showed that C. elegans tolerates up to 4 h of CS exposure, as this had no significant effect on the rate of mortality measured at 48 h post exposure. Furthermore, CS exposure had profound effects on gene expression and resulted in impaired bacterial clearance. Several genes, which had human orthologues were selected for further investigation using RNAi techniques. This approach lead to the conclusion that CS-induced downregulation of lbp-7 (human orthologue: fatty acid binding protein 5 (FAB5)) plays a major role in bacterial clearance in C. elegans. In order to determine the relevance of this finding for COPD, the authors demonstrated that FAB5 mRNA expression is increased by bacteria in primary human bronchial epithelial cells and that cells isolated from COPD patients show lower FAB5 mRNA levels than cells from healthy smokers.

Another recent study used a similar approach to investigate the role of hypercapnia - which may occur in COPD as a result of impaired gas exchange - in

gene expression as well as motility and muscle morphology (205). Exposure of C. elegans to increased CO_2 levels resulted in altered gene expression and decreased motility, which was associated with abnormal muscle fiber organization. These data support a role for hypercapnia in skeletal muscle wasting observed in COPD (46, 48, 49).

These two studies show that C. elegans can be used to study potential mechanisms involved in COPD. Exposure of C. elegans to conditions or factors relevant to human disease could be a powerful source of information due to its ease of use, low cost, fully sequenced genome and availability of RNAi tools, providing the obtained results can be translated to mammalian models and human disease. The use C. elegans as a model species for COPD does however have major drawbacks. Most importantly, since C. elegans is a nematode, it does not have lungs. Therefore, although C. elegans offers many possibilities for investigating functional implications of altered gene expression and could thus be used for studying specific processes related to human disease, it is not a disease model of COPD.

Cellular and molecular mechanisms

The understanding of cellular and molecular mechanisms underlying COPD is limited compared to other obstructive lung diseases such as asthma. A major goal of our investigations was to define specific mechanisms involved in inflammation and airway remodelling in this disease, using CSE- and LPS-exposed airway smooth muscle cells in vitro, as well as a newly developed guinea pig model of LPS-induced COPD in vivo and ex vivo. The in vitro studies were focused on signalling mechanisms involved in CSE-, LPS- and growth factor-induced changes in airway smooth muscle phenotype and function. The animal model studies addressed the role of the cholinergic system and the NO-arginase axis as two major, interrelated pathways that are likely to be involved in the inflammatory, structural and functional changes observed in COPD.

Cholinergic mechanisms

Increased cholinergic tone has been identified as the major reversible component of airflow limitation in COPD (206). Acetylcholine release from parasympathetic nerve endings results in contraction of airway smooth muscle and increased mucus release. This contribution of acetylcholine to airflow limitation is the basis for the use and effectiveness of anticholinergics as bronchodilators in COPD (207). However, recent studies have suggested that anticholinergics, in addition to bronchodilatation, may have other beneficial effects. Data from the UPLIFT

trial show that the long-acting anticholinergic tiotropium bromide reduces exacerbation frequency, all-cause mortality and the number of adverse respiratory and cardiac events in COPD patients (208, 209). In addition, a subgroup analysis indicated that tiotropium reduces the rate of lung function decline in patients with mild COPD (210), young patients (211) and in those not on other controller medication (212). Recent observations in an acute mouse model of CS exposure suggest that tiotropium may reduce pulmonary neutrophilia by reducing the expression of pro-inflammatory cytokines and chemokines in the lung (213). The effects of tiotropium on structural remodelling in COPD are still unknown.

However, tiotropium has been shown to inhibit allergen-induced airway eosinophilia as well as airway smooth muscle thickening and increased mucin expression, suggesting that acetylcholine contributes to airway inflammation and remodelling in asthma (214, 215). Muscarinic receptors are expressed in both inflammatory and structural cells in the lung (207). Muscarinic agonists have been shown to induce the release of pro-inflammatory chemokines from macrophages and epithelial cells (216, 217) and to augment CSE-induced IL-8 release from airway smooth muscle cells (62). In addition, the observation that muscarinic receptor stimulation induces proliferation and collagen synthesis in lung fibroblasts suggests a role for acetylcholine in fibrosis (218, 219). Interestingly, the acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) is also widely expressed in the lung (207). The observations that muscarinic receptors are present on cells which have no parasympathetic innervation and that acetylcholine can be synthesized by non-neuronal cells indicate that non-neuronal acetylcholine may also be involved in lung physiology and pathophysiology. Indeed, ChAT expression is increased in lung fibroblasts from smokers and COPD patients, suggesting that non-neuronal acetylcholine may be involved in the pathogenesis of COPD (220).

Arginase

Arginase is an enzyme which converts L-arginine to L-ornithine and urea. It is involved in the urea cycle in the liver but is also expressed in extrahepatic tissues lacking a complete urea cycle (221). Two isoforms of arginase have been identified; arginase I is a cytosolic enzyme primarily expressed in the liver, whereas arginase II is a mitochondrial enzyme expressed in extrahepatic tissue (222). Both arginase isoforms are constitutively expressed in the lung, particularly in epithelial cells, (myo)fibroblasts and endothelial cells, as well as in macrophages and neutrophils (223-226). Arginase expression and/or activity was shown to be increased in animal models of asthma (221) as well as in

asthmatic patients (227-229). In a guinea pig model of allergic asthma, increased arginase activity was shown to contribute to allergen-induced airways obstruction, hyperresponsiveness, and inflammation (230). Increased arginase activity was found to result in a decreased L-arginine bioavailability for the enzyme nitric oxide synthase (NOS). This leads to a decreased production of the bronchodilatory, anti-inflammatory nitric oxide (NO) as well as an increased production of the pro-contractile, pro-inflammatory oxidant species, peroxynitrite (231-236). Decreased production of NO may contribute to airway obstruction, inflammation and hyperresponsiveness (235). Under inflammatory conditions, inducible NOS (iNOS) is upregulated and arginase-induced low L-arginine bioavailability results in concomitant production of NO and superoxide anions (O_2^-) by this enzyme, leading to rapid formation of peroxynitrite (237). Peroxynitrite production by iNOS may contribute to allergen-induced airway hyperresponsiveness as well as inflammation in allergic asthma (230, 238). Furthermore, peroxynitrite also induces MUC5A/C expression in airway epithelium and may therefore contribute to mucus hypersecretion (239). Arginase has also been shown to play a role in TGF-β-induced collagen synthesis, due to increased formation of the collagen precursor L-proline downstream of L-ornithine (222, 240, 241). Recent studies from our group indicate that increased arginase activity contributes to airway remodelling in asthma, which may involve increased synthesis of L-proline and polyamines downstream L-ornithine, as well as reduced production of NO (242).

Although many studies have indicated an important role of arginase in the pathophysiology of asthma, there are very few studies that have focused on its potential role in COPD. Only recently, increased arginase activity was demonstrated in BAL fluid from COPD patients (243). Remarkably, similar observations were made in sputum from patients with asthma or chronic bronchitis in the late 1970s (244, 245), but this was interpreted as leaking of hepatic arginase. Increased arginase gene expression in the lung has been observed in CS-exposed rats (246) and in LPS-exposed mice (191, 247). In addition, smoking was shown to further increase arginase gene expression and immunostaining in the airways of mild asthmatics (248). Increased arginase expression and/or activity has also been found in pulmonary endothelial cells and serum of patients with pulmonary arterial hypertension (249), which may occur as a co-morbidity of COPD (5, 41). Increased arginase activity may contribute to endothelial dysfunction in pulmonary arterial hypertension by causing reduced production of eNOS-derived vasodilatory NO (249). This is further supported by the observation that oral L-arginine or inhaled NO decrease pulmonary arterial pressures in this disease (250). Despite accumulating

evidence suggesting a potential increase of arginase activity or expression in COPD, no studies have thus far demonstrated a role for arginase in the pathophysiology of this disease.

TGF-β-activated kinase 1

TGF-β-activated kinase 1 (TAK1) is a serine/threonine kinase and a member of the MAP kinase kinase kinase family (MAP3K7). It was originally identified as a mediator of Smad-independent TGF-β signalling, but has since also been shown to play a key role in pro-inflammatory signalling pathways downstream of TLR, IL-1R and TNFR (Figure 1) (251-256). TAK1 is an important activator of the NF-κB pathway, as well as the ERK 1/2 and p38 MAP kinase pathways (251-255, 257-260). Activation of TAK1 contributes to the initiation of immune responses by inducing the release of pro-inflammatory cytokines, such as IL-8, IL-6 and TNF-α, and has also been shown to promote proliferation or survival of B-cells, T-cells and neutrophils (257, 261-264). In addition to stimuli of the above mentioned receptors, TAK1 is also activated by diesel exhaust particles as well as osmotic stress and hypoxia, indicating a role in stress-induced signalling (265-267). Considering the major role of TAK1 in TLR-signalling and the evidence suggesting a role for TLRs in the pathophysiology of COPD as well as in CS-induced signalling and inflammation, an important role for TAK1 in the development of COPD can be envisaged. However, no direct evidence has yet been reported.

In addition to its role in inflammation, several studies have indicated a major involvement of TAK1 in embryonal development (268-271). Interestingly, aberrant TAK1 signalling was shown to result, among others, in impaired development of the lung as well as abnormal vascular development lacking smooth muscle (269, 270). TAK1 has also been implicated in tissue remodelling. Increased TAK1 expression was associated with cardiac hypertrophy resulting from pressure overload in mice or with myocardial infarction in rats (272, 273). Expression of activated TAK1 in the mouse myocardium is sufficient to induce cardiac hypertrophy (273). Despite this evidence for the role of TAK1 in the development of the lung and vascular tissue as well as the remodelling of cardiac muscle tissue, there are no reports addressing the potential role of TAK1 in airway smooth muscle function.

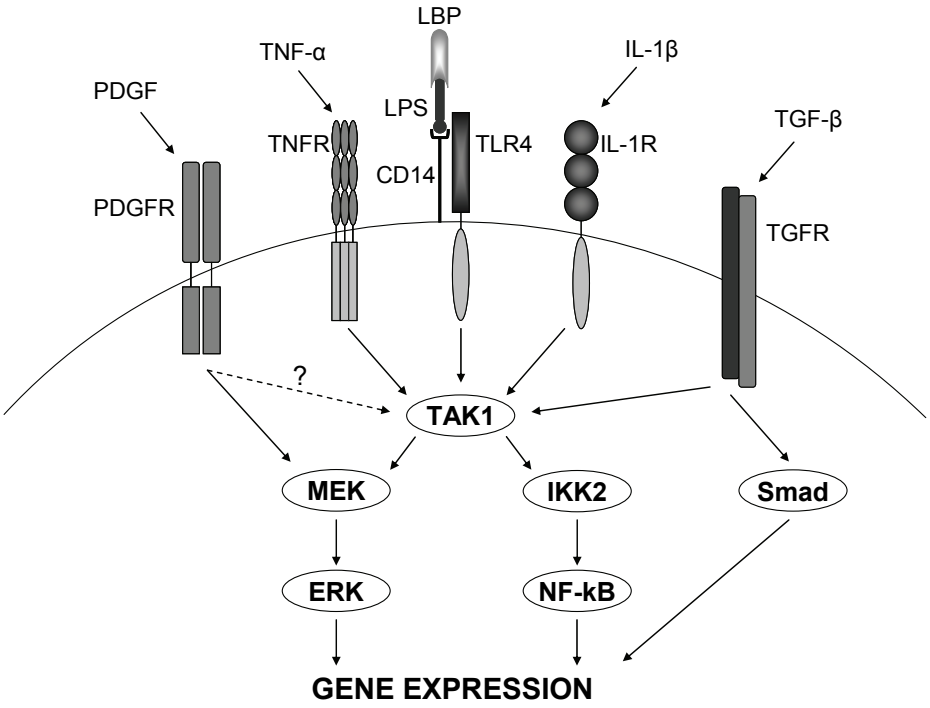

Figure 1: Pro-inflammatory and pro-fibrotic signalling in COPD.
Inhalation of cigarette smoke or other invoking factors of COPD, including lipopolysaccharide (LPS), leads to the release of pro-inflammatory cytokines and pro-fibrotic growth factors from structural and inflammatory cells in the lung. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), activate TNF- and IL-1-receptors (TNFR and IL-1R), respectively. LPS binds LPS binding protein (LBP) and CD14, thereby activating Toll-like receptor 4 (TLR4). Subsequently, intracellular adaptor proteins of the three receptors activate interleukin-1 receptor associated kinase (IRAK) and TNF-receptor-associated factor (TRAF), leading to activation of transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1). TAK1 initiates NF-κB signalling via activation of IκB kinase 2 (IKK2) and extracellular signal-regulated kinase (ERK) signalling via activation of mitogen activated protein (MAP) kinase kinase (MEK). Among others, both signalling pathways are involved in the transcription of genes encoding for pro-inflammatory chemokines such as IL-8, which may perpetuate inflammation of the lung. Growth factors, including platelet-derived growth factor (PDGF), activate MAP kinase signalling via the small GTPase Ras and induce proliferative responses involved in airway remodelling. Although a link between PDGF and TAK1 has thus far not been demonstrated, we hypothesize that PDGFR stimulation may also lead to activation of TAK1. TGF-β activates the TGF-β receptor (TGFR), initiating both Smad and TAK1 signalling (either directly by TGFR or via the X-linked inhibitor of apoptosis (XIAP)), which may cause tissue remodeling by inducing proliferative and fibrotic responses.

Scope of the thesis

As indicated above, the objective of this thesis was to investigate mechanisms of pulmonary inflammation and remodeling in COPD by using *in vitro* and *in vivo* approaches. The focus of **Chapters 2 and 3** is phenotypic modulation of airway smooth muscle by stimuli involved in the pathogenesis of COPD. As discussed above, mitogens induce a proliferative, hypocontractile phenotype of airway smooth muscle. Proliferation of airway smooth muscle cells may cause increased airway smooth muscle mass, which has been demonstrated in COPD (18). Using cultured BTSM cells and tissue, the studies in **Chapter 2** explore the mitogenic capacity of CSE and LPS as well as the MAP kinase pathways potentially involved in the CSE- and LPS-induced responses. In **Chapter 3** the role of TAK1 in growth factor-induced ERK 1/2 signalling and modulation of airway smooth muscle cells and tissue to a proliferative, hypocontractile phenotype is investigated. The study was performed in BTSM cells and tissue as well as in primary human airway smooth muscle cells, the role of TAK1 being explored by the pharmacological TAK1 inhibitor LL-Z-1640-2 as well as the expression of dominant-negative TAK1. **Chapter 4** focuses on the potential role of airway smooth muscle cells as a source of pro-inflammatory cytokines in COPD. The studies described in this chapter investigate the role of TAK1, NF-κB and ERK 1/2 pathways in CSE-induced IL-8 release by cultured human airway smooth muscle cells. Studies described in **Chapter 5** address the role of endogenous acetylcholine in pulmonary inflammation and remodelling using an animal model of COPD. For this purpose, a guinea pig model of LPS-induced COPD was developed. Animals were intranasally instilled, twice weekly for 12 weeks, with sterile saline or LPS and pre-treated with either saline or the long-acting anticholinergic drug tiotropium. The effect of tiotropium on LPS-induced neutrophilia, MUC5A/C expression and changes in hydroxyproline content, mean linear intercept (MLI) and lung vasculature were evaluated. **Chapter 6** is a review of the literature describing the role of arginase in various pulmonary diseases. Evidence from animal models as well as from clinical studies is presented. **Chapter 7** is dedicated to unraveling the role of arginase in the pathophysiology of COPD. Using the guinea pig model of COPD described in **Chapter 5**, the effects of pretreatment by inhalation of the specific arginase inhibitor (2)S-amino-boronohexanoic acid (ABH) on LPS-induced pulmonary inflammation and remodelling were investigated. In addition to some of the parameters evaluated in **Chapter 5** (neutrophilia, MUC5A/C expression, hydroxyproline content and pulmonary vascular dimensions), arginase activity and IL-8 levels in the lung as well as right ventricle mass were evaluated in this disease model.

References

1. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.
2. Eisner MD, Anthonisen N, Coultas D, Kuenzli N, Perez-Padilla R, Postma D, Romieu I, Silverman EK, Balmes JR. An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;182:693-718.
3. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 1997;349:1498-1504.
4. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;8:183-192.
5. Hogg JC, Timens W. The pathology of chronic obstructive pulmonary disease. *Annu Rev Pathol* 2009;4:435-459.
6. Tetley TD. Inflammatory cells and chronic obstructive pulmonary disease. *Curr Drug Targets Inflamm Allergy* 2005;4:607-618.
7. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol* 2003;81:289-296.
8. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010;363:2233-2247.
9. Leopold PL, O'Mahony MJ, Lian XJ, Tilley AE, Harvey BG, Crystal RG. Smoking is associated with shortened airway cilia. *PLoS ONE* 2009;4:e8157
10. Verra F, Escudier E, Lebargy F, Bernaudin JF, De CH, Bignon J. Ciliary abnormalities in bronchial epithelium of smokers, ex-smokers, and nonsmokers. *Am J Respir Crit Care Med* 1995;151:630-634.
11. Barnes PJ. Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev* 2004;56:515-548.
12. Barnes PJ. Alveolar macrophages as orchestrators of COPD. *COPD* 2004;1:59-70.
13. Di Stefano A, Caramori G, Ricciardolo FLM, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy* 2004;34:1156-1167.
14. van der Vliet A, Eiserich JP, Shigenaga MK, Cross CE. Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999;160:1-9.
15. Finkelstein R, Fraser RS, Ghezzo H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;152:1666-1672.
16. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:822-826.
17. van der Strate BW, Postma DS, Brandsma CA, Melgert BN, Luinge MA, Geerlings M, Hylkema MN, van den BA, Timens W, Kerstjens HA. Cigarette smoke-induced emphysema: A role for the B cell? *Am J Respir Crit Care Med* 2006;173:751-758.
18. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004;350:2645-2653.
19. Gadgil A, Duncan SR. Role of T-lymphocytes and pro-inflammatory mediators in the pathogenesis of chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 2008;3:531-541.
20. Chrysofakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, Klimathianaki M, Siafakas NM. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest* 2004;125:71-76.
21. Gadgil A, Zhu X, Sciurba FC, Duncan SR. Altered T-cell phenotypes in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006;3:487-488.

22. Bosken CH, Hards J, Gatter K, Hogg JC. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. *Am Rev Respir Dis* 1992;145:911-917.
23. Gosman MME, Willemse BWM, Jansen DF, Lapperre TS, van Schadewijk A, Hiemstra PS, Postma DS, Timens W, Kerstjens HAM, the Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) Study Group. Increased number of B-cells in bronchial biopsies in COPD. *Eur Respir J* 2006;27:60-64.
24. Brusselle GG, Demoor T, Bracke KR, Brandsma CA, Timens W. Lymphoid follicles in (very) severe COPD: beneficial or harmful? *Eur Respir J* 2009;34:219-230.
25. Baraldo S, Lokar OK, Turato G, Zuin R, Saetta M. The role of lymphocytes in the pathogenesis of asthma and COPD. *Curr Med Chem* 2007;14:2250-2256.
26. Lee SH, Goswami S, Grudo A, Song Lz, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, Bakaeen F, Coxson HO, Cogswell S, Storness-Bliss C, Corry DB, Kheradmand F. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat Med* 2007;13:567-569.
27. Postma DS, Kerstjens HA. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:S187-S192.
28. Chung KF. The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:347-354.
29. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:S28-S38.
30. Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Nakahara K, Umeda A. Increased expression of transforming growth factor-β1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med* 2001;163:1476-1483.
31. Morty RE, Konigshoff M, Eickelberg O. Transforming growth factor-β signaling across ages: from distorted lung development to chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2009;6:607-613.
32. Churg A, Tai H, Coulthard T, Wang R, Wright JL. Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. *Am J Respir Crit Care Med* 2006;174:1327-1334.
33. Wang RD, Tai H, Xie C, Wang X, Wright JL, Churg A. Cigarette smoke produces airway wall remodeling in rat tracheal explants. *Am J Respir Crit Care Med* 2003;168:1232-1236.
34. Wang RD, Wright JL, Churg A. Transforming growth factor-beta1 drives airway remodeling in cigarette smoke-exposed tracheal explants. *Am J Respir Cell Mol Biol* 2005;33:387-393.
35. Wang RD, Wright JL, Churg A. Transforming growth factor-beta1 drives airway remodeling in cigarette smoke-exposed tracheal explants. *Am J Respir Cell Mol Biol* 2005;33:387-393.
36. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J* 2008;31:1334-1356.
37. Prescott E, Lange P, Vestbo J. Chronic mucus hypersecretion in COPD and death from pulmonary infection. *Eur Respir J* 1995;8:1333-1338.
38. Vestbo J, Prescott E, Lange P. Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med* 1996;153:1530-1535.
39. Caramori G, Di Gregorio C, Carlstedt I, Casolari P, Guzzinati I, Adcock IM, Barnes PJ, Ciaccia A, Cavallesco G, Chung KF, Papi A. Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. *Histopathology* 2004;45:477-484.
40. Barbera JA, Peinado VI, Santos S. Pulmonary hypertension in chronic obstructive pulmonary disease. *Eur Respir J* 2003;21:892-905.
41. MacNee W. Right heart function in COPD. *Semin Respir Crit Care Med* 2010;31:295-312.

42. Peinado VI, Pizarro S, Barbera JA. Pulmonary vascular involvement in COPD. *Chest* 2008;134:808-814.
43. Santos S, Peinado VI, Ramirez J, Melgosa T, Roca J, Rodriguez-Roisin R, Barbera JA. Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD. *Eur Respir J* 2002;19:632-638.
44. Wright JL, Lawson L, Pare PD, Hooper RO, Peretz DI, Nelems JM, Schulzer M, Hogg JC. The structure and function of the pulmonary vasculature in mild chronic obstructive pulmonary disease. The effect of oxygen and exercise. *Am Rev Respir Dis* 1983;128:702-707.
45. Wright JL, Petty T, Thurlbeck WM. Analysis of the structure of the muscular pulmonary arteries in patients with pulmonary hypertension and COPD: National Institutes of Health nocturnal oxygen therapy trial. *Lung* 1992;170:109-124.
46. Barnes PJ, Celli BR. Systemic manifestations and comorbidities of COPD. *Eur Respir J* 2009;33:1165-1185.
47. Sevenoaks MJ, Stockley RA. Chronic obstructive pulmonary disease, inflammation and co-morbidity--a common inflammatory phenotype? *Respir Res* 2006;7:70
48. Wouters EFM. Chronic obstructive pulmonary disease. 5: Systemic effects of COPD. *Thorax* 2002;57:1067-1070.
49. Wouters EFM. Local and systemic inflammation in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:26-33.
50. Qaseem A, Snow V, Shekelle P, Sherif K, Wilt TJ, Weinberger S, Owens DK. Diagnosis and management of stable chronic obstructive pulmonary disease: a clinical practice guideline from the American College of Physicians. *Ann Intern Med* 2007;147:633-638.
51. Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, Maslen TK. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. *BMJ* 2000;320:1297-1303.
52. Lapperre TS, Snoeck-Stroband JB, Gosman MM, Jansen DF, van SA, Thiadens HA, Vonk JM, Boezen HM, Ten Hacken NH, Sont JK, Rabe KF, Kerstjens HA, Hiemstra PS, Timens W, Postma DS, Sterk PJ. Effect of fluticasone with and without salmeterol on pulmonary outcomes in chronic obstructive pulmonary disease: a randomized trial. *Ann Intern Med* 2009;151:517-527.
53. Celli BR, Thomas NE, Anderson JA, Ferguson GT, Jenkins CR, Jones PW, Vestbo J, Knobil K, Yates JC, Calverley PM. Effect of pharmacotherapy on rate of decline of lung function in chronic obstructive pulmonary disease: results from the TORCH study. *Am J Respir Crit Care Med* 2008;178:332-338.
54. Barnes PJ. Inhaled corticosteroids in COPD: a controversy. *Respiration* 2010;80:89-95.
55. Rabe KF. Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 2011;
56. Bosken CH, Wiggs BR, Pare PD, Hogg JC. Small airway dimensions in smokers with obstruction to airflow. *Am Rev Respir Dis* 1990;142:563-570.
57. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1993;148:1220-1225.
58. De Jongste JC, Mons H, Block R, Bonta IL, Frederiksz AP, Kerrebijn KF. Increased in vitro histamine responses in human small airways smooth muscle from patients with chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1987;135:549-553.
59. Opazo Saez AM, Seow CY, Pare PD. Peripheral airway smooth muscle mechanics in obstructive airways disease. *Am J Respir Crit Care Med* 2000;161:910-917.
60. Dekkers BG, Maarsingh H, Meurs H, Gosens R. Airway structural components drive airway smooth muscle remodeling in asthma. *Proc Am Thorac Soc* 2009;6:683-692.
61. Fong CY, Pang L, Holland E, Knox AJ. TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L201-L207.

62. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J, Kolahian S, Halayko AJ, Hiemstra PS, Zuyderduyn S. Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. *Eur Respir J* 2009;34:1436-1443.
63. Oenema TA, Kolahian S, Nanninga JE, Rieks D, Hiemstra PS, Zuyderduyn S, Halayko AJ, Meurs H, Gosens R. Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle. *Respir Res* 2010;11:130
64. Oltmanns U, Chung KF, Walters M, John M, Mitchell JA. Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle. *Respir Res* 2005;6:74
65. Oltmanns U, Walters M, Sukkar M, Xie S, Issa R, Mitchell J, Johnson M, Chung KF. Fluticasone, but not salmeterol, reduces cigarette smoke-induced production of interleukin-8 in human airway smooth muscle. *Pulm Pharmacol Ther* 2008;21:292-297.
66. Panettieri RA, Tan EM, Ciocca V, Luttmann MA, Leonard TB, Hay DW. Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and contraction In vitro: differential sensitivity to cysteinyl leukotriene receptor antagonists. *Am J Respir Cell Mol Biol* 1998;19:453-461.
67. Pang L, Knox AJ. Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: role of cyclooxygenase products. *J Immunol* 1998;161:2509-2515.
68. Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis* 1993;148:720-726.
69. Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, Carter R, Wong HH, Cadbury PS, Fahy JV. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* 2004;169:1001-1006.
70. Amishima M, Munakata M, Nasuhara Y, Sato A, Takahashi T, Homma Y, Kawakami Y. Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway. *Am J Respir Crit Care Med* 1998;157:1907-1912.
71. Aubert JD, Hayashi S, Hards J, Bai TR, Pare PD, Hogg JC. Platelet-derived growth factor and its receptor in lungs from patients with asthma and chronic airflow obstruction. *Am J Physiol Lung Cell Mol Physiol* 1994;266:L655-L663.
72. Gosens R, Meurs H, Bromhaar MM, McKay S, Nelemans SA, Zaagsma J. Functional characterization of serum- and growth factor-induced phenotypic changes in intact bovine tracheal smooth muscle. *Br J Pharmacol* 2002;137:459-466.
73. Krymskaya VP, Orsini MJ, Eszterhas AJ, Brodbeck KC, Benovic JL, Panettieri RA, Jr., Penn RB. Mechanisms of proliferation synergy by receptor tyrosine kinase and G protein-coupled receptor activation in human airway smooth muscle. *Am J Respir Cell Mol Biol* 2000;23:546-554.
74. Dekkers BG, Schaafsma D, Nelemans SA, Zaagsma J, Meurs H. Extracellular matrix proteins differentially regulate airway smooth muscle phenotype and function. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1405-L1413.
75. Fernandes DJ, Ravenhall CE, Harris T, Tran T, Vlahos R, Stewart AG. Contribution of the p38MAPK signalling pathway to proliferation in human cultured airway smooth muscle cells is mitogen-specific. *Br J Pharmacol* 2004;142:1182-1190.
76. Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H. Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol* 2003;28:257-262.
77. Gosens R, Schaafsma D, Meurs H, Zaagsma J, Nelemans SA. Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype. *Eur J Pharmacol* 2004;483:71-78.
78. Gosens R, Roscioni SS, Dekkers BGJ, Pera T, Schmidt M, Schaafsma D, Zaagsma J, Meurs H. Pharmacology of airway smooth muscle proliferation. *Eur J Pharmacol* 2008;585:385-397.

79. Halayko AJ, Salari H, Ma X, Stephens NL. Markers of airway smooth muscle cell phenotype. *Am J Physiol* 1996;270:L1040-L1051.
80. Halayko AJ, Solway J. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. *J Appl Physiol* 2001;90:358-368.
81. Hirst SJ, Twort CH, Lee TH. Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* 2000;23:335-344.
82. Dekkers BG, Schaafsma D, Tran T, Zaagsma J, Meurs H. Insulin-induced laminin expression promotes a hypercontractile airway smooth muscle phenotype. *Am J Respir Cell Mol Biol* 2009;41:494-504.
83. Schaafsma D, McNeill KD, Stelmack GL, Gosens R, Baarsma HA, Dekkers BG, Frohwerk E, Penninks JM, Sharma P, Ens KM, Nelemans SA, Zaagsma J, Halayko AJ, Meurs H. Insulin increases the expression of contractile phenotypic markers in airway smooth muscle. *Am J Physiol Cell Physiol* 2007;293:C429-C439.
84. Karpova AY, Abe MK, Li J, Liu PT, Rhee JM, Kuo WL, Hershenson MB. MEK1 is required for PDGF-induced ERK activation and DNA synthesis in tracheal myocytes. *Am J Physiol Lung Cell Mol Physiol* 1997;272:L558-L565.
85. Orsini MJ, Krymskaya VP, Eszterhas AJ, Benovic JL, Panettieri RA, Jr., Penn RB. MAPK superfamily activation in human airway smooth muscle: mitogenesis requires prolonged p42/p44 activation. *Am J Physiol Lung Cell Mol Physiol* 1999;277:L479-L488.
86. Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* 1997;155:1770-1776.
87. Numanami H, Koyama S, Nelson DK, Hoyt JC, Freels JL, Habib MP, Amano J, Haniuda M, Sato E, Robbins RA. Serine protease inhibitors modulate smoke-induced chemokine release from human lung fibroblasts. *Am J Respir Cell Mol Biol* 2003;29:613-619.
88. Pace E, Ferraro M, Siena L, Melis M, Montalbano AM, Johnson M, Bonsignore MR, Bonsignore G, Gjomarkaj M. Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells. *Immunology* 2008;124:401-411.
89. Karimi K, Sarir H, Mortaz E, Smit JJ, Hosseini H, De Kimpe SJ, Nijkamp FP, Folkerts G. Toll-like receptor-4 mediates cigarette smoke-induced cytokine production by human macrophages. *Respir Res* 2006;7:66
90. Mortaz E, Adcock IM, Ito K, Kraneveld AD, Nijkamp FP, Folkerts G. Cigarette smoke induces CXCL8 production by human neutrophils via activation of TLR9 receptor. *Eur Respir J* 2009;1143-1154.
91. Mortaz E, Rad M, Johnson M, Raats D, Nijkamp F, Folkerts G. Salmeterol with fluticasone enhances the suppression of IL-8 release and increases the translocation of glucocorticoid receptor by human neutrophils stimulated with cigarette smoke. *J Mol Med* 2008;86:1045-1056.
92. Hu W, Xie J, Zhao J, Xu Y, Yang S, Ni W. Involvement of Bcl-2 family in apoptosis and signal pathways induced by cigarette smoke extract in the human airway smooth muscle cells. *DNA Cell Biol* 2008;
93. Ishii T, Matsuse T, Igarashi H, Masuda M, Teramoto S, Ouchi Y. Tobacco smoke reduces viability in human lung fibroblasts: protective effect of glutathione S-transferase P1. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1189-L1195.
94. Slebos DJ, Ryter SW, van der TM, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X, Lee JS, Postma DS, Kauffman HF, Choi AM. Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am J Respir Cell Mol Biol* 2007;36:409-417.
95. van der Toorn M, Slebos DJ, de Bruin HG, Leuvenink HG, Bakker SJ, Gans RO, Koeter GH, van Oosterhout AJ, Kauffman HF. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1211-L1218.

96. Rusznak C, Sapsford RJ, Devalia JL, Justin John R, Hewitt EL, Lamont AG, Wood AJ, Shah SS, Davies RJ, Lozewicz S. Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells in vitro. *Am J Respir Cell Mol Biol* 1999;20:1238-1250.
97. van der Toorn M, Smit-de Vries MP, Slebos DJ, de Bruin HG, Abello N, van Oosterhout AJM, Bischoff R, Kauffman HF. Cigarette smoke irreversibly modifies glutathione in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1156-L1162.
98. Streck E, Jorres RA, Huber RM, Bergner A. Effects of cigarette smoke extract and nicotine on bronchial tone and acetylcholine-induced airway contraction in mouse lung slices. *J Investig Allergol Clin Immunol* 2010;20:324-330.
99. Wang RD, Wright JL, Churg A. Transforming growth factor-beta1 drives airway remodeling in cigarette smoke-exposed tracheal explants. *Am J Respir Cell Mol Biol* 2005;33:387-393.
100. Wright JL, Sun JP, Churg A. Cigarette smoke exposure causes constriction of rat lung. *Eur Respir J* 1999;14:1095-1099.
101. Wang RD, Wright JL, Churg A. Transforming growth factor-beta1 drives airway remodeling in cigarette smoke-exposed tracheal explants. *Am J Respir Cell Mol Biol* 2005;33:387-393.
102. Cahn Z, Siegel M. Electronic cigarettes as a harm reduction strategy for tobacco control: a step forward or a repeat of past mistakes? *J Public Health Policy* 2011;32:16-31.
103. Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol Lung Cell Mol Physiol* 1999;276:L549-L555.
104. Facchinetti F, Amadei F, Geppetti P, Tarantini F, Di Serio C, Dragotto A, Gigli PM, Catinella S, Civelli M, Patacchini R. α,β-Unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. *Am J Respir Cell Mol Biol* 2007;37:617-623.
105. Haswell LE, Hewitt K, Thorne D, Richter A, Gaţa MD. Cigarette smoke total particulate matter increases mucous secreting cell numbers in vitro: a potential model of goblet cell hyperplasia. *Toxicol in Vitro* 2010;24:981-987.
106. Moretto N, Facchinetti F, Southworth T, Civelli M, Singh D, Patacchini R. {alpha},{beta}-Unsaturated aldehydes contained in cigarette smoke elicit IL-8 release in pulmonary cells through mitogen-activated protein kinases. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L839-L848.
107. Patel IS, Seemungal TA, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha JA. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax* 2002;57:759-764.
108. Wilkinson TM, Hurst JR, Perera WR, Wilks M, Donaldson GC, Wedzicha JA. Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest* 2006;129:317-324.
109. Sethi S, Maloney J, Grove L, Wrona C, Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;173:991-998.
110. Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, Nagai S, Izumi T. The potential of various lipopolysaccharides to release IL-8 and G-CSF. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L658-L666.
111. Shan X, Hu A, Veler H, Fatma S, Grunstein JS, Chuang S, Grunstein MM. Regulation of Toll-like receptor 4-induced proasthmatic changes in airway smooth muscle function by opposing actions of ERK1/2 and p38 MAPK signaling. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L324-L333.
112. Sukkar MB, Xie S, Khorasani NM, Kon OM, Stanbridge R, Issa R, Chung KF. Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle. *J Allergy Clin Immunol* 2006;118:641-648.

113. Li Q, Zhou X, Kolosov VP, Perelman JM. Nicotine suppresses inflammatory factors in HBE16 airway epithelial cells after exposure to cigarette smoke extract and lipopolysaccharide. *Transl Res* 2010;156:326-334.
114. Nishioka M, Ogawa E, Kinose D, Haruna A, Ohara T, Ito I, Hoshino Y, Ito Y, Matsumoto H, Niimi A, Mio T, Chin K, Hirai T, Muro S, Mishima M. Lipopolysaccharide induced connective tissue growth factor gene expression in human bronchial epithelial cells. *Respirology* 2010;15:669-676.
115. Janoff A. Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. *Am Rev Respir Dis* 1985;132:417-433.
116. Bedard M, McClure CD, Schiller NL, Francoeur C, Cantin A, Denis M. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *Am J Respir Cell Mol Biol* 1993;9:455-462.
117. Carroll TP, Greene CM, Taggart CC, Bowie AG, O'Neill SJ, McElvaney NG. Viral inhibition of IL-1- and neutrophil elastase-induced inflammatory responses in bronchial epithelial cells. *J Immunol* 2005;175:7594-7601.
118. Hiemstra PS, van Wetering S, Stolk J. Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. *Eur Respir J* 1998;12:1200-1208.
119. Nakamura H, Yoshimura K, McElvaney NG, Crystal RG. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 1992;89:1478-1484.
120. Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. *Am J Pathol* 2005;167:651-661.
121. Shao MXG, Nadel JA. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-α-converting enzyme. *J Immunol* 2005;175:4009-4016.
122. Song JS, Cho KS, Yoon HK, Moon HS, Park SH. Neutrophil elastase causes MUC5AC mucin synthesis via EGF receptor, ERK and NF-kB pathways in A549 cells. *Korean J Intern Med* 2005;20:275-283.
123. Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, Stockley RA, Cole PJ. Effects of human neutrophil elastase and Pseudomonas aeruginosa proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 1991;4:26-32.
124. Chignard M, Pidard D. Neutrophil and pathogen proteinases versus proteinase-activated receptor-2 lung epithelial cells: more terminators than activators. *Am J Respir Cell Mol Biol* 2006;34:394-398.
125. Suzuki T, Yamashita C, Zemans RL, Briones N, Van Linden A, Downey GP. Leukocyte elastase induces lung epithelial apoptosis via a PAR-1-, NF-κB-, and p53-dependent pathway. *Am J Respir Cell Mol Biol* 2009;41:742-755.
126. DiCamillo SJ, Carreras I, Panchenko MV, Stone PJ, Nugent MA, Foster JA, Panchenko MP. Elastase-released epidermal growth factor recruits epidermal growth factor receptor and extracellular signal-regulated kinases to down-regulate tropoelastin mRNA in lung fibroblasts. *J Biol Chem* 2002;277:18938-18946.
127. DiCamillo SJ, Yang S, Panchenko MV, Toselli PA, Naggar EF, Rich CB, Stone PJ, Nugent MA, Panchenko MP. Neutrophil elastase-initiated EGFR/MEK/ERK signaling counteracts stabilizing effect of autocrine TGF-β on tropoelastin mRNA in lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L232-L243.
128. Geraghty P, Rogan MP, Greene CM, Boxio RMM, Poiriert T, O'Mahony M, Belaaouaj A, O'Neill SJ, Taggart CC, McElvaney NG. Neutrophil elastase up-regulates cathepsin B and matrix metalloprotease-2 expression. *J Immunol* 2007;178:5871-5878.
129. Greene CM, Carroll TP, Smith SGJ, Taggart CC, Devaney J, Griffin S, O'Neill SJ, McElvaney NG. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol* 2005;174:1638-1646.
130. Canning BJ, Chou Y. Using guinea pigs in studies relevant to asthma and COPD. *Pulm Pharmacol Ther* 2008;21:702-720.

131. Wright JL, Cosio M, Churg A. Animal models of chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L1-L15.
132. Hecht SS. Carcinogenicity studies of inhaled cigarette smoke in laboratory animals: old and new. *Carcinogenesis* 2005;26:1488-1492.
133. Mauderly JL, Bechtold WE, Bond JA, Brooks AL, Chen BT, Cuddihy RG, Harkema JR, Henderson RF, Johnson NF, Rithidech K, . Comparison of 3 methods of exposing rats to cigarette smoke. *Exp Pathol* 1989;37:194-197.
134. Wright JL, Postma DS, Kerstjens HA, Timens W, Whittaker P, Churg A. Airway remodeling in the smoke exposed guinea pig model. *Inhal Toxicol* 2007;19:915-923.
135. Wright JL, Zhou S, Preobrazhenska O, Marshall C, Sin DD, Laher I, Golbidi S, Churg AM. Statin reverses smoke-induced pulmonary hypertension and prevents emphysema but not airway remodeling. *Am J Respir Crit Care Med* 2011;183:50-58.
136. Coggins CR. A review of chronic inhalation studies with mainstream cigarette smoke in rats and mice. *Toxicol Pathol* 1998;26:307-314.
137. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin Exp Allergy* 2007;37:1467-1479.
138. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *J Immunol* 2006;177:4350-4359.
139. Churg A, Cosio M, Wright JL. Mechanisms of cigarette smoke-induced COPD: insights from animal models. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L612-L631.
140. Wright JL, Churg A. Animal models of cigarette smoke-induced COPD. *Chest* 2002;122:301S-306S.
141. Wright JL, Churg A. Animal models of cigarette smoke-induced chronic obstructive pulmonary disease. *Expert Rev Respir Med* 2010;4:723-734.
142. Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzo H, Triantafillopoulos A, Whittaker K, Hoidal JR, Cosio MG. The development of emphysema in cigarette smoke-exposed mice is strain dependent. *Am J Respir Crit Care Med* 2004;170:974-980.
143. Sekhon HS, Wright JL, Churg A. Cigarette smoke causes rapid cell proliferation in small airways and associated pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol* 1994;267:L557-L563.
144. Wright JL, Sun JP. Dissociation of chronic vascular cell proliferation and vascular contractility after chronic cigarette smoke exposure. *Eur Respir J* 1999;14:832-838.
145. Wright JL, Tai H, Churg A. Vasoactive mediators and pulmonary hypertension after cigarette smoke exposure in the guinea pig. *J Appl Physiol* 2006;100:672-678.
146. Nadziejko C, Fang K, Bravo A, Gordon T. Susceptibility to pulmonary hypertension in inbred strains of mice exposed to cigarette smoke. *J Appl Physiol* 2007;102:1780-1785.
147. Gross P, Pfitzer EA, TOLKER E, Babyak MA, Kaschak M. Experimental emphysema: its production with papain in normal and silicotic rats. *Arch Environ Health* 1965;11:50-58.
148. Stone PJ, Lucey EC, Calore JD, McMahon MP, Snider GL, Franzblau C. Defenses of the hamster lung against human neutrophil and porcine pancreatic elastase. *Respiration* 1988;54:1-15.
149. Kaplan PD, Kuhn C, Pierce JA. The induction of emphysema with elastase. I. The evolution of the lesion and the influence of serum. *J Lab Clin Med* 1973;82:349-356.
150. Kuhn C, Yu SY, Chraplyvy M, Linder HE, Senior RM. The induction of emphysema with elastase. II. Changes in connective tissue. *Lab Invest* 1976;34:372-380.
151. Niehaus GD, Reddan WG. Development of a canine model of experimental emphysema by intratracheal instillation of elastase. *Chest* 1980;77:276-278.
152. Gudapaty SR, Liener IE, Hoidal JR, Padmanabhan RV, Niewoehner DE, Abel J. The prevention of elastase-induced emphysema in hamsters by the intratracheal administration of a synthetic elastase inhibitor bound to albumin microspheres. *Am Rev Respir Dis* 1985;132:159-163.

153. Ip MP, Kleinerman J, Ranga V, Sorensen J, Powers JC. The effects of small doses of oligopeptide elastase inhibitors on elastase-induced emphysema in hamsters: a dose-response study. *Am Rev Respir Dis* 1981;124:714-717.
154. Kleinerman J, Ranga V, Rynbrandt D, Sorensen J, Powers JC. The effect of the specific elastase inhibitor, alanyl alanyl prolyl alanine chloromethylketone, on elastase-induced emphysema. *Am Rev Respir Dis* 1980;121:381-387.
155. Stone PJ, Lucey EC, Calore JD, Snider GL, Franzblau C, Castillo MJ, Powers JC. The moderation of elastase-induced emphysema in the hamster by intratracheal pretreatment or post-treatment with succinyl alanyl alanyl prolyl valine chloromethyl ketone. *Am Rev Respir Dis* 1981;124:56-59.
156. Couillin I, Vasseur V, Charron S, Gasse P, Tavernier M, Guillet J, Lagente V, Fick L, Jacobs M, Coelho FR, Moser R, Ryffel B. IL-1R1/MyD88 signaling is critical for elastase-induced lung inflammation and emphysema. *J Immunol* 2009;183:8195-8202.
157. Gamze K, Mehmet HM, Deveci F, Turgut T, Ilhan F, Ozercan I. Effect of bosentan on the production of proinflammatory cytokines in a rat model of emphysema. *Exp Mol Med* 2007;39:614-620.
158. Lucey EC, Keane J, Kuang PP, Snider GL, Goldstein RH. Severity of elastase-induced emphysema is decreased in tumor necrosis factor-alpha and interleukin-1beta receptor-deficient mice. *Lab Invest* 2002;82:79-85.
159. Stein J, Fenigstein H. Pathological anatomy of hunger disease. *Curr Concepts Nutr* 1979;7:207-234.
160. Harkema JR, Mauderly JL, Gregory RE, Pickrell JA. A comparison of starvation and elastase models of emphysema in the rat. *Am Rev Respir Dis* 1984;129:584-591.
161. Kerr JS, Riley DJ, Lanza-Jacoby S, Berg RA, Spilker HC, Yu SY, Edelman NH. Nutritional emphysema in the rat. Influence of protein depletion and impaired lung growth. *Am Rev Respir Dis* 1985;131:644-650.
162. Sahebjami H, Vassallo CL, Wirman JA. Lung mechanics and ultrastructure in prolonged starvation. *Am Rev Respir Dis* 1978;117:77-83.
163. Sahebjami H, Wirman JA. Emphysema-like changes in the lungs of starved rats. *Am Rev Respir Dis* 1981;124:619-624.
164. Sahebjami H. Nutrition and lung structure and function. *Exp Lung Res* 1993;19:105-124.
165. Coxson HO, Chan IH, Mayo JR, Hlynsky J, Nakano Y, Birmingham CL. Early emphysema in patients with anorexia nervosa. *Am J Respir Crit Care Med* 2004;170:748-752.
166. Massaro D, Alexander E, Reiland K, Hoffman EP, Massaro GD, Clerch LB. Rapid onset of gene expression in lung, supportive of formation of alveolar septa, induced by refeeding mice after calorie restriction. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1313-L1326.
167. Sahebjami H, Domino M. Effects of starvation and refeeding on elastase-induced emphysema. *J Appl Physiol* 1989;66:2611-2616.
168. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
169. Rylander R. Endotoxin and occupational airway disease. *Curr Opin Allergy Clin Immunol* 2006;6:62-66.
170. Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, Tielen MJ, van Schayck CP. Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am J Respir Crit Care Med* 1998;157:15-18.
171. Wang XR, Zhang HX, Sun BX, Dai HL, Hang JQ, Eisen EA, Wegman DH, Olenchock SA, Christiani DC. A 20-year follow-up study on chronic respiratory effects of exposure to cotton dust. *Eur Respir J* 2005;26:881-886.
172. Nagai H, Tsuji F, Shimazawa T, Goto S, Yoshitake K, Koda A. Participation of collagenase and elastase in LPS-induced airway hyperresponsiveness in guinea pigs. *Inflammation* 1991;15:317-330.

173. Spond J, Case N, Chapman RW, Crawley Y, Egan RW, Fine J, Hey JA, Kreutner W, Kung T, Wang P, Minnicozzi M. Inhibition of experimental acute pulmonary inflammation by pirfenidone. *Pulm Pharmacol Ther* 2003;16:207-214.
174. Toward TJ, Broadley KJ. Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *Br J Pharmacol* 2000;131:271-281.
175. Vernooy JHJ, Dentener MA, van Suylen RJ, Buurman WA, Wouters EFM. Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells . No role for tumor necrosis factor-α and infiltrating neutrophils. *Am J Respir Cell Mol Biol* 2001;24:569-576.
176. Vincent D, Lefort J, Chatelet F, Bureau MF, Dry J, Vargaftig BB. Intratracheal E. coli lipopolysaccharide induces platelet-dependent bronchial hyperreactivity. *J Appl Physiol* 1993;74:1027-1038.
177. Chapman RW, Minnicozzi M, Celly CS, Phillips JE, Kung TT, Hipkin RW, Fan X, Rindgen D, Deno G, Bond R, Gonsiorek W, Billah MM, Fine JS, Hey JA. A novel, orally active CXCR1/2 receptor antagonist, Sch527123, inhibits neutrophil recruitment, mucus production, and goblet cell yperplasia in animal models of pulmonary inflammation. *J Pharmacol Exp Ther* 2007;322:486-493.
178. Chen L, Wang T, Zhang JY, Zhang SF, Liu DS, Xu D, Wang X, Chen YJ, Wen FQ. Toll-like receptor 4 relates to lipopolysaccharide-induced mucus hypersecretion in rat airway. *Arch Med Res* 2009;40:10-17.
179. Harkema JR, Hotchkiss JA. Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways. *Toxicol Lett* 1993;68:251-263.
180. Toward TJ, Broadley KJ. Goblet cell hyperplasia, airway function, and leukocyte infiltration after chronic lipopolysaccharide exposure in conscious guinea pigs: effects of rolipram and dexamethasone. *J Pharmacol Exp Ther* 2002;302:814-821.
181. Yanagihara K, Seki M, Cheng PW. Lipopolysaccharide induces mucus cell metaplasia in mouse lung. *Am J Respir Cell Mol Biol* 2001;24:66-73.
182. Brass DM, Savov JD, Gavett SH, Haykal-Coates N, Schwartz DA. Subchronic endotoxin inhalation causes persistent airway disease. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L755-L761.
183. Brass DM, Savov JD, Whitehead GS, Maxwell AB, Schwartz DA. LPS binding protein is important in the airway response to inhaled endotoxin. *J Allergy Clin Immunol* 2004;114:586-592.
184. Brass DM, Hollingsworth JW, Fessler MB, Savov JD, Maxwell AB, Whitehead GS, Burch LH, Schwartz DA. The IL-1 type 1 receptor is required for the development of LPS-induced airways disease. *J Allergy Clin Immunol* 2007;120:121-127.
185. Savov JD, Gavett SH, Brass DM, Costa DL, Schwartz DA. Neutrophils play a critical role in development of LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L952-L962.
186. Savov JD, Brass DM, Berman KG, McElvania E, Schwartz DA. Fibrinolysis in LPS-induced chronic airway disease. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L940-L948.
187. Savov JD, Brass DM, Lawson BL, Elvania-Tekippe E, Walker JK, Schwartz DA. Toll-like receptor 4 antagonist (E5564) prevents the chronic airway response to inhaled lipopolysaccharide. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L329-L337.
188. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, Wouters EF. Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. *Am J Respir Cell Mol Biol* 2002;26:152-159.
189. Rutgers SR, Postma DS, Ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Chest* 2000;117:262S
190. Willemse BW, Ten Hacken NH, Rutgers B, Lesman-Leegte IG, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J* 2005;26:835-845.

191. Brass DM, Yang IV, Kennedy MP, Whitehead GS, Rutledge H, Burch LH, Schwartz DA. Fibroproliferation in LPS-induced airway remodeling and bleomycin-induced fibrosis share common patterns of gene expression. *Immunogenetics* 2008;60:353-369.
192. Brass DM, Hollingsworth JW, Cinque M, Li Z, Potts E, Toloza E, Foster WM, Schwartz DA. Chronic LPS inhalation causes emphysema-like changes in mouse lung that are associated with apoptosis. *Am J Respir Cell Mol Biol* 2008;39:584-590.
193. Kaneko Y, Takashima K, Suzuki N, Yamana K. Effects of theophylline on chronic inflammatory lung injury induced by LPS exposure in guinea pigs. *Allergol Int* 2007;56:445-456.
194. Rudolphus A, Stolk J, Dijkman JH, Kramps JA. Inhibition of lipopolysaccharide-induced pulmonary emphysema by intratracheally instilled recombinant secretory leukocyte proteinase inhibitor. *Am Rev Respir Dis* 1993;147:442-447.
195. Stolk J, Rudolphus A, Davies P, Osinga D, Dijkman JH, Agarwal L, Keenan KP, Fletcher D, Kramps JA. Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *J Pathol* 1992;167:349-356.
196. Brass DM, Hollingsworth JW, Elvania-Tekippe E, Garantziotis S, Hossain I, Schwartz DA. CD14 is an essential mediator of LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L77-L83.
197. Puljic R, Benediktus E, Plater-Zyberk C, Baeuerle PA, Szelenyi S, Brune K, Pahl A. Lipopolysaccharide-induced lung inflammation is inhibited by neutralization of GM-CSF. *Eur J Pharmacol* 2007;557:230-235.
198. Ulich TR, Watson LR, Yin SM, Guo KZ, Wang P, Thang H, del CJ. The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. *Am J Pathol* 1991;138:1485-1496.
199. Becker S, Mundandhara S, Devlin RB, Madden M. Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: further mechanistic studies. *Toxicol Appl Pharmacol* 2005;207:269-275.
200. Shibata S, Takahashi G, Shioya N, Inada K, Endo S. Suppressive effects of sivelestat on interleukin 8 and TNF-alpha production from LPS-stimulated granulocytes in whole blood culture. *J Anesth* 2010;24:901-907.
201. Ulich TR, Yin SM, Guo KZ, del CJ, Eisenberg SP, Thompson RC. The intratracheal administration of endotoxin and cytokines. III. The interleukin-1 (IL-1) receptor antagonist inhibits endotoxin- and IL-1-induced acute inflammation. *Am J Pathol* 1991;138:521-524.
202. Doz E, Noulin N, Boichot E, Guenon I, Fick L, Le BM, Lagente V, Ryffel B, Schnyder B, Quesniaux VF, Couillin I. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J Immunol* 2008;180:1169-1178.
203. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int Arch Allergy Immunol* 2006;141:354-368.
204. Green RM, Gally F, Keeney JG, Alper S, Gao B, Han M, Martin RJ, Weinberger AR, Case SR, Minor MN, Chu HW. Impact of cigarette smoke exposure on innate immunity: a *Caenorhabditis elegans* model. *PLoS ONE* 2009;4:e6860
205. Sharabi K, Hurwitz A, Simon AJ, Beitel GJ, Morimoto RI, Rechavi G, Sznajder JI, Gruenbaum Y. Elevated CO2 levels affect development, motility, and fertility and extend life span in Caenorhabditis elegans. *Proc Natl Acad Sci* 2009;106:4024-4029.
206. Gross NJ, Skorodin MS. Role of the parasympathetic system in airway obstruction due to emphysema. *N Engl J Med* 1984;311:421-425.
207. Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* 2006;7:73
208. Celli B, Decramer M, Kesten S, Liu D, Mehra S, Tashkin DP. Mortality in the 4 year trial of tiotropium (UPLIFT) in patients with COPD. *Am J Respir Crit Care Med* 2009;180:948-955.

209. Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, Decramer M. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med* 2008;359:1543-1554.
210. Decramer M, Celli B, Kesten S, Lystig T, Mehra S, Tashkin DP. Effect of tiotropium on outcomes in patients with moderate chronic obstructive pulmonary disease (UPLIFT): a prespecified subgroup analysis of a randomised controlled trial. *Lancet* 2009;374:1171-1178.
211. Morice AH, Celli B, Kesten S, Lystig T, Tashkin D, Decramer M. COPD in young patients: A pre-specified analysis of the four-year trial of tiotropium (UPLIFT). *Respir Med* 2010;104:1659-1667.
212. Troosters T, Celli B, Lystig T, Kesten S, Mehra S, Tashkin DP, Decramer M. Tiotropium as a first maintenance drug in COPD: secondary analysis of the UPLIFT-trial. *Eur Respir J* 2010;36:65-73.
213. Wollin L, Pieper MP. Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. *Pulm Pharmacol Ther* 2010;23:345-354.
214. Bos IST, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J* 2007;30:653-661.
215. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med* 2005;171:1096-1102.
216. Profita M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Pieper MP, Gjomarkaj M. Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism. *Eur J Pharmacol* 2008;582:145-153.
217. Sato E, Koyama S, Okubo Y, Kubo K, Sekiguchi M. Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. *Am J Physiol Lung Cell Mol Physiol* 1998;274:L970-L979.
218. Haag S, Matthiesen S, Juergens UR, Racke K. Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. *Eur Respir J* 2008;32:555-562.
219. Matthiesen S, Bahulayan A, Kempkens S, Haag S, Fuhrmann M, Stichnote C, Juergens UR, Racke K. Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. *Am J Respir Cell Mol Biol* 2006;35:621-627.
220. Profita M, Bonanno A, Siena L, Bruno A, Ferraro M, Montalbano AM, Albano GD, Riccobono L, Casarosa P, Pieper MP, Gjomarkaj M. Smoke, choline acetyltransferase, muscarinic receptors, and fibroblast proliferation in chronic obstructive pulmonary disease. *J Pharmacol Exp Ther* 2009;329:753-763.
221. Maarsingh H, Zaagsma J, Meurs H. Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives. *Br J Pharmacol* 2009;3:652-664.
222. Wu G, Morris SM. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336:1-17.
223. Klasen S, Hammermann R, Fuhrmann M, Lindemann D, Beck KF, Pfeilschifter J, Racke K. Glucocorticoids inhibit lipopolysaccharide-induced up-regulation of arginase in rat alveolar macrophages. *Br J Pharmacol* 2001;132:1349-1357.
224. Lindemann D, Racke K. Glucocorticoid inhibition of interleukin-4 (IL-4) and interleukin-13 (IL-13) induced up-regulation of arginase in rat airway fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol* 2003;368:546-550.
225. Que LG, Kantrow SP, Jenkinson CP, Piantadosi CA, Huang YC. Induction of arginase isoforms in the lung during hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 1998;275:L96-102.
226. Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R, Truini M, Fabbi M, Ferrini S, Barbieri O. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. *Int J Cancer* 2009;125:887-893.

227. Morris CR, Poljakovic M, Lavrisha L, Machado L, Kuypers FA, Morris SM, Jr. Decreased arginine bioavailability and increased serum arginase activity in asthma. *Am J Respir Crit Care Med* 2004;170:148-153.
228. North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L911-L920.
229. Zimmermann N, King NE, Laporte J, Yang M, Mishra A, Pope SM, Muntel EE, Witte DP, Pegg AA, Foster PS, Hamid Q, Rothenberg ME. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J Clin Invest* 2003;111:1863-1874.
230. Maarsingh H, Zuidhof AB, Bos IS, Van Duin M, Boucher JL, Zaagsma J, Meurs H. Arginase inhibition protects against allergic airway obstruction, hyperresponsiveness and inflammation. *Am J Respir Crit Care Med* 2008;178:565-573.
231. de Boer J, Meurs H, Coers W, Koopal M, Bottone AE, Visser AC, Timens W, Zaagsma J. Deficiency of nitric oxide in allergen-induced airway hyperreactivity to contractile agonists after the early asthmatic reaction: an ex vivo study. *Br J Pharmacol* 1996;119:1109-1116.
232. de Boer J, Meurs H, Flendrig L, Koopal M, Zaagsma J. Role of nitric oxide and superoxide in allergen-induced airway hyperreactivity after the late asthmatic reaction in guinea-pigs. *Br J Pharmacol* 2001;133:1235-1242.
233. Maarsingh H, Leusink J, Bos IST, Zaagsma J, Meurs H. Arginase strongly impairs neuronal nitric oxide-mediated airway smooth muscle relaxation in allergic asthma. *Respir Res* 2006;7:6
234. Maarsingh H, Bossenga BE, Bos IST, Volders HH, Zaagsma J, Meurs H. L-Arginine deficiency causes airway hyperresponsiveness after the late asthmatic reaction. *Eur Respir J* 34:191-199.
235. Meurs H, McKay S, Maarsingh H, Hamer MA, Macic L, Molendijk N, Zaagsma J. Increased arginase activity underlies allergen-induced deficiency of cNOS-derived nitric oxide and airway hyperresponsiveness. *Br J Pharmacol* 2002;136:391-398.
236. Schuiling M, Zuidhof AB, Bonouvrie MA, Venema N, Zaagsma J, Meurs H. Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. *Br J Pharmacol* 1998;123:1450-1456.
237. Maarsingh H, Pera T, Meurs H. Arginase and pulmonary diseases. *Naunyn Schmiedebergs Arch Pharmacol* 2008;378:171-184.
238. Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AK, van der Linde HJ, van A, I, Coene A, Nijkamp FP. Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* 1996;153:1697-1701.
239. Fischer B, Voynow J. Neutrophil elastase induces MUC5AC messenger RNA expression by an oxidant-dependent mechanism. *Chest* 2000;117:317S-320S.
240. Kitowska K, Zakrzewicz D, Konigshoff M, Chrobak I, Grimminger F, Seeger W, Bulau P, Eickelberg O. Functional role and species-specific contribution of arginases in pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2007;294:L34-L45.
241. Warnken M, Haag S, Matthiesen S, Juergens UR, Racke K. Species differences in expression pattern of arginase isoenzymes and differential effects of arginase inhibition on collagen synthesis in human and rat pulmonary fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol* 2010;381:297-304.
242. Maarsingh H, Dekkers BG, Zuidhof AB, Bos IS, Menzen MH, Klein T, Flik G, Zaagsma J, Meurs H. Increased arginase activity contributes to airway remodelling in chronic allergic asthma. *Eur Respir J* 2011;In Press-doi: 10.1183/09031936.00146610.
243. Hodge S, Matthews G, Mukaro V, Ahern J, Shivam A, Hodge G, Holmes M, Jersmann H, Reynolds PN. Cigarette smoke-induced changes to alveolar macrophage phenotype and function is improved by treatment with procysteine. *Am J Respir Cell Mol Biol* 2010;2009-0459OC.
244. Chachaj W, Gladysz A, Puchala-Liebhart E, Wrzyszcz, Ostrowska E. [Carbamoyl ornithine transferase, arginase and cobalt-activated acylase in patients during asthma attacks or patients with respiratory failure]. *Pol Tyg Lek* 1978;33:85-88.

245. Kochanski L, Kossmann S, Rogala E, Dwornicki J. [Sputum arginase activity in bronchial asthma]. *Pneumonol Pol* 1980;48:329-332.
246. Gebel S, Gerstmayer B, Kuhl P, Borlak J, Meurrens K, Muller T. The kinetics of transcriptomic changes induced by cigarette smoke in rat lungs reveals a specific program of defense, inflammation, and circadian clock gene expression. *Toxicol Sci* 2006;93:422-431.
247. Gungor N, Pennings JL, Knaapen AM, Chiu RK, Peluso M, Godschalk RW, Van Schooten FJ. Transcriptional profiling of the acute pulmonary inflammatory response induced by LPS: role of neutrophils. *Respir Res* 2010;11:24
248. Bergeron C, Boulet LP, Page N, Laviolette M, Zimmermann N, Rothenberg ME, Hamid Q. Influence of cigarette smoke on the arginine pathway in asthmatic airways: increased expression of arginase I. *J Allergy Clin Immunol* 2007;119:391-397.
249. Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, Thunnissen FB, Farver C, Hazen SL, Jennings C, Dweik RA, Arroliga AC, Erzurum SC. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J* 2004;18:1746-1748.
250. Morris CR, Morris SM, Jr., Hagar W, van WJ, Claster S, Kepka-Lenhart D, Machado L, Kuypers FA, Vichinsky EP. Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? *Am J Respir Crit Care Med* 2003;168:63-69.
251. Irie T, Muta T, Takeshige K. TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor-[kappa]B in lipopolysaccharide-stimulated macrophages. *FEBS Letters* 2000;467:160-164.
252. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue Ji, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-I[kappa]B as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* 1999;398:252-256.
253. Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, Doi T, Saiki I. Tumor necrosis factor-α-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J Biol Chem* 2003;278:36916-36923.
254. Shim JH, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, Gygi S, Glimcher LH. TAK1 is an essential regulator of BMP signalling in cartilage. *EMBO J* 2009;28:2028-2041.
255. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* 1995;270:2008-2011.
256. Yamaguchi K, Nagai Si, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J* 1999;18:179-187.
257. Ear T, Fortin CF, Simard FA, McDonald PP. Constitutive association of TGF-β-activated kinase 1 with the IκB kinase complex in the nucleus and cytoplasm of human neutrophils and its impact on downstream processes. *J Immunol* 2010;184:3897-3906.
258. Nishimura M, Shin MS, Singhirunnusorn P, Suzuki S, Kawanishi M, Koizumi K, Saiki I, Sakurai H. TAK1-mediated serine/threonine phosphorylation of EGFR via p38/ERK: NF-κB-independent survival pathways in TNF-α signaling. *Mol Cell Biol* 2009;MCB
259. Sakurai H, Shigemori N, Hasegawa K, Sugita T. TGF-[beta]-activated kinase 1 stimulates NF-[kappa]B activation by an NF-[kappa]B-inducing kinase-independent mechanism. *Biochem Biophys Res Commun* 1998;243:545-549.
260. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev* 2005;19:2668-2681.
261. Cheng T, Peng XC, Li FF, Zhang XL, Hu KZ, Zhu JF, Zeng BF. Transforming growth factor-β activated kinase 1 signaling pathways regulate TNF-α production by titanium alloy particles in RAW 264.7 cells. *J Biomed Mater Res A* 2010;93A:1493-1499.

262. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* 2005;6:1087-1095.
263. Sato S, Sanjo H, Tsujimura T, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Takeuchi O, Akira S. TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. *Int Immunol* 2006;18:1405-1411.
264. Zhou C, Ma FZ, Deng XJ, Yuan H, Ma HS. Lactobacilli inhibit interleukin-8 production induced by Helicobacter pylori lipopolysaccharide-activated Toll-like receptor 4. *World J Gastroenterol* 2008;14:5090-5095.
265. Blanco S, Santos C, Lazo PA. Vaccinia-related kinase 2 modulates the stress response to hypoxia mediated by TAK1. *Mol Cell Biol* 2007;27:7273-7283.
266. HuangFu WC, Omori E, Akira S, Matsumoto K, Ninomiya-Tsuji J. Osmotic stress activates the TAK1-JNK pathway while blocking TAK1-mediated NF-κB activation. *J Biol Chem* 2006;281:28802-28810.
267. Yun YP, Joo JD, Lee JY, Nam HY, Kim YH, Lee KH, Lim CS, Kim HJ, Lim YG, Lim Y. Induction of nuclear factor-[kappa]B activation through TAK1 and NIK by diesel exhaust particles in L2 cell lines. *Toxicol Lett* 2005;155:337-342.
268. Jadrich JL, O'Connor MB, Coucouvanis E. Expression of TAK1, a mediator of TGF-[beta] and BMP signaling, during mouse embryonic development. *Gene Expression Patterns* 2003;3:131-134.
269. Jadrich JL, O'Connor MB, Coucouvanis E. The TGF-β activated kinase TAK1 regulates vascular development in vivo. *Development* 2006;133:1529-1541.
270. Komatsu Y, Shibuya H, Takeda N, Ninomiya-Tsuji J, Yasui T, Miyado K, Sekimoto T, Ueno N, Matsumoto K, Yamada G. Targeted disruption of the Tab1 gene causes embryonic lethality and defects in cardiovascular and lung morphogenesis. *Mech Dev* 2002;119:239-249.
271. Ohkawara B, Shirakabe K, Hyodo-Miura J, Matsuo R, Ueno N, Matsumoto K, Shibuya H. Role of the TAK1-NLK-STAT3 pathway in TGF-β-mediated mesoderm induction. *Genes Dev* 2004;18:381-386.
272. Matsumoto-Ida M, Takimoto Y, Aoyama T, Akao M, Takeda T, Kita T. Activation of TGF-β1-TAK1-p38 MAPK pathway in spared cardiomyocytes is involved in left ventricular remodeling after myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* 2006;290:H709-H715.
273. Zhang D, Gaussin V, Taffet GE, Belaguli NS, Yamada M, Schwartz RJ, Michael LH, Overbeek PA, Schneider MD. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. *Nat Med* 2000;6:556-563.