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FACULTY OF MEDICINE AND HEALTH SCIENCES

ROLE OF LYMPHOID FOLLICLES AND TRANSFORMING GROWTH FACTOR (TGF)-β SUPERFAMILY IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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[PART I: INTRODUCTION]

[**CHAPTER 1:** CHRONIC OBSTRUCTIVE PULMONARY DISEASE]

1.1. Definition

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as *"a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients* [1]*."*

Spirometry, the most reproducible test for pulmonary function, is the gold standard for the clinical diagnosis of COPD and should be considered in any patient presenting with dyspnea, chronic cough or sputum production and with a history of exposure to risk factors. A ratio of the Forced Expiratory Volume in one second (FEV_1) to the Forced Vital Capacity (FVC) below 0.70 determines the presence of airflow limitation. FEV_1 is the maximum volume of air that can be forcedly exhaled in one second after complete inspiration, while FVC represents the maximum volume of air that can be forcedly exhaled. A classification of GOLD severity is made based on post-bronchodilator FEV_1 values and is subdivided into 4 stages by the GOLD initiative **(Table 1)** [1].

Table 1. Classification of severity of airflow limitation in COPD.
Table adapted with permission of Vestbo et al. Am J Respir Crit Care Med 2013;187(4):347-365 [1].

GOLD stage	COPD	FEV_1/FVC		FEV_1
	Absent	≥ 0.70		
GOLD 1	Mild	< 0.70	and	≥ 80% predicted
GOLD 2	Moderate	< 0.70	and	50% ≤ FEV_1 < 80% predicted
GOLD 3	Severe	< 0.70	and	30% ≤ FEV_1 < 50% predicted
GOLD 4	Very severe	< 0.70	and	< 30% predicted

COPD results from a complex interplay between genetic susceptibility and environmental exposure, most importantly tobacco smoke. The best known genetic risk factor linked to COPD is a deficiency of the serine protease α1 antitrypsin, causing early-onset emphysema, but is only present in a small percentage of patients [2]. The genetics underlying COPD are currently unknown, although recent studies have identified interesting genetic loci associated with lung function [3;4]. While tobacco smoke is the most important environmental risk factor in western countries, exposure to biomass smoke is the most prevalent risk factor in developing countries. Also occupational exposure to dusts and gases, respiratory infections and asthma are associated with COPD [5].

To date, COPD is treatable, but not curable, since none of the existing medications for COPD has been shown to slow down the disease progression. Its management consists of controlling symptoms and reducing exacerbations [1]. Smoking cessation is the single intervention that can slow down the accelerated decline in lung function [6]. Pharmacologic treatment, including long-acting bronchodilators and inhaled corticosteroids is used to reduce symptoms and exacerbations [1]. Although COPD is a chronic inflammatory disease, effective anti-inflammatory drugs are lacking, due to a poor understanding of the underlying mechanisms and heterogeneity of the disease [7].

1.2. Epidemiology

COPD is a highly prevalent disorder and represents a substantial economic burden throughout the world **(Figure 1)**. COPD prevalence, morbidity and mortality are highly variable across countries, probably because of differences in defining and diagnosing COPD. Moreover, the prevalence and incidence of COPD are underestimated, since COPD is frequently underdiagnosed and undertreated [1].

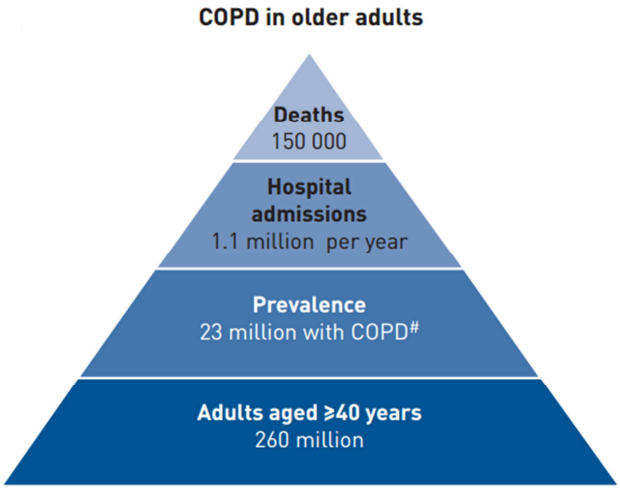

Figure 1. The burden of COPD, around 2010, in the 28 countries of the European Union.
#: Global Initiative for Chronic Obstructive Lung Disease stages II–IV. An additional 17 million adults aged ≥40 years had stage I chronic obstructive pulmonary disease (COPD).
Figure reproduced with permission of the European Respiratory Society © The European Lung White Book Respiratory Health and Disease in Europe. Published 2013, Print ISBN 978-1-84984-042-2; online ISBN 978-1-84984-043-9 [8].

An increasing number of epidemiological studies on COPD prevalence has been published. The Latin American Project for the Investigation of Obstructive Lung Diseases (PLATINO) study estimated prevalence rates of COPD ranging from 7.8% in Mexico City (Mexico) to 19.7% in Montevideo (Uruguay)[9]. The Burden of Obstructive Lung Diseases (BOLD) study, a population-based study in 12 different countries, found a prevalence of stage 2 or higher COPD of 10.1% overall, 11.8% for men, and 8.5% for women [10]. In a cohort study in the Netherlands (Rotterdam Study), the overall incidence of COPD was estimated around 9.2/1,000 person-years in an elderly population (> 55 years), with a remarkably high incidence in the youngest women [11].

Morbidity measurements include physician visits, emergency department visits and hospitalizations. COPD is also associated with significant comorbid diseases, such as cardiovascular disease, cancer and musculoskeletal impairment [1]. COPD has a substantial morbidity that is underestimated by both the healthcare providers and by the patients [12].

Approximately 2.7 million deaths from COPD occurred worldwide in 2000 [13]. The WHO World Health Report listed COPD as the fifth leading cause of death worldwide in 2002. It is estimated that COPD will be ranked as the third leading cause of death by 2020, due to continued exposure to risk factors and the aging of the world's population [14].

Keeping these data in mind, it is not surprising that COPD is associated with significant economic burden **(Figure 2)**. According to the ERS European Lung White Book, the overall annual cost for healthcare and lost productivity due to COPD are estimated as €48.4 billion in the 28 countries of the European Union. Remarkably, approximately half of the economic burden of respiratory disease is attributable to smoking [15].

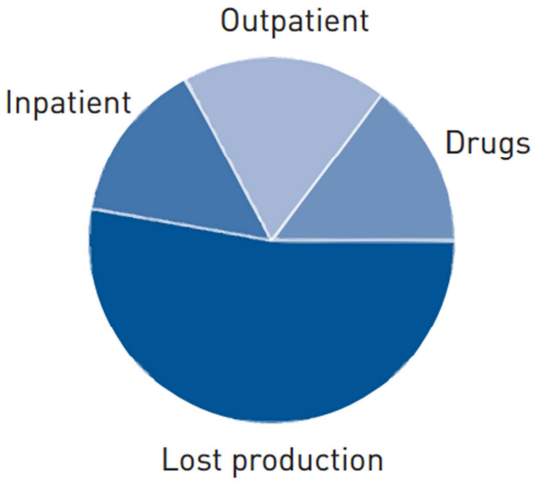

Figure 2. The economic burden of COPD. Distribution of direct and indirect costs by category.
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1.3. Clinical manifestations

COPD is a heterogeneous disease and patients typically present with symptoms such as chronic and progressive dyspnea, cough and sputum production. These symptoms vary over time and not all patients display all the symptoms [1].

Cough and sputum production are an important respiratory defense mechanism against possibly dangerous inhaled particulates. It is usually the first symptom of COPD to develop and may precede the development of airflow limitation by many years [16]. Regular production of sputum for 3 months or more in 2 consecutive years is the epidemiological definition of chronic bronchitis, but this definition does not really reflect the symptoms as seen in patients with COPD [12;17]. Breathlessness is usually the reason why patients seek medical help. It is characteristically persistent and progressive in the course of COPD [12]. Additionally, fatigue, weight loss and anorexia are common problems in severe disease [18]. To assess these symptoms, the COPD Assessment Test (CAT) is a reliable and responsive questionnaire to measure the impact on the patient's daily life and well-being [16].

Another important contributor to the morbidity and progression of COPD are exacerbations. An exacerbation of COPD is an acute event characterized by a worsening of the patient's respiratory symptoms that is beyond normal day-to-day variations and leads to a change in medication [19]. Several different definitions of exacerbations exist and exacerbations can be classified according to severity or etiology. Several factors can evoke an exacerbation, such as respiratory tract infections (viral and/or bacterial), noninfective causes (air pollution and cold temperatures); however, in some patients, no etiological factor can be identified. Acute exacerbations are the main cause of hospitalization among COPD patients and are associated with long-lasting consequences [20].

Patients with COPD also suffer from comorbidities, such as osteoporosis, cardiovascular diseases, psychiatric illness (depression) and lung cancer [21]. Comorbidities can occur at any stage during the course of COPD, irrespective of the degree of airflow limitation [22]. Cardiovascular diseases are the most frequent diseases coexisting with COPD and include ischemic heart disease, heart failure, atrial fibrillation and hypertension [1;23]. Importantly, the majority of patients with (especially mild to moderate) COPD die of other conditions than of COPD itself, therefore comorbidities deserve specific attention [24].

COPD is generally a progressive disease with an irreversible decline in lung function, however not all individuals follow the same clinical course over time. The concept that smoking causes irreversible obstructive changes in susceptible people, was developed by Fletcher and Peto [25]. Furthermore, impaired lung development can predispose to COPD **(Figure 3)**. Stopping exposure to noxious agents may slow the progression of airflow obstruction. However, COPD cannot be cured. The degree of airflow limitation in COPD is only loosely related to symptom severity [12]. End-stage patients have a progressively worsening dyspnoea and often present with multiple comorbidities, resulting in poor quality of life [26].

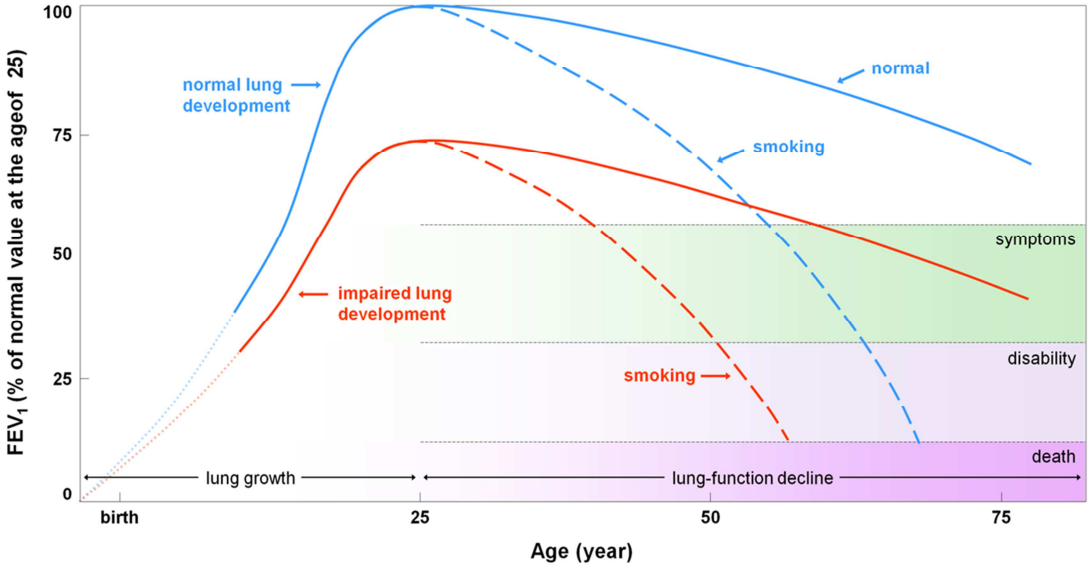


Figure 3. "New" Fletcher and Peto Curve. Natural progress of COPD in smokers. Host characteristics (such as genetic susceptiblity) and environmental exposures (such as smoking) influence lung growth in childhood and adolescence and determine the rate of decline in lung function in adulthood. FEV_1:forced expiratory volume in one second.

Figure adapted with permission of Brusselle N Engl J Med 2009;361(27):2664-2665 [27]. Copyright © 2009 Massachusetts Medical Society.

1.4. Pathology of COPD

Inhaled particles cause an abnormal inflammatory response in persons who will develop COPD. This response will lead to pathological changes in the airways, lung parenchyma and pulmonary vasculature, which are responsible for the airflow limitation and symptoms that define COPD. Importantly, these inflammatory and structural changes - illustrated in **Figure 4** - persist upon smoking cessation [1].

The main site of mucus hypersecretion is the central airways (larger than 2 mm in diameter). Enlarged bronchial mucous glands and an increased number of goblet cells have been found in patients with COPD [28], although an increase in the number of goblet cells has not been consistently reported [29]. **Chronic bronchitis** is also characterized by inflammation in the subepithelial region of the bronchial wall, consisting of neutrophils, macrophages and $CD8^+$ T lymphocytes [30;31].

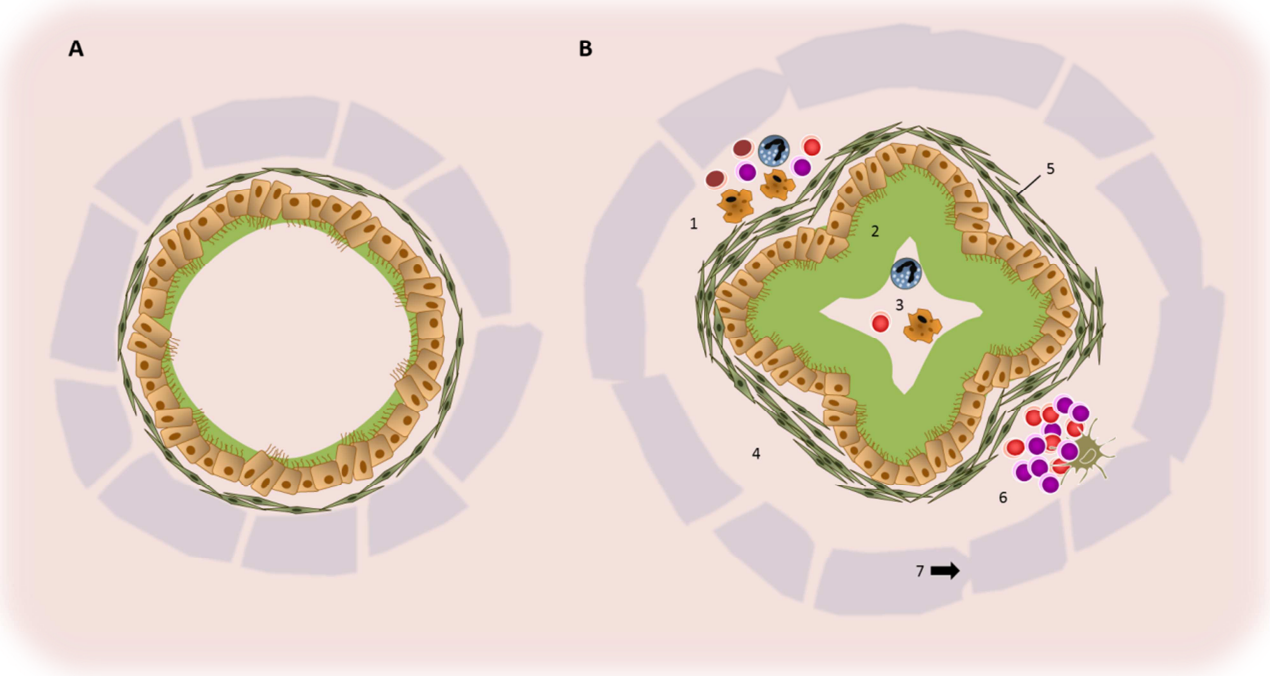


Figure 4. Pathophysiological processes involved in COPD. A) Normal airway. **B)** Airway of a patient with COPD, characterized by 1) inflammatory cellular infiltration, 2) mucus hypersecretion, 3) airway lumen narrowing, 4) subepithelial fibrosis, 5) smooth muscle cell hypertrophy, 6) development of lymphoid follicles and 7) destruction of the alveolar walls or emphysema.

The smaller conducting airways (less than 2 mm in diameter) are considered to be the major sites of increased airway resistance in patients with COPD [32]. A process called "**obstructive bronchiolitis**" which encompasses inflammatory cellular infiltration, goblet cell metaplasia, mucus plugging in the airway lumen, subepithelial fibrosis and smooth muscle cell hypertrophy, is present [29;33-35]. The inflammatory cell infiltrates in the airway wall consist of neutrophils, macrophages, B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes [33]. The increase in lymphocytes, which organize into lymphoid follicles in advanced stages of COPD, is associated with a decline in FEV_1 (see **Chapter 2**) [33;36]. All together, these changes lead to an increase in airway wall thickness, the subsequent narrowing of the airway lumen and decreased FEV_1.

In the alveolar compartment, a pathological process called **emphysema** occurs. Emphysema is defined as an abnormal enlargement of airspaces, distal to the terminal bronchioles, accompanied by alveolar destruction [1]. Centrilobular emphysema affects the respiratory bronchioles in the central area of the acinus. This type of emphysema is more prominent in the upper lung zones and is characteristically associated with tobacco smoking. Panlobular emphysema involves dilatation of the entire acinus. This form of emphysema is associated with deficiency of a1-antitrypsin and early onset [37;38]. Emphysema reduces the elastic recoil, which predisposes the airways to collapse prematurely during exhalation [39]. Both the narrowing of peripheral airways and parenchymal destruction will progressively lead to gas trapping during expiration, resulting in hyperinflation [1].

Anatomic abnormalities in the pulmonary vasculature consist of remodelling of pulmonary arteries and arterioles. In studies using necropsy material and surgical specimens, a thickening of the intima of pulmonary arteries in patients with mild to moderate COPD and a thickening of both the intima and media in patients with severe COPD have been described [40]. Also muscularisation of pulmonary arterioles, which have no muscular layer in normal subjects, is shown in patients with emphysema [41]. In contrast to patients with pulmonary arterial hypertension, no plexiform lesions have been found in patients with COPD. Consequently, endothelial dysfunction in pulmonary arteries of COPD is present [42;43]. Similar to the inflammation in the airway wall, mainly $CD8^+$ T lymphocytes infiltrate the adventitia of pulmonary muscular arteries and this inflammatory reaction is associated with an impaired vascular relaxation and thickening of the intimal layer [44].

1.5. Pathogenesis of COPD

Effective therapy for patients with COPD is still lacking. Despite multiple efforts worldwide, the pathogenetic mechanisms underlying COPD are still not fully elucidated. The above- mentioned pathology appears to be an amplification of the normal inflammatory response to inhaled irritants. The inflammation persists after smoking cessation in patients with COPD, indicating that the inflammation is maintained through autonomous mechanisms. Oxidative stress and protease-antiprotease and apoptosis-proliferation imbalances further modify lung inflammation. Importantly, genetic susceptibility, involving a broad set of known (such as TGF-β1, MMP-9 and hedgehog interacting protein…) and unknown genes and epigenetic changes also contribute to the development of COPD. These mechanisms do not operate separately and several interactions occur, making the pathogenesis even more complex **(Figure 5)**.

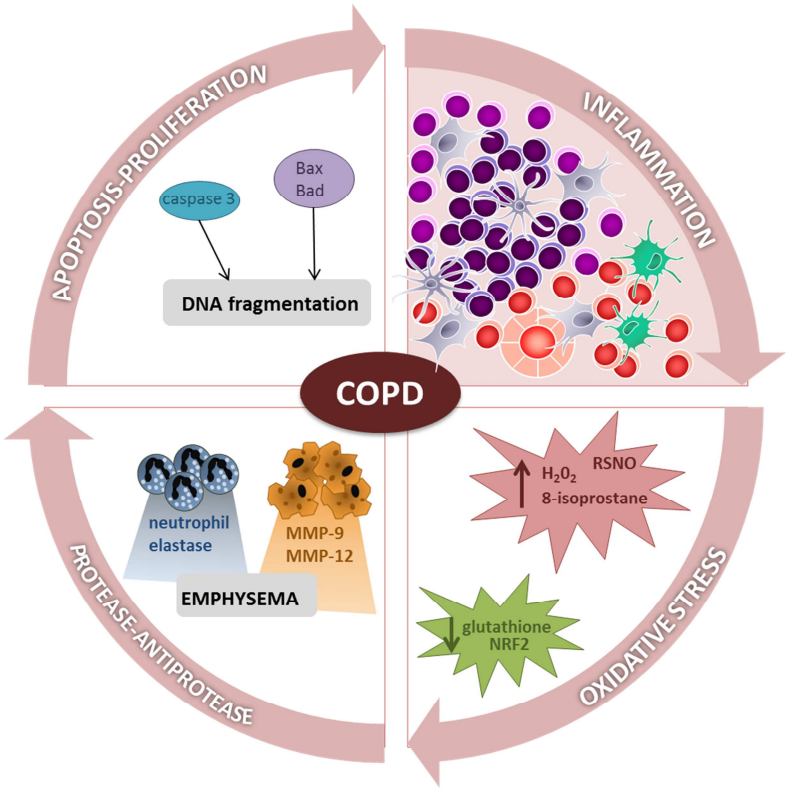


Figure 5. Schematic overview of the pathogenetic mechanisms underlying COPD. Four important mechanisms take part in the development of COPD: 1) inflammation: influx of innate and adaptive immune cells, leading to the development of lymphoid follicles in severe disease, 2) oxidative stress: imbalance between oxidants (including hydrogen peroxide [H_2O_2], nitrosothiol [RSNO], lipid peroxidation products) and anti-oxidants will result in oxidative stress, 3) imbalance between proteases (e.g. neutrophil elastase and MMP-9, MMP-12) and antiproteases: the net result is an increased proteolytic activity and breakdown of connective tissue and emphysema, 4) imbalance between apoptosis and proliferation: increased apoptosis of endothelial and epithelial cells not counteracted by proliferation can trigger matrix destruction. These disease mechanisms all interact with each other leading to the pathophysiology and symptoms seen in COPD. MMP: matrix metalloproteinase, NRF2: nuclear factor erythoid 2-related factor.

1.5.1. Chronic inflammation

As a first line of defence, the lungs have innate immune mechanisms encompassing the epithelial barrier and innate immune cells, specifically macrophages/monocytes, neutrophils, eosinophils, mast cells, natural killer (NK) cells and dendritic cells. These cells recognize microbes (Pathogen Associated Molecular Patterns or PAMPS) and danger signals (Damage Associated Molecular Patterns or DAMPs) by Pattern Recognition Receptors (PRR) [45]. Importantly, the innate immune system is sufficient to develop pulmonary inflammation and emphysema, as shown by studies using Severe Combined Immune Deficient (SCID) and Recombination Activating Gene (RAG)1 deficient mice, which lack adaptive immunity [46;47].

Exposure to cigarette smoke (CS) leads to activation of several PRRs on **epithelial cells** and production of a broad range of chemokines and inflammatory mediators (IL-8, IL-1β, TGF-β1, MCP-1, LTB_4) [48-51]. In addition, cigarette smoke can directly cause injury to epithelial cells, which will release DAMPs. Elevated levels of DAMPS, such as extracellular adenosine triphosphate (ATP) and high-mobility group box 1 (HMGB1) have been found in the bronchoalveolar lavage (BAL) fluid of patients with COPD compared to never smokers and smokers without COPD [52;53]. All together, these mechanisms will activate the innate immune system **(Figure 6)**.

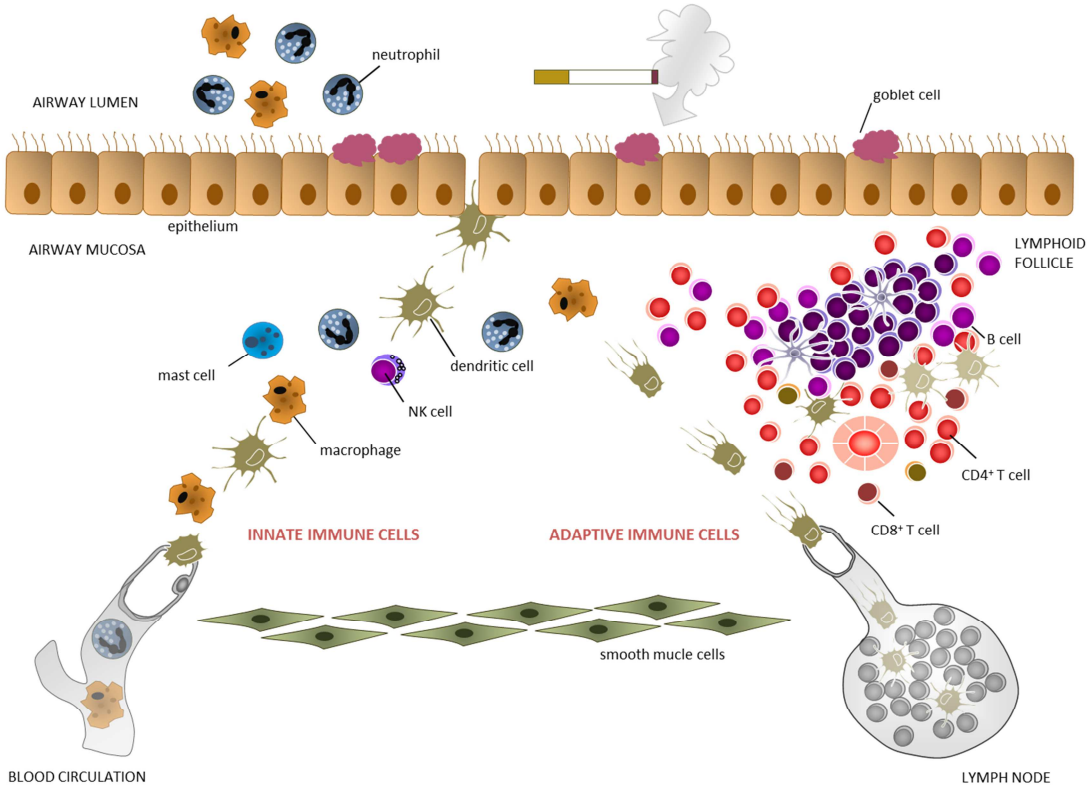


Figure 6. Innate and adaptive immune cells in the pathogenesis of COPD. *Figure adapted with permission of Brusselle et al Lancet 2011;378 (9795):1015-1026* [54]. Copyright © 2011 Elsevier Ltd.

Macrophages contribute to most hallmarks of COPD. Increased numbers of macrophages are present in patients with COPD and there is a correlation between the numbers of macrophages and increasing severity of COPD [33;55;56]. This increase may be due to increased recruitment of CXCR2+ monocytes, increased proliferation and reduced cell death of macrophages [57;58]. Activated macrophages release inflammatory mediators and chemokines, adding to the inflammation and recruitment of other immune cells [59;60]. Macrophages also contribute to tissue damage through the production of reactive oxygen species and the release of proteolytic enzymes such as matrix metalloproteinases (MMP-1, -9 and -12) [61-63]. MMP-12 (or macrophage elastase) knockout (KO) mice are protected against CS-induced airspace enlargement, indicating the importance of MMP-12 in the development of emphysema [64]. Furthermore, alveolar macrophages from patients with COPD have reduced phagocytic capacity, which may result in increased bacterial load [65].

The presence of neutrophilic inflammation in the airways is a characteristic feature of COPD. **Neutrophils** are increased in sputum and airway smooth muscle cells of patients with COPD and to a lesser extent in the lung parenchyma [66;67]. Neutrophil numbers in sputum and bronchial mucosa are associated with peripheral airflow obstruction and a lower FEV_1 value [55;68]. The functional role of neutrophils in COPD is not yet clear. Neutrophils can contribute to alveolar destruction by the production of neutrophil elastase, proteinase-3, cathepsin G and MMP-8 and -9. In this context, animal studies using neutrophil elastase KO mice revealed a requirement for neutrophil elastase in CS-induced emphysema [69]. However, no association was found between airway neutrophilia and severity of emphysema in patients with COPD [68]. Additionally, neutrophils can release neutrophil extracellular traps (NETs), which are fibrillary networks of nuclear components (DNA and histones), decorated by granule proteins with antimicrobial capacity. Although NETs are important for the host immunity against infections, they can simultaneously be harmful and are linked to COPD [70;71]. It has been suggested that neutrophils are involved in mucus hypersecretion, since neutrophil elastase and cathepsin G, serine proteases produced by neutrophils, can stimulate mucus secretion from airway gland serous cells [72]. Similar to macrophages, the phagocytic capacity of neutrophils is impaired upon cigarette smoke extract (CSE) treatment [73].

Eosinophil numbers are elevated in blood, BAL fluid, sputum and biopsy specimens of a subgroup of patients with COPD compared to healthy controls [74;75]. During exacerbations of chronic bronchitis, increased numbers of eosinophils are present in bronchial biopsies and sputum [76]. The role of eosinophils in the pathogenesis of COPD is not clear (in contrast to their well-known role in asthma), but they may represent a distinct COPD phenotype. In patients with pulmonary emphysema, eosinophilic inflammation in the airways has been related to the reversible part of the airflow obstruction in response to glucocorticoids [75].

While **mast cells** have a key role in asthma through the release of several bronchoconstrictors, their role in COPD is unknown. Increased, reduced or unchanged numbers of mast cells have been reported in COPD airways and lungs. The morphology and molecular expression of mast cells are changed in lungs of patients with COPD, but the functional consequences of these alterations are yet to be determined [55;77;78].

Natural killer (NK) cells were originally classified as lymphocytes based on their morphology, but are now generally accepted as components of the innate immune system, because they lack antigen-specific cell surface receptors [79]. Patients with COPD have increased numbers of NK cells in the subepithelium and induced sputum compared with healthy smokers, while in a mouse model of chronic cigarette smoke exposure no changes in the numbers of NK cells were demonstrated [55;80;81]. The increased proportion of NK cells is inversely correlated with the degree of airflow limitation in smokers [55]. Cytotoxic activity of NK cells is reduced in the peripheral blood of patients with COPD, while in the airways the cytotoxic activity is elevated [81;82].

Dendritic cells (DCs) are specialized antigen-presenting cells that form the link between innate and adaptive immunity, by taking up antigen (Ag), migrating to the lymph nodes and presenting the Ag to the T lymphocytes via expression of major-histocompatibility-complex (MHC) proteins. By immunohistochemistry, our group showed selective accumulation of langerin positive myeloid DCs in the epithelium of small airways of patients with COPD, associated with increased expression of the chemokine CCL20 [83;84]. Langerin positive DCs are very efficient in inducing $CD8^+$ T cell responses by cross-presentation [85]. On the other hand, an electron microscopy study found decreased bronchial mucosal DC numbers in current smokers with COPD compared to ex-smokers with COPD [86]. COPD progression is associated with increases in costimulatory molecule expression by lung DCs [87]. The exact role of DCs in COPD is unclear as the antigenic stimulants have not yet been identified.

The adaptive immune system functions through antigen-specific receptors and comprises T and B lymphocytes. **$CD8^+$ cytotoxic T lymphocytes** predominate over $CD4^+$ T lymphocytes in the airways, lung parenchyma and pulmonary arteries of patients with COPD and their numbers are significantly correlated with the degree of airflow limitation in smokers [88;89]. The CXCL10/CXCR3 chemokine axis, increased in peripheral airways of smokers with COPD, might be implicated in $CD8^+$ T cell recruitment [90]. These cytotoxic T cells are highly activated in COPD and release proteolytic enzymes, such as perforin and granzyme b and show increased cytotoxic potential [91;92]. There is an association between apoptosis and CD8+ T cells in the alveolar wall, suggesting that cytotoxic T cells could contribute to the process of emphysema [93].

Adoptive transfer of pathogenic $CD4^+$ T cells into naïve mice results in the development of emphysema, indicating that also $CD4^+$ T cells are involved in the pathogenesis of COPD [94]. The $CD4^+$ T lymphocytes that accumulate in the airways and lungs of patients with COPD are mainly **Th1 cells**. Th1 cells expressing CCR5 and CXCR3 secrete more interferon-γ (IFN-γ) in COPD patients than in control smokers [95]. However, the response of Th1 cells to endotoxin is impaired in smokers with and without airflow limitation, which may account for the increased susceptibility to bacterial infections in COPD [96].

Th17 cells are a distinct lineage of $CD4^+$ T cells that mediate immunity against extracellular pathogens and are involved in autoimmunity by production of IL17A and IL17F [97]. Patients with COPD have higher numbers of IL17A+ cells in the bronchial submucosa than smokers and never smokers [98;99]. By flow cytometry, the Th17 subset was reported to be increased in the peripheral blood of patients with COPD, which is inversely correlated with the FEV_1 value [100]. IL17R KO mice failed to develop emphysema after 6 months of CS exposure [101]. Th17 cells are known for stimulating neutrophilic inflammation upon LPS treatment [102].

Another subtype of $CD4^+$ T lymphocytes that may be involved in COPD are **regulatory T cells** (Tregs). Tregs have immunoregulatory functions and inhibit autoimmunity by producing anti-inflammatory cytokines such as IL-10 and TGF-β1 [103]. In BAL fluid, $CD4^+$-$CD25^{bright}$ cells were increased in smokers with normal lung function compared with healthy never-smokers and patients with moderate COPD in one study [104], whereas another study demonstrated that smoking induces $CD4^+$ - CD25 bright cells independent of lung function [105]. Tregs are decreased in the lungs of patients with emphysema and this is associated with reduced levels of FoxP3 (transcription factor characteristic for Tregs) and IL-10 [106]. In lymphoid follicles on the other hand, the number of $CD4^+$ $FoxP3^+$ cells is raised in patients with COPD, indicating that Tregs might be involved in dampening excessive inflammation (and autoimmunity) in COPD [107]. Apparently, the number of Tregs is dependent of the anatomical location that is studied. An upregulation of FOXP3-positive cells in large airways of patients with COPD was present, but a downregulation was found in small airways that correlated with airflow limitation [108].

Next to T lymphocytes, **B lymphocytes** are also implicated in the pathogenesis of COPD. B cells are increased in bronchial biopsies of large airways of patients with COPD and are higher in more severe COPD [109]. Progression of COPD is associated with the development of organized lymphoid follicles, consisting of B and T lymphocytes **(Figure 6)** [33]. The mechanisms behind this process - called lymphoid neogenesis - will be discussed extensively in **Chapter 2**.

1.5.2. Oxidative stress

Oxidative stress may be an important amplifying mechanism in COPD. The lungs are continuously exposed to oxidants, either generated endogenously or exogenously. Lung cells are protected against this oxidative challenge by antioxidant systems. Oxidative stress arises when there is a disequilibrium between oxidants and antioxidants in favor of the former [110].

Numerous studies have shown that oxidative stress is present in patients with COPD. Several markers of oxidative stress, such as hydrogen peroxide, nitrosothiol, lipid peroxidation products (such as 8-isoprostane,…) are increased in exhaled breath condensate of patients with COPD [111-113]. Moreover, oxidant production increases further during exacerbations [112]. There is evidence for systemic oxidative stress, since products of lipid peroxidation are elevated in plasma in smokers with COPD [114]. There may also be a reduction in antioxidants in COPD. Glutathione and nuclear factor erythoid 2-related factor (NRF2) protein levels are reduced in lungs of patients with COPD [115]. NRF2 is a redox-sensitive transcription factor that is involved in the regulation of many antioxidant genes [110]. Epidemiological studies have shown a significant association between reduced dietary intake of antioxidants (vitamin E and C) and lung function and demonstrated that antioxidant supplementation reduced the risk of developing chronic lung disease in women [116;117].

The environmental sources of airborne oxidative stress include oxidant gasses, ultrafine particulate matter and most importantly CS. CS contains around 10^{17} oxidant molecules per puff [110]. However, cessation of smoking does not stop the continued oxidant burden, indicating that oxidative stress most likely arises from endogenous sources [118]. Inflammatory cells (neutrophils, eosinophils and macrophages) and structural cells (e.g. epithelial cells) that are activated in the airways of patients with COPD produce reactive oxygen species [119].

Oxidative stress occurs in the presence of inflammation, but can also enhance the inflammation. Through the upregulation of redox-sensitive transcription factors, such as nuclear factor κB (NF-κB) and activating protein 1 (AP-1), oxidative stress may induce neutrophilic inflammation via increased expression of IL-8 [120-122]. Oxidative stress is also implicated in mucus hypersecretion, inactivation of antiproteases and apoptosis of structural cells and subsequently emphysema [123-125]. Ablation of Nrf2 in mice resulted in increased susceptibility to CS-induced inflammation and emphysema compared to wild-type mice [126]. Furthermore, there is a link between oxidative stress and the relative resistance to corticosteroids in patients with COPD. Histone deacetylase activity, required for the anti-inflammatory action of corticosteroids, is reduced upon CS exposure and this is correlated with a reduction in glucocorticoid responsiveness *in vitro* [127].

1.5.3. Protease-antiprotease imbalance

It has been long proposed that there is an imbalance between proteases and antiproteases, resulting in an increased proteolytic activity, breakdown of connective tissue and emphysema. This imbalance may occur either by an excessive release of proteases or by an increased breakdown of antiproteases. This hypothesis was proposed after the observation that smokers with a deficiency of α1-antitrypsin developed early onset pulmonary emphysema [128].

Neutrophil elastase (NE) is a serine protease, produced by neutrophils, which is inhibited by α1-antitrypsine. Intratracheal instillation of human NE in mice resulted in the development of emphysema, whereas NE deficient mice are protected against CS-induced emphysema [69;129]. Exogenous α1-antitrypsin was able to inhibit matrix degradation, as assessed by lower desmosine (elastin breakdown) and hydroxyproline (collagen breakdown) levels [130]. NE is also involved in mucus production, stimulates neutrophil influx and increases oxidative stress [131;132]. Given these observations, it was thought that neutrophils and neutrophil elastase were the main effector cell and protease respectively, however no significant correlation between neutrophil numbers and the degree of tissue destruction was found in humans, suggesting other proteases may be involved [133].

The idea that macrophages and macrophage-derived matrix metalloproteinases (MMPs) are important, emerged when several studies found that MMP-1, -2 and -9 levels are higher in lungs or lavaged macrophages of patients with emphysema [134;135]. Interestingly, MMP-12 deficient mice do not develop airspace enlargement upon exposure to CS [64]. MMP-12 levels and activity are elevated in induced sputum of patients with COPD compared to healthy smokers and never smokers [61]. However the function of MMPs as matrix-degrading proteases is still unproven. Most likely, emphysema is the result of different proteases working together and interacting with other types of mediators. It was shown that both MMP12 and neutrophils are required for matrix breakdown in mice exposed to CS [136].

Normally proteases are counteracted by endogenous antiproteases. α1-antitrypsin and secretory leukocyte protease inhibitor (SLPI) are the major inhibitors of serine proteases. As mentioned above, oxidative stress can inactivate these inhibitors [124]. Cleaved SLPI is found in BAL fluid of patients with emphysema in contrast to healthy controls, indicating that cleavage is another mechanism whereby antiproteases can be inhibited [137]. Tissue inhibitor of MMPs (TIMP)1-4 inhibit MMPs. The inflammation driven TIMP-1 secretion from alveolar macrophages is blunted in cells derived from patients with COPD [138]. TIMP-3 KO mice demonstrate a spontaneous, age-related emphysema [139]. Finally, genetic mutations that result in loss of function or reduced protein levels of antiproteases have been described, of which genetic deficiency of α1-antitrypsin is the best known example [128;140].

1.5.4. Apoptosis-proliferation imbalance

It is generally accepted that CS induces an exaggerated influx of inflammatory cells into the lungs and that these inflammatory cells produce proteases and reactive oxygen species that cause tissue destruction and eventually emphysema. A new concept in the pathogenesis of emphysema has been proposed that loss of epithelial and endothelial cells via apoptosis can trigger matrix destruction and alveolar space enlargement. Apoptosis is a tightly regulated mechanism leading to programmed cell death and is critical for the maintenance of normal tissue homeostasis.

This hypothesis was confirmed by several studies in animal models. In the study of Kasahara and colleagues, intravascular administration of a vascular endothelial growth factor (VEGF)-receptor antagonist generated non-inflammatory emphysema [141]. Adding to this, a single intratracheal injection of active caspase 3 (pro-apoptotic enzyme) induced airspace enlargement in rats, even without inflammation [142]. In a CS-induced mouse model of COPD, apoptosis of type II alveolar epithelial cells was higher compared to the air-exposed littermates [143]. Similar data are found in human studies. Apoptotic cells (alveolar epithelial cells, interstitial cells and inflammatory cells) are elevated in lungs of patients with COPD and emphysema and this increase persisted despite smoking cessation [144-146]. Also, expression of Bax and Bad (pro-apoptotic proteins) was detected in emphysema [145]. On the other hand, other studies did not demonstrate a difference in apoptosis between COPD patients and healthy controls, but described an association between apoptosis and pack years [93].

In physiologic circumstances, apoptosis and proliferation are in balance to maintain tissue homeostasis. In COPD, this balance is disturbed. While apoptosis is increased in patients with emphysema, proliferation rate is not different [147]. On the other hand, increased proliferation together with apoptosis has been reported in emphysema compared to smokers and never smokers [148]. VEGF is a growth factor required for endothelial cell survival. Expression of VEGF and its receptor are reduced in emphysema, suggesting that a decrease of endothelial cell maintenance factors may be part of the pathogenesis of emphysema [149].

Although it has been shown in animal studies that apoptosis of structural walls is sufficient to cause emphysema, it is probable that interaction between apoptosis and inflammation (and other pathogenetic mechanisms) will take place in patients with COPD. In this context, alveolar macrophages from patients with COPD have decreased phagocytic ability, contributing to inefficient clearance of apoptotic cells [150].

[**CHAPTER 2:** CXCL13 AND LYMPHOID NEOGENESIS IN COPD]

2.1. Lymphoid neogenesis in COPD

The primary purpose of the immune system is to defend the human body against potential hazardous antigens and pathogens. In tissues that harbor a persistent source of antigen, the immune system has evolved in a way that lymphocyte infiltrates will organize themselves in lymphoid follicles. This process is called 'lymphoid neogenesis' and refers to the development of ectopic lymphoid tissue which resemble secondary lymphoid organs (e.g. lymph nodes, mucosal-associated lymphoid tissue and spleen) in non-lymphoid tissues that are targeted by chronic inflammatory processes or auto-immunity [151].

A distinction should be made between lymphoid aggregates and follicles. Aggregates lack functional architecture and their primary function is local priming and activation. Following criteria have to be fulfilled before the term lymphoid follicles can be used [152;153]:

a) Specific arrangement of anatomically distinct yet adjacent B and T cell compartments
b) The presence of follicular dendritic cells (FDCs) that present antigen-antibody immune complexes to B lymphocytes
c) The development of high endothelial venules (HEV) that allow continuous transmigration of lymphocytes
d) Evidence of B cell class switching, as demonstrated by the presence of activation-induced cytidine deaminase (AID) enzyme
e) Germinal center reactions in the B cell area, which are the principal site of B cell maturation

An alternative name for lymphoid follicles are tertiary lymphoid organs (TLOs) as opposed to primary lymphoid organs (thymus and bone marrow), where lymphocytes are generated, and secondary lymphoid organs. While secondary lymphoid organs are pre-programmed during ontogeny, tertiary lymphoid organs arise under environmental influences in adults. TLOs are architecturally similar to secondary lymphoid organs, share the same molecular and cellular requirements for their formation and contain all the necessary cell types to elicit adaptive immune responses. In contrast to lymph nodes, TLOs are not encapsulated and are not supplied by afferent lymph vessels [151;154].

In the lung, it was originally stated that lymphoid structures in close association with the bronchial epithelium were constitutively present as part of bronchus-associated lymphoid tissue (BALT), similar to the gut-associated lymphoid tissue (GALT). However, in normal human lung no lymphoid tissue was observed, except in lungs of children and adolescents [155;156].

BALT can be induced (iBALT) upon antigenic stimuli, such as CS or bacteria and viruses, but also in pathological situations, such as rheumatoid arthritis or lung transplantation. More than 20 years ago, the presence of BALT has been reported in smokers, being significantly more prominent in smokers compared to never smokers. Moreover, these authors reported that BALT is not per se associated with a specialized epithelium, therefore the term 'lymphoid follicles' will be used instead of BALT [36;157].

Higher numbers of small airways containing lymphoid follicles were found in patients with severe COPD compared to healthy subjects and patients with mild-to-moderate COPD **(Figure 7)**. Importantly, the number of lymphoid follicles is associated with the progression of COPD [33]. B-cell follicles were not only demonstrated in association with small airways but also in the lung parenchyma of patients with COPD [158]. Furthermore, individual B cells were found to be increased in the lungs of patients with COPD [109]. Similarly, mice exposed to long-term CS develop lymphoid follicles around airways and blood vessels as well as in the lung parenchyma [158;159] **(Figure 7)**. The number and size of the follicles correlates with airspace enlargement in CS-exposed mice [158].

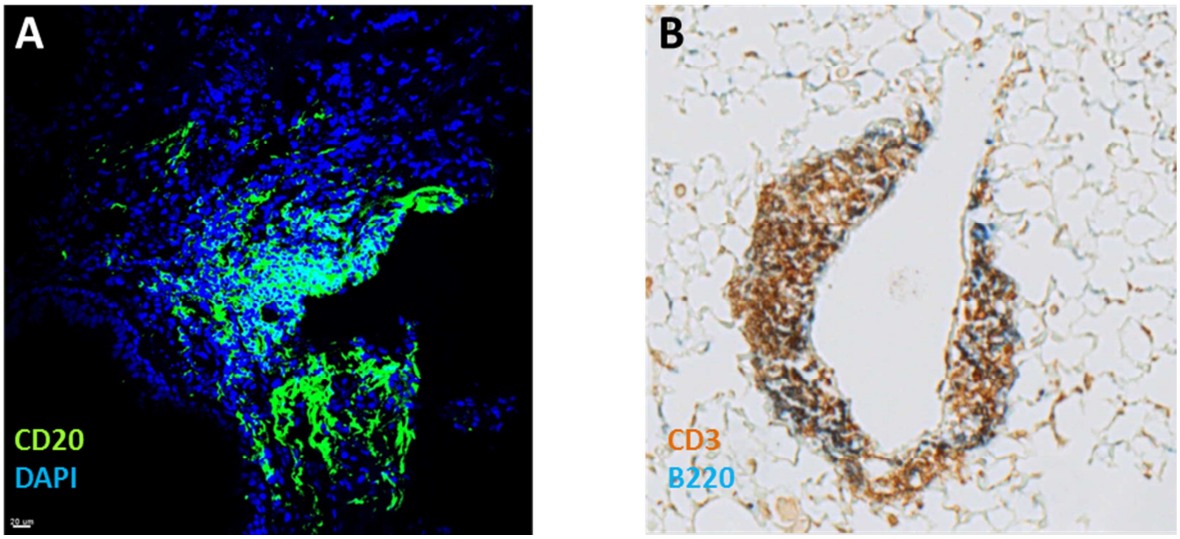


Figure 7. Lymphoid follicles in severe COPD and cigarette smoke (CS)-exposed mice. A) Representative image of immunofluorescence staining with anti-CD20 antibody (green) in lung tissue of patients with severe chronic obstructive pulmonary disease (COPD) GOLD III. Counterstaining with 4',6-diamidino-2-phenylindole (DAPI) is shown in blue. **B)** Representative image of immunohistochemical staining with anti-CD3 antibody (brown) for T lymphocytes and anti-B220 antibody (blue) for B lymphocytes in lung tissue of CS-exposed mice.

Several studies have been published where the cellular organization of these structures has been investigated. The follicles consist of a B cell core, which comprises the majority of cells, surrounded by T cells. The B cells are predominantly $CD27^+$ memory B cells, but also $CD138^+$ plasma cells are detected in the direct vicinity of the follicles. The B cells within the follicle are mainly IgM positive. Immunoglobulin gene analysis of B cells isolated from lymphoid follicles demonstrated ongoing mutations in individual B cell contigs, suggestive for oligoclonal B cell proliferation, stimulated by an unidentified antigen. These data, together with the expression of the proliferation marker Ki-67 within lymphoid follicles, are suggestive for the presence of an active germinal center [158]. In this context, numbers of memory B cells in the lungs of patients with COPD are significantly higher compared to non-COPD controls, also suggestive for antigen-specific stimulation [160].

The T cell periphery of lymphoid follicles consists mainly of $CD4^+$ T cells (80-90 %), and to a lesser extent $CD8^+$ T cells [158]. Also increased numbers of $CD4^+$ $FoxP3^+$ immunomodulatory Treg cells were observed in follicles of patients with COPD [107]. The density of $CD57^+$ cells, important effectors of cytotoxicity, within lymphoid follicles of patients with COPD is increased compared to that of never smokers and smokers without COPD [161]. Antigen presentation in the lymphoid follicles is mediated by FDCs and DCs. FDCs are specialized reticular fibroblasts and are located within the B cell center as shown by staining for CD21 and CD35, 2 markers for FDCS [152]. Myeloid and plasmacytoid DCs are also found within the T cell zone [152;162] **(Figure 8)**. Moreover, the number of pDCs is higher in lymphoid follicles of patients with mild-to-moderate COPD compared to smokers without airflow limitation [162].

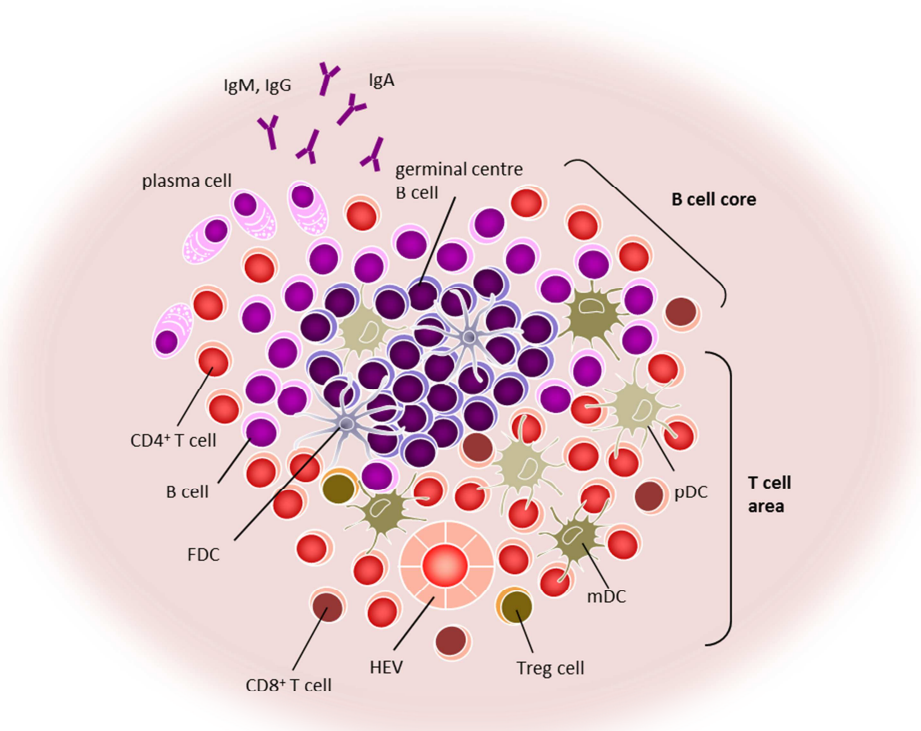


Figure 8. Structure of pulmonary lymphoid follicles in COPD. Treg cell: regulatory T cell, FDC: follicular dendritic cell, HEV: high endothelial venule, mDC: myeloid dendritic cell, pDC: plasmacytoid dendritic cell, CD: cluster of differentiation, Ig: Immunoglobulin. *Figure adapted with permission of Brusselle et al Lancet 2011;378 (9795):1015-1026* [54]. Copyright © 2011 Elsevier Ltd.

The pathogenic role of lymphoid follicles in COPD is not yet clear. The unique organization and cellular composition (presence of lymphocytes and Ag presenting DCs) create the optimal conditions for an effective immune response at the site of antigen deposition. In addition, lymphoid follicles are not encapsulated, making them directly accessible for encountering antigens. In that regard, ectopic lymphoid follicles may develop as protective structures against infectious agents and prevent infections from spreading to other parts of the body. This makes sense, knowing that the presence of pathogens in the airways/lungs of patients with COPD is a common occurrence and also increases with worsening airflow obstruction, in parallel with lymphoid follicles [163]. Animal studies with influenza infection confirm that tertiary lymphoid organs are protective in an infectious setting. Genetic targeted mice without peripheral lymphoid organs but with iBALT can clear influenza infection and even survive higher doses of virus [164]. Another approach, where iBALT was depleted by elimination of DCs, but where the secondary lymphoid organs were intact, emphasizes the role of DCs and iBALT in humoral immunity upon influenza infection in mice [165].

Consistent with these data, IgM and IgA, which are essential for mucosal immune responses are produced within lymphoid follicles in patients with COPD [157;158]. Accordingly, there was a strong negative association between oral and/or inhaled corticosteroid therapy and the percentage of airways containing lymphoid follicles in COPD [166]. It can be hypothesized that the steroid-induced suppression of lymphoid follicles may account for the increased risk of pneumonia in patients who use corticosteroids, pointing towards a protective role of lymphoid follicles [167].

On the other hand, lymphoid follicles may contribute to the tissue destruction and poorly reversible progression of COPD, since their numbers increase with disease severity [33]. Lymphoid follicles are a concentrated source for inflammatory cells and concomitant pro-inflammatory mediators and proteases. The persistent inflammation and destruction may give rise to injury-related neo- or self-antigens in the lung tissue, triggering the development of lymphoid follicles. Numerous studies demonstrated the presence of autoantibodies in COPD, such as auto-antibodies against HEp-2^{+} epithelial cells, endothelial cells and elastin [106;168;169]. Higher numbers of anti-decorin IgG antibody producing cells were present in peripheral blood mononuclear cells (PBMCs) derived from patients with COPD compared to healthy controls [160]. Injection of xenogeneic endothelial cells in rats resulted in the development of emphysema [170]. In contrast, others could not confirm auto-antibody responses in COPD [171]. Although Tregs are increased in lymphoid follicles of patients with moderate COPD, it is possible that they fail to suppress autoimmunity in severe COPD [107]

2.2. CXCL13

The organization of tertiary lymphoid organs is the result of a coordinated interplay between haematopoietic and stromal cells, adhesion molecules, cytokines and chemokines [151]. CXC-chemokine ligand (CXCL)13 is one of those lymphoid chemokines. Lymphoid or homeostatic chemokines are constitutively expressed in lymphoid tissues and mediate the formation and maintenance of lymphoid tissues, through the trafficking of leukocytes to the lymphoid organs [172].

Formation of lymphoid follicles follows many of the pathways used for the organogenesis of secondary lymphoid organs. Therefore, it is important to first summarize the mechanisms involved in secondary lymphoid tissue development. Crucial is the mutual interaction between haematopoietic inducer cells and stromal organizer cells. Binding of the cytokine lymphotoxin (LT)$\alpha_1\beta_2$, expressed by inducer cells, to lymphotoxin β receptor (LTβR) on the surface of organizer cells induces the expression of lymphoid chemokines [CXCL12/13, CC-chemokine ligand (CCL)19/21] and adhesion molecules [vascular cell adhesion molecule (VCAM)1, intercellular adhesion molecule (ICAM)1, mucosal addressin cell adhesion molecule (MADCAM)1] in organizer cells. This will attract additional haematopoietic cells and will initiate clustering of the cells. This is followed by HEV differentiation, further recruiting naïve lymphocytes from the blood stream. As a result, a well-organized structure with a distinct B and T cell zone is formed [153;172].

The necessity of LTβR signaling for the development of secondary lymphoid tissue is further substantiated by the absence of lymph nodes and Peyer's patches and impaired spleen organization in LT-deficient mice [173]. The chemokines and adhesion molecules are involved in attracting and retaining the cells required for formation. CCL19 and CCL21 are produced by stromal cells and attract naïve T cells and mature DCs via CCR7 to T cell areas. The secondary lymphoid organs of CCR7 deficient mice have a disturbed architecture due to the impaired entry of T lymphocytes and DCs [174].CXCL12 and CXCL13 attract $CXCR4^+$ and $CXCR5^+$ B cells into the B cell zone. CXCR5 deficient mice lack inguinal lymph nodes and have only a few Peyer's patches, which are phenotypically abnormal [175]. CXCL13 deficient mice have a more severe deficiency and lack most lymph nodes [176]. CXCL13 has a role in B cell compartmentalization by mediating B cell attraction. Additionally, CXCL13 induces LT$\alpha_1\beta_2$ expression on B cells, subsequent LTβR stimulation and up regulation of its own expression through this positive feedback loop [175;176].

Tertiary lymphoid structures develop in a broad variety of human chronic inflammatory disorders, including autoimmune diseases (rheumatoid arthritis, multiple sclerosis…), infectious disease, cancer and COPD [151;153]. The cellular and molecular mechanisms behind the formation of lymphoid follicles

remain to be fully elucidated, but it is clear that many features are shared with the formation of peripheral lymphoid organs. Instead of the specific inducer cells, B and T cells and DCs could express $LT\alpha_1\beta_2$ and provide the initial trigger [176-178]. An organizer cell type of mesenchymal origin that promotes the development of lymphoid follicles has not yet been identified, but stromal cells are detected in lymphoid follicles of influenza-infected mice [179]. Increased expression of homeostatic chemokines, such as CXCL12, CXCL13 and CCL19, is found in tissues harboring lymphoid follicles ranging from patients with Sjörgen's syndrome to the CS-induced murine model of COPD [180-183]. Overexpression of LT-α, CXCL13 or CCL21 is sufficient to induce the development of ectopic lymphoid tissue, while CXCL12 and CCL19 promote leukocyte accumulation [184-186].

In COPD, the underlying mechanisms of lymphoid follicle formation remain to be elucidated. It has been hypothesized that lymphoid follicles are formed by the recruitment of CXCR3-expressing T and B lymphocytes, which increase with disease severity. The CXCR3 ligands CXCL9 and CXCL10 are expressed by cells in and around lymphoid follicles [187]. In our CS-induced model of airway disease, we have proposed the following mechanism of lymphoid neogenesis [180;181] **(Figure 9)**:

a) **Initiation** of inflammation
   - CS induces pulmonary inflammation with lymphocytes that express $LT\alpha_1\beta_2$
   - $LT\beta R$ signaling on nearby stromal cells induces the expression of lymphoid chemokines: CXCL13 will attract B cells via CXCR5 and CCL19 will attract T cells and DCs via CCR7
   - Positive feedback loop via CXCL13-induced $LT\alpha_1\beta_2$ expression on B cells

b) **Clustering** of lymphocytes and DCs into unorganized lymphoid aggregates at inflamed site

c) **Organization** of lymphoid follicles in the presence of chronic inflammation
   - Separation into T and B cell zones upon persisting expression of CXCL13 and CCL19
   - Differentiation of activated endothelial cells into HEVs, allowing additional supply of naïve lymphocytes
   - Differentiation of stromal cells into FDCs in the B cell core which function as a scaffold for B cell organization and germinal center formation

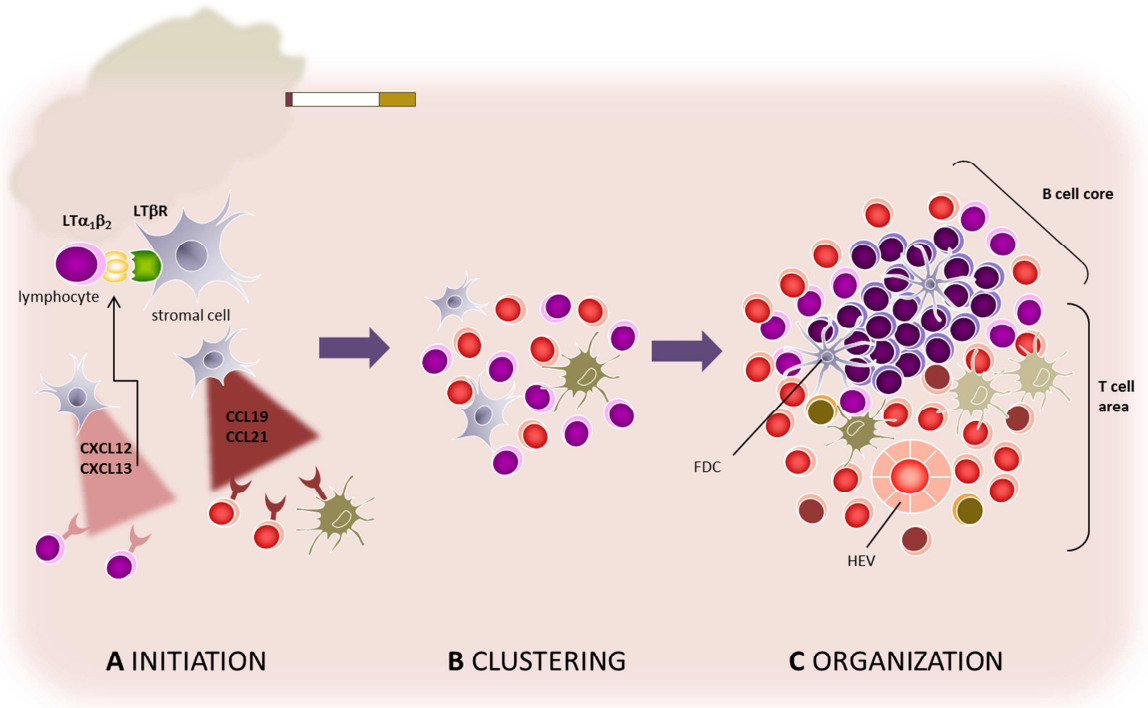


Figure 9. Proposed mechanism of lymphoid neogenesis in COPD. A) Cigarette smoke (CS) induces LTβR signaling which in turn stimulates the production of chemokines CXCL12, CXCL13, CCL19 en CCL21. **B)** Clustering of lymphocytes and DCs into aggregates. **C)** Organization into organized lymphoid follicles with the presence of follicular dendritic cells (FDCs) and high endothelial venules (HEVs).

Our research group has already studied the involvement of CCR7 and LT-α in CS-induced inflammation and lymphoid follicle formation. CCR7 signalling was not required for the development of lymphoid follicles. CCR7 deficiency modulated the accumulation of innate inflammatory cells in lungs and airways; in contrast, the influx of airway derived DCs and T cells into the lymph nodes was CCR7-dependent [181]. The development of CS-induced lymphoid follicles is not impaired by LT-α deficiency, but the production of secretory IgA is attenuated in LT-α deficient mice, suggesting a possible role of LT-α in the functionality of CS-induced follicles. Moreover, CXCL13 expression is present in lymphoid follicles and the induction of CXCL13 upon CS exposure is dependent on intact lymphotoxin signaling, pointing towards a role of CXCL13 in the organization of CS-induced lymphoid neogenesis [180].

Indeed, another approach to target lymphoid follicles is to neutralize CXCL13, blocking the recruitment of B lymphocytes. This has been tested in experimental models of several chronic inflammatory and autoimmune diseases. Genetic deficiency of CXCL13 in an experimental mouse model of multiple sclerosis resulted in a mild, self-limited form of disease with an attenuation of white matter inflammation and gliosis, which may be partly due to poor lymphocyte priming [188]. In a model of type 1 diabetes using non-obese diabetic (NOD) mice, neutralization of CXCL13 unraveled B lymphocyte organization in islet lymphoid follicles, without impairing their functionality or having an impact on disease progression [189].

Deficiency in CXCR5, the receptor for CXCL13, leads to a complete lack of gastric lymphoid follicles upon *Helicobacter pylori* infection and an overall reduction of chronic gastric inflammation [190]. The same CXCR5 knockout mice were protected from antigen-induced arthritis and also demonstrated impaired development and organization of tertiary lymphoid tissue [191]. Also in collagen-induced arthritis, mice treated prophylactically with a neutralizing antibody to CXCL13 developed less severe arthritis in combination with reduced follicular response [192]. In the therapeutic setting, these mice were still protected against disease, although formation of germinal centers was not altered [193].

The effect of CXCL13 neutralization on the development of pulmonary lymphoid follicles was also studied in the context of influenza infection and asthma. CXCL13 is not necessary for the formation or function of lymphoid follicles upon influenza infection. The only apparent defect is a loosely organized B cell zone that lacks FDCs [179]. In the murine model of OVA-induced allergic asthma, CXCL13 neutralization abrogated the allergic reaction as measured by IgE production and pulmonary inflammation [194]. Clearly, these preclinical studies suggest that interfering with the CXCL13/CXCR5 axis might provide a therapeutic target in several diseases.

[CHAPTER 3: TGF-β SUPERFAMILY IN COPD]

Based on 'TGF-β superfamily in obstructive lung diseases: more suspects than TGF-β alone' Verhamme FM, Bracke KR, Joos GF, Brusselle GG. American Journal of Respiratory Cell and Molecular Biology. 2014 November 14. Epub ahead of print [195].

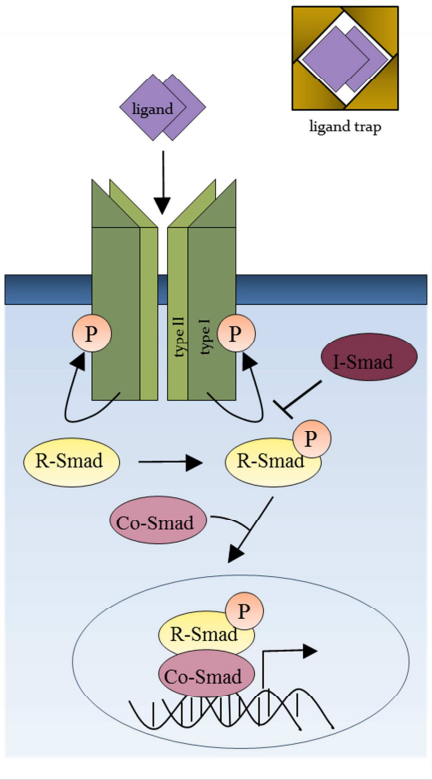
3.1. The TGF-β superfamily

The transforming growth factor (TGF)-β pathway is one of the most evolutionarily conserved signal transduction pathways within the animal kingdom [196]. There are more than 30 TGF-β superfamily ligands in mammals, including the TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs).

All ligands of the TGF-β superfamily are synthesized as prepropeptide precursors and then processed and secreted as homodimers or heterodimers. The mature ligands are dominated by the cystine-knot architecture that results from 6-9 conserved cysteine residues forming intra- and intermolecular disulfide bonds. Next to structural features, this protein family also shares a similar signal transduction cascade. For signalling, each ligand requires a set of type I and type II serin/threonine kinase receptors. In humans, 5 type II and 7 type I receptors have been identified. The activated receptor complex propagates the signal into the canonical pathway through phosphorylation of the receptor-regulated Smads (R-Smads). TGF-βs and activins signal through Smad2/3, whereas Smad1/5/8 act downstream of BMP and GDF signalling. Subsequently, the R-Smads form complexes with Co-Smad4 and translocate to the nucleus to regulate gene expression (**Figure 10**). Other pathways can also be activated by the TGF-β family, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), and are referred to as 'non-canonical' signalling [197-200].

Proteins of the TGF-β superfamily are pleiotropic mediators, playing key roles in diverse developmental and physiological pathways, owing to ubiquitous expression of their signalling components. It is not surprising that disruptions in this tightly regulated pathway are associated with a wide range of human diseases, including respiratory disorders [201-203]. Most research has focussed on the TGF-β1 ligand, showing its involvement in chronic sinus disease, pulmonary fibrosis, asthma and COPD [204-206].

In this thesis, I will start with a short overview on what is currently known on TGF-β1 and then focus on other members of the TGF-β superfamily, more specifically activin-A and BMP-6.



	TGF-β	activin	BMP	GDF
Ligands	TGF-β1 TGF-β2 TGF-β3	Activin-A Activin-B Activin-AB	BMP-1 BMP-2/4 BMP-3 BMP-5/6/7/8 BMP-9/10 BMP-15	GDF-1/3 GDF-5/6/7 GDF-8/11 GDF-9/15
Ligand traps	Follistatin and related proteins Hyaluronan Decorin Latency associated peptide α2-macroglobulin	Follistatin and related proteins Inhibin	Dan Twisted gastrulation Chordin/Noggin Others (follistatin and related proteins, …)	Dan Twisted gastrulation Chordin/Noggin Others (follistatin and related proteins, …)
Type II receptors	TGFBR2	ActR2A ActR2B	ActR2 ActR2B BMPR2	TGFBR2 ActR2A ActR2B BMPR2
Type I receptors	ALK1 ALK2 ALK5	ALK1 ALK2 ALK4 ALK7	ALK 1 ALK2 ALK3 ALK6	ALK3 ALK4 ALK5 ALK6 ALK7
R-Smad	Smad2/3	Smad2/3	Smad1/5/8	Smad1/5/8 Smad2/3
Co-Smad	Smad4	Smad4	Smad4	Smad4
I-Smad	Smad7	Smad7	Smad6 Smad7	Smad6 Smad7

Figure 10. Signalling components of the TGF-β superfamily. The canonical signalling pathway through the SMADs is shown for the transforming growth factor-βs (TGF-βs), activins, bone morphogenetic proteins (BMPs) and growth differentation factors (GDFs). ALK (activin receptor-like kinase), R-Smad (receptor-regulated Smad), I-Smad (inhibitory Smad).

Figure reprinted with permission of Verhamme et al. Am J Respir Cell Mol Biol 2014 November 14 (Epub ahead of print) [195].

3.2. TGF-β1

The TGF-β subfamily has 3 different isoforms, TGF-β1, TGF-β2 and TGF-β3, encoded by different genes. They signal through a similar cascade, exhibit overlapping properties, but can have distinct functions as demonstrated by different phenotypes of the respective knock-out mice [207-209]. Each isoform is synthesized as an inactive precursor, targeted to the extracellular matrix (ECM), in complex with the latency associated peptide (LAP) and latent TGF-β binding protein (LTBP). Activation of TGF-β involves release of the complex from the ECM, proteolysis and release from LAP, by which TGF-β is now able to interact with its receptors [210].

TGF-β1 is the most studied and most prevalent isoform with a widespread expression and broad functionality. As TGF-β1 deficient mice display excessive inflammatory lesions in multiple organs and die prematurely, it became clear that TGF-β1 is essential for **immune regulation** [207]. TGF-β1 serves as a regulatory cytokine by inducing tolerance as well as containing and resolving inflammation. It does so by controlling the chemotaxis, activation, proliferation and survival of a broad range of inflammatory cells, including mast cells, DCs, macrophages and lymphocytes [211]. Most importantly, TGF-β1 is essential for the development and maintenance of FoxP3 expressing $CD4^+CD25^+$ Treg cells [212]. In the presence of IL-6, TGF-β1 is also able to induce the differentiation of pathogenic Th17 cells [213]. Moreover, TGF-β1 is a potent chemokine, which allows the rapid accumulation of macrophages, neutrophils, eosinophils and other cells at the site of inflammation [211]. In summary, TGF-β1 has a pivotal role in immune regulation, imbalance between the pro- and anti-inflammatory action is associated with immune-mediated pathologies such as autoimmune diseases, inflammatory diseases and cancer.

Furthermore, TGF-β1 is involved in the **repair** process of damaged tissue after inflammatory reactions, dysregulation of this process leads to tissue fibrosis and impaired organ functionality. Overexpression of TGF-β1 results in tissue fibrosis [214]. TGF-β1 has multiple roles in this process, it can induce proliferation and differentiation of fibroblasts into myofibroblasts, is a potent stimulator of ECM production, such as fibronectin and collagen and can decrease the production of ECM-degrading proteases. TGF-β1 target genes include connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), collagen Ia2 and plasminogen activator inhibitor (PAI)-2 [214-216]. Another important action of TGF-β1 involves epithelial-to-mesenchymal transition (EMT), a process in which epithelial cells differentiate into fibroblasts and myofibroblasts. EMT has been proposed to contribute to the remodelling process in asthma and COPD [216].

Keeping these data in mind, TGF-β1 has emerged as an interesting regulator in the pathogenesis of COPD. First of all, several independent genetic studies identified single-nucleotide polymorphisms (SNPs) of TGF-β1 associated with COPD susceptibility or with dyspnea [217-220]. Evidence of altered TGF-β1 signalling in COPD has manifested, but the data are somewhat contradictory. TGF-β1 is present in airway and alveolar epithelial cells, airway and vascular smooth muscle cells and macrophages. mRNA and protein levels of TGF-β1 are significantly higher in the bronchiolar and alveolar epithelium of patients with COPD compared to subjects without COPD. Moreover, a significant correlation was found between TGF-β1 levels and smoking history and lung function [48;221]. In contrast, when a subdivision of COPD severity was made based on the GOLD criteria, immunohistochemical staining demonstrated reduced TGF-β1 protein levels in epithelial and stromal cells and type 2 pneumocytes in COPD GOLD stage II compared to controls [222]. The release of TGF-β1 and TIMP-1 by alveolar macrophages is impaired in COPD, suggestive for lower anti-inflammatory and anti-proteolytic activity in patients with COPD [223].

Secondly, exposure to CS, the most important risk factor for COPD, also affects TGF-β1 signalling. Rat tracheal explants that were exposed to CS *in vitro* exhibited elevated TGF-β1 secretion [224]. This has also been confirmed *in vivo*, in mice exposed to CS in acute and subacute exposure models [225-227]. After long term exposure of 6 months CS, TGF-β1 levels declined both in the small airways as in the lung parenchyma [226;228].

Persistent repair due to increased TGF-β1 signalling may contribute to airway remodelling and fibrosis. Correspondingly, the enhanced number of TGF-β1 expressing epithelial and submucosal cells of patients with chronic bronchitis correlated with basement membrane thickness and fibroblast number [229]. Adding to this, CSE exposure of differentiated bronchial epithelial cells induced EMT by activation of ROS and through the release and autocrine action of TGF-β1 [230]. On the other hand, inadequate repair with decreased TGF-β1 signalling may predispose to the development of emphysema. Loss of integrin-mediated TGF-β1 activation causes age-dependent emphysema through increased macrophage expression of MMP-12 [231]. Moreover, Smad3 deficiency in mice resulted in progressive age-related airspace enlargement, associated with a high expression of MMP-9 and MMP-12 [232]. Exposure of these Smad3 null mice to cigarette smoke aggravated air space enlargement and alveolar cell apoptosis [233]. Thus, TGF-β1 is an important regulator of inflammation and remodelling and acts in a spatiotemporal manner in COPD.

3.3. Activin-A

Activins are dimeric proteins consisting of 2 β subunits, βA and βB, that form the hetero- or homodimers, activin-A (βA:βA), activin-B (βB:βB), or activin-AB (βA:βB). Two other subunits (βC and βE) have also been identified [234]. Activin-A is originally isolated from ovarian fluid as a stimulator of follicle-stimulating hormone, but recently, activin-A has emerged as a multi-faceted cytokine.

3.3.1. Activin-A in inflammation and disease

Activin-A can display pro- and anti-inflammatory properties depending on the cellular and temporal context [235-237]. Activin-A inhibited the processing of IL-1β precursor into the mature cytokine in monocytic cell lines [238]. Activin-A also stimulated expression of inducible nitric oxide synthase (iNOS) and production of NO in unactivated macrophages, but this effect was reversed in LPS-activated macrophages [239]. Furthermore, activin-A is able to skew the macrophage polarization towards an M1 phenotype [240]. Similarly, neutrophils are a significant source of mature, stored activin-A and activin-A secretion by neutrophils is stimulated by the inflammatory cytokine TNF-α [241]. Next to innate immune cells, activin-A also regulates adaptive immune cells. Activin-A acts on resting B cells by increasing cell growth and IgG production and acts indirectly on activated B cells through modulation of other immune cells [242].

On the other hand, activin-A is known to stimulate the development of FoxP3$^+$ regulatory T cells [243]. Although there are many conflicting results, the current hypothesis is that activin-A exerts a pro-inflammatory effect early on in the course of inflammation, but once the inflammatory response has been established, activin-A functions as an anti-inflammatory cytokine. In the light of these observations, it is not surprising that activin-A is implicated in several inflammatory and fibrotic diseases from which we will discuss Crohn Disease and inflammatory arthropathies [234].

In patients with Crohn's Disease or ulcerative colitis, expression of the activin βA subunit was increased and located in the mucosa and sub-mucosa of inflamed tissue, correlating with high expression of IL-1β, whereas no expression could be detected in the normal human digestive tract [244;245]. The activin type I and II receptors were expressed in intestinal tissues of both patients with Crohn Disease and ulcerative colitis, as well as of those from healthy controls [245]. Activin-A enhanced the migration, inhibited the proliferation and induced apoptosis of intestinal epithelial cell lines, and this growth inhibition was largely reversed by follistatin, suggesting a role for activin-A in intestinal epithelial cell function [245;246]. In several murine models of colitis, both local and systemic up-

regulation of activin-A occurred. Importantly, *in vivo* administration of follistatin attenuated inflammation, reduced histologic score and increased survival rate, presumably by promoting the proliferation of epithelial cell proliferation/repair [246;247].

Increased expression of activin-A, both on the RNA and protein level, has been found in synovial fluid from patients with inflammatory arthropathies but not in patients with non-inflammatory osteoarthritis [248-250]. The majority of the activin-A producing cells in synovial tissues are $CD68^+$ macrophage-like cells and fibroblast-like synoviocytes, which also express the activin receptors [251]. Serum levels of activin-A were higher in patients with rheumatoid arthritis, systemic lupus erythrematosus and osteoarthritis compared to controls and correlated positively with disease activity parameters [248]. It is still a question whether activin-A is beneficial, suggested by the fact that IL-6 mediated activities are suppressed by activin-A, or whether activin-A is harmful in the pathogenesis of rheumatoid arthritis, as activin-A promotes cell proliferation of synovial fibroblasts [249;251]. More functional studies in animal models are necessary to establish the pathophysiological role of activin-A in rheumatoid arthritis.

3.3.2. Activin-A signalling in the lung

Activin-A is expressed in alveolar and bronchiolar epithelium, alveolar macrophages and vascular smooth muscle cells [252;253]. The less studied βB, βC and βE subunits have a lower expression, but can be detected in lung tissue [254].

Activins interact with activin type II receptor A and B (ActR2A and ActR2B), which recruits the type I receptor or activin receptor-like kinase (ALK)4, but also ALK7 [255]. The type II receptors are broadly expressed in every structural cell type of the lung [253;254;256]. Lung fibroblasts and bronchial epithelial cells are known to express the type I receptor ALK4 [254].

The activated type I receptor propagates the signal by phosphorylation of R-Smad2 and R-Smad3. Smad2/3 expression has been seen in several cell types in the lung, such as bronchial epithelial cells, fibroblasts, pulmonary artery smooth muscle cells and alveolar type 2 cells; the phosphorylated form of Smad2 is especially detected in bronchial epithelial cells. Also Smad4, a signalling mediator which is shared by different members of the TGF-β superfamily, is abundantly expressed in the lung [222;254;257].

Activin signalling is negatively regulated both extracellularly and intracellularly by diverse mechanisms **(Figure 10)**. Inhibitory Smad6 and 7 function as a negative feedback mechanism in TGF-β signalling [258]. Smad7 has been detected in airway epithelial cells, stromal cells and alveolar type 2

cells [222]. Follistatin is an extracellular glycoprotein that binds and neutralizes activin with high affinity by blocking the receptor binding sites. Follistatin also binds with lower affinity to some other members of the TGF-β superfamily, including GDF-8/9 and BMP-2/5/7/8 [234;258]. Follistatin expression has been found in alveolar macrophages, bronchial and alveolar epithelial cells and vascular smooth muscle cells [253]. Follistatin-related protein (or follistatin-like 3), which has a high degree of structural homology with follistatin, also binds and inhibits activins and BMPs and is expressed in lung tissue [259].

3.3.3. Activin-A in respiratory disorders

Bleomycin treatment is commonly used to study **pulmonary fibrosis** in animal models. Almost 20 years ago, Matsuse and colleagues found enhanced levels of activin-A, particularly in alveolar macrophages and fibroblasts in the fibrotic area upon bleomycin treatment [252;253]. Follistatin was only slightly upregulated, suggesting that the effect of activin-A may dominate the antagonistic effects of follistatin, whereas the levels of ActR2B were not altered [253]. These data were subsequently confirmed in lung samples from patients with pulmonary fibrosis, where activin-A is detected in alveolar macrophages, metaplastic epithelium, hyperplastic smooth muscle cells and in desquamated cells [260].

Functional studies showed that activin-A stimulates the proliferation and differentiation of lung fibroblasts into myofibroblasts **(Figure 11)**[261;262]. Furthermore, activin-A facilitates the capability of lung fibroblasts to contract collagen gels [263]. Follistatin administration to bleomycin-treated rats was effective in reducing infiltration of macrophages and neutrophils and reducing lung permeability at an early phase and resulted in attenuated lung fibrosis at a late phase [253]. Consistent with these findings, deficiency of Smad3, the common mediator of TGF-β/activin-A signalling, attenuated bleomycin-mediated lung fibrosis, as measured by deposition of collagen and fibronectin [264]. Similarly, transient gene transfer of Smad7 partially prevented bleomycin-induced pulmonary fibrosis in mice [265].

A first hint for a possible role of activin-A in **asthma** was offered by the discovery that activin-A was strongly upregulated in a mouse model of ovalbumin (OVA)-induced allergic airway inflammation, together with an increase of ALK4 and Smad mediators [254]. Similarly, other studies in this allergic asthma model have shown elevated levels of activin-A in bronchoalveolar lavage fluid, released from airway epithelial cells or inflammatory cells, such as activated mast cells and macrophages **(Figure 11)** [266-268]. Importantly, follistatin concentrations also increased, but not as dramatically as activin-A, suggesting that there is a relative excess of activin-A [266]. In human disease, activin-A was elevated in the lungs, serum and peripheral blood $CD4^+$ T cells of patients with asthma [261;269]. Furthermore,

patients with asthma exhibited less inhibitory Smad7 immunoreactivity in bronchial epithelial cells than normal subjects. After allergen challenge of asthma patients, P-Smad2, ALK4 and ActR2A positive cells were upregulated, whereas activin-A and follistatin levels showed no change [270]. In contrast, Semitekolou and coworkers demonstrated, despite increased activin-A levels, declined ALK4 and ActR2A positive cells [269]. Overall, these data suggest an activation of the activin-A pathway in asthma.

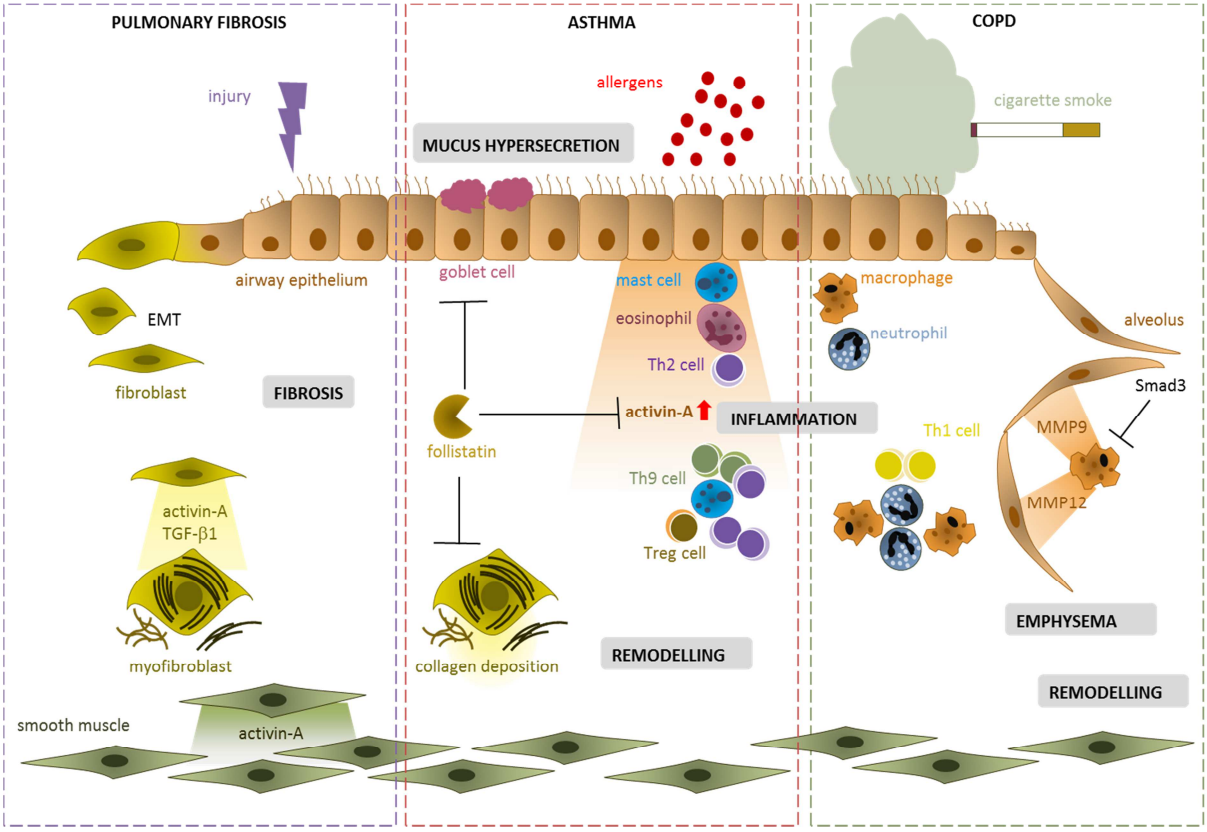


Figure 11. Mechanisms of action of activin-A in lung diseases. Dysregulation of activin-A can have deleterious effects on normal lung homeostasis, leading to pathogenetic mechanisms in chronic lung diseases (pulmonary fibrosis/asthma/COPD), including inflammation, airway remodelling, emphysema and mucus hypersecretion. *Figure adapted with permission of Verhamme et al. Am J Respir Cell Mol Biol 2014 November 14 (Epub ahead of print)* [195].

Mechanistically, activin-A appears to provide a link between early Th2-driven allergic responses and long-term structural changes in the airways that are characteristic for asthma. Intranasal delivery of follistatin in an acute OVA mouse model inhibited the allergen-specific Th2 immune response and mucus production [266]. IL-13, the key cytokine for mucus production, controlled the activin-A secretion by bronchial epithelial cells [271]. Systemic administration of a neutralizing antibody against activin-A exacerbated OVA-induced allergic airway disease, whereas recombinant activin- A reduced the symptoms, through the modulation of antigen-specific regulatory T cells [269]. Remarkably, it thus seems that activin-A may exert distinct functions depending on the route of administration and type

of inhibitor. Finally, a role for activin-A together with TGF-β1 was suggested in Th9-mediated allergic inflammation upon house dust mite instillation [272].

In vitro studies demonstrating that activin-A promotes the proliferation of airway smooth muscle cells and bronchial epithelial cells, pointed towards a possible role of activin-A in the process of airway remodelling [267;270]. Activin-A neutralization by intranasal instillation of follistatin in a murine model of chronic allergic asthma confirmed that activin-A is crucial for mucus hypersecretion, subepithelial collagen deposition and thickening of the subepithelial smooth muscle **(Figure 11)** [273]. Mice deficient in Smad3 have significantly decreased airway remodelling, which is associated with decreased numbers of peribronchial myofibroblasts [268]. Smad2 overexpression in the airway epithelium resulted in enhanced airway hyperreactivity after allergen challenge concomitant with changes in smooth muscle and matrix remodelling. Remodelling was markedly inhibited by injection of an activin-A neutralizing antibody, providing direct evidence for a role of activin-A in remodelling [274].

Several studies reported dysregulated Smad signalling in the lungs of cigarette smoke-exposed mice and of patients with **COPD**. [222;226-228;275]. As mentioned above, Smad3 deficient mice develop progressive age-related airspace enlargement, which is aggravated upon CS exposure [232;233]. These effects are thought to be primarily mediated by TGF-β1, but activin-A can also be involved.

3.4. BMP-6

Originally identified as proteins that stimulate bone morphogenesis, it is now clear that BMPs regulate the development and homeostasis of nearly all organs and tissues [276]. With over 20 mammalian members identified, BMPs constitute the largest subfamily of the TGF-β superfamily **(Figure 10)**. Based on their amino acid sequence similarity, BMPs can be classified into at least 4 subgroups: BMP-2/4, BMP-5/6/7/8, BMP-9/10, BMP-12/13/14 [277]. BMP-6 can signal through three type II receptors (ActR2A, ActR2B and BMPR2) and three type I receptors (ALK2, ALK3 and ALK6) [278].

BMP-6 was discovered to be an essential stimulator of hepcidin expression and is thereby important for **iron homeostasis** [279;280]. The hormone hepcidin was originally identified as an antimicrobial peptide. It functions as the "hypoferremia hormone" by degrading the iron exporter ferroportin and inhibiting the release of iron into the circulation. Through this mechanism, hepcidin negatively impacts bacterial growth by decreasing the iron availability [281]. BMP-6 deficient mice develop iron overload in the liver, pancreas, heart and kidney by reduced hepcidin synthesis. Interestingly, BMP-6 deficient mice have still the ability to induce hepcidin in the presence of inflammation as demonstrated by treatment with LPS [279;280]. In a T cell transfer colitis model, treatment with an anti-BMP-6 antibody inhibited liver hepcidin expression and colon IL-17 expression [282].

BMP-6 can also have a direct impact on cells of the **immune system**. Macrophages express all three known type II BMP receptors and two of the three known type I receptors (ALK2 and ALK3). *In vitro* treatment of the macrophage cell line RAW264.7 with recombinant BMP-6 inhibited cell proliferation and induced expression of inducible nitric oxide synthase (iNOS),TNF-α and IL-1β [283;284]. Similarly, $CD4^+$ T cells express ALK2, ALK3 and BMPR2. When Jurkat TAg cells are incubated with recombinant BMP-6, the BMP-6 signalling pathway was activated and proliferation was reduced. This anti-proliferative effect was thought to be mediated via increased inhibitor of differentiation (Id)1 protein levels [285]. Furthermore, BMP-6 has an inhibitory effect on the proliferation of both naïve and memory B cells and on the differentiation of plasmablasts. In activated memory B cells, BMP-6 induced cell death. BMP-6 was also the most potent BMP protein in the inhibition of Ig production [286;287]. Backwards, BMP-6 itself is regulated by inflammatory signals. In cultures of fibroblast-like synoviocytes, the presence of IL-1β and TNF-α induced BMP-6 expression [288].

Finally, the BMP family is implicated in tissue **remodelling**. The first link was found in a kidney model, where it was discovered that BMP-7, which shares 87 % amino acid identity with BMP-6, reversed TGF-β1 induced EMT *in vitro* and reduced renal injury *in vivo* [289]. Similar findings were reported in

fibrosis models of liver and lungs, although contradictory results were found [290-293]. The therapeutic potential of BMP-7 in pulmonary fibrosis has been confirmed in asbestos- and silica-induced animal models [292;293]. Conversely, Murray and colleagues failed to establish a protective effect of BMP-7 in lung fibrosis either *in vivo* or *in vitro* [291]. *In vitro* studies with BMP-7 demonstrated the pro-fibrotic function of BMP-7 in airway epithelial cells, but in cultured lung fibroblasts BMP-7 opposed TGF-β1 dependent fibrogenic activity [294-296]. In an OVA-induced mouse model of asthma, intranasal treatment with BMP-7 reduced lung inflammation and type 1 collagen deposition [297].

Although initial studies could not demonstrate the same function for BMP-6, recent studies do point towards a role for BMP-6 in fibrosis [289;298-300]. Increased numbers of myofibroblast progenitor cells in patients with type 1 diabetes were found to be negatively correlated with reduced BMP-6 expression [300]. The antifibrotic action of BMP-6 was confirmed in BMP-6 deficient mice which underwent unilateral ureteral obstruction [299]. Correspondingly, BMP-6 deficiency alleviated non-alcoholic fatty liver disease in mice , although in this model, BMP-6 was upregulated [298] **(Figure 12)**.

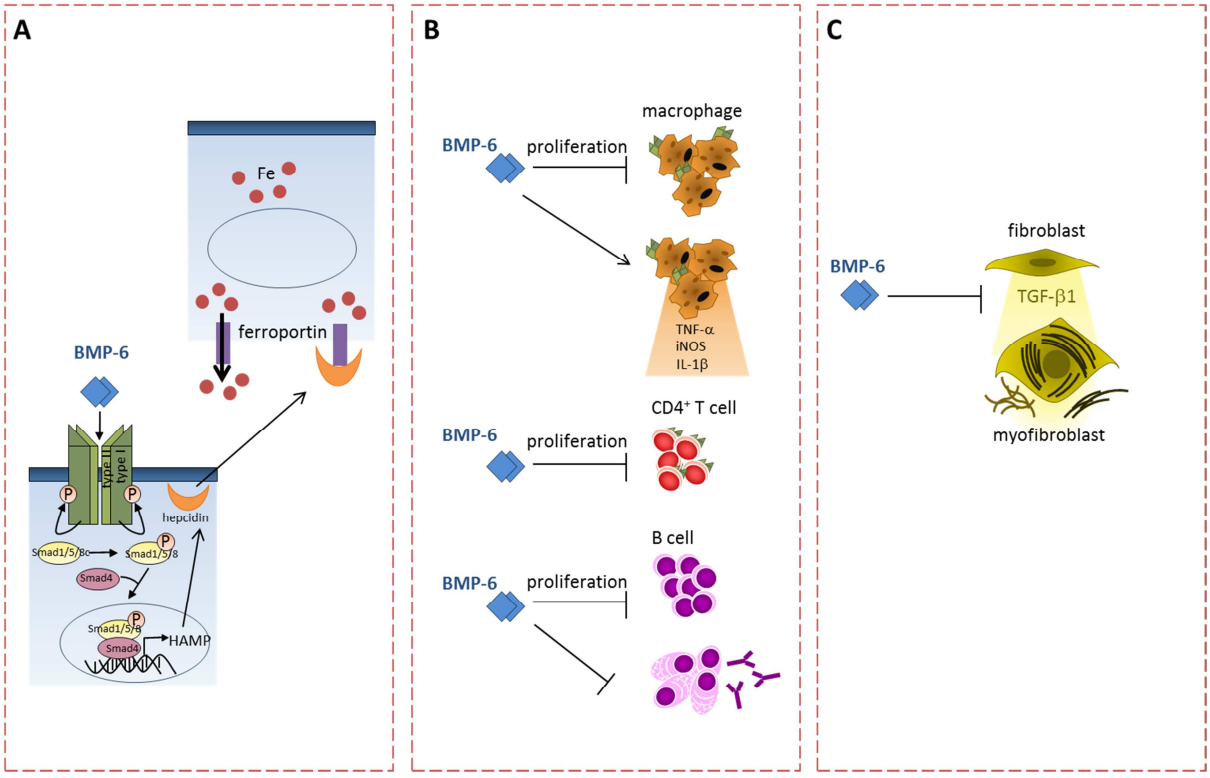


Figure 12. Functions of BMP-6. A) Iron homeostasis: BMP-6 stimulates the expression of hepcidin (HAMP) which degrades the iron exporter ferroportin, and inhibits the release of iron from the cell. **B)** Immune system: BMP-6 inhibits the proliferation of macrophages, B and T cells. Moreover, BMP-6 stimulates the release of TNF-α, iNOS and IL-1β from macrophages and inhibits immunoglobulin production. **C)** BMP-6 inhibits the TGF-β1 induced pro-fibrotic actions.

3.4.1. BMP-6 signalling in the lung

Few studies have addressed the specific localization of BMP-6 in lung tissue. Expression of BMP-6 has been detected in murine lung tissue and more specifically in bronchiolar epithelium and pulmonary artery smooth muscle cells **(Figure 13)** [257;301;302]. Its receptors are abundantly expressed in the pulmonary vasculature, airway epithelium and lung fibroblasts [256;303-305].

BMP-6 passes the signal on through R-Smad1, R-Smad5 and R-Smad8 (canonical pathway). Phosphorylation of these Smad proteins, hence an active BMP signalling, exists in airway epithelial cells, alveolar macrophages and lung fibroblasts, next to the pulmonary endothelium and vascular smooth muscle [304;306]. In addition, non-canonical signalling through p38, ERK1/2 and JNK has been observed in pulmonary artery smooth muscle cells, lung fibroblasts and airway epithelial cells [304;307;308].

One of the most important targets of BMP signalling are the basic helix-loop-helix Id proteins. There are 4 known members in mammals (Id1 through 4), exhibiting similar, but not identical biological functions [309]. Id4 is barely detectable in the lung, but Id1 and Id3 are expressed in pulmonary vacular endothelial cells and alveolar epithelium, while Id2 is detected in bronchial epithelium and smooth muscle cell compartments of the lung [310].

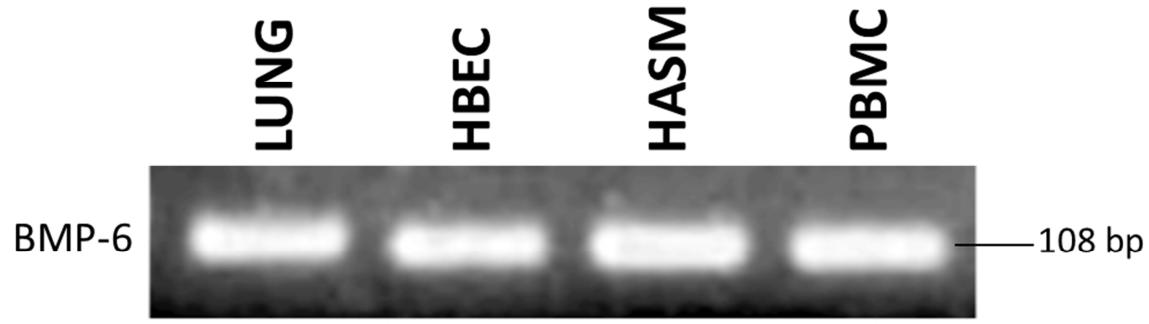


Figure 13. Expression of BMP-6 in human lung tissue and human primary cells. mRNA expression profiling of BMP-6 in human lung tissue, human bronchial epithelial cells (HBECs), human airway smooth muscle cells (HASM) and peripheral blood mononuclear cells (PBMCs).
Figure adapted with permission of Loth et al. Nat Genet 2014;46(7):669-677 [3].

3.4.2. BMP-6 in respiratory disorders

Very little information exists regarding BMP-6 and respiratory disorders. Already more than 10 years ago, an induction of pulmonary BMP-6 was observed upon OVA-induced airway inflammation in mice [301]. In addition, genetic studies revealed a possible contribution of BMP-6. Gene expression analysis of lung tissue from smokers without COPD and smokers with COPD demonstrated reduced BMP-6 expression [311]. Recently, the BMP-6 locus was found to be associated with FVC in a large genome wide association study [3]. Therefore, it is interesting to study the *in vivo* functional role of BMP-6 in COPD.

[**CHAPTER 4:** TRANSLATIONAL RESEARCH IN COPD – MATERIALS AND METHODS]

4.1. Rationale

Translation originates from Latin and means to 'carry across'. Translational research refers to the bench-to-bedside (and vice versa) principle where new knowledge, mechanisms and techniques generated by basic research are used to provide new drugs and treatment options for human disease (**Figure 14**).

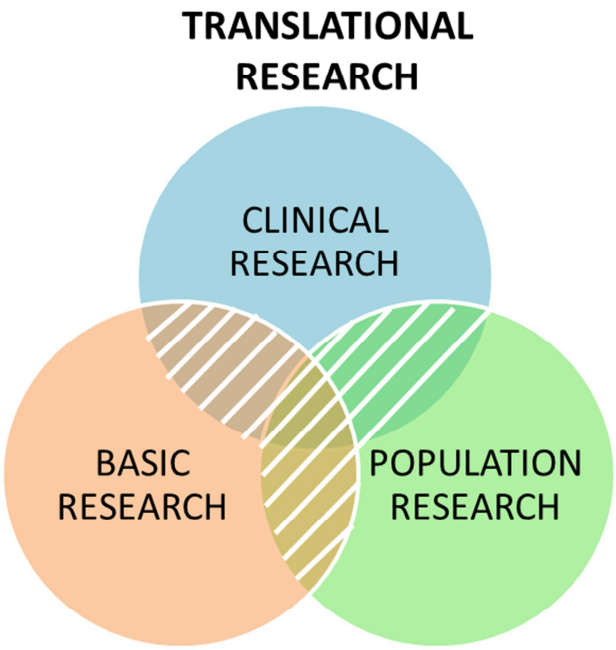


Figure 14. Translational Research (in hatched area). An integrated approach where laboratory discoveries (via *in vitro* cell systems and *in vivo* animal models) are translated in clinical and population based research.

Animal models act as a bridge between *in vitro* studies and studies in humans and enable to answer fundamental research questions. Mice are highly preferred as an animal model, because – besides that they are easy to breed, house and handle – the mouse genome has been entirely sequenced and is highly homologue to the human genome. Another advantage is the opportunity to manipulate gene expression in mice by the generation of gene overexpressing, knockin and knockout mice. Several limitations need to be taken into account, when using murine models of COPD. No model mimics all the hallmarks of COPD. Moreover, there are certain differences in respiratory physiology/anatomy and immune system between mice and human. Our research group developed a murine model, in which CS inhalation – the primary cause of COPD in humans – triggers most pathological manifestations of COPD [312]. For the detailed description, see **Chapter 4.2.**

To overcome the limitations of murine studies, our research group has collected and studied human-derived biological samples (lung tissue and induced sputum) from patients with COPD, smokers without airflow limitation and never smokers. These samples were processed to study gene and protein expression. This approach allows us to confirm the data generated in the mice model. For the detailed description, see **Chapter 4.3.1.**

Airway epithelium is an important regulator of inflammatory processes in the lung through the production of a broad range of inflammatory mediators. In order to study the effect of CS on human epithelium, a third, *in vitro* approach was used with primary human bronchial epithelial cell cultures. Bronchial epithelial cells were grown on air-liquid interface to obtain mucociliary differentiation. Most studies examining airway epithelial cells *in vitro* were performed on either immortalized epithelial cell lines or submerged cultures of primary bronchial epithelial cells. Submerged cultures are however limited in their ability to develop into fully differentiated epithelial layers [313]. Ease of operation, small test sample needed and the fact that specific mechanisms of action can be tested, make this model an ideal system to perform mechanistic studies. However, as with any *in vitro* cell culture model, cells grow in a milieu different from that encountered *in vivo*, which may result in outcomes irrelevant for the *in vivo* situation. For the detailed description, see **Chapter 4.3.3.**

4.2. Murine model of COPD

4.2.1. Protocol

Using a smoking apparatus and a plexiglass smoke chamber (7500 cm^3) (**Figure 15**), groups of 8-10 mice are subjected whole body to mainstream CS of 5 simultaneously lit cigarettes (Kentucky Reference Cigarette 3R4F without filter; University of Kentucky, Lexington, KY, USA). This exposure is repeated 4 times a day, with 30 minutes smoke-free intervals and 5 days a week. Because mice do not tolerate undiluted CS, the smoke chamber has an air supply, establishing an optimal smoke/air ratio of 1/6. The control groups were exposed to room air. Using this protocol, carboxyhemoglobin levels in serum of CS-exposed mice reached 8.35 ± 0.46 % (versus 0.65 ± 0.25 % in air-exposed mice), corresponding to serum levels in human smokers [314].

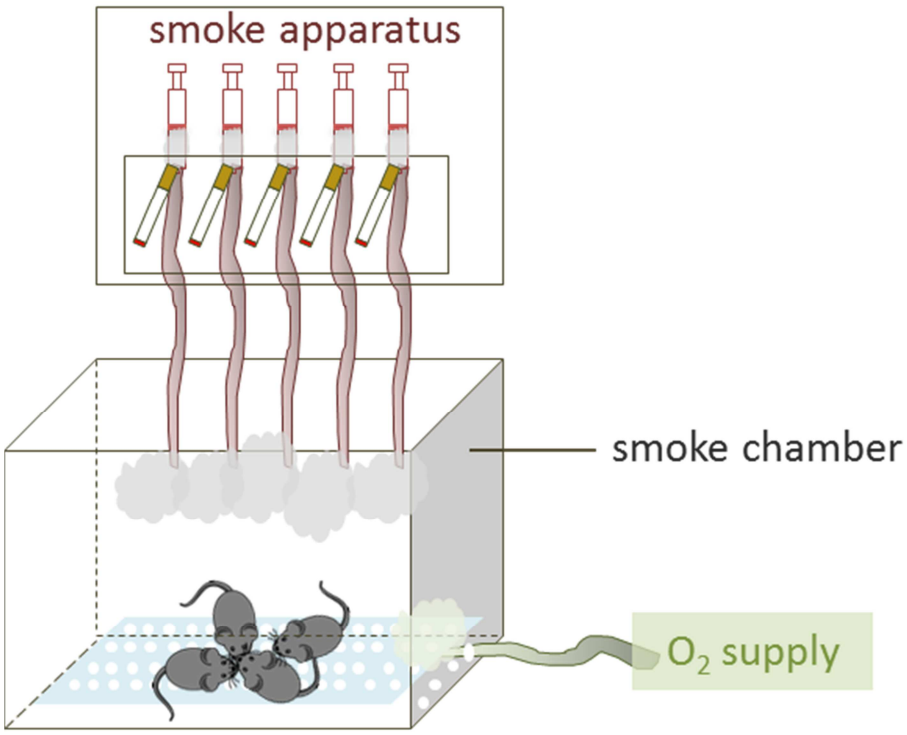


Figure 15. Schematic overview of the cigarette smoke exposure model. Mice receiving whole body exposure of cigarette smoke in a plexiglass chamber connected to the smoke apparatus.

The mice were exposed for 3 days (acute exposure), 4 weeks (subacute exposure) or 24 weeks (chronic exposure). The acute and subacute timepoint are sufficient to mimic the CS-induced pulmonary inflammation, ideally to elucidate key mechanisms of CS-induced inflammation. Chronically-exposed mice exhibit additional hallmarks of COPD, including emphysema, lymphoid

follicles and airway wall remodeling [47;312]. This chronic model is highly relevant because the exposure period corresponds with one quarter of the murine life span, similar to the development of COPD in humans after at least 15 to 20 years of smoking (≈ one quarter of the human life span). This murine model is standardized in the C57BL/6 mouse strain, which is moderately sensitive to CS and is widely used in numerous genetically modified mice [315].

24 hours after the last exposure, mice are sacrificed by an overdose pentobarbital by intraperitoneal injection and blood, tissue (lung tissue and lung draining mediastinal lymph nodes) and bronchoalveolar lavage (BAL) samples are collected to study the CS-induced effects. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences, Ghent University.

4.2.2. Inflammation in BAL, lung tissue and mediastinal lymph nodes

BAL is performed to harvest and analyze the cell population within the alveolar spaces and airway lumina. Processing of the BAL cells into cytospins allows discrimination between morphologically different cell types such as monocytes/macrophages, neutrophils and lymphocytes, using a May-Grünwald-Giemsa staining (**Figure 16**). For more detailed information of cell type markers, flow cytometry is used (see below).

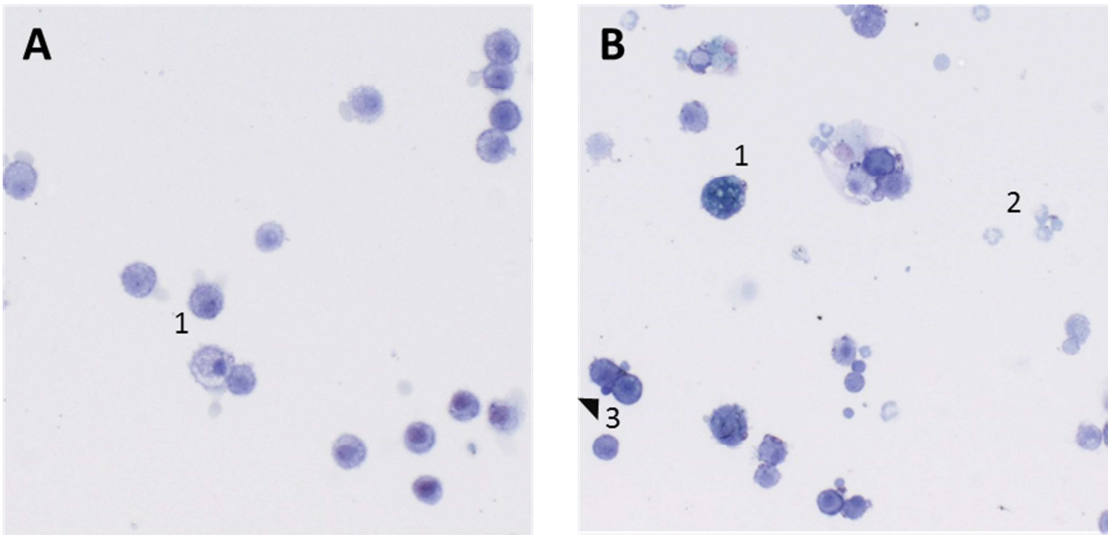


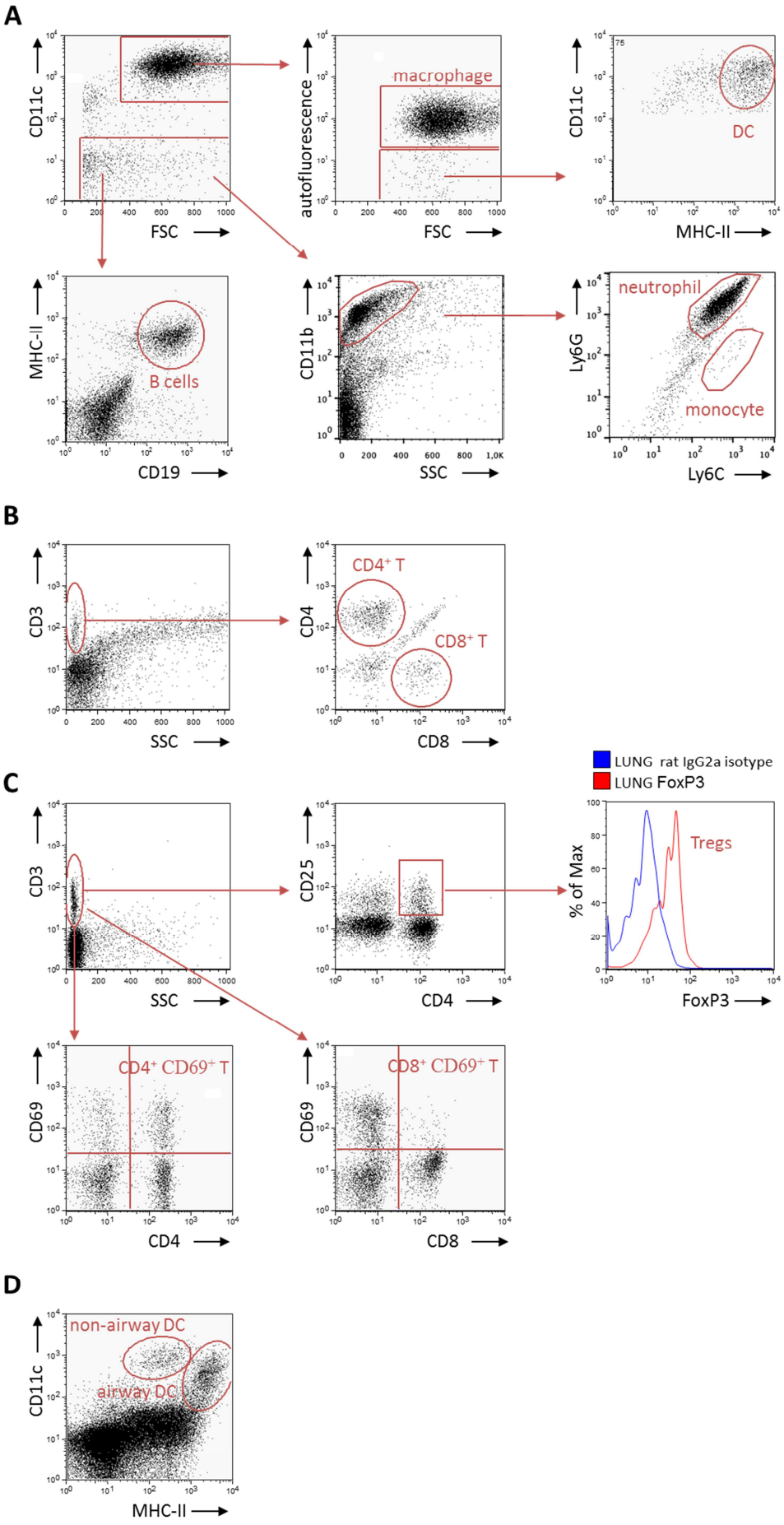
Figure 16. Cytospins of bronchoalveolar lavage cells stained with May-Grünwald-Giemsa. Photomicrographs of BAL cells of C57BL/6 mice exposed to air **A)** or cigarette smoke for 24 weeks **B)**. **A)** In air-exposed mice, resident alveolar macrophages (1) are the main cell type in the airway lumen. **B)** Upon CS exposure, different inflammatory cells populate the airway lumen, including activated macrophages (1), neutrophils (2) with segmented nuclei and small lymphocytes (3).

To obtain insights into the inflammatory cell population in the lungs and lymph nodes (LN), the lung (major lobe of the right lung) and lung draining lymph nodes are harvested for the preparation of single cell suspensions.

Flow cytometric analysis is performed on BAL cells and cell digests of LN and lung. Flow cytometry is a technique for the characterization of different cell populations based on cell-specific markers, by using a combination of antibodies conjugated to fluorescent dyes that bind these markers (surface or intracellular). The number and characterization of dendritic cells (DCs), macrophages, inflammatory monocytes and neutrophils, T lymphocytes ($CD4^+$, $CD8^+$ and Tregs) and B lymphocytes are defined (**Figure 17**).

Inflammatory mediators are measured on the RNA level by qRT-PCR using RNA extracted from total lung tissue (small lobe of the right lung). Analysis of protein levels is performed on BAL supernatant using ELISA or the multiplex Cytometric Bead Array (CBA). For the detailed description, see **Chapter 4.4.**

Figure 17. Gating strategy for the characterization of inflammatory cells in bronchoalveolar lavage, lung and lymph node. A) Macrophages are identified as $CD11c^+$ and high-autofluorescent cells, whereas the $CD11c^+$, low-autofluorescent, MHC-II^+ cell population is identified as DCs. B lymphocytes are identified as $CD11c^{low}$ and $CD19^{bright}$ population. Inflammatory monocytes are defined as $CD11c^-CD11b^+Ly6G^-Ly6C^+$ and inflammatory neutrophils as $CD11c^-CD11b^+Ly6G^+Ly6C^+$. **B)** T lymphocytes are characterized as $CD3^+$ producing low side scatter. We identify $CD4^+$ and $CD8^+$ T lymphocytes **B)** and use CD69 as activation marker in lung tissue **C)**. **C)** Regulatory T cells (Tregs) are $CD25^+$ and $FoxP3^+$ (intracellular staining). **D)** In the LN, we distinguished the $CD11c^{int/high}$MHC-II^{high} airway derived DCs from the $CD11c^{high}$MHC-II^{int} non-airway derived DCs. →


A
CD11c
FSC
autofluorescence
macrophage
FSC
CD11c
DC
MHC-II
MHC-II
B cells
CD19
CD11b
SSC
neutrophil
Ly6G
monocyte
Ly6C
B
CD3
SSC
CD4+ T
CD8+ T
CD4
CD8
C
LUNG rat IgG2a isotype
LUNG FoxP3
CD3
SSC
CD25
CD4
% of Max
Tregs
FoxP3
CD69
CD4+ CD69+ T
CD4
CD69
CD8+ CD69+ T
CD8
D
CD11c
non-airway DC
airway DC
MHC-II

4.2.3. Lymphoid neogenesis

Mice develop lymphoid tissue in the lungs, upon 24 weeks of CS exposure (chronic exposure) [159;180;316]. To localize and quantify the number of lymphoid follicles in lung tissue, lymphocytes are visualized on paraffin sections obtained from the left lung with an immunohistochemical CD3/B220 double-staining (**Figure 18**) [159]. All lymphoid infiltrates in the proximity of airways and in the lung parenchyma are counted. Lymphoid follicles are defined as dense organized accumulations of at least 50 cells. We also quantified the lymphoid aggregates which are loose not-organized accumulations of less than 50 cells. The number of lymphoid structures was normalized for the number of airways (for structures around the airways) or for the area of lung parenchyma (for structures in the parenchyma). All measurements are performed in a blinded fashion.

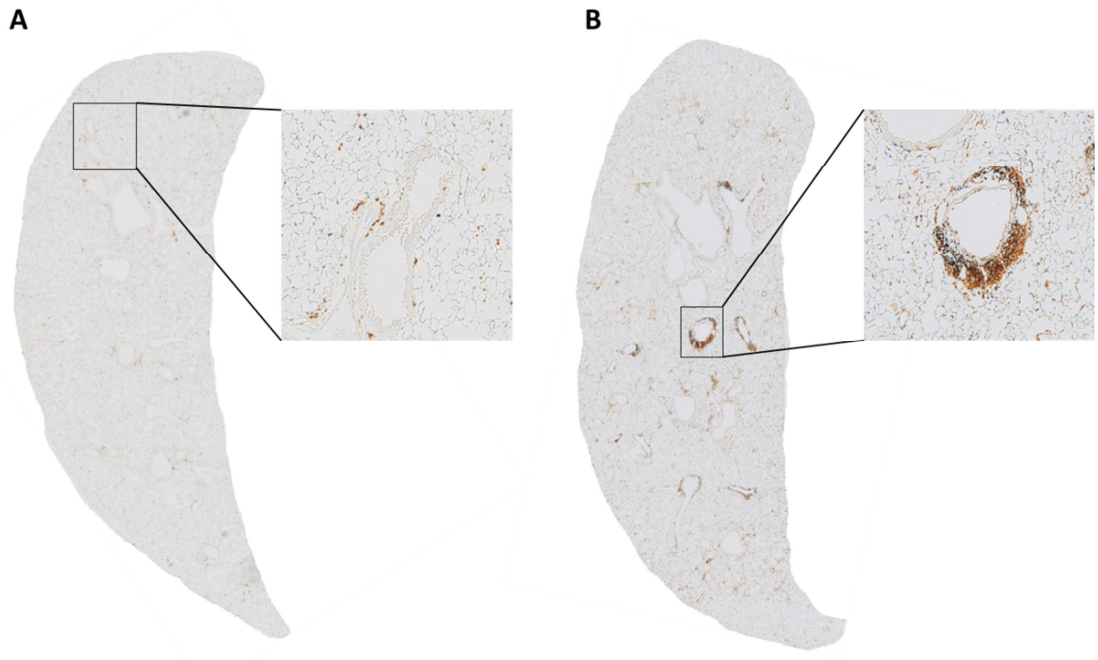


Figure 18. Lymphoid follicles in cigarette smoke-exposed mice. Photomicrographs of murine lung sections stained for CD3 (T lymphocytes shown in brown) and B220 (B lymphocytes shown in blue). **A)** In air-exposed mice, sparse lymphocytes are spread throughout the lung. **B)** In CS-exposed mice (chronic exposure) lymphocytes organize into lymphoid structures both around the airways and in the lung parenchyma.

4.2.4. Emphysema

Emphysema is characterized by destruction of lung parenchyma and permanent enlargement of the alveolar spaces. These emphysematous lesions, interspersed by apparently normal parenchyma, are mimicked in C57BL/6 mice. The morphometric assessment of emphysema is based on the measurement of airspace enlargement (**mean linear intercept** or **L_m**) and destruction of alveolar walls (**destructive index** or **DI**) [317;318]. All measurements are performed in a blinded fashion.

Lung sections obtained from the left lung are stained with haematoxylin and eosin. Photomicrographs of the lung parenchyma are captured using a Zeiss AxioVision image analysis system. Only sections without cutting artefacts, compression or hilar structures are used in this analysis. For quantifications, a grid (100 x 100 µm) is placed over photomicrographs using image analysis software (Image J 1.47v). Each intercept of the horizontal and vertical grid lines with the alveolar walls is marked and counted. The total length of each line is divided by the number of intercepts and then averaged to calculate the L_m (**Figure 19**).

DI is measured by superimposing a grid with 42 points on the photomicrographs using image analysis software (Image J 1.47v). Structures lying under these points are classified as normal (N) or destroyed (D) alveolar and/or duct spaces. Points falling over other structures (duct walls, alveolar wals, etc.) are not included. The DI is calculated with the formula: DI = D / (D + N) x 100.

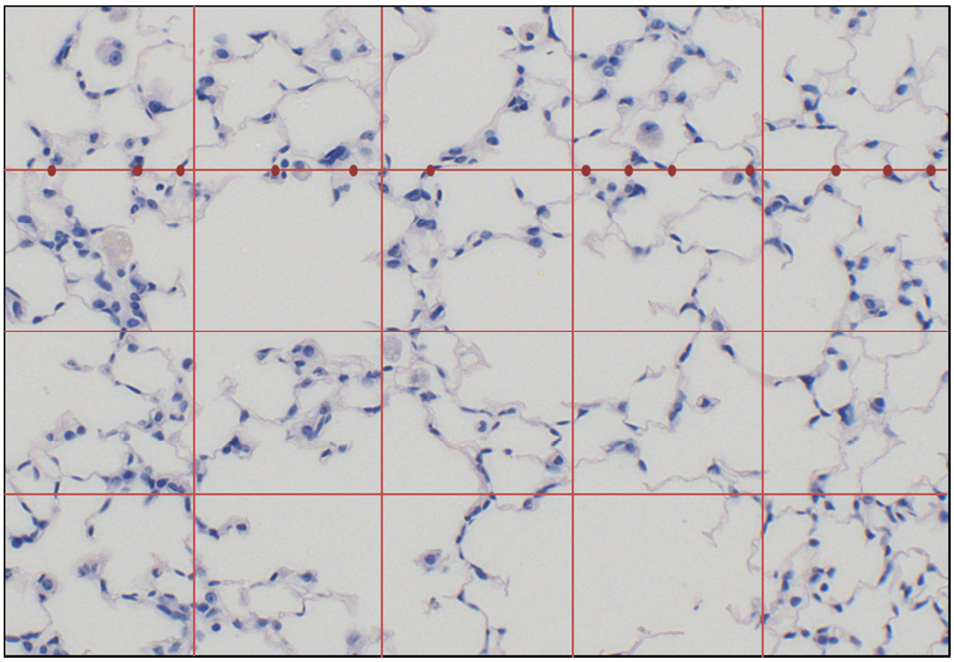


Figure 19. Quantification of pulmonary emphysema in mice. The mean linear intercept (L_m) is measured by placing a 100 x 100 µm grid over each field (red lines). The total length of each line of the grid divided by the number of alveolar intercepts (red dots) per line gives the average distance between alveolated surfaces or the L_m.

4.2.5. Airway wall remodelling

To determine the degree of airway remodelling, the deposition of collagen and fibronectin in the airway wall is measured on lung sections (left lung) using staining with Sirius Red and anti-fibronectin antibody [319]. The following morphometrical parameters are marked manually on the digital representation of the airway (**Figure 20**): the length of the basement membrane (Pbm), the area defined by the basement membrane (Abm) and the area defined by the total adventitial perimeter (Ao). The total bronchial wall area (WAt) is calculated as WAt = Ao – Abm and normalized to the squared Pbm. The area in the total airway wall covered by the stain is determined by the software (Axiovision, Zeiss). The area of collagen or fibronectin is normalized to Pbm. All airways with a Pbm smaller than 2000 µm and cut in reasonable cross sections are included. Quantitative measurements are performed in a blinded fashion.

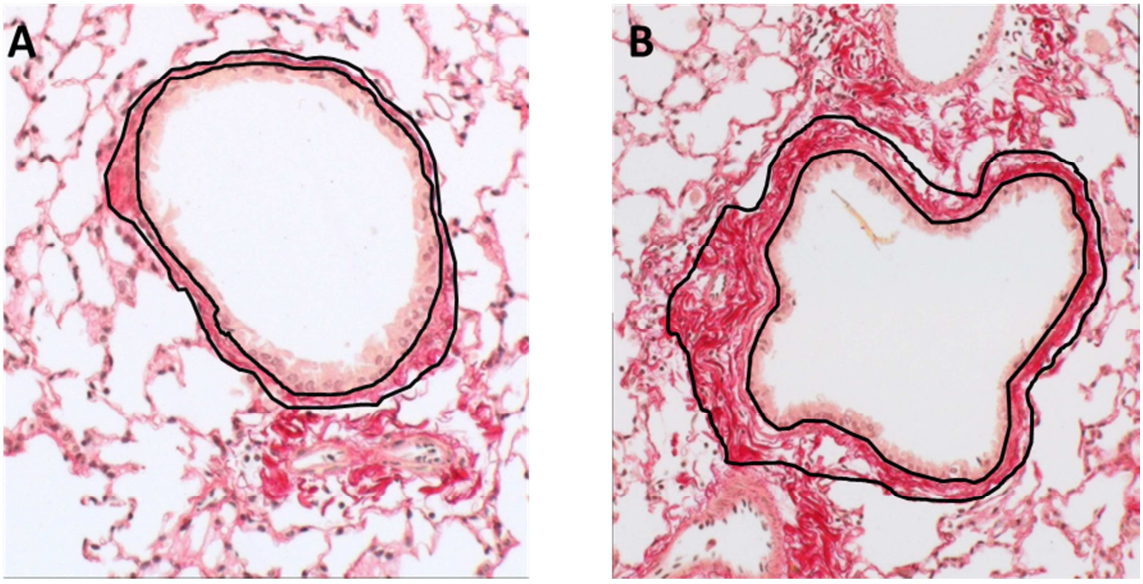


Figure 20. Quantification of airway wall remodelling in mice. Assessment of collagen deposition in the airway wall on Sirius Red stained lung sections. The area between the 2 black lines that stains positively for collagen is quantified by the Axiovision software (Zeiss) using an image analysis system. **A)** Air-exposed mice, **B)** CS-exposed mice.

4.3. Human studies

4.3.1. Lung tissue

Lung tissue is obtained from patients who undergo lobectomy or pneumectomy for various reasons (mostly solitary pulmonary tumours) at the Ghent University Hospital. None of the patients were treated with neo-adjuvant chemotherapy or had recently encountered a COPD exacerbation. COPD diagnosis and severity is defined using pre-operative spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification [1]. Written informed consent is obtained from all subjects prior to surgery according to protocols approved by the medical ethical committee of the Ghent University Hospital. All patients are interviewed about their smoking habits and medication use and are carefully characterized. Patients are classified as ex-smokers when they have quit smoking at least 1 year prior to surgery.

Lung tissue at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumour invasion is collected by a pathologist. Part of the resection specimens is sampled for immunohistochemical analysis from the peripheral lung, ensuring the sampling of small airways. These samples are fixed in paraformaldehyde and embedded in paraffin (More details on immunohistochemistry, see **Chapter 4.4.2.**). Another part of the lung tissue is stored in RNA stabilizing agent (RNAlater, Qiagen, Hilden, Germany) at minus 80 °C for later RNA extraction and qRT-PCR (More details on qRT-PCR , see **Chapter 4.4.1.**). This procedure is schematically presented in **Figure 21**.

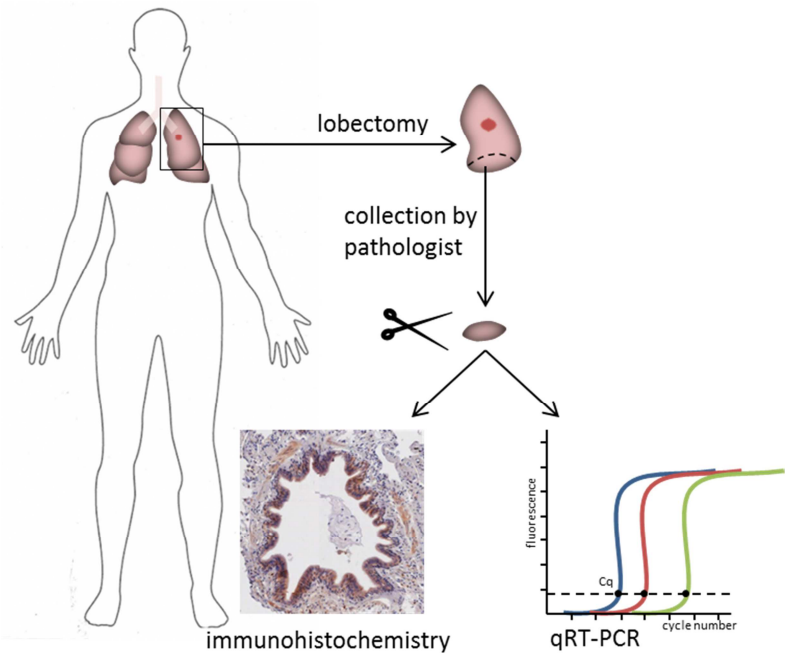


Figure 21. Protocol for the collection of human lung tissue. Lung tissue is collected by a pathologist from lung lobes of patients who underwent lobectomy. In the lab, we sample a part of the resection specimens for immunohistochemical analysis and a part for RNA extraction and qRT-PCR analysis.

4.3.2. Induced sputum

Patients are recruited from the outpatient pulmonary clinic of the Ghent University Hospital, while controls are recruited by advertising as well as from the outpatient clinic. Patients with a recent exacerbation (preceding 2 months), with (very) severe COPD, asthma, bronchiectasis or a respiratory infection (preceding 4 weeks) are excluded. Written informed consent is obtained from all subjects and the patients are interviewed as described above. This study is approved by the Medical Ethical Committee of the Ghent University Hospital.

Sputum induction and processing are performed as previously described [61]. Sterile, pyrogen-free, hypertonic saline at increasing concentrations of NaCl (3 %, 4 % and 5 %) is inhaled during a 5 minute period after premedication with salbutamol (2 x 200 μg). The saline is nebulized via an ultrasonic nebulizer (Ultra-Neb, Devilbiss Health Care Inc, Somerset, PA, USA). Each time after the inhalation, subjects are encouraged to cough and expectorate an adequate sample. Monitoring of pulmonary function has been performed before and after induction. Sputum plugs are selected, transferred in a polystyrene tube and mixed with dithiotreitol (DTT; 10 % Sputalysin, Boehringer-Calbiochem Corp, San Diego, CA, USA) for 30 seconds by vortex and for 15 minutes on a tube rocker. After the addition of PBS, samples are filtered through a 70 μm cell strainer and centrifuged for separation between the cell fraction and cell-free supernatant fraction. The supernatant is stored at minus 80 °C until further analysis.

The cell fraction is further processed for counting in a haemocytometer and the cell concentration is adjusted to 0.7 x 10^6 cells/ml. Cytospins are prepared on Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA, USA) and spun for 5 minutes at 300 rpm. The cytospins are stained with May-Grünwald-Giemsa for differential cell counts by 2 blinded investigators. 400 nucleated cells per slide are counted and expressed as the percentage of intact round nucleated cells, excluding squamous epithelial cells.

4.3.3. Bronchial epithelial cell cultures

Primary human bronchial epithelial cells (HBECs) are obtained by enzymatic digestion from lung resection specimens obtained from anonymous donors during surgery for lung cancer [320]. Bronchial rings are incubated overnight with proteinase type XIV in HBSS. Afterwards, epithelial cells are gently scraped off the luminal surface and cultured in supplemented keratinocyte serum-free medium (KSFM) on pre-coated 6-well plates until 80-90 % confluency (1-2 weeks). After near confluency is reached, the cells are trypsinized. The cultures are stored in liquid nitrogen until further culture.

Cells (passage 2) are transferred to pre-coated 9 cm petri dishes and cultured for 4-6 days in supplemented KSFM medium until near confluency. After trypsinization, cells are seeded on pre-coated semi-permeable Transwell membranes and grown submerged for 4-7 days until they reach confluence in medium containing a high concentration of retinoic acid (15 ng/ml; Lonza, Breda, The Netherlands). Thereafter, cells are cultured at an air-liquid interface for another 2 weeks. The apical side of the epithelial layers is washed with warm PBS three times a week, while the medium is refreshed at the same time. Mucociliary differentiation is usually observed between day 7 and 10 after exposure to air-liquid interface. All cells are cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cell culture protocol is shown in **Figure 22**.

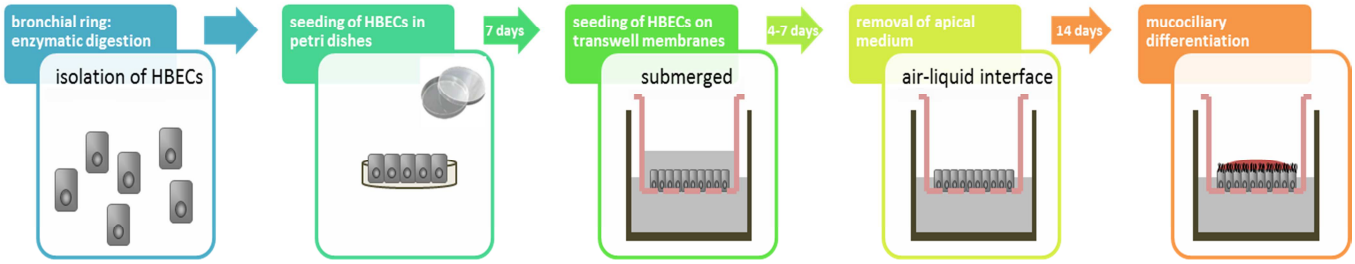


Figure 22. Culture of primary human bronchial epithelial cells (HBECs). Primary HBECs are isolated by enzymatic digestion of bronchial rings. After reaching confluence, the cells are transferred to 9 cm petri dishes for 7 days until the cells are confluent. Then the cells are transferred to Transwell inserts. For the first 4-7 days, the cells are grown submerged in medium both on the apical and basal side. Thereafter, the medium on the apical side is removed and the cells are grown in air-liquid interface for 14 days during which mucociliary differentiation occurs.

After the 14-day air-liquid interface culture period, HBECs are exposed to whole volatile smoke or air as a negative control, using a modification of the system reported by Beisswenger and coworkers (Amatngalim *et al.*, manuscript in preparation) [321]. In short, a pump generates a continuous flow of CS by burning Reference Cigarettes 3R4F (University of Kentucky, Lexington, KY, USA) and the smoke stream is guided into the exposure chamber placed inside a CO_2 incubator. The pump is set on a constant voltage and a flow meter is used to assure a constant flow of 1 litre/minute. A ventilator inside the exposure chamber distributes the CS equally over the cells. A filter is used at the outlet of the exposure chamber to reduce smoke particles reaching the pump. It takes 5 minutes for a cigarette to burn, after which smoke is removed from the exposure chamber for 10 minutes. Immediately after the CS exposure, the medium is refreshed and the cells, together with the basal medium, are harvested 3, 6 and 24 hours after the CS exposure. The CS protocol is schematically presented in **Figure 23**.

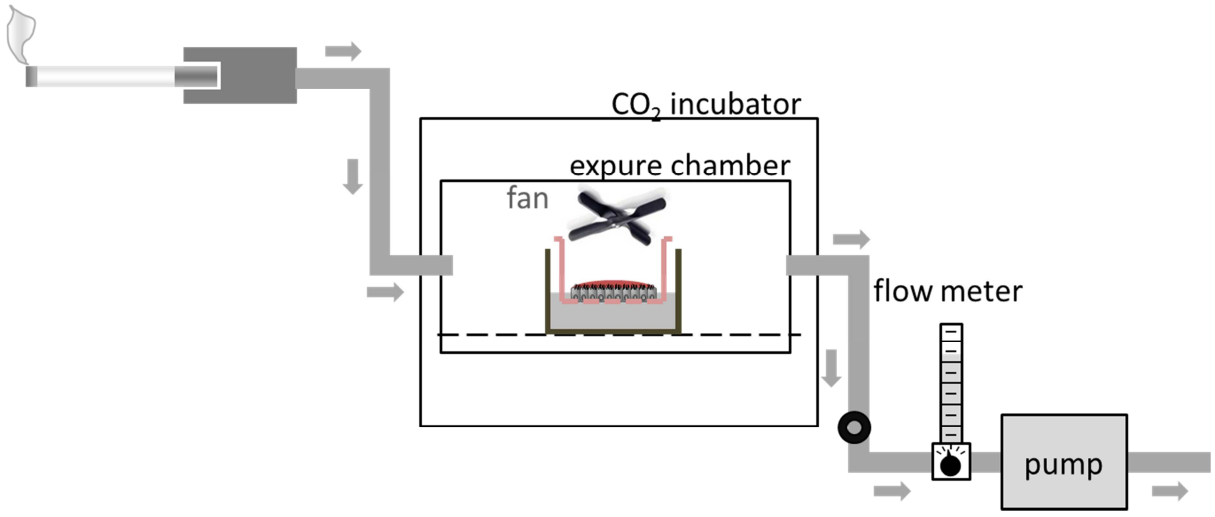


Figure 23. Schematic overview of the cigarette smoke exposure model for *in vitro* use. A pump generates a continuous flow of CS and the smoke stream is guided into the exposure chamber in the CO_2 incubator through a plastic tube. The pump is set on a constant voltage and together with a flow meter, this assures a constant flow. A ventilator inside the exposure chamber distributes the CS equally over the cells. Also a filter is used to reduce smoke particles reaching the pump. It took 5 minutes for a cigarette to burn. Immediately after the exposures, the medium was refreshed and the cells were harvested 3, 6 and 24 hours after the exposure.

4.4. Experimental techniques

Several techniques have been applied, both on human and mouse lung tissue to quantify gene expression and to measure protein levels and localize proteins in lung tissue. In this section, these methods will be described. Quantitative real-time reverse transcriptase (qRT-PCR) has proven to be a powerful tool to quantify gene expression profiles. In order to obtain more insights into the protein profile, ELISA and CBA are performed on supernatant fractions (BAL or sputum). To visualize proteins, lung sections are subjected to immunohistochemical staining.

4.4.1. Measurement of mRNA expression

Lung mRNA is extracted using the miRNeasy Mini kit from Qiagen (Hilden, Germany) and cDNA is prepared with the Transcriptor Universal cDNA Master Kit (Roche, Basel, Switzerland) following manufacturer's instructions. The expression of the gene of interest relative to 3 reference genes is analysed using TaqMan Gene Expression assays including specific primers/fluorogenic probe mix (Applied Biosystems, Halle, Belgium). All reactions are set up in duplicate using identical amplification conditions. The amplification conditions consist of 10 minutes at 95 °C and 50 cycles of 95 °C for 10 seconds and 60 °C for 15 seconds. A standard curve derived from serial dilutions of a mixture of all samples is included in each run. Amplifications are performed using a LightCycler 480 detection system (Roche). Expression of target genes is corrected by a normalization factor that is calculated based on the expression of three reference genes, using the geNorm applet according to the guidelines and theoretical framework previously described (http:// medgen.ugent.be / ~jvdesomp / genorm/) [322].

4.4.2. Measurement of protein levels

Using ELISA, we can quantify the protein levels in lung tissue or in the airways by analyzing BAL supernatant. The ELISAs used in this thesis are commercially available and are performed following the manufacturer's instructions. The disadvantage of this technique is that large amounts of sample are needed and for that reason CBA is sometimes preferred.

Protein levels of cytokines and chemokines in BAL fluid are determined by CBA. Multiple proteins can be analyzed in one assay using beads of known size and fluorescence, which can be detected by flow cytometry. A mixture of capture bead populations, each with distinct fluorescence intensities and coated with a capture antibody specific for a soluble protein are prepared. The beads are incubated

together with PE-conjugated detection antibodies, test samples or standards to form sandwich complexes. Following acquisition of sample data using the flow cytometer (FACSCalibur™ flow cytometer, BD Biosciences), the data are analyzed using the CBA Analysis Software (BD Biosciences). The intensity of the fluorescent signal of each sandwich complex reveals the concentration of that particular protein.

For information about the specific localization of a specific protein, immunohistochemical staining is performed. The quantification of activin-a in the airway epithelium by colour recognition, is shown in **Figure 24**. The epithelium is manually marked between the airway lumen and the basement membrane, using KS400 software (Zeiss). The epithelial area with positive activin-A staining is normalized to the length of the basement membrane.

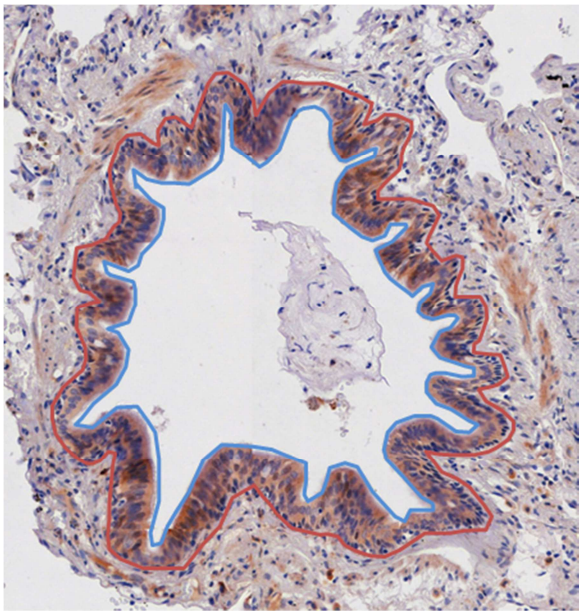


Figure 24. Quantification of immunohistochemical staining. Positive staining is quantified in the airway epithelium by Axiovision software (Zeiss) using an image analysis system. The airway epithelium is manually marked by 2 lines, the airway lumen in blue and the epithelial basement membrane in red.

[PART II: RESEARCH WORK]

[**CHAPTER 5:** RESEARCH OBJECTIVES]

COPD is a highly prevalent disorder and represents a significant burden worldwide. COPD susceptibility is dependent of both environmental exposures (most importantly tobacco smoke) and genetic susceptibility (including genes involved in the transforming growth factor-β superfamily). The abnormal inflammatory response in the lungs of patients with COPD results in airway wall remodelling and emphysema. The pathogenesis consists of a complex and not fully understood interplay between inflammatory cells, proteolytic activity, oxidative stress and apoptosis. Current treatment is pure symptomatic, indicating that novel treatments are urgently needed.

In a first study, we focused on the functional role of **lymphoid follicles** in the pathogenesis of COPD, since it is unclear whether lymphoid follicles are harmful or beneficial in COPD. COPD is hallmarked by a persisting inflammation, that continues despite smoking cessation and triggers the development of lymphoid follicles in severe disease. Importantly, the number of follicles correlates positively with disease severity, suggesting a role of lymphoid follicles in the persistent nature of COPD. CXCL13 is crucial in B cell recruitment and formation of lymphoid tissue. **We hypothesized that neutralizing CXCL13 abrogates the development of lymphoid follicles, enabling us to study the function of lymphoid follicles in COPD.** This approach allows us to assess the contribution of these local (auto)immune responses in the development of pulmonary inflammation, emphysema and airway wall remodeling. Furthermore, we aimed to analyze the expression of CXCL13 in the airways and lungs of patients with COPD compared to control groups (never smokers and smokers without airflow obstruction).

Members of the transforming growth factor (TGF)-β superfamily, which include TGF-βs, activins and bone morphogenetic proteins (BMPs) are implicated in fundamental physiological processes. Dysregulation of these pathways is associated with numerous diseases. The prototypical ligand TGF-β1 has been extensively studied in the pathogenesis of COPD, especially considering its role in immune regulation and airway remodeling. However, little is known about the other ligands of this superfamily in the pathogenesis of COPD. **Activin-A** signaling is activated in chronic inflammatory airway diseases, such as pulmonary fibrosis and asthma. **We hypothesized that activin-A plays a crucial role in the induction of pulmonary inflammation after CS exposure.** We first analyzed the expression of activin-A and its natural antagonist, follistatin in the lungs of CS-exposed mice, in lungs of patients with COPD and in primary human bronchial epithelial cell cultures exposed to CS. The functional role of activin-A was investigated in CS-exposed mice, using follistatin to antagonize activin-A.

Bone morphogenetic proteins represent another subfamily of the TGF-β superfamily and were originally identified as proteins that stimulate bone morphogenesis. It is now becoming clear that these proteins regulate the development of nearly all organs and tissues. More specifically, we were interested in the role of **BMP-6**, since a recent genome wide association study (GWAS) has found an association between genetic variants in the gene encoding BMP-6 and the forced vital capacity (FVC). **We hypothesized that reduced BMP-6 expression in lungs of COPD patients facilitates the development of CS-induced inflammation, emphysema and airway wall remodelling, mainly through iron-dependent mechanisms.** We measured the pulmonary expression of BMP-6 in patients with COPD and mice exposed to CS. Next, we exposed BMP-6 deficient mice to subacute and chronic CS and investigated the role of BMP-6 in CS-induced hallmarks of COPD, including pulmonary inflammation, emphysema and airway wall remodelling. Moreover, since BMP-6 is essential to iron homeostasis, we studied the effect of BMP-6 deficiency on CS-induced changes in content, storage and transport of iron.

[CHAPTER 6: ROLE OF CXCL13 IN CIGARETTE SMOKE-INDUCED LYMPHOID FOLLICLE FORMATION AND COPD]

Lymphoid follicles are present in lungs of patients with severe COPD. However, the role of these tertiary lymphoid organs in the pathogenesis of COPD is still unknown. CXCL13 is a chemokine attracting B lymphocytes and is crucial for lymphoid neogenesis. In this study, we aimed to abrogate the formation of CS-induced lymphoid follicles by antibody mediated neutralization of CXCL13 in a CS-induced murine model of COPD.

Bracke KR*, Verhamme FM*, Seys LJM, Bantsimba-Malanda C, Cunoosamy MD, Herbst R, Hammad H, Lambrecht BN, Joos GF, Brusselle GG. (* equal contribution). Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and COPD. American Journal of Respiratory and Critical Care Medicine. 2013;188:343-355 [323].

ABSTRACT

Rationale: The B-cell attracting chemokine CXCL13 is an important mediator in the formation of tertiary lymphoid organs (TLOs). Increased numbers of ectopic lymphoid follicles have been observed in lungs of patients with severe COPD. However, the role of these TLOs in the pathogenesis of COPD remains unknown.

Objectives: By neutralizing CXCL13 in a mouse model of chronic cigarette smoke (CS)-exposure, we aimed at interrogating the link between lymphoid follicles and development of pulmonary inflammation, emphysema and airway wall remodelling.

Methods: We first quantified and localized CXCL13 in lungs of air- or CS-exposed mice and in lungs of never smokers, smokers without airflow obstruction and patients with COPD by RT-PCR, ELISA and immunohistochemistry. Next, CXCL13 signaling was blocked by prophylactic or therapeutic administration of anti-CXCL13 antibodies in mice exposed to air or CS for 24 weeks and several hallmarks of COPD were evaluated.

Measurements and main results: Both mRNA and protein levels of CXCL13 were increased in lungs of CS-exposed mice and patients with COPD. Importantly, expression of CXCL13 was observed within B-cell areas of lymphoid follicles. Prophylactic and therapeutic administration of anti-CXCL13 antibodies completely prevented the CS-induced formation of pulmonary lymphoid follicles in mice. Interestingly, absence of TLOs attenuated destruction of alveolar walls and inflammation in bronchoalveolar lavage (BAL), but did not affect airway wall remodelling.

Conclusions: CXCL13 is produced within lymphoid follicles of patients with COPD and is crucial for the formation of TLOs. Neutralization of CXCL13 partially protects mice against CS-induced inflammation in BAL and alveolar wall destruction.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) affects the airways and lungs and is characterized by a progressive and not fully reversible airflow limitation [1;54]. Cigarette smoking is by far the most important risk factor for COPD. Importantly, although smoking cessation is currently the only effective treatment for COPD, it only partially attenuates the accelerated decline in lung function. Pathology of COPD includes obstruction of the small airways and destruction of lung parenchyma (emphysema) [12]. Until now, the mechanisms that are responsible for the development and progression of COPD are not well understood. Although the most common hypotheses focus on the role of the innate immune system, there are clear signs of adaptive immune responses in patients with COPD [54]. $CD8^+$ T-lymphocytes are associated with smoking and subsequent risk for COPD [88] and moreover, organisation of T- and B-lymphocytes in lymphoid follicles has been observed in advanced stages of COPD [33].

Ectopic lymphoid follicles, also called tertiary lymphoid organs (TLOs), have a composition and organization reminiscent of the secondary lymphoid organs, such as lymph nodes, Peyer's patches or spleen; however, their function remains largely unknown. They occur in several chronic inflammatory diseases, and are formed by lymphoid neogenesis, a mutual interaction between hematopoietic 'inducer' cells and stromal 'organizer' cells [153;172]. Briefly, chronic inflammation activates lymphocytes to express the cytokine lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) on their membrane. Signaling through the lymphotoxin β receptor on stromal cells triggers the release of chemokines that orchestrate lymphocyte homing and compartmentalization of TLOs. CCL19 and CC21 regulate homing of $CCR7^+$ naïve T-cells and mature dendritic cells, while CXCL13 attracts $CXCR5^+$ B-cells into the lymphoid follicles. Additionally, CXCL13 induces $LT\alpha_1\beta_2$ expression on B-cells, creating a positive feedback loop[176].

Increased numbers of small airways containing lymphoid follicles have been found in patients with severe COPD, compared to healthy subjects and patients with mild-to-moderate COPD [33;324]. In addition, lymphoid follicles have also been detected in the lung parenchyma of patients with COPD and in lungs of mice upon chronic cigarette smoke (CS) exposure [158;159]. Although the demonstration of oligoclonality in follicular B-cells suggests a local antigen-specific immune response, the role of lymphoid follicles in COPD is still controversial, especially since it is not known which antigens are involved [152]. The immune response may be directed against CS-derived antigens or lung tissue components and thus be of a harmful (autoimmune) nature. In contrast, they may be beneficial for the host by providing protection against microbial colonization and infection of the lower respiratory tract.

In this study we determined the presence of the B-cell attracting chemokine CXCL13 in the lungs – and more specific in the lymphoid follicles – of both mice exposed to CS and patients with COPD. Next, using the chronic CS model of COPD, we aimed at disrupting lymphoid neogenesis by neutralizing CXCL13, both in a prophylactic and a therapeutic setting. This allowed us to study the contribution of lymphoid follicles in the CS-induced development of pulmonary inflammation, emphysema and airway wall remodeling and to elucidate the role of these TLOs in the pathogenesis of COPD.

MATERIALS AND METHODS

Human study populations

Written informed consent was obtained from all subjects according to protocols approved by the medical ethical committee of the Ghent University Hospital. Lung resection specimens were obtained from 53 patients diagnosed with solitary pulmonary tumours at Ghent University Hospital (Ghent, Belgium). Lung tissue at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumour invasion was collected by a pathologist. Sputum induction was performed on 44 subjects who were recruited from the outpatient pulmonary clinic of the Ghent University Hospital or by advertising. There was no overlap between the subjects of lung tissue and sputum analysis. Sputum induction and processing was performed as described previously [61].

Animals

Male C57BL/6J mice, 6-8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), maintained in standard conditions under a 12 h light-dark cycle and provided a standard diet (Pavan, Brussels, Belgium) and chlorinated tap water *ad libitum*. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences (Ghent University).

Cigarette smoke (CS) exposure

C57BL/6 mice were exposed whole body to CS, as described previously [159;312]. Briefly, groups of 10 mice were exposed to the mainstream CS of 5 cigarettes (Reference Cigarette 3R4F without filter, University of Kentucky, Lexington, KY, USA), four times a day with 30 minutes smoke-free intervals, 5 days a week for 4 or 24 weeks. An optimal smoke/air ratio of 1/6 was obtained. The control groups were exposed to room air.

Anti-CXCL13 treatment

8 groups of 10 C57BL/6J mice were exposed for 24 weeks (chronic exposure) to air or CS. 6 groups were injected intraperitoneally (i.p.) every 2 weeks from the start of the experiment (prophylactic treatment) with 200 µl PBS, rat IgG_{2a} isotype control antibody (500 µg/mouse, R&D Systems, Abingdon, UK) or anti-CXCL13 monoclonal antibody (500 µg/mouse, R&D Systems)**.** A further 2 groups were injected i.p. every 2 weeks from week 13 onwards (therapeutic treatment) with 200 µl rat IgG_{2a} isotype control antibody (500 µg/mouse) or anti-CXCL13 monoclonal antibody (500 µg/mouse). All injections were performed 1 h before air- or CS-exposure.

Quantification and detection of CXCL13

CXCL13 mRNA expression in total lung tissue of mice and human subjects was analyzed using TaqMan Gene Expression Assays (Applied Biosystems, Forster City, CA, USA), as described previously [84]. CXCL13 protein levels were determined in BAL fluid supernatant of mice and induced sputum supernatant of human subjects using commercially available ELISA kits (R&D Systems). Immunofluorescence staining with anti-CXCL13 (R&D Systems), anti-CXCR5 (R&D Systems) and anti-CD20 (eBioscience) was performed on frozen sections of human lung tissue, as described previously [325]. Immunohistochemical staining with anti-CXCL13 (R&D Systems) was performed on paraffin sections of the left lung of mice, as described previously [180].

Quantification of lymphoid follicles

To evaluate the presence of lymphoid follicles in lung tissue, paraffin sections of the left lung were subjected to an immunohistolchemical CD3/B220 double-staining [159]. All lymphoid infiltrates in the proximity of airways and in the parenchyma were counted. Dense accumulations of at least 50 cells were defined as lymphoid follicles, whereas accumulations of less than 50 cells were defined as lymphoid aggregates. Counts of infiltrates in the proximity of airways were normalized for the number of airways per lung section. Counts of infiltrates in the parenchyma were normalized for the area of lung parenchyma.

Quantification of pulmonary inflammation

Numbers of inflammatory cells in bronchoalveolar lavage (BAL), lung tissue and mediastinal lymph nodes of mice were determined as described previously [180;326-328]. Protein levels of cytokines and chemokines were determined in BAL of mice using the Cytometric Bead Array (BD Biosciences, San Diego, CA, USA) [47]. mRNA expression levels of 83 target genes were determined in total lung tissue by RT-PCR with the Biomark System (Fluidigm, San Francisco, CA, USA) using TaqMan Gene Expression Assays (Applied Biosystems).

Quantification of emphysema and airway wall remodelling

To evaluate pulmonary emphysema, we determined enlargement of alveolar spaces by quantifying the mean linear intercept (L_m)[318] and destruction of alveolar walls by measuring the destructive index (DI)[317], as described previously [159;312]. Immunohistochemistry for matrix metalloproteinase 12 (MMP12) was performed as described previously [159]. Airway wall remodelling was defined by measuring the amount of fibronectin and collagen in the airway wall [319].

Statistical analysis

Statistical analysis was performed with Sigma Stat software (SPSS 19.0, Chicago, IL, USA) using non-parametric tests (Kruskall-Wallis; Mann-Whitney U). Reported values are expressed as mean ± SEM. P-values < 0.05 were considered to be significant.

RESULTS

Expression of CXCL13 in cigarette smoke-exposed mice and patients with COPD

CXCL13 expression was analysed in a mouse model of cigarette smoke (CS)-exposure, as well as in 2 independent human COPD study populations.

RNA expression of CXCL13 was significantly increased in total lung tissue of mice exposed to CS for 24 weeks, compared to air-exposed mice **(Fig. 1A)**. Accordingly, CXCL13 protein levels were significantly elevated in BAL supernatant of mice exposed for 24 weeks to CS **(Fig. 1B)**. No differences in CXCL13 expression were found upon 4 weeks of CS-exposure **(Fig. 1A-B)**. Importantly, immunohistochemistry on lung tissue revealed CXCL13 expression inside lymphoid follicles, which appear upon chronic CS-exposure **(Fig. 1C-D)**.

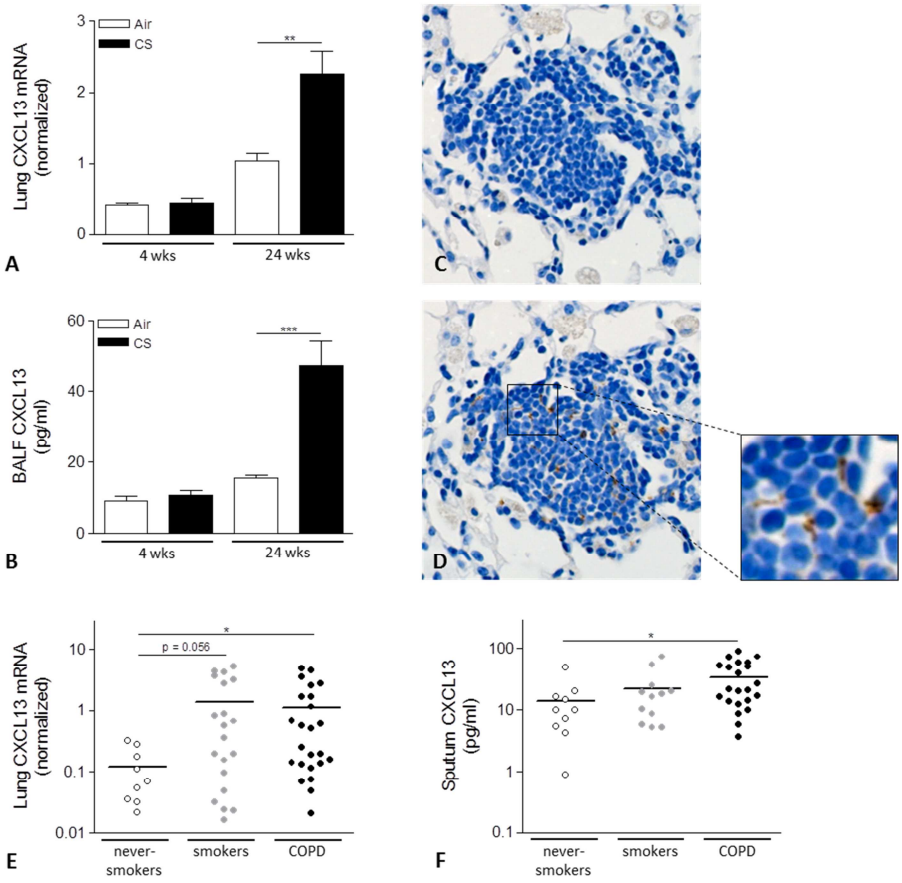


Figure 1. Expression of CXCL13 in CS-exposed mice and patients with COPD. (A) RT-PCR analysis showing CXCL13 mRNA expression in total lung tissue of mice exposed to air- or CS for 4 or 24 weeks (n=10/group). **(B)** Protein levels of CXCL13 in BAL supernatant of mice exposed to air- or CS for 4 or 24 weeks, as analyzed by ELISA (n=10/group). Representative images of immunohistochemical staining for isotype control **(C)** or CXCL13 **(D)** in pulmonary lymphoid follicles of mice exposed to CS for 24 weeks. **(E)** RT-PCR analysis showing CXCL13 mRNA expression in total lung tissue of never-smokers (n=9), smokers without airflow obstruction (n=20) and patients with COPD (n=24). **(F)** Protein levels of CXCL13 in induced sputum supernatant of never-smokers (n=10), smokers without airflow obstruction (n=12) and patients with COPD (n=22). Values are expressed as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

In a translational approach, mRNA and protein expression of CXCL13 was analysed in total lung tissue of 53 subjects and induced sputum supernatant of 44 subjects, respectively. The characteristics of these 2 independent study populations are given in **Tables 1** and **2**. Both populations consisted of never-smokers, smokers without airflow obstruction and patients with COPD (GOLD stage II). Compared to never-smokers, RT-PCR analysis revealed increased expression of CXCL13 mRNA in lung tissue of smokers without airflow obstruction and patients with COPD, although only the latter reached statistical significance **(Fig. 1E)**. Accordingly, CXCL13 protein levels were significantly elevated in induced sputum supernatant of patients with COPD, compared to never-smokers **(Fig. 1F)**.

Table 1: Characteristics of study subjects for lung mRNA analysis

	never smokers	smokers	COPD
Number	9	20	24
Gender (male/female)	2/7 #	16/4 #	20/0 #
Age (years)	61 (51-70)	64 (55-71)	65 (59-69)
Current-smoker / Ex-smoker	NA	11/9	14/10
Smoking history (pack years)	0 (0-0)	33 (15-50)*	45 (40-60)*§
FEV1 post-bronchodilator (L)	2,4 (2,1-3,4)	3,1 (2,6-3,5)	2,1 (1,8-2,4)§
FEV1 post-bronchodilator % predicted	103 (92-119)	96 (93-113)	68 (61-73)*§
FEV1 / FVC post-bronchodilator (%)	77 (75-81)	76 (73-79)	56 (54-60)*§
ICS (yes/no)	0/11 #	0/20 #	10/14 #

Footnote
NA (not applicable); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids).
Data are presented as median (IQR)
Mann-Whitney U test: * $P < 0,05$ versus never smokers; § $P < 0,05$ versus smokers
Fisher's exact test: # $P < 0,001$

Importantly, immunofluorescence staining and confocal microscopy revealed expression of CXCL13 and its receptor CXCR5 inside lymphoid follicles of patients with severe and very severe COPD (GOLD stages III and IV) **(Fig. 2A-B)**. Moreover, merged images of CXCL13, CXCR5 and CD20 immunofluorescence staining provided clear evidence of CXCL13 expression within B-cell areas of lymphoid follicles **(Fig. 2D)**.

Table 2: Characteristics of study subjects for sputum analysis (by ELISA)

	never smokers	smokers	COPD
Number	10	12	22
Gender (male/female)	4/6 #	5/7 #	21/1 #
Age (years)	47 (24-57)	59 (51-64)*	70 (61-74)*§
Current-smoker / Ex-smoker	NA	2/10	4/18
Smoking history (pack years)	0,0 (0,0-0,0)	34 (23-46)*	29 (21-44)*
FEV1 post-bronchodilator (L)	3,1 (2,7-3,6)	3,2 (2,4-3,9)	2,1 (1,7-2,2)*§
FEV1 post-bronchodilator % predicted	98 (95-106)	110 (101-120)	66 (63-70)*§
FEV1 / FVC post-bronchodilator (%)	78 (76-92)	79 (75-82)	51 (46-59)*§
ICS (yes/no)	0/10 #	0/12 #	12/10 #
Sputum differential cell count:			
% Macrophages	43 (37-63)	40 (26-55)	21 (10-32)*§
% Neutrophils	55 (32-60)	56 (43-71)	76 (62-85)*§
% Eosinophils	0,2 (0,0-1,2)	0,5 (0,2-1,7)	1,1 (0,6-3,1)
% Lymphocytes	1,0 (0,5-1,8)	0,5 (0,0-0,7)*	0,2 (0,0-0,6)*
% Bronchial epithelial cells	0,4 (0,0-1,6)	0,2 (0,0-0,7)	0,2 (0,0-0,3)
% Squamous epithelial cells	1,3 (0,4-3,7)	1,0 (0,5-4,3)	0,4 (0,0-2,9)
Viability (%)	91 (83-96)	90 (85-93)	91 (81-92)

Footnote

NA (not applicable); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids)

Data are presented as median (IQR)

Mann-Whitney U test: * $P < 0,05$ versus never smokers; § $P < 0,05$ versus smokers

Fisher's exact test # $P < 0,001$

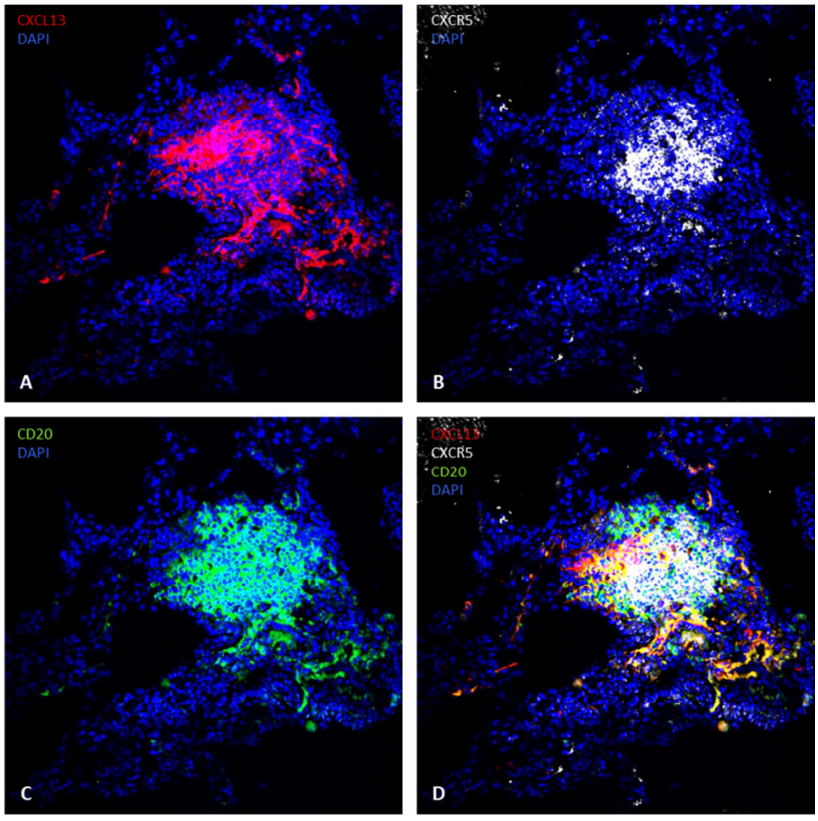


Figure 2. Immunofluorescence microscopy for CXCL13, CXCR5 and CD20 in lymphoid follicles of patients with COPD.

Representative images of immunofluorescence staining with antibodies against CXCL13 **(A)**, CXCR5 **(B)**, CD20 **(C)** and CXCL13/CXCR5/CD20 **(D)** in pulmonary lymphoid follicles of patients with severe to very severe COPD (GOLD stages III and IV). The yellow color in **(D)** represents co-localisation of CD20 and CXCL13. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Neutralization of CXCL13 in cigarette smoke-exposed mice

Briefly, 8 groups of 10 mice were exposed for 24 weeks to air or CS. 6 groups were injected i.p. every 2 weeks from the start of the experiment (prophylactic treatment) with PBS, rat IgG_{2a} isotype control antibody or anti-CXCL13 monoclonal antibody. For clarity reasons and since for none of the analyses differences were observed between PBS or rat IgG_{2a} isotype treated groups, the results of the PBS treated groups are not shown. In addition, 2 groups were injected i.p. every 2 weeks from week 13 onwards (therapeutic treatment) with rat IgG_{2a} isotype control antibody or anti-CXCL13 monoclonal antibody. Again, for clarity reasons and since no differences were observed between prophylactic or therapeutic IgG_{2a} isotype treated groups, the results of the latter group are not shown.

Since CXCL13 creates a positive feedback loop by up-regulating lymphotoxin on B-cells, which in turn further induces CXCL13 [176;329], we examined the CXCL13 mRNA expression levels as a control of effective CXCL13 neutralization. Importantly, the CS-induced increase in CXCL13 mRNA expression in total lung tissue was significantly attenuated upon both prophylactic and therapeutic treatment with anti-CXCL13 antibodies **(Fig. 3)**.

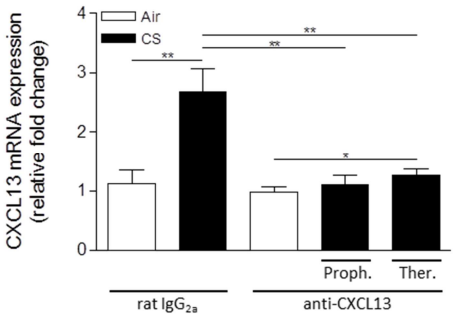


Figure 3. Effect of CXCL13 neutralization on CXCL13 mRNA expression in total lung tissue. mRNA expression of CXCL13 in total lung tissue of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=8/group). Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$.

Effect of CXCL13 neutralization on cigarette smoke-induced lymphoid follicle formation

Immunohistochemistry using anti-CD3 and anti-B220 monoclonal antibodies to stain T- and B-lymphocytes respectively, was used to identify the presence of lymphoid follicles and aggregates in lung tissue of mice. Representative images of a lung lobe from each of the 5 experimental groups are shown in **Fig. 4A-E**.

Lymphoid follicles were absent in air-exposed mice. In isotype treated, CS-exposed mice there was a significant increase in the number of both peribronchial and parenchymal lymphoid follicles **(Fig. 4F-G)**. However, both prophylactic and therapeutic administration of anti-CXCL13 antibodies resulted in a very strong and significant inhibition of lymphoid follicle formation around the airways and in the lung parenchyma **(Fig. 4F-G)**. In contrast, there was no obvious effect of CXCL13 neutralization on the presence of lymphoid aggregates, defined as loose infiltrations of less than 50 lymphocytes **(Fig. 4H-I)**.

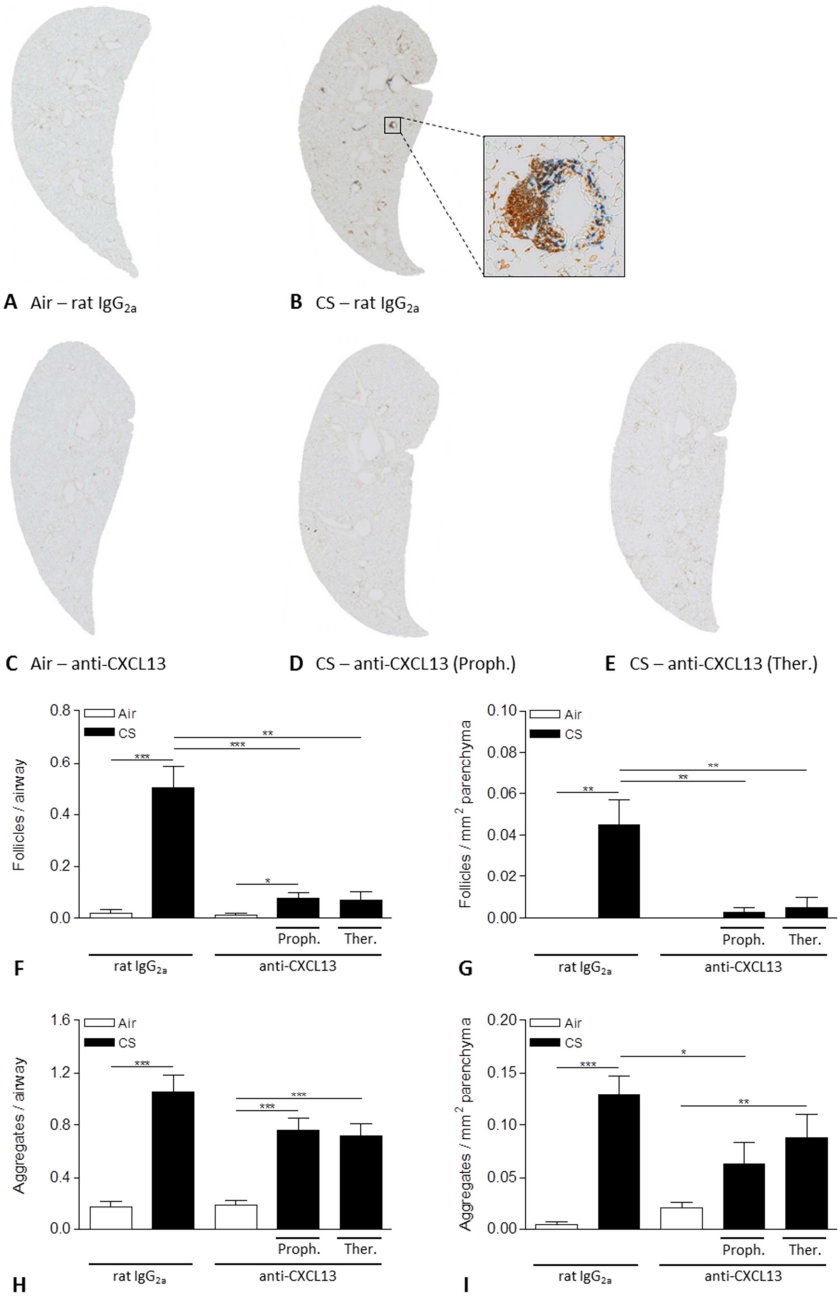


Figure 4. Effect of CXCL13 neutralization on lymphoid follicle formation.

Representative images of CD3/B220 stained lung lobes of **(A)** air-exposed, isotype treated, **(B)** CS-exposed, isotype treated, **(C)** air-exposed, anti-CXCL13 treated, **(D)** CS-exposed, prophylactic anti-CXCL13 treated and **(E)** CS-exposed, therapeutic anti-CXCL13 treated mice. Quantification of **(F)** peribronchial lymphoid follicles, **(G)** parenchymal lymphoid follicles, **(H)** peribronchial lymphoid aggregates and **(I)** parenchymal lymphoid aggregates in lung tissue of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Consistent with the occurrence of lymphoid follicles, the levels of immunoglobulins IgM, IgG and IgA were significantly elevated in BAL supernatant of isotype treated, CS-exposed mice **(Fig. 5A-C)**. Both prophylactic and therapeutic neutralization of CXCL13 significantly attenuated the CS-induced increase in BAL immunoglobulins **(Fig. 5A-C)**. Importantly, upon both prophylactic and therapeutic treatment with anti-CXCL13 antibodies, we found a significant attenuation of the CS-induced increase in B-cell maturation antigen (BCMA), a marker for plasma cells [330] **(Fig. 5D)**. No obvious differences were observed in serum levels of IgM, IgG or IgA **(Fig. 5E-G)**.

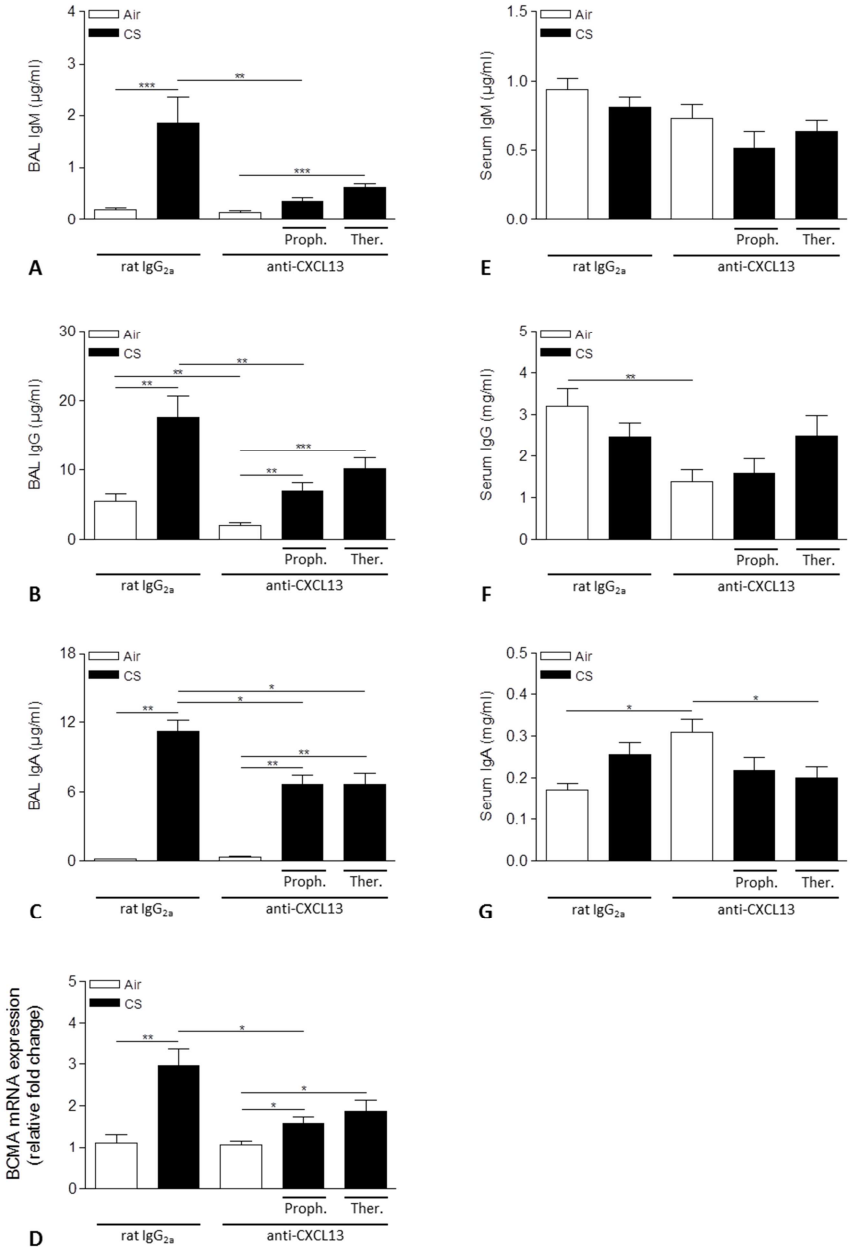


Figure 5. Effect of CXCL13 neutralization on immunoglobulin levels in BAL supernatant and serum. Levels of **(A)** IgM, **(B)** IgG and **(C)** IgA in BAL supernatant of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies, as measured by ELISA (n=10/group). **(D)** mRNA expression of B-cell maturation antigen (BCMA) in total lung tissue of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=8/group). Levels of **(E)** IgM, **(F)** IgG and **(G)** IgA in serum of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies, as measured by ELISA (n=10/group).Values are expressed as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Effect of CXCL13 neutralization on cigarette smoke-induced inflammation in broncho-alveolar lavage, lung tissue and mediastinal lymph nodes

In bronchoalveolar lavage (BAL) of isotype treated mice, CS-exposure resulted in a significant increase in the number of total BALF cells, macrophages, neutrophils, dendritic cells, B-lymphocytes and $CD4^+$ and $CD8^+$ T-lymphocytes **(Fig. 6A-H)**. Prophylactic neutralization of CXCL13 significantly attenuated the CS-induced increase in total BAL cells, macrophages, neutrophils, dendritic cells and especially B-lymphocytes **(Fig. 6A-E)**. The latter were also significantly reduced upon therapeutic administration of CXCL13 **(Fig. 6E)**. No effect of prophylactic or therapeutic treatment with anti-CXCL13 was observed on the CS-induced increase in CXCL1/KC, CCL2/MCP-1, CCL5/RANTES, TNFα and IL-6 in BAL supernatant (data not shown).

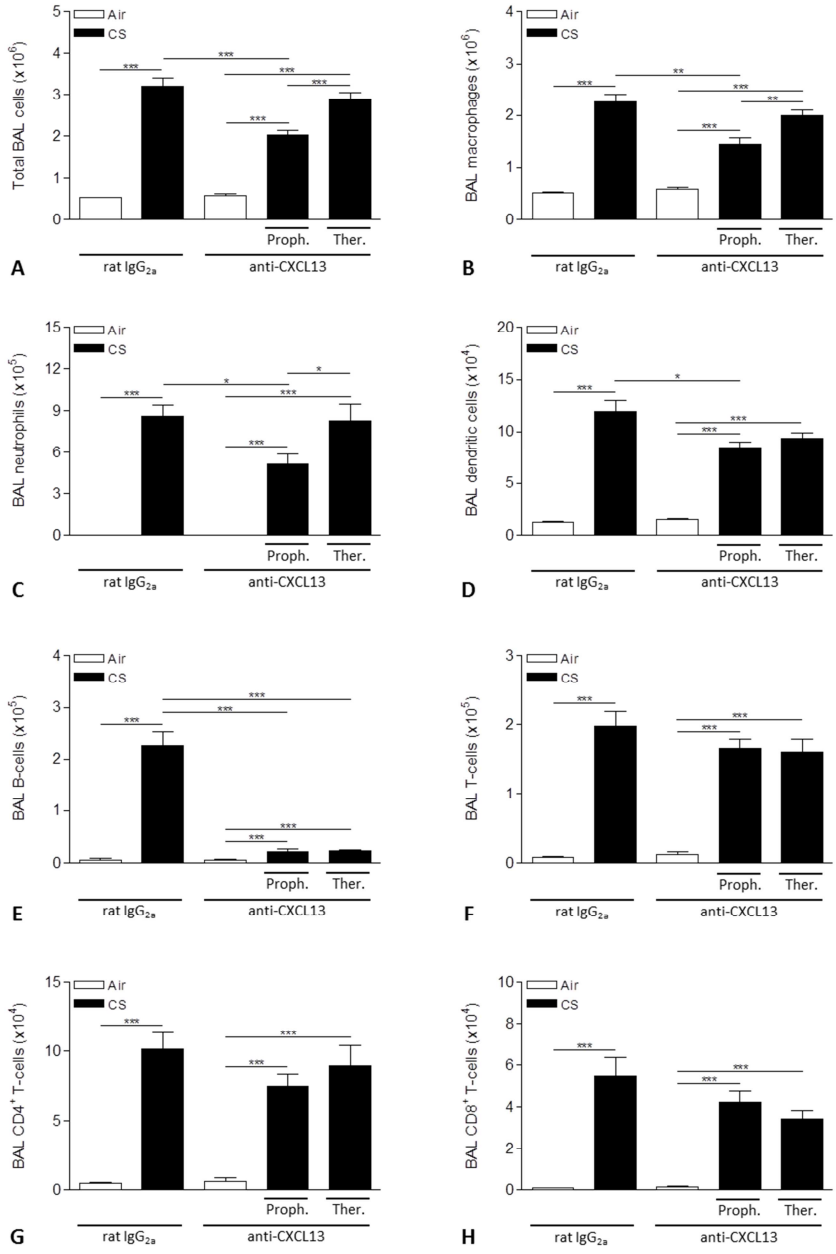


Figure 6. Effect of CXCL13 neutralization on inflammatory cell numbers in bronchoalveolar lavage (BAL). Numbers of **(A)** total cells, **(B)** macrophages, **(C)** neutrophils, **(D)** dendritic cells, **(E)** B-lymphocytes, **(F)** T-lymphocytes, **(G)** $CD4^+$ T-lymphocytes and **(H)** $CD8^+$ T-lymphocytes in BAL of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In lung tissue, neutralization of CXCL13 had no significant effect on the CS-induced increase of macrophages, dendritic cells, B-lymphocytes, Tregs and activated ($CD69^+$) $CD4^+$ and $CD8^+$ T-lymphocytes **(Fig. 7A-H)**. Consistently, the CS-induced increase in mRNA expression of several inflammatory cytokines and chemokines, including IL-1α, TNF-α, CCL19, CCL20, CXCL1 and CXCL9 was not influenced by anti-CXCL13 treatment (data not shown).

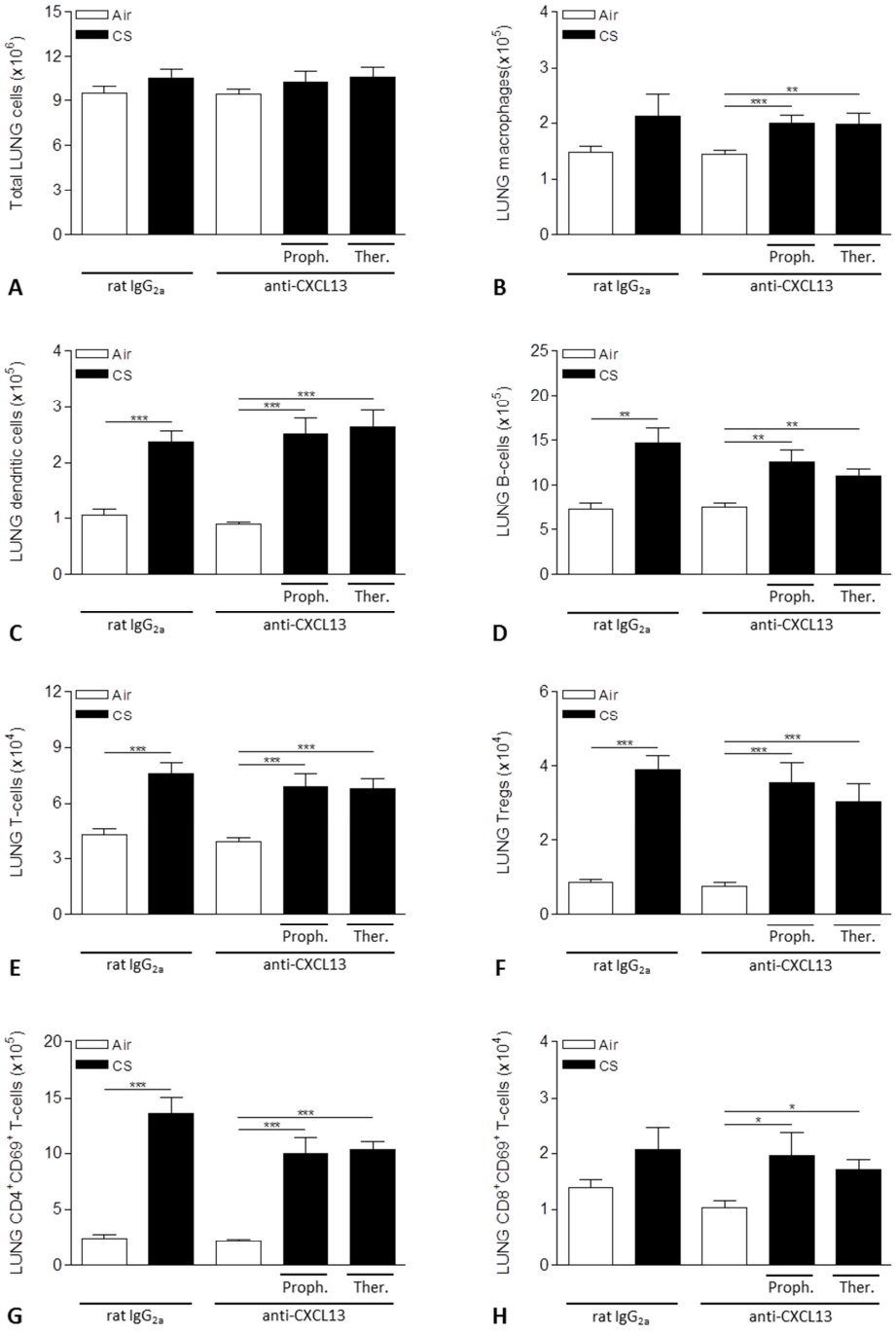


Figure 7. Effect of CXCL13 neutralization on inflammatory cell numbers in lung tissue. Numbers of **(A)** total cells, **(B)** macrophages, **(C)** dendritic cells, **(D)** B-lymphocytes, **(E)** T-lymphocytes, **(F)** Tregs, **(G)** $CD4^+CD69^+$ T-lymphocytes and **(H)** $CD8^+CD69^+$ T-lymphocytes in lung tissue of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In mediastinal lymph nodes (MLN), neutralization of CXCL13 significantly attenuated the CS-induced increase in the number of B-lymphocytes, but not of total MLN cells, airway-derived dendritic cells, T-lymphocytes and Tregs **(Fig. 8A-F)**.

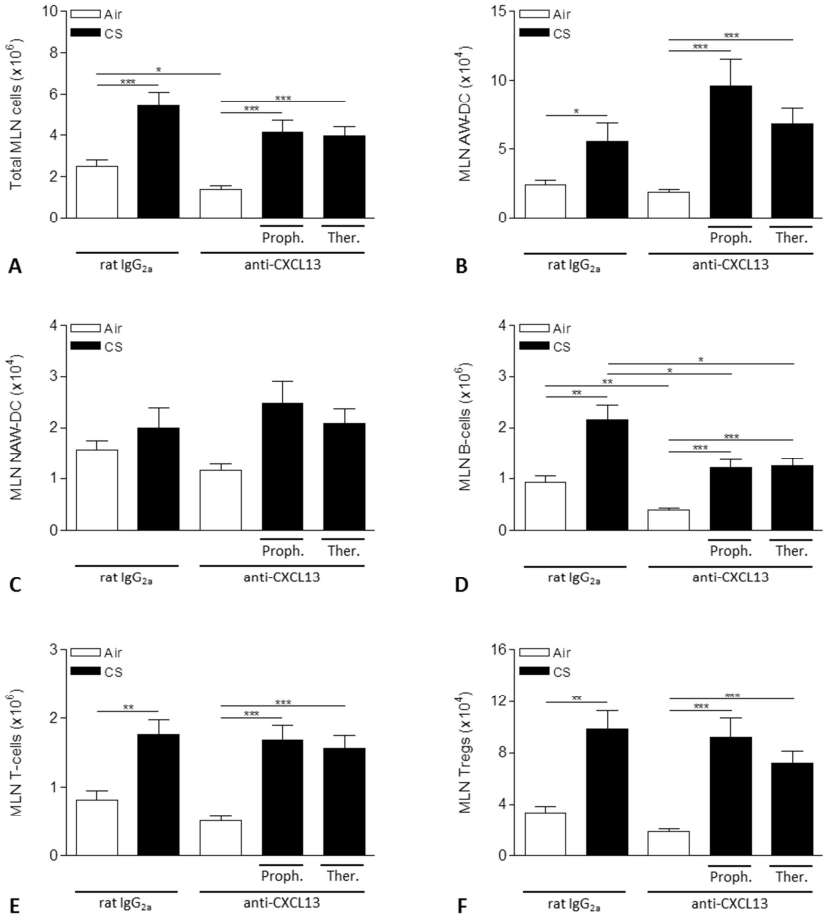


Figure 8. Effect of CXCL13 neutralization on inflammatory cell numbers in mediastinal lymph nodes (MLN). Numbers of **(A)** total cells, **(B)** airway-derived dendritic cells (AW-DC), **(C)** non airway-derived dendritic cells (NAW-DC), **(D)** B-lymphocytes, **(E)** T-lymphocytes and **(F)** Tregs in MLN of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Values are expressed as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Effect of CXCL13 neutralization on cigarette smoke-induced pulmonary emphysema and airway wall remodelling

To evaluate pulmonary emphysema, we determined enlargement of alveolar spaces by quantifying the mean linear intercept (Lm) and destruction of alveolar walls by measuring the destructive index (DI). Both isotype and (prophylactic or therapeutic) anti-CXCL13 treated, CS-exposed groups showed a significant increase in the Lm and DI, compared to air-exposed control groups **(Fig. 9A-G)**. Importantly, upon chronic CS-exposure no significant differences in Lm were observed between isotype and anti-CXCL13 treated mice **(Fig. 9A)**. In contrast, mice treated prophylactic with anti-CXCL13 antibodies showed a significantly lower DI, indicating a partial protection against destruction

of alveolar walls **(Fig. 9B)**. Interestingly, mRNA expression levels of matrix metalloproteinase 12 (MMP12) were lower in lung tissue of prophylactic anti-CXCL13 treated mice **(Fig. 9H)** compared to isotype treated mice, although this trend did not reach statistical significance. However, semi-quantitative analysis of MMP12 immunohistochemistry revealed significantly less intense MMP12 staining in macrophages of anti-CXCL13 treated mice **(Fig. 9I-N)**.

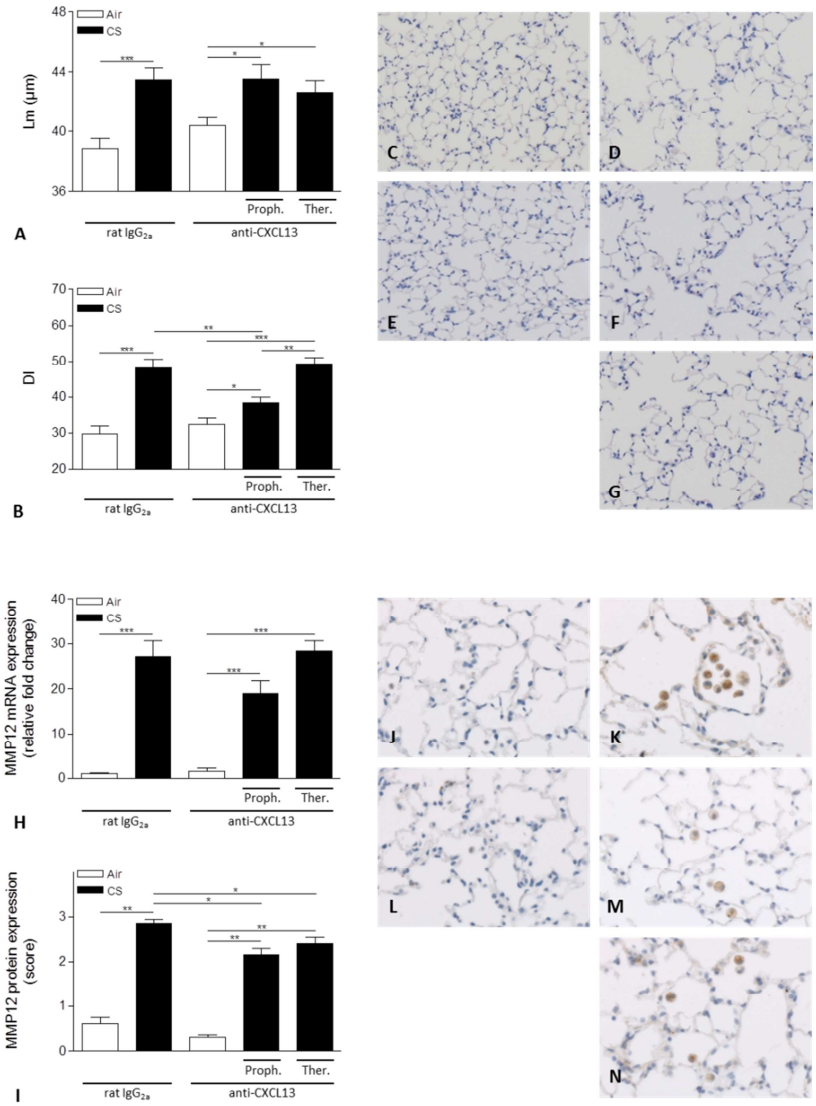


Figure 9. Effect of CXCL13 neutralization on development of pulmonary emphysema. Measurement of **(A)** mean linear intercept (Lm) and **(B)** destructive index (DI) in lungs of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Representative images of H&E stained lung sections of **(C)** air-exposed, isotype treated, **(D)** CS-exposed, isotype treated, **(E)** air-exposed, anti-CXCL13 treated, **(F)** CS-exposed, prophylactic anti-CXCL13 treated and **(G)** CS-exposed, therapeutic anti-CXCL13 treated mice. **(H)** mRNA expression of matrix metalloproteinase 12 (MMP12) in total lung tissue and **(I)** semi-quantitative analysis of MMP12 immunostaining in tissue macrophages of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=8/group). Representative images of MMP12 stained lung sections of **(J)** air-exposed, isotype treated, **(K)** CS-exposed, isotype treated, **(L)** air-exposed, anti-CXCL13 treated, **(M)** CS-exposed, prophylactic anti-CXCL13 treated and **(N)** CS-exposed, therapeutic anti-CXCL13 treated mice. Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Airway wall remodelling was investigated by measuring the deposition of fibronectin and collagen in the airway wall. Both isotype and (prophylactic or therapeutic) anti-CXCL13 treated, CS-exposed groups showed a significant increase in the deposition of fibronectin, compared to air-exposed control groups **(Fig. 10A-F)**. Importantly, upon chronic CS-exposure no significant differences in fibronectin deposition were observed between isotype and anti-CXCL13 treated mice **(Fig. 10A)**. Although deposition of collagen in the airway wall showed similar results as fibronectin deposition, no significant differences were observed between the study groups (data not shown).

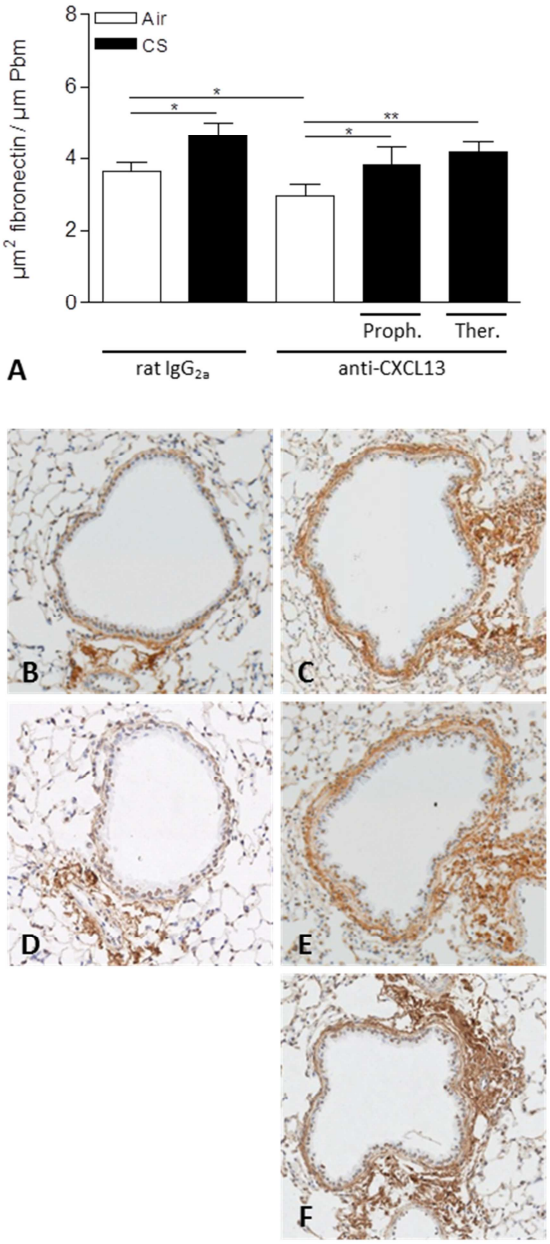


Figure 10. Effect of CXCL13 neutralization on airway wall remodelling. (A) Quantification of fibronectin deposition in the airway wall of mice exposed to air or CS and treated with rat IgG_{2a} isotype control or anti-CXCL13 antibodies (n=10/group). Representative images of fibronectin-stained lung sections of **(B)** air-exposed, isotype treated, **(C)** CS-exposed, isotype treated, **(D)** air-exposed, anti-CXCL13 treated, **(E)** CS-exposed, prophylactic anti-CXCL13 treated and **(F)** CS-exposed, therapeutic anti-CXCL13 treated mice. Values are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$.

DISCUSSION

In this study we show increased levels of CXCL13 in lung tissue and induced sputum of patients with COPD. Moreover, we clearly demonstrate expression of CXCL13 inside lymphoid follicles of patients with severe and very severe COPD. Neutralizing CXCL13 in a mouse model of chronic cigarette smoke (CS)-exposure, completely disrupted the formation of peribronchial and parenchymal lymphoid follicles. Importantly, the absence of these lymphoid follicles attenuated inflammatory cell recruitment in bronchoalveolar lavage (BAL) and partially protected the mice against destruction of alveolar walls upon chronic CS-exposure, but it had no effect on the development of airway wall remodelling.

We found increased levels of CXCL13, a potent B-cell attracting chemokine, in bronchoalveolar lavage (BAL) fluid and lung tissue of mice chronically exposed to CS, which is accompanied by an elevated accumulation of B-lymphocytes in the respective pulmonary compartments of these mice [180]. In this manuscript we also show increased levels of CXCL13 in induced sputum and lung tissue of patients with COPD, compared to non-smoking controls. Similar observations have been made in other inflammatory diseases of the lower and upper airways such as asthma and chronic rhinosinusitis [194;331]. Importantly, CXCL13 is critically involved in organisation of secondary lymphoid organs such as spleen and lymph nodes, but also in the formation of ectopic lymphoid tissue [176]. Indeed, CXCL13 overexpression from the rat insulin promoter induces TLOs in pancreatic islands [184], while neutralization of CXCL13 or its receptor CXCR5 disrupts lymphoid neogenesis in several infectious and autoimmune models, as well as in OVA-induced airway inflammation [179;189-191;194]. Interestingly, we demonstrate clear expression of CXCL13 in B-cell areas within lymphoid follicles of patients with severe to very severe COPD. This is in line with recent observations by Litsiou and colleagues, who identified pulmonary B-cells of patients with COPD as major cellular sources of CXCL13 [329].

Administration of anti-CXCL13 antibodies, either prophylactic – during the entire course of the chronic (6 months) exposure to CS – or therapeutic – starting from established inflammation at 3 months of CS-exposure – almost completely prevented the formation of peribronchial and parenchymal lymphoid follicles. As neutralizing CXCL13 did not affect the numbers of lymphocytes in the lungs, nor the presence of lymphoid aggregates (defined as loose accumulations of less than 50 lymphocytes), it is clear that CXCL13 plays a crucial role in the organization of CS-induced lymphoid follicles, but is not required for the recruitment of B-cells to the lung.

The increase in immunoglobulins IgM, IgG and IgA in the BAL fluid, that coincides with the occurrence of lymphoid follicles upon chronic CS-exposure, was significantly attenuated upon both prophylactic and therapeutic neutralization of CXCL13. Interestingly, CXCL13 neutralization significantly attenuated the CS-induced increase in B-cell maturation antigen (BCMA), a receptor which is highly expressed in plasma cells [330]. In addition, expression of myeloid cell leukemia sequence 1 (MCL1) by plasma cells, which promotes plasma cell survival, is sustained through the BCMA receptor [332]. This suggests that the inhibition of follicle formation by anti-CXCL13 treatment leads to reduced numbers of plasma cells (due to reduced plasma cell survival and/or reduced plasma cell differentiation). Moreover, these data support the presence of germinal centers and plasma cell differentiation in CS-induced lymphoid follicles.

The role of lymphoid follicles in COPD is still unknown and controversial. The finding of oligoclonal B-cells in the pulmonary lymphoid follicles points to a local antigen specific immune reaction [158]. However, until now it is not known which antigens are involved. They may originate from CS components or derivatives, or they can be auto-antigens originating from epithelial damage or degradation of extracellular matrix. Either way, these antigens may elicit harmful (auto)immune responses that may contribute to the – even after smoking cessation – ongoing pulmonary inflammation. In contrast, the immune responses induced by lymphoid follicles in COPD may be protective when directed against antigens from microbial origin. Infection and colonization of the lower respiratory tract by bacteria is a common feature of COPD, especially in the more severe stages where it often parallels the occurrence of lymphoid follicles [163]. Moreover, lymphoid neogenesis can be induced in mice by infection with *Haemophilus influenzae* or by chronic exposure to bacterial products [333;334].

Preventing the formation of lymphoid follicles by neutralizing CXCL13 in CS-exposed mice offers a platform to assess the contribution of harmful local (auto)immune responses in the development of pulmonary inflammation, emphysema and airway wall remodelling. Both prophylactic and therapeutic administration of anti-CXCL13 completely abrogated the recruitment of B-cells to the bronchoalveolar compartment and mediastinal lymph nodes (MLN). Importantly, prophylactic treatment with anti-CXCL13 antibodies significantly attenuated the increase in macrophages, dendritic cells and neutrophils in the BAL upon chronic CS-exposure. This is consistent with reported results in other models of airway and non-airway diseases, such as OVA-induced allergic airway inflammation or intestinal nematode infection, where targeting CXCL13 or CXCR5 also results in reduced immune responses [194;335].

We assessed the development of pulmonary emphysema by quantifying the mean linear intercept (Lm) – as a measure of airspace enlargement – and the destructive index (DI) – as a measure of alveolar wall destruction. Although the CS-induced increase in Lm was not influenced by CXCL13 neutralization, we did observe a partial but significant protection against destruction of alveolar walls, reflected in an attenuated increase in the DI. The discrepancy between these two methods may be due to the relative small increase in Lm upon CS-exposure, making it difficult to study the effects of an intervention such as CXCL13 neutralization. In contrast, the DI offers a more distinct increase upon CS-exposure. Nevertheless, confirmation of the observed effect of anti-CXCL13 treatment on the development of emphysema requires more robust methods, such as micro-CT or pulmonary function tests, which is a limitation of the current study. However, in line with the observed effect of CXCL13 neutralization on alveolar wall destruction, we found reduced expression of matrix metalloproteinase 12 (MMP12) in macrophages of CS-exposed, anti-CXCL13 treated mice, compared to isotype-treated controls. MMP12, also known as macrophage metalloelastase, has been implicated as one of the key proteinases in the development of emphysema upon CS-exposure [64;336]. On the other hand, we have previously shown that the innate immune system suffices to develop specific hallmarks of COPD, as severe combined immunodeficiency (*scid*) mice – which lack B- and T-lymphocytes – still develop pulmonary emphysema [47]. Still, this does not rule out the presence of an autoimmune component in COPD, which could aggravate inflammation and alveolar destruction in chronic disease.

In conclusion, we have shown that the B-cell attracting chemokine CXCL13 is increased in lungs of CS-exposed mice and patients with COPD, and that this chemokine is crucial for the CS-induced development of ectopic TLOs. Importantly, disrupting lymphoid follicles by neutralizing CXCL13 had a sustained effect on inflammation in BAL and on destruction of alveolar walls, but did not affect the development of airway wall remodelling. These findings have important consequences for future therapeutic strategies that target adaptive immune responses and lymphoid neogenesis in COPD.

[CHAPTER 7: ROLE OF ACTIVIN-A IN CIGARETTE SMOKE-INDUCED INFLAMMATION AND COPD]

Activin-A is a pleiotropic cytokine, belonging to the TGF-β superfamily and has been implicated in respiratory disorders such as acute lung injury, asthma and pulmonary fibrosis. However, the role of activin-A and its endogenous inhibitor, follistatin, in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) is unknown.

Verhamme FM, Bracke KR, Amatngalim GD, Verleden GM, Van Pottelberge GR, Hiemstra PS, Joos GF, Brusselle GG. Role of activin-A in cigarette smoke-induced inflammation and COPD. European Respiratory Journal. 2014; 43: 1028-1041 [337].

ABSTRACT

Objective: Activin-A is a pleiotropic cytokine belonging to the TGF-β superfamily and has been implicated in asthma and pulmonary fibrosis. However, the role of activin-A and its endogenous inhibitor, follistatin, in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) is unkown.

Methods: We first quantified activin-A and follistatin in lungs of air- or CS-exposed mice and in lungs of patients with COPD by immunohistochemistry, ELISA and qRT-PCR. We subsequently studied the effect of CS on primary human bronchial epithelial cells (HBECs) *in vitro*. Next, activin-A signalling was antagonized *in vivo* by administration of follistatin in mice exposed to air or CS for 4 weeks.

Results: Protein levels of activin-A were increased in the airway epithelium of patients with COPD compared with never-smokers and smokers. CS-exposed HBECs expressed higher levels of activin-A and lower levels of follistatin. Both mRNA and protein levels of activin-A were increased in lungs of CS-exposed mice, whereas follistatin levels were reduced upon CS exposure. Importantly, administration of follistatin attenuated the CS-induced increase of inflammatory cells and mediators in the bronchoalveolar lavage fluid in mice.

Conclusions: These results suggest that an imbalance between activin-A and follistatin contributes to the pathogenesis of CS-induced inflammation and COPD.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a chronic respiratory disease with obstruction of the small airways (obstructive bronchiolitis) and destruction of lung parenchyma (emphysema). The major risk factor for COPD is tobacco smoking, but air pollution and occupational exposure to dusts and chemicals are also important contributors together with genetic susceptibility [1]. COPD is a leading cause of morbidity and mortality worldwide [14]. Besides smoking cessation, current treatment of COPD is mainly symptomatic. It is believed that a complex interaction between a dysregulated immune response, oxidative stress, imbalanced proteolytic activity and increased apoptosis leads to the pathology seen in patients with COPD [37;54;338]. To date, the precise molecular mechanisms underlying the pathogenesis of COPD are not fully understood.

Members of the transforming growth factor (TGF)-β superfamily, which include TGF-βs, activins and bone morphogenetic proteins (BMPs) are pleiotropic cytokines regulating fundamental physiological processes and have been linked to numerous diseases [201]. Since TGF-β has a key role in immune regulation and tissue remodelling in COPD, other members of the TGF-β superfamily may also be involved in the pathogenesis of COPD [48;211;221]. Activin-A is a homodimeric protein comprising 2 inhibin βA subunits (INHBA) and signals via a constitutively active activin type II receptor (Act-RIIA and Act-RIIB) that recruits and phosphorylates an activin type I receptor (activin-receptor-like kinases (ALK) -4 and/or -7). The receptor complex propagates the signal by phosphorylating the same intracellular Smads (Smad2 and Smad3) as TGF-β. Phosporylated Smad2/3 subsequently translocate to the nucleus in association with Smad4 to initiate gene transcription [339;340]. Regulation of activin-A signalling is achieved by the endogenous inhibitor, follistatin that binds activin-A with high affinity and blocks the interaction between activin-A and the type II receptor [341]. Follistatin can also bind and inhibit several BMPs (BMP-2, -4, -5, -6, -7, -8) and TGF-β3, although the binding affinity of follistatin is much higher for activin-A than for BMPs [342;343].

Several groups have demonstrated activation of the activin-A signalling pathway in an experimental model of allergic asthma and in patients with asthma [254;261;266;270]. Similarly, patients with acute respiratory distress syndrome or pulmonary fibrosis have higher levels of activin-A in bronchoalveolar lavage (BAL) fluid and lung tissue, respectively, compared to control patients [260;344].

Since activin-A is an important inflammatory mediator in several respiratory diseases, we hypothesized that activin-A plays a significant role in the pathogenesis of COPD. To elucidate this, we investigated the expression of activin-A and follistatin in lungs of never-smokers and smokers with and without COPD and in primary human bronchial epithelial cell (HBEC) cultures exposed to cigarette smoke (CS). Finally, we used a CS-induced mouse model of COPD to examine the *in vivo* functional role of activin-A by administering its endogenous inhibitor follistatin.

MATERIALS AND METHODS

Human study populations

Lung resection specimens were obtained from 100 patients, of which 89 from surgery for solitary pulmonary tumours (Ghent University Hospital, Ghent, Belgium) and 11 from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue of 64 (out of 100) patients, including 11 never-smokers, 24 smokers without airflow limitation and 29 patients with COPD GOLD II was used for mRNA expression analysis **(Table 1)**. Lung tissue of 71 (out of 100) patients, including 10 never-smokers, 26 smokers without airflow limitation and 35 patients with COPD (21 GOLD II, 4 GOLD III and 10 GOLD IV) was used for immunohistochemical analysis **(Table 2)**. Lung tissue of 35 (out of 100) patients was used for both mRNA and immunohistochemical analysis.

Written informed consent was obtained from all subjects. This study was approved by the medical ethical committees of the Ghent University Hospital and the University Hospital Gasthuisberg Leuven.

Activin-A immunohistochemistry and quantification

Paraffin-embedded sections of human lung tissue were subjected to activin-A staining using anti-activin-A antibody as previously described [84]. Immunohistochemical staining with anti-activin-A (R&D Systems, Abingdon, UK) was performed on paraffin sections of the left lung of mice. The area with positive activin-A staining was quantified using KS400 software (Zeiss) and was normalized to the length of the basement membrane.

Phospho-Smad2 immunohistochemistry

Paraffin sections were incubated overnight with primary phospho-Smad2 (Ser465/467) Ab (Cell Signaling Technology, Danvers, USA) or isotype rabbit IgG (Abcam, Cambridge, UK). Next, the slides were incubated with PowerVision poly-horseradish peroxidase-anti-rabbit (Immunovision Technologies, Burlingame, USA) and stained with 3,3'-diaminobenzidine substrate (Dako, Glostrup, Denmark). Finally, sections were rinsed in demineralised water, counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, USA) and mounted in DPX (Klinipath, Duiven, The Netherlands).

Culture of human bronchial epithelial cells

Primary human bronchial epithelial cells (HBECs) were obtained by enzymatic digestion from lung resection specimens obtained from anonymous donors during surgery for lung cancer, as described previously [320]. Cells from passage 2 were cultured at an air-liquid interface in medium containing a high concentration of retinoic acid to induce mucociliary differentiation, as described previously [345].

After the 14-day air-liquid interface culture period, HBECs were exposed to whole volatile smoke or air as a negative control, using a modification of the system reported by Beisswenger and coworkers [321]. In other experiments, HBECs were stimulated both on the apical and basal side with recombinant human activin-A (R&D Systems) or with recombinant human TGF-β1 (R&D Systems) as positive control.

Cigarette smoke exposure

C57BL/6 mice (Charles River Laboratories) were exposed to CS, as described previously [312].

Administration of follistatin

C57BL/6 mice were injected i.p. with 1 µg of recombinant mouse follistatin-288 (R&D Systems) or PBS (controls) [246]. Mice were injected 3 times a week in a subacute (4 weeks) CS experiment, 30 minutes before air or CS exposure.

Statistical analysis

Statistical analysis was performed with Sigma Stat software (SPSS 19.0, Chicago, IL, USA), using Kruskal-Wallis, Mann-Whitney U, Wilcoxon Signed Rank, Fisher's exact test and Spearman correlation analysis. Characteristics of the study population are expressed as median and interquartile range. Pearson correlation and linear regression analysis were performed on log-transformed data. Values from the *in vitro* study with HBECs are reported relatively to the control group. Reported values are expressed as mean ± SEM. Differences at p-values < 0.05 were considered to be significant (*P<0.05, **P<0.01 and *** P<0.001).

RESULTS

Activin-A and follistatin mRNA expression in lungs of patients with COPD

To characterize the expression of activin-A and follistatin in human lung tissue, we extracted mRNA from total lung tissue of a study population containing 11 never-smokers, 24 smokers without airflow limitation and 29 patients with COPD, GOLD II. The characteristics of the different study groups are summarized in **Table 1**. Activin-A is a homodimer of inhibin βA subunits. By measuring the mRNA expression of the inhibin βA subunit (INHBA), we observed higher mRNA levels of activin-A in lung tissue of current smokers both with and without COPD, compared to never-smokers. Moreover, current smokers had significantly higher activin-A mRNA expression, compared to ex-smokers **(Figure 1A)**. The mRNA expression of follistatin **(Figure 1B)** was similar between the different study groups. Taken together, the ratio of activin-A to follistatin is increased in current smokers both with and without COPD in comparison with never-smokers. **(Figure 1C)**.

Table 1: Characteristics of study subjects for lung mRNA analysis (by qRT-PCR)

	never smokers	smokers	COPD II
Number	11	24	29
Age (years)	60 (47-70)	64 (55-71)	65 (59-69)
Gender (m/f)	3/8 #	18/6 #	29/0 #
Current-smoker/Ex-smoker	NA	12/12 °	17/12 °
Pack-years	NA	28 (14-49) †	45 (40-60) †*
FEV_1 (% predicted)	104 (92-119)	96 (92-113)	69 (63-74) †§
FEV_1/FVC (%)	77 (75-80)	76 (73-78)	56 (54-60) †§
ICS (yes/no)	0/11 °	1/23 °	12/17 °

Footnote

m (male); f (female); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids); NA (not applicable)

Data are presented as median (IQR)

Mann-Whitney U test: † P<0.001 vs never-smokers, * P<0.01 vs smokers, § P<0.001 vs smokers

Fisher's exact test: ° P<0.01 and # P<0.001

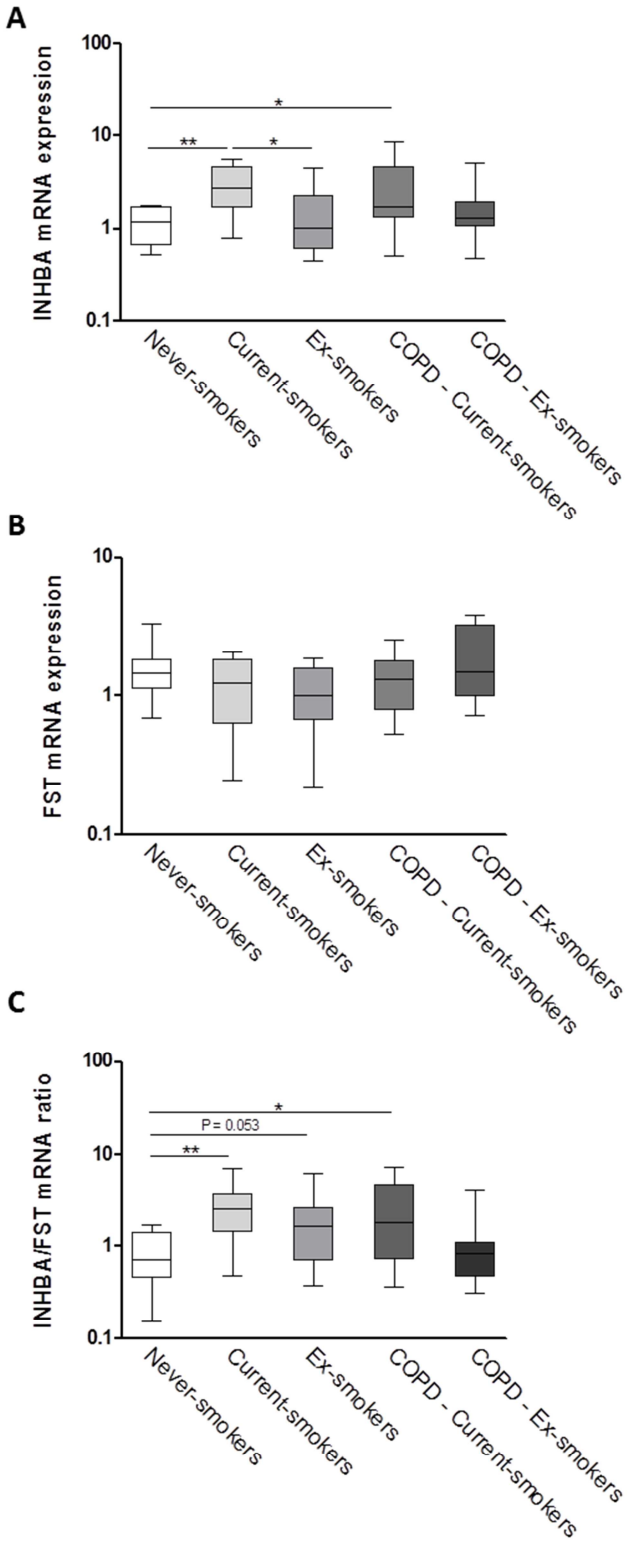


Figure 1. Pulmonary mRNA expression of INHBA (activin-A) and follistatin in human subjects. mRNA levels of activin-A (INHBA: inhibin βA subunit) **(A)** and follistatin **(B)** in total lung of never-smokers (N = 11) , current-smokers (N = 12), ex-smokers (N = 12), current-smokers with COPD (N = 17) and ex-smokers with COPD (N = 12), as measured by qRT-PCR. In **(C)** the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, PPIA, HPRT-1). Data are expressed as box-and whisker plots (*P<0.05 and **P<0.01).

Increased activin-A signalling in airway epithelium of patients with COPD

In order to localize pulmonary activin-A signalling, we performed immunohistochemical staining for activin-A and phospho-Smad2 on lung tissue sections of never-smokers, smokers and patients with COPD GOLD II-IV. The demographic, clinical and lung functional characteristics of the study subjects are presented in **Table 2**. **Figure 2** shows representative lung sections stained for activin-A in never-smokers **(Figure 2A)**, smokers **(Figure 2B)**, patients with COPD GOLD II **(Figure 2C)** and patients with COPD GOLD IV **(Figure 2D and 2F)**. We observed positive staining for activin-A in bronchiolar epithelial cells with a minor staining of smooth muscle cells and alveolar macrophages in the lungs of never-smokers **(Figure 2A)**. Using imaging analysis software, we quantified the activin-A positive staining in the airway epithelium. Protein levels of activin-A in the airway epithelium were significantly increased in patients with COPD GOLD II and GOLD III-IV, compared to never-smokers and smokers without airflow limitation **(Figure 2G)**. The expression of activin-A in the airway epithelium is significantly associated with disease severity, expressed as post-bronchodilator FEV_1 (% predicted) and FEV_1/FVC (%) (data not shown). After adjustment for various confounders, the association with FEV_1 remained significant (data not shown). After semi-quantitative scoring, we also demonstrated higher scores for activin-A in the airway smooth muscle cell layer and alveolar macrophages of patients with COPD compared to never smokers (data not shown).

Table 2: Characteristics of study subjects for immunohistochemical study

	never smokers	smokers	COPD II	COPD III-IV
Number	10	26	21	14
Age (years)	59 (51-70)	57 (51-65)	66 (60-70)	52 (50-59) ¤
Gender (m/f)	2/8 #	20/6 #	20/1 #	10/4 #
Current-smoker/Ex-smoker	NA	13/13 °	12/9 °	3/11 °
Pack-years	NA	24 (15-36) †	50 (40-61) †*	30 (25-39) †~
FEV_1 (% predicted)	103 (93-118)	102 (90-113)	68 (59-74) †§	25 (24-42) †§¶
FEV_1/FVC (%)	76 (75-82)	76 (72-80)	56 (53-60) †§	36 (32-42) †§¶
ICS (yes/no)	0/10 #	0/26 #	7/14 #	13/1 #

Footnote

m (male); f (female); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids); NA (not applicable)

Data are presented as median (IQR)

Mann-Whitney U test: † P<0.001 vs never-smokers, * P<0.01 vs smokers, § P<0.001 vs smokers, ~ P<0.05 vs GOLD II, ¤ P<0.01 vs GOLD II and ¶ P<0.001 vs GOLD II

Fisher's exact test: ° P<0.01 and # P<0.001

We evaluated the phosphorylation status and nuclear accumulation of Smad2 as a hallmark for activated activin-A signalling. Almost no positive nuclear staining was present in the airway epithelial cells of never smokers and smokers without COPD **(Figure 2H-I)**, whereas, nuclei of airway epithelial cells of patients with COPD stained strongly **(Figure 2J-M)**. Importantly, there was a significant, positive correlation between the semi-quantitative levels of p-Smad2 and activin-A in the airway epithelium (data not shown).

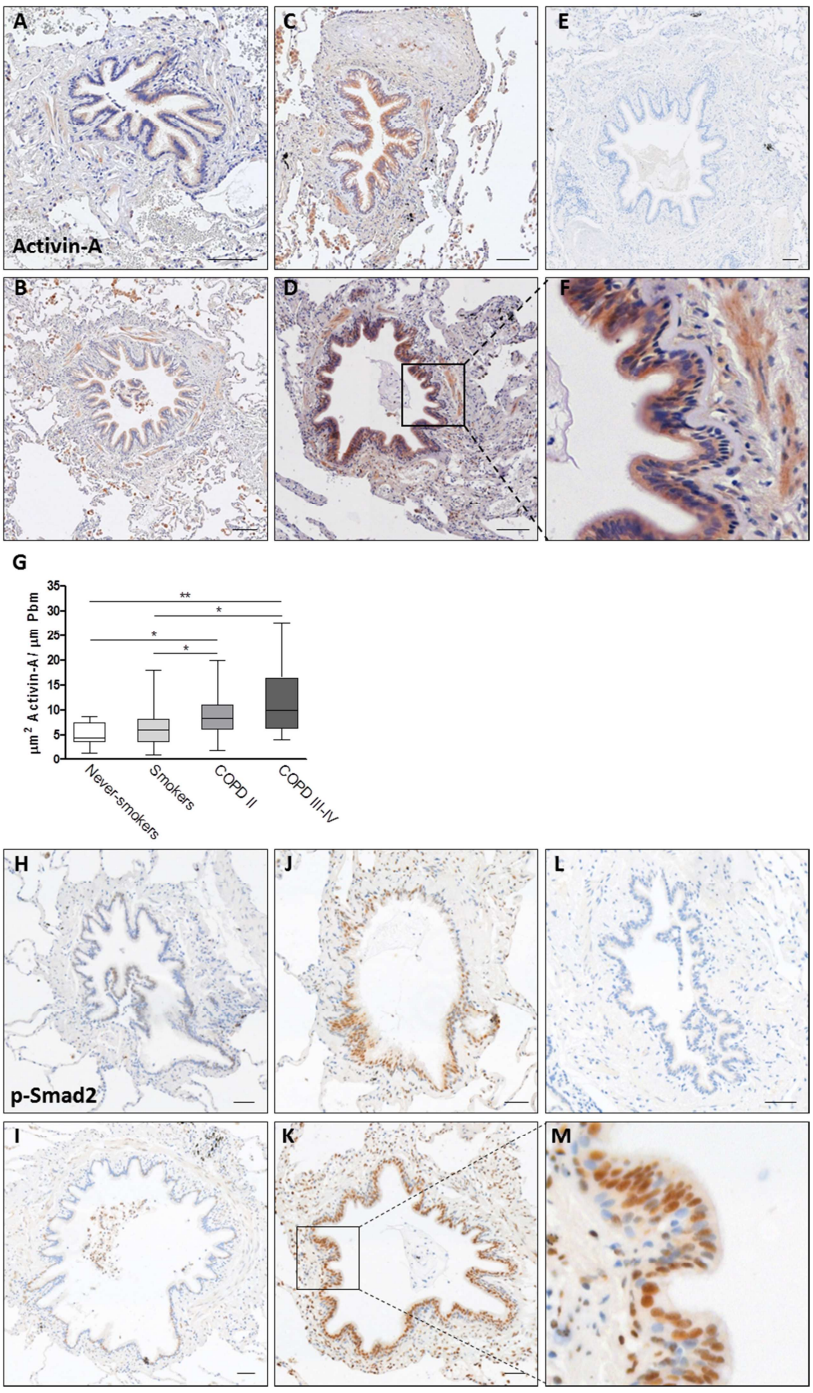


Figure 2. Pulmonary activin-A and phospho-Smad2 expression in human lungs by immunohistochemistry.

Representative photomicrographs for activin-A of human lung sections of a never-smoker **(A)**, smoker without airflow limitation **(B)**, patient with COPD GOLD II **(C)** and patient with COPD GOLD IV **(D)**. Isotype control is presented in **(E)**. **(F)** is a detailed picture from **(D)**. Quantification of activin-A protein expression in airway epithelium of never-smokers (N = 10), smokers (N = 26) and patients with COPD GOLD II (N = 21) and COPD GOLD III-IV (N = 14) is shown in **(G)**. Results are expressed as the epithelial area positive for activin-A normalized to the length of the basement membrane (Pbm). Data are expressed as box-and whisker plots (*P<0.05, **P<0.01).

Representative photomicrographs for p-Smad2 of human lung sections of a never-smoker **(H)**, smoker without airflow limitation **(I)**, patient with COPD GOLD II **(J)** and patient with COPD GOLD IV **(K)**. Isotype control is presented in **(L)**. **(M)** is a detailed picture from **(K)**. Original magnification 200x. Scale bar = 100 µm.

Cigarette smoke exposure increases expression of activin-A in human bronchial epithelial cells

To confirm the results seen in patients with COPD, primary human bronchial epithelial cells (HBECs), grown *in vitro* at air-liquid interface, were exposed to mainstream CS. HBECs and basal media were harvested 3, 6 and 24 hours after CS exposure. RT-PCR analysis on mRNA extracted from HBECs revealed a significant increase in expression of activin-A 3, 6 and 24 hours after CS exposure, compared to air-exposed cells **(Figure 3A)**. The follistatin mRNA expression was slighty lower in CS-exposed HBECs **(Figure 3B)**. This resulted in a significantly increased ratio of activin-A to follistatin in CS-exposed HBECs compared to air-exposed HBECs **(Figure 3C)**. Accordingly, the release of activin-A in the basal medium from CS-exposed HBEC cultures was augmented, especially 24 hours after CS exposure **(Figure 3D)**, whereas the release of follistatin was decreased after CS exposure **(Figure 3E)**. In conclusion, the ratio of activin-A to follistatin was significantly increased in basal medium of CS-exposed HBECs 24 hours after CS exposure **(Figure 3F)**. These data show that expression and release of activin-A in response to CS is enhanced in human HBECs.

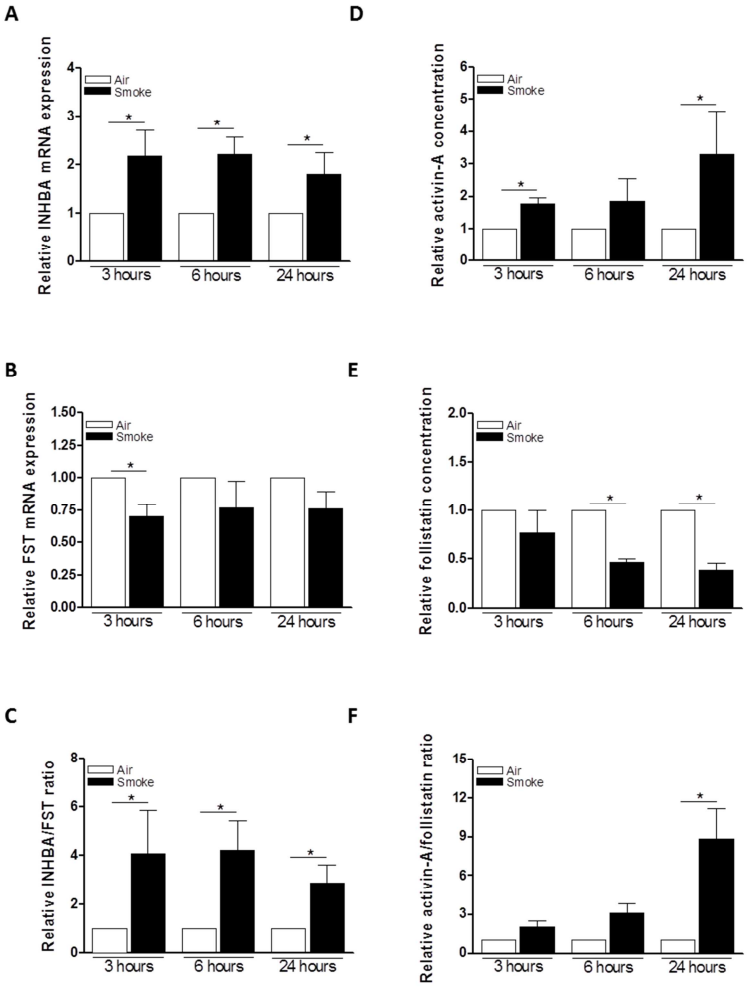


Figure 3. Expression of activin-A and follistatin in air-liquid interface human bronchial epithelial cell (HBEC) cultures upon exposure to air or cigarette smoke. Basal media and cells were harvested 3, 6 and 24 hours after exposure. mRNA from HBECs was isolated and analyzed for the expression of INHBA (inhibin βA subunit of activin-A) **(A)**, FST (follistatin) **(B)** by RT-PCR. In **(C)** the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of two reference genes (RPL13A, ATP5B). Protein levels of activin-A **(D)** and follistatin **(E)** in the basal medium were analyzed by ELISA. In **(F)** the ratio of activin-A to follistatin protein is shown. Data are reported relatively to the air-exposed group. Results are depicted as mean ± SEM of 5 different experiments each performed in duplicate and using cells from different donors (*P<0.05).

Increased pulmonary expression of activin-A upon CS exposure in mice

As previously shown by our group [312], exposure of C57BL/6 mice to CS for 4 weeks or 24 weeks lead to inflammation in the bronchoalveolar lavage compartment and lung tissue, characterized by increased numbers of macrophages, neutrophils, dendritic cells and B and T lymphocytes. The effect of CS on the pulmonary expression of activin-A and follistatin was determined in total lung tissue by quantitative RT-PCR and immunohistochemistry and in BAL fluid supernatant by ELISA. Activin-A mRNA expression in lung tissue was significantly increased upon chronic CS exposure, compared to air-exposed littermates **(Figure 4A)**, while CS significantly downregulated follistatin mRNA expression **(Figure 4B)**. Importantly, immunohistochemistry on lung tissue revealed activin-A staining in airway epithelium **(Figure 4H)**, which was increased upon chronic (24 weeks) CS exposure **(Figure 4D)**, similar to our observations in patients with COPD. Activin-A protein levels were increased significantly in BAL fluid at all timepoints, with an especially marked increase after 24 weeks CS exposure **(Figure 4E)**. In contrast, the protein levels of follistatin in BAL fluid were similar between air- and CS-exposed mice **(Figure 4F)**. Taken together, the ratio of activin-A to follistatin was increased after CS exposure at all timepoints, both on mRNA and protein level **(Figure 4C and 4G)**.

To determine if activin-A levels persist after smoke cessation, we analyzed protein levels of activin-A and numbers of inflammatory cells in BAL fluid of C57BL/6 mice on day 1, 14 and 56 following subacute exposure to air or CS **(Figure 5)**. CS-induced inflammation (monocytes, dendritic cells, $CD4^+$ T lymphocytes) was mostly resolved on day 14 *post*-exposure, except for neutrophils and $CD8^+$ T lymphocytes **(Figure 5B-D)**. Inflammation in the BAL fluid was completely cleared 56 days after exposure **(Figure 5)**. Consistent with the inflammation data, there was a notable persistence of activin-A in the BAL fluid on day 14, whereas by day 56, activin-A levels were restored to normal **(Figure 5A)**.

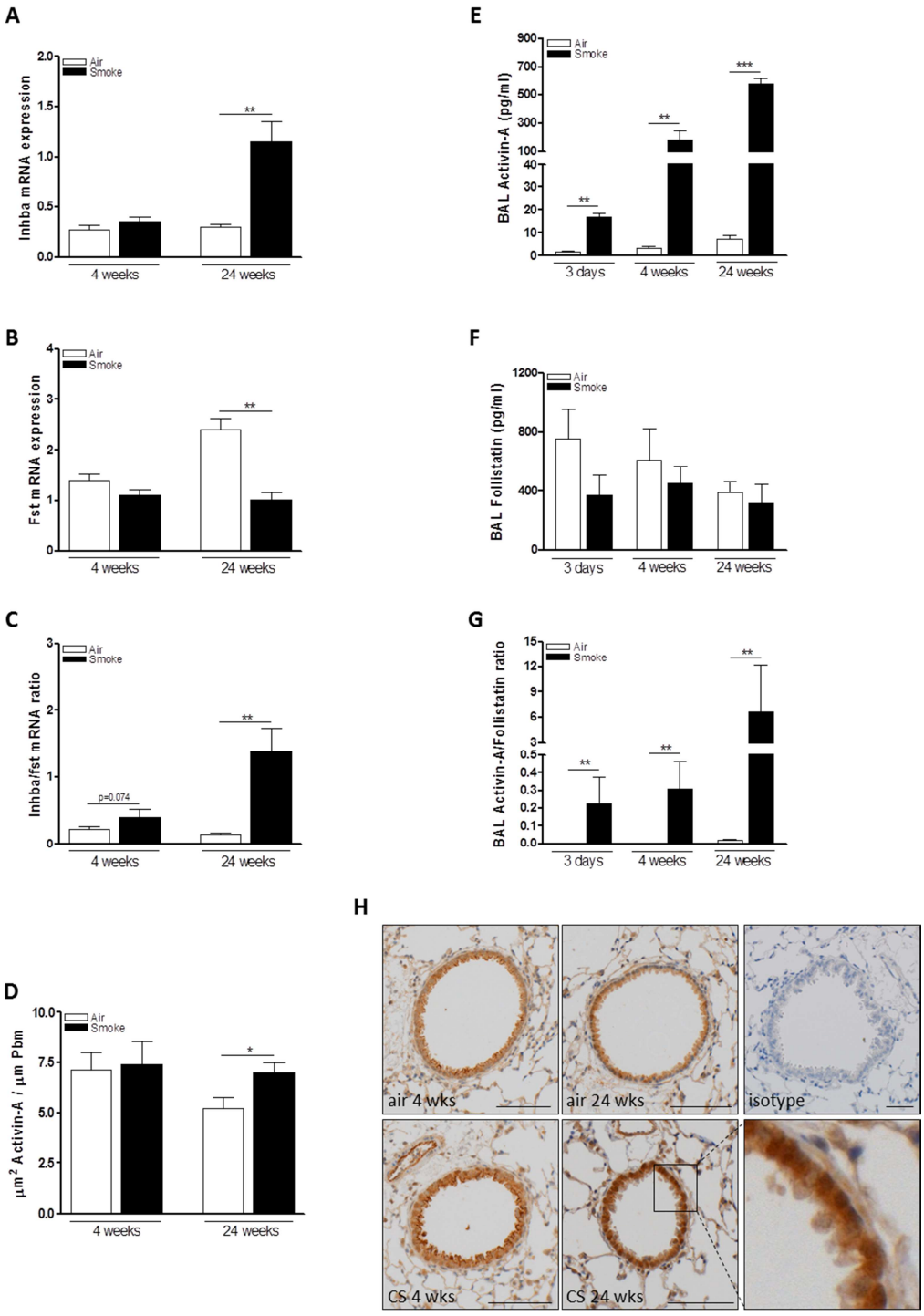


Figure 4. Expression of activin-A and follistatin in C57BL/6 mice upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) exposure to air or cigarette smoke. mRNA levels of Inhba (inhibin βA subunit of activin-A) **(A)** and follistatin **(B)** in total lung, as measured by qRT-PCR. In **(C)** the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, TFRC, HPRT-1). Quantification of activin-A protein expression in airway epithelium of mice upon exposure to air or CS is shown in **(D)**. Results are expressed as the epithelial area positive for activin-A normalized to the length of the basement membrane (Pbm). **(H)** Representative images of activin-A-stained lung sections of air- and CS-exposed mice. Original magnification 200x. Scale bar = 100 µm. Protein levels of activin-A **(E)** and follistatin **(F)** in bronchoalveolar lavage (BAL) fluid measured by ELISA. In **(G)** the ratio of activin-A to follistatin protein is shown. Data are expressed as mean ± SEM (N = 8-10 animals/group; *P<0.05, **P<0.01 and *** P<0.001).

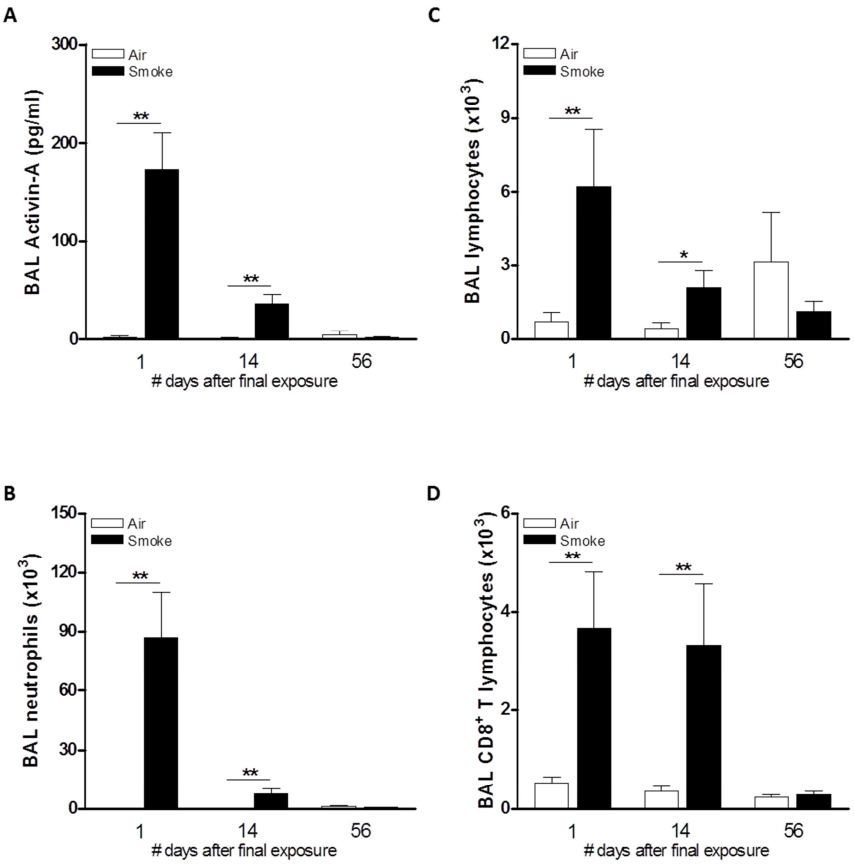


Figure 5. Pulmonary expression of activin-A and pulmonary inflammation in C57BL/6 mice on day 1, 14 and 56 after subacute exposure to air or cigarette smoke. Protein levels of activin-A **(A)** in bronchoalveolar lavage (BAL) fluid measured by ELISA. **(B-D)** Cell differentiation in bronchoalveolar lavage (BAL) fluid. Neutrophils **(B)**, lymphocytes **(C)** were counted on cytospins and $CD8^+$ T lymphocytes **(D)** were enumerated by flow cytometry. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01).

CS-induced inflammation in BAL is attenuated upon neutralization of activin-A by follistatin

To elucidate whether activin-A is involved in the pulmonary inflammation upon CS exposure, we exposed C57BL/6 mice to CS for 4 weeks (subacute exposure) and injected the mice i.p. 3 times a week with 1 μg follistatin or PBS. In PBS treated mice, exposure to CS significantly increased the total number of BAL cells and absolute numbers of monocytes, neutrophils and $CD4^+$ and $CD8^+$ T lymphocytes in BAL fluid **(Figure 6A-F)**. In contrast, follistatin treatment significantly attenuated the accumulation of monocytes and $CD8^+$ T lymphocytes in CS-exposed mice **(Figure 6B, 6D and 6F)**.

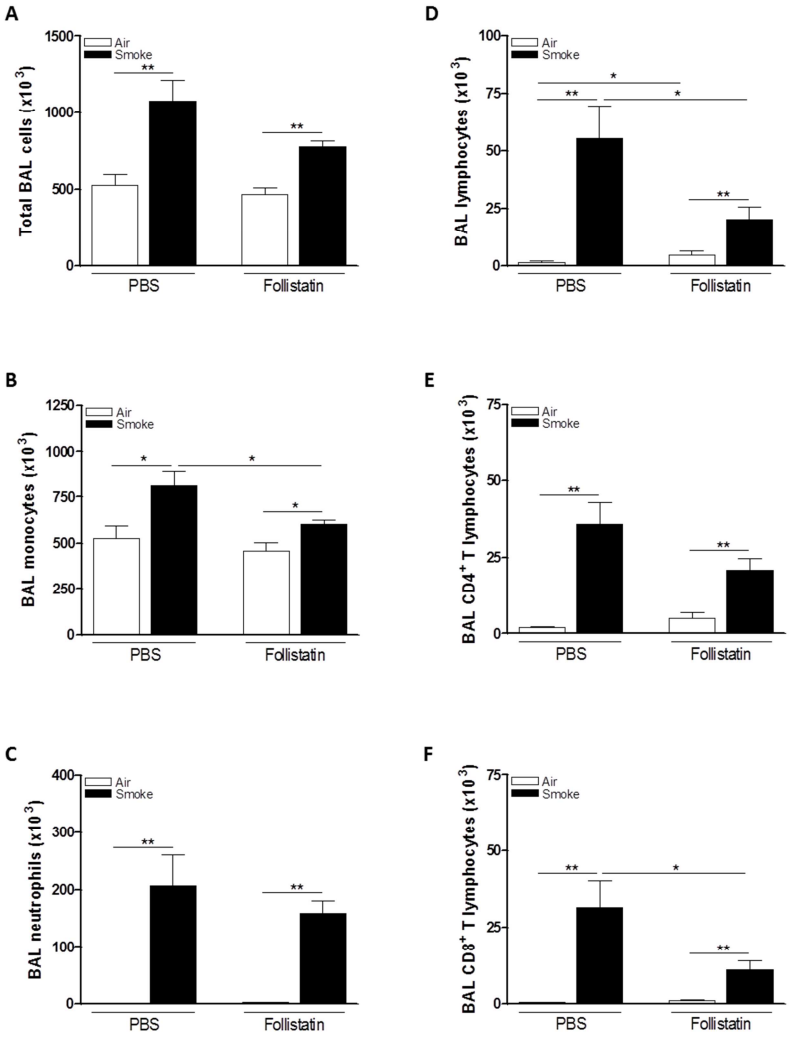


Figure 6. Effect of CS exposure and neutralization of activin-A with follistatin on the total number of bronchoalveolar lavage (BAL) cells and cell subsets in BAL fluid (A-F). Total BAL cells **(A)**, monocytes **(B)**, neutrophils **(C)**, lymphocytes **(D)**, $CD4^+$ T lymphocytes **(E)** and $CD8^+$ T lymphocytes **(F)** in C57BL/6 mice upon 4 weeks exposure to air or CS, injected intraperitoneally 3 times a week with follistatin or PBS. All cell types were enumerated by cytospin counts, except for the $CD4^+$ and $CD8^+$ T lymphocytes which were determined by flow cytometry. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01).

Follistatin affects cytokine and chemokine expression upon CS exposure

Because we observed a decreased inflammatory cell recruitment in BAL of follistatin treated mice upon 4 weeks CS exposure, we measured cytokines IL-6, TNFα and IL-10 and chemokines MCP-1 and KC in BAL fluid by cytometric bead array. In PBS treated mice, CS exposure significantly increased the protein levels of IL-6, TNFα, MCP-1 and KC. In follistatin treated mice, the increase of IL-6, MCP-1 and KC was significantly attenuated, compared to PBS treated mice **(Figure 7A and 7D-E)**. Follistatin treated mice showed a trend towards an attenuation of the CS-induced increase of TNFα in BAL fluid (P=0.061; **Figure 7B**). In contrast, IL-10 protein levels did not increase upon CS exposure in PBS-treated mice, while follistatin treatment significantly increased IL-10 protein expression upon CS **(Figure 7C)**.

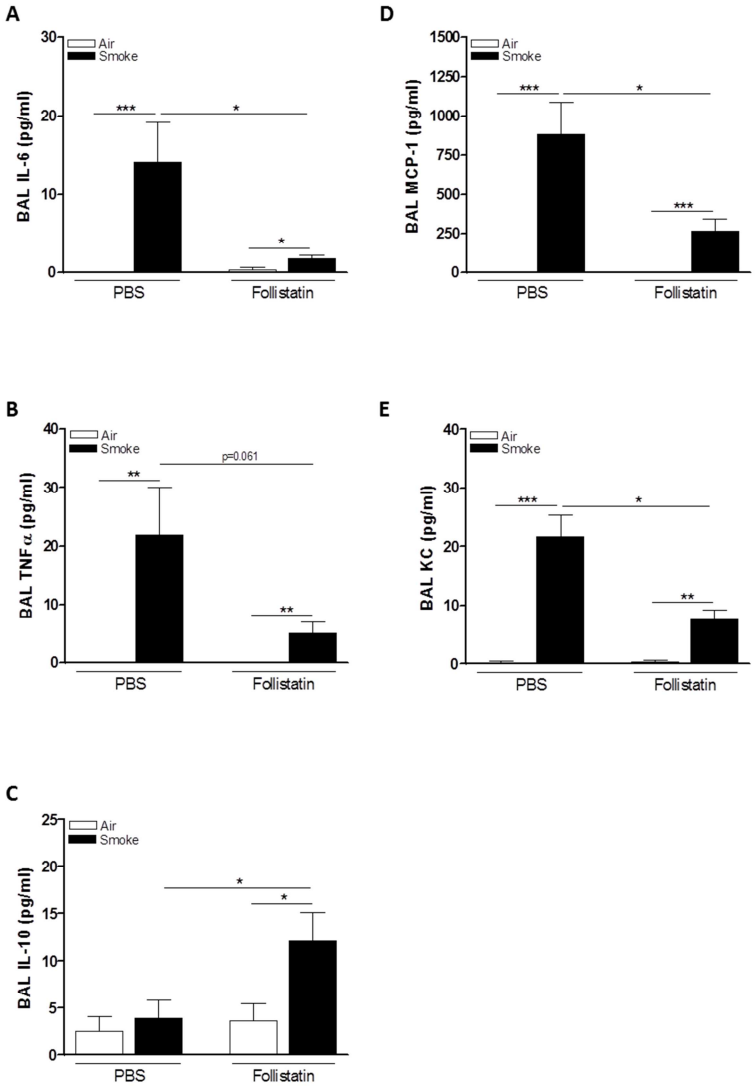


Figure 7. Inflammatory cytokines and chemokines in the bronchoalveolar lavage (BAL) fluid of C57BL/6 mice upon 4 weeks exposure to air or CS, injected i.p. 3 times a week with follistatin or PBS. IL-6 **(A)**, TNFα **(B)**, IL-10 **(C)** MCP-1 **(D)** and KC **(E)** protein levels, measured by cytometric bead array. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01 and *** P<0.001).

Follistatin reduces CS-induced pulmonary activin-A/TGF-β signalling in mice

We determined activin-A and TGF-β1 protein levels in BAL fluid of PBS and follistatin treated mice by ELISA. In follistatin treated mice, the CS-induced increase of activin-A and TGF-β1 was significantly attenuated, compared to PBS treated mice **(Figure 8A-B)**. In contrast, the mRNA expression of activin-A was not influenced by the follistatin treatment **(Figure 8D)**. Using ELISA, we also quantified the phosphorylation state of Smad2 as a marker of active signalling. CS exposure induced a significant increase of p-Smad2 in the lung homogenates of PBS-treated mice, which was strongly suppressed in follistatin-treated mice **(Figure 8C)**. These results indicate that follistatin was effective as an antagonist for activin-A.

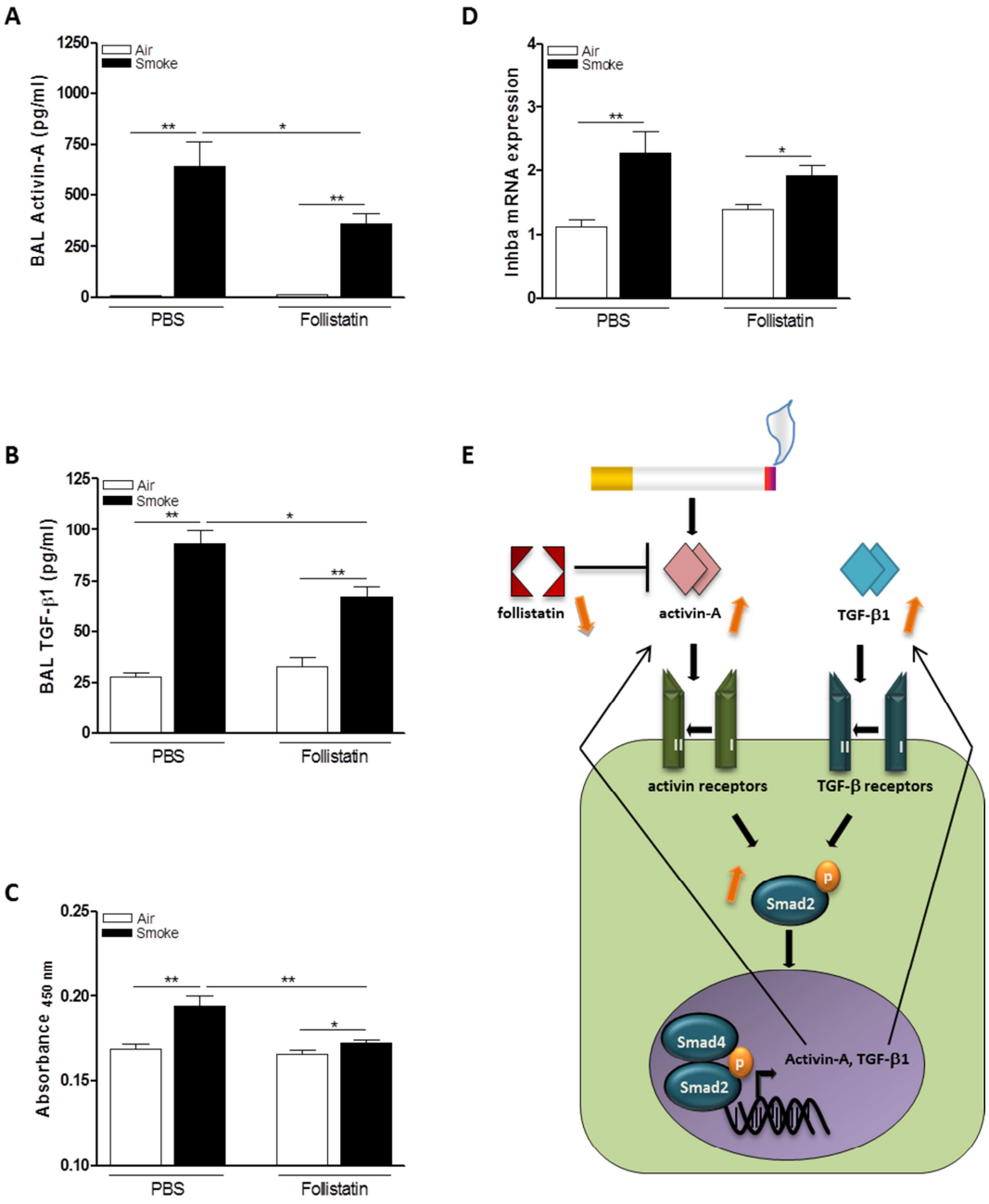


Figure 8. Activin-A/TGF-β signalling in the bronchoalveolar lavage (BAL) fluid (A-B) and lungs (C) of C57BL/6 mice upon 4 weeks exposure to air or CS, injected with follistatin or PBS. Activin-A **(A)**, TGF-β1 **(B)** and phosphorylated-Smad2 (**C**-expressed as raw optical density values at 450 nm) levels were quantified by ELISA. Activin-A (Inhba: inhibin βA subunit) mRNA levels **(D)** were measured using qRT-PCR. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05 and **P<0.01). In **(E)** a schematic overview of the effect of cigarette smoke (CS) exposure on pulmonary activin-A signalling is shown. CS induces activin-A expression in airway epithelial cells, but attenuates its endogenous antagonist follistatin. Activin-A binds to type II receptors on the surface of target cells, which oligomerize with type I receptors. The activated type I receptor phosphorylates intracellular protein Smad2, which forms a complex with Smad4 and translocates to the nucleus to initiate gene expression of target genes such as TGF-β1, which signals through the same Smad proteins. TGF-β1 in turn can induce the expression of activin-A, creating a positive feedback loop mechanism. TGF-β1: transforming growth factor-β1, I: activin type I receptors, II: activin type II receptors, P: phosphorylated form.

DISCUSSION

In this study, we demonstrate a marked role for activin-A in CS-induced inflammation and COPD using a combination of *ex vivo* studies on human subjects, *in vitro* studies on CS-exposed primary human bronchial epithelial cell (HBEC) cultures and *in vivo* studies using CS-exposed mice. We show for the first time increased levels of activin-A in the lungs of CS-exposed mice and patients with COPD. Moreover, we can mimic this *in vitro* by exposing HBEC cultures to CS. Neutralizing activin-A with follistatin, its natural inhibitor, in a mouse model of subacute CS exposure, attenuates inflammation in the bronchoalveolar lavage fluid.

We demonstrated a higher activin-A mRNA expression in the lungs of current smokers irrespective of airflow limitation, suggesting a CS-driven effect, rather than an effect of the disease itself. In addition, at the protein level, epithelial activin-A was solely augmented in patients with COPD and not in smokers without airflow limitation, implicating a disease effect. The discrepancy between mRNA and protein levels may be explained by epigenetic mechanisms and/or differences in translational control that contribute to the translation of mRNA into protein. Also in the CS-driven murine model of COPD, we revealed increased levels of activin-A in BAL fluid and lung tissue. Importantly, activin-A levels, together with neutrophil and $CD8^+$ T lymphocyte counts, were still elevated 14 days after final CS exposure, suggesting that activin-A release is not merely due to an acute CS effect. After CS cessation, neutrophil counts and activin-A levels dropped more rapidly than $CD8^+$ T lymphocytes. The persistence of $CD8^+$ T lymphocytes in the airways can be explained by the recruitment of effector T cells which are generated in the draining lymph nodes after stimulation with airway-derived DCs.

Since the increase in activin-A in patients with COPD and CS-exposed mice is not accompanied by an up-regulation of follistatin, there is a relative excess of activin-A, unopposed by follistatin. Similarly, there is evidence from several studies that activin-A is increased in pulmonary diseases such as pulmonary fibrosis and asthma, but also in other non-pulmonary inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis [244;251;260;261]. Accordingly, in airway epithelium of patients with COPD phosphorylation of Smad2, a downstream mediator of activin-A, was elevated. However, we should keep in mind that Smad2 can also be activated by other ligands of the TGF-β superfamily and that other non-canonical pathways (e.g. NF-κB, …) can propagate the activin-A signal [346].

To support the finding that epithelial cells produce activin-A upon CS exposure, we exposed primary human bronchial epithelial cells (HBECs) *in vitro* to CS. *In vitro*, it has been shown that CS can down-regulate the TGF-β pathway in bronchial epithelial cells [347]. However, we demonstrated that mRNA levels of activin-A increase upon CS exposure while follistatin expression is hardly influenced. Furthermore, CS exposure stimulates the secretion of activin-A and inhibits the secretion of follistatin, resulting in a relative excess of activin-A. Interestingly, activin-A is also increased in cultured airway epithelial cells challenged with human rhinovirus, which is frequently associated with COPD exacerbations [348].

In accordance with the data in patients with COPD, activin-A was increased in the lungs and BAL fluid of mice exposed to CS. To elucidate whether activin-A is an innocent bystander or plays an active role in pulmonary inflammation upon CS, we blocked activin-A by administering follistatin in CS-exposed mice. We demonstrated a significant attenuation of the CS-induced increase of $CD8^+$ T lymphocytes in the BAL fluid, which is the predominant T cell population in patients with COPD [88]. Strikingly, the secretion of several chemokines (MCP-1 and KC) and cytokines (IL-6 and TNFα) was reduced in follistatin treated CS-exposed mice compared to the PBS treated group. Interestingly, the secretion of IL-10, an anti-inflammatory cytokine is increased upon follistatin treatment in CS-exposed mice. CS exposure induced increased regulatory T cell numbers in the lung and in the draining lymph nodes, however this was not affected by follistatin treatment (data not shown) [326].

This is the first study exploring the potential of blocking activin signalling to inhibit inflammation in a CS-driven murine model. Our *in vivo* data are in line with experimental data in animal models of other respiratory diseases, showing that the pro-inflammatory effects of activin-A can be blocked in an *in vivo* settting. Exogenous follistatin reduced the number of macrophages and neutrophils in BAL fluid of a bleomycin-induced rat model of pulmonary fibrosis [253]. Furthermore, neutralization of endogenous activin-A with ActRIIB-Fc protein reduced the acute lung injury-like pathology in LPS-instilled mice, diminishing neutrophilic inflammation and IL-6 protein levels in BAL [344]. Several reports studied blockade of the activin-A pathway as a treatment for allergic asthma, which resulted in conflicting outcomes. In one study, intranasal administration of follistatin reduced ovalbumin-induced Th2 immune responses in mediastinal lymph nodes of mice, while another study showed that systemic depletion of activin-A by a neutralizing antibody exacerbated ovalbumin-induced asthmatic disease [266;269]. These last papers suggest that the route of administration of follistatin may influence the outcome. In our study set-up – systemic administration of follistatin by intraperitoneal injection – it is not possible to differentiate between a local or extrapulmonary effect.

An intriguing aspect of activin-A is that - like TGF-β – it has both pro- and anti-inflammatory activities depending on the type of tissue and injury [235]. A pro-inflammatory activity is suggested by the capability of activin-A to skew the macrophage polarization towards an M1 phenotype [240]. In contrast, activin-A is known to stimulate the development of FoxP3$^+$ regulatory T cells [243]. The current hypothesis is that activin-A exerts a pro-inflammatory effect early on in the course of inflammation, but once the inflammatory response has been established, activin-A may stimulate anti-inflammatory effects [234]. Overall, our data suggest that activin-A promotes the pulmonary inflammation after CS. Accordingly, when we stimulated HBECs with increasing doses of activin-A for 24 or 48 hours, a dose-dependent, but not significant increase of IL-1β, TGF-β1 and IL-8 mRNA expression was observed, together with an enhanced secretion of IL-8 (data not shown).

One of the strengths of our study is its translational character. Observations made *ex vivo* in patients with COPD were confirmed *in vitro* in HBEC cultures and *in vivo* in CS-exposed mice. A limitation is that only pulmonary inflammation was investigated which is only one aspect of the complex pathogenesis of COPD. Accumulating data suggest that activin-A is also involved in airway remodelling. *In vitro* results indicate that activin-A can stimulate proliferation of human lung fibroblasts and smooth muscle cells [262;267]. Moreover blocking activin-A inhibited collagen deposition and thickening of the subepithelial smooth muscle layer in two different murine models of allergic asthma [273;274]. Similarly, it would be helpful to study the relation between activin-A and peribronchial fibrosis in patients with COPD. In the murine model of COPD it is practically not feasable to study the effect of activin-A silencing on airway remodelling using a pharmacological approach (follistatin or a neutralizing antibody), since it is only after 24 weeks of CS exposure that airway wall remodelling appears [312]. Interestingly, we demonstrated that follistatin treatment suppresses protein levels of the pro-fibrotic cytokine TGF-β1 in BAL supernatant of CS-exposed mice. Since follistatin does not antagonize TGF-β1, the attenuated TGF-β1 levels are likely the result of reduced activin-A based stimulation of TGF-β1 secretion [261]. Importantly, TGF-β1 can also stimulate activin-A expression, inducing a positive feedback loop [261]. In **Figure 8E** we schematically show the effect of CS exposure on activin-A signalling in the lungs.

In conclusion, these data demonstrate that activin-A is increased in the airway epithelium of patients with COPD and in the lungs of CS-exposed mice. Also, *in vitro* we confirmed that exposing HBEC cultures to CS modulates the delicate balance between activin-A and follistatin. Moreover, our *in vivo* studies in a CS-driven murine model indicate that activin-A contributes to the pathogenesis of CS-induced pulmonary inflammation.

[CHAPTER 8: MODULATION OF PULMONARY INFLAMMATION AND IRON HOMEOSTASIS BY BONE MORPHOGENETIC PROTEIN 6 IN COPD]

Bone morphogenetic protein (BMP)-6 is a member of the TGF-β superfamily. An association between the BMP-6 locus and forced vital capacity has been found in a genome-wide association study. However, the role of BMP-6 in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) is unknown.

Verhamme FM, Bracke KR, Delanghe J, Verleden GM, Joos GF, Brusselle GG. Modulation of pulmonary inflammation and iron homeostasis by bone morphogenetic protein 6 in COPD.
Manuscript in preparation.

ABSTRACT

Rationale: Chronic Obstructive Pulmonary Disease (COPD) is associated with abnormal inflammatory responses and airway wall remodelling, leading to reduced lung function. An association between the bone morphogenetic protein (BMP-6) locus and forced vital capacity has been found in a genome-wide association study. However, the role of BMP-6 in the pathogenesis of COPD remains unknown.

Methods: The expression and localization of BMP-6 was analyzed in lungs of never smokers, smokers without COPD and patients with COPD and in lungs of air- or cigarette smoke (CS)-exposed mice, by qRT-PCR and immunohistochemistry. Next, we exposed BMP-6 KO mice and control littermates to air or CS for 4 (subacute) or 24 weeks (chronic) and evaluated pulmonary inflammation, airway wall remodelling and iron homeostasis.

Results: mRNA levels of BMP-6 were decreased in lungs of smokers with and without COPD compared with never smokers and in lungs of CS-exposed mice. Importantly, BMP-6 mRNA expression declined with disease severity. Expression of BMP-6 protein was observed in airway smooth muscle cells, endothelial cells and pulmonary macrophages by immunohistochemistry. After subacute CS exposure, the CS-induced increase in macrophage and neutrophil numbers in BAL fluid was aggravated in BMP-6 KO mice, compared to control littermates. Accordingly, the CS-induced increase in MCP-1 and KC mRNA levels, chemokines attracting monocytes and neutrophils, and protein levels of TGF-β1, were higher in BMP-6 deficient mice. After chronic CS exposure, however, there was no difference in pulmonary inflammation and airway wall remodelling between WT and BMP-6 KO mice. Finally, BMP-6 KO mice develop massive iron overload in the lungs.

Conclusions: These results suggest that down-regulation of BMP-6 - as observed in lungs of smokers and patients with COPD - contributes to pulmonary inflammation after subacute CS exposure, but appears less important in chronic CS-induced inflammation and remodelling.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by a progressive and not fully reversible airflow limitation that is associated with an enhanced chronic inflammatory response in the airways and the lung. The major risk factor for COPD is tobacco smoking. Pathology of COPD includes obstruction of the small airways (obstructive bronchiolitis) and destruction of lung parenchyma (emphysema). The diagnosis of COPD is made using spirometry. A ratio of the Forced Expiratory Volume in one second (FEV_1) to the Forced Vital Capacity (FVC) below 0.70 determines the presence of airflow limitation [1].

COPD results from a complex interplay between genetic susceptibility and environmental exposures. The genetics underlying COPD are currently unknown, although recent studies have identified interesting genetic loci associated with impaired lung function [3;4]. In a recent meta-analysis of genome-wide association studies by the CHARGE and SpiroMeta consortia six new loci were identified that were associated with FVC, including the gene encoding bone morphogenetic protein (BMP)-6 [3].

BMP-6 is a member of the transforming growth factor (TGF)-β superfamily, which includes TGF-βs, activins, BMPs and growth differentiation factors (GDFs) [349]. The BMP subfamily was initially identified as a family of proteins that induce ectopic cartilage and bone formation, but during the last years, data has accumulated demonstrating the involvement of BMPs in development and homeostasis in various organs [350;351]. BMP-6 is essential for iron homeostasis, as it is an important regulator of hepcidin, a hormone that controls iron export [279;280]. An increased iron sequestration has been observed in macrophages of patients with COPD, which correlated positively with emphysema severity, suggesting that iron dysregulation might contribute to oxidative stress [352]. Whether BMP-6 is simply a non-specific bystander or whether BMP-6 actively contributes to disease pathology, remains to be elucidated.

We hypothesized that BMP-6 contributes to the pathogenesis of COPD by modulating pulmonary inflammation, airway wall remodelling and iron homeostasis. First, we determined the expression of BMP-6 in the lungs of smokers and patients with COPD and in the lungs of mice exposed to CS [312]. Next, we exposed BMP-6 KO mice and control littermates to CS for 4 weeks (subacute exposure) and 24 weeks (chronic exposure). We analyzed the inflammatory responses in both bronchoalveolar lavage (BAL) and lung tissue and quantified the extent of airway wall remodelling. Finally, we studied the effect of BMP-6 deficiency on CS-induced changes in content, storage and transport of iron.

MATERIALS AND METHODS

Human study populations

Lung resection specimens were obtained from 84 patients, of which 70 had surgery for solitary pulmonary tumours (Ghent University Hospital, Ghent, Belgium) and 14 underwent lung transplantation for end-stage COPD (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumour invasion was collected by a pathologist. None of the patients operated for malignancy were treated with neo-adjuvant chemotherapy.

COPD diagnosis and severity were defined according the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification, using pre-operative spirometry [1]. Written informed consent was obtained from all subjects. This study was approved by the medical ethical committees of the Ghent University Hospital and the University Hospital Gasthuisberg Leuven.

Animals

Male C57Bl/6Jx129Sv BMP-6 knockout (KO) (*Bmp6*^{m1rob}) mice were mated with female C57Bl/6J mice in the animal facility at the Faculty of Medicine and Health Sciences, Ghent University (Ghent, Belgium). Genotyping of the offspring was performed on toe DNA following a standard procedure, as already described [353]. From the offspring, male BMP-6 KO mice and their WT littermates were used for the present study. C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in sterilized cages with filter tops under 12h light/dark cycle and *ad libitum* access to food (Pavan, Brussels, Belgium) and chlorinated tap water. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences, Ghent University.

Cigarette smoke exposure

Mice were exposed to CS, as described previously [312]. Briefly, animals were exposed whole body to mainstream CS of 5 cigarettes (Reference Cigarette 3R4F without filter; University of Kentucky) 4 times a day with 30-min smoke free intervals, for 4 weeks (subacute), 12 weeks or 24 weeks (chronic). The control groups were exposed to room air. An optimal smoke/air ratio of 1/6 was obtained. 24 hours after the last exposure, mice were sacrificed by an intraperitoneal (i.p.) injection of pentobarbital (CEVA-Sanofi, Paris, France).

Collection of serum

Blood was sampled from the orbital sinus and processed for the collection of serum.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as previously described [225]. Briefly, lungs were first lavaged using 3 times 300 µl HBSS, without Ca^{2+} and Mg^{2+} and supplemented with 1% BSA (for protein measurement), followed by 3 times 1 ml HBSS without Ca^{2+} and Mg^{2+} supplemented with 0.6 mM EDTA, via a tracheal cannula. The lavage fractions were pooled. Total BAL counts were obtained using a Bürker chamber.

Lung harvest and preparation of single-cell suspensions

Following BAL, the pulmonary and systemic circulation was rinsed with saline, supplemented with 5 mM EDTA. The left lung was used for histology, as previously described [312]. The right lung was clamped and removed for RNA extraction (small lobe), preparation of lung homogenates (middle lobe) and preparation of single-cell suspensions (major lobe). For the latter, the lung tissue was thoroughly minced, digested and subjected to red blood cell lyses. After passage through a 50 µm cell strainer, cells were counted with a Z2 particle counter (Beckman-Coulter, Inc., Fullerton, CA, USA) [312].

Flow cytometry

Cells (both BAL and lung single-cell suspensions) were first incubated with FcR blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce non-specific binding. Secondly, labelling reactions were performed to identify the macrophages, inflammatory monocytes, inflammatory neutrophils and lymphocytes, using the following Abs: APC conjugated CD11c (clone HL3), PE conjugated MHC class II (clone AF6-120.1), biotin conjugated CD11b (clone M1/70), FITC conjugated Ly6C (clone AL -21), PE conjugated Ly6G (clone 1A8), FITC conjugated CD4 (clone GK1.5), FITC or PE conjugated CD8 (clone 53-6.7), APC conjugated CD3 (clone 145-2C11) and PE conjugated CD69 (H1.2F3). All reactions were performed on ice in FACS-EDTA buffer. All monoclonal Abs were purchased from BD Biosciences (San Diego, CA, USA). The macrophages were identified as the $CD11c^{bright}$, high autofluorescent cell population, as described by Vermaelen and Pauwels [328]. Inflammatory monocytes were defined as $CD11c^{-}CD11b^{+}Ly6G^{-}Ly6C^{+}$ and inflammatory neutrophils as $CD11c^{-}CD11b^{+}Ly6G^{+}Ly6C^{+}$. T lymphocytes were characterized as small, CD3+, which are further separated in $CD4^{+}$ and $CD8^{+}$ T lymphocytes in BAL and lung and add CD69 as activation marker for the lung T lymphocytes. Finally, samples were incubated with 7-Amino-actinomycin D for exclusion of dead cells (BD Biosciences).

Flow cytometry data acquisition was performed on a dual-laser FACS Calibur™ flow cytometer running CellQuest™ software (BD Biosciences). FlowJo Software (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

Additionally, macrophages populations were sorted from lung $CD11c^+$ cells (after enrichment with anti-CD11c microbeads and passage through a VarioMACS magnetic cell separator) and total BAL cells on a FACS Vantage with a Sort Enhancement Module, according to the gating strategy detailed above [336].

Preparation of lung tissue homogenate

The middle lobe of the right lung was snap-frozen (in liquid nitrogen) and stored at -80°C until further analysis. The lobes were transferred to tubes containing 1 ml RIPA buffer (Cell Signaling Technology, Danvers, USA) containing Halt™ Protease Inhibitor Cocktail Kit (Thermo Scientific, Waltham, MA, USA) and homogenized on ice using TissueRuptor (Qiagen, Hilden, Germany). The homogenates were sonicated (4 times for 5 seconds) and centrifuged (14000 rpm for 10 minutes at 4°C) and the middle layer was transferred to microcentrifuge tubes. Total protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Lung tissue homogenates were diluted with RIPA buffer to a final protein concentration of 500 μg/ml.

ELISA

We determined BMP-6 levels in lung homogenate using a commercially available kit (USCNK Life Science Inc, Hubei, China). TGF-β1 and ferritin protein levels in the BAL fluid were determined by an ELISA kit purchased from R&D Systems (Abingdon, UK) for TGF-β1 and from Abcam (Cambridge, UK) for ferritin. All ELISAs were performed following the manufacturer's instructions.

BMP-6 immunohistochemistry and quantification

To evaluate BMP-6 expression in lung tissue (both human and mice), paraffin-embedded sections were subjected to BMP-6 staining using an anti-BMP-6 antibody (Abcam). 3μm thick paraffin embedded sections were cut on poly-L-lysin coated slides. After dewaxing with Ultra Clear (Klinipath, Duiven, The Netherlands) and rehydrations, antigen retrieval was performed using citrate-buffer. After blocking for endogenous peroxidase activity and application of Fc-block, slides were incubated with rabbit monoclonal anti-BMP-6 or isotype rabbit IgG (Abcam) during 24h, followed by incubation with PowerVision poly-horseradish peroxidase-anti-rabbit (Immunovision Technologies, Burlingame, USA). After rinsing with PBS containing 0.3% Triton X-100, diaminobenzidine substrate (Dako, Glostrup, Denmark) was added for 30 minutes, sections were rinsed in demineralised water, counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, USA), dehydrated and mounted in DPX (Klinipath, Duiven, The Netherlands).

Photomicrographs were taken of all airways and the area of the airway smooth muscle layer was marked manually on the digital presentation of the airways using Axiovision software (Zeiss, Oberkochen, Germany) and was normalized to the total bronchial wall area (WAt). All airways with a Pbm larger than 500 μm and smaller than 2000 μm and cut in reasonable cross sections are included. Quantitative measurements are performed in a blinded fashion.

Quantification of airway wall remodelling

To determine the degree of airway remodelling, the deposition of collagen and fibronectin in the airway wall is measured on lung sections (left lung) using staining with Sirius Red or anti-fibronectin antibody [319]. The following morphometrical parameters are marked manually on the digital representation of the airway: the length of the basement membrane (Pbm), the area defined by the basement membrane (Abm) and the area defined by the total adventitial perimeter (Ao). The total bronchial wall area (WAt) is calculated as WAt = Ao – Abm and normalized to the squared Pbm. The area in the total airway wall covered by the stain is determined by the software (Axiovision). The area of collagen or fibronectin is normalized to Pbm. All airways with a Pbm smaller than 2000 μm and cut in reasonable cross sections are included. Quantitative measurements are performed in a blinded fashion.

RNA extraction and real-time PCR-analysis

Total RNA was extracted from isolated macrophages and from total lung tissue (human and mice) with the miRNeasy Mini kit (Qiagen). cDNA was obtained by the Transcriptor First Strand cDNA synthesis kit (Roche, Basel, Switzerland), following manufacturer's instructions. Expression of target genes BMP-6, CCL2, CXCL1, Id1, hepcidin and reference genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase-1 (HPRT-1) and Peptidylpropyl isomerase 1 (PPIA) were analyzed using Taqman Gene Expression Assays (Applied Biosystems, Forster City, CA, USA). Real-time PCR reactions were set up in duplicate using diluted cDNA using identical amplification conditions for each of the target and reference genes. A standard curve derived from serial dilutions of a mixture of all samples were included in each run. The amplification conditions consisted of: 10 minutes at 95°C and 50 cycles of 95°C for 10 seconds and 60°C for 15 seconds. Amplifications were performed using a LightCycler 480 detection system (Roche). Data were processed using the standard curve method. Expression of target genes was corrected by a normalization factor that was calculated based on the expression of two or three reference genes, using the geNorm applet according to the guidelines and theoretical framework previously described (http://medgen.ugent.be/~jvdesomp/genorm/) [322].

Quantification of iron concentration

The iron concentration in lung homogenates and serum was assayed using a ferrozine-based colorimetric method on a Cobas 8000 analyzer (Roche).

Statistical analysis

Statistical analysis was performed with Sigma Stat software (SPSS 19.0, Chicago, IL, USA), using Kruskal-Wallis, Mann-Whitney U and Fisher's exact test. Characteristics of the study population are expressed as median and interquartile range. Reported values are expressed as mean ± SEM. Differences at p-values < 0.05 were considered to be significant (*P<0.05, **P<0.01 and *** P<0.001).

RESULTS

BMP-6 expression and localization in lungs of patients with COPD

To characterize the expression of BMP-6 in human lung tissue, we extracted mRNA from lung tissue of 84 subjects (16 never-smokers, 24 smokers without airflow limitation, 30 patients with COPD GOLD II and 14 patients with COPD GOLD III-IV). The demographic and clinical characteristics of the different study groups are summarized in **Table 1**. The mRNA levels of BMP-6 are significantly lower in smokers without COPD and patients with COPD compared to never-smokers. Moreover, the BMP-6 mRNA expression was significantly lower in severe COPD compared to smokers without COPD **(Figure 1A)**. Interestingly, immunohistochemical staining on human lung tissue sections revealed strong positive staining for BMP-6 in airway smooth muscle cells, endothelial cells and pulmonary macrophages, as well as a weak staining in airway epithelial cells **(Figure 1B-E)**.

Table 1: **Characteristics of study subjects for lung mRNA analysis (by qRT-PCR)**

	never smokers	smokers	COPD II	COPD III-IV
Number	16	24	30	14
Age (years)	64 (51-71)	65 (55-71)	65 (59-69)	56 (54-60) $^{\bullet ç ¶}$
Gender (m/f)	3/13$^{\#}$	19/5$^{\#}$	29/1$^{\#}$	8/6$^{\#}$
Current-smoker/Ex-smoker	0/0$^{\#}$	12/12$^{\#}$	17/13$^{\#}$	0/14$^{\#}$
Pack-years	NA	33 (15-50) †	45 (40-60) $^{\dagger ç}$	30 (25-30) $^{\dagger ¶}$
FEV_1 (% predicted)	110 (92-118)	96 (92-113)	69 (64-74) $^{\dagger §}$	23 (20-33) $^{\dagger §¶}$
FEV_1/FVC (%)	78 (75-83)	76 (73-78)	56 (53-60) $^{\dagger §}$	32 (27-35) $^{\dagger §¶}$
ICS (yes/no)	0/16$^{\#}$	1/23$^{\#}$	13/17$^{\#}$	13/1$^{\#}$

Footnote

m (male); f (female); FEV_1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids); NA (not applicable)

Data are presented as median (IQR)

Mann-Whitney U test: $^{\bullet}$ P<0.05 vs never-smokers, † P<0.001 vs never-smokers, ^{ç} P<0.05 vs smokers, § P<0.001 vs smokers and ¶ P<0.001 vs GOLD II

Fisher's exact test: $^{\#}$ P<0.001

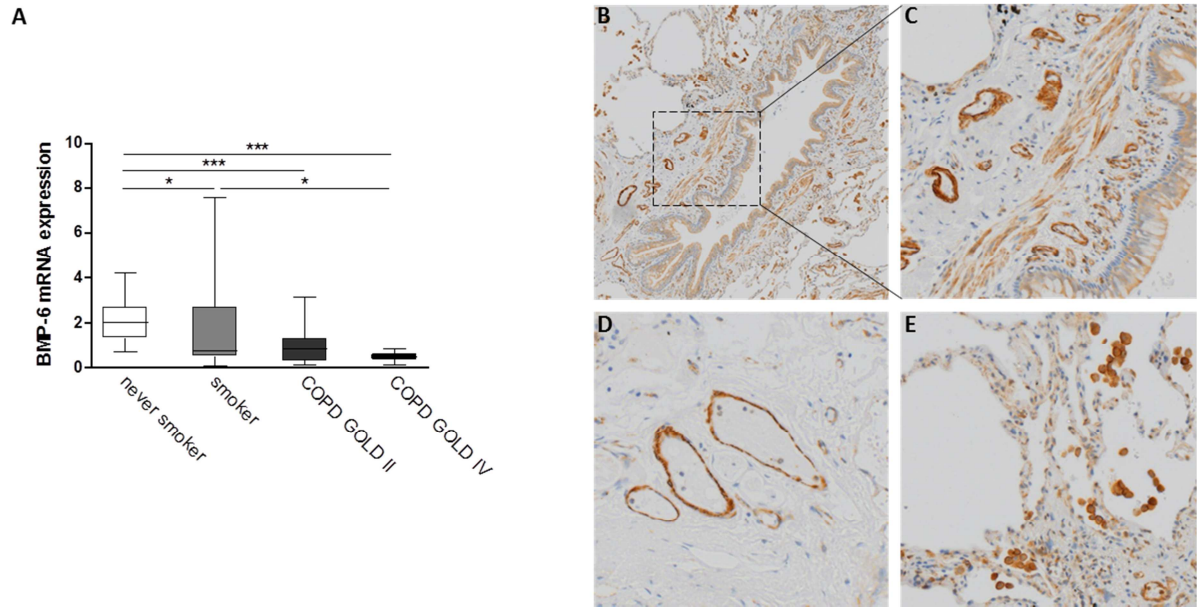


Figure 1. Pulmonary mRNA expression and localization of BMP-6 in human subjects. mRNA levels of BMP-6 **(A)** in lung of never-smokers (N = 16) , smokers without airflow limitation (N = 24), patients with COPD GOLD II (N = 30) and patients with COPD GOLD III-IV (N = 14), as measured by qRT-PCR. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, PPIA, HPRT-1). Data are expressed as box-and whisker plots (*P<0.05 and ***P<0.001). In **(B-E)** representative photomicrographs of immunohistochemical staining for BMP-6 of lung sections of a patient with COPD (GOLD II) is shown. Detail on **C)** airway smooth muscle cells and bronchial epithelial cells, **D)** endothelial cells and **E)** alveolar macrophages. Original magnification 200x.

BMP-6 expression and localization in lungs of CS-exposed mice

The effect of CS on the pulmonary expression of BMP-6 was determined in lung tissue by quantitative RT-PCR, ELISA and immunohistochemistry. mRNA expression of BMP-6 was significantly decreased in lung tissue of mice exposed to CS for 24 weeks, compared with air-exposed controls. Accordingly, the expression of Id1, a target gene of BMP-6 signalling, was also reduced in mice exposed to CS **(Figure 2A-B)**. Immunohistochemistry on murine lung tissue demonstrated BMP-6 staining in airway smooth muscle cells, endothelial cells and pulmonary macrophages, similar to our observations in human lung tissue. **Figure 2E-F** shows representative lung sections stained for BMP-6 in air-exposed mice **(Figure 2E)** and CS-exposed mice **(Figure 2F)**. Using imaging analysis software, we quantified the BMP-6 positive staining in the airway smooth muscle cell layer. No differences in protein levels of BMP-6 in the airway smooth muscle cells were found after 24 weeks of CS exposure **(Figure 2D)**. In fluorescence-activated cell sorted lung macrophages from CS-exposed mice, BMP-6 expression was significantly lower compared to lung macrophages from the air-exposed control group **(Figure 2C)**.

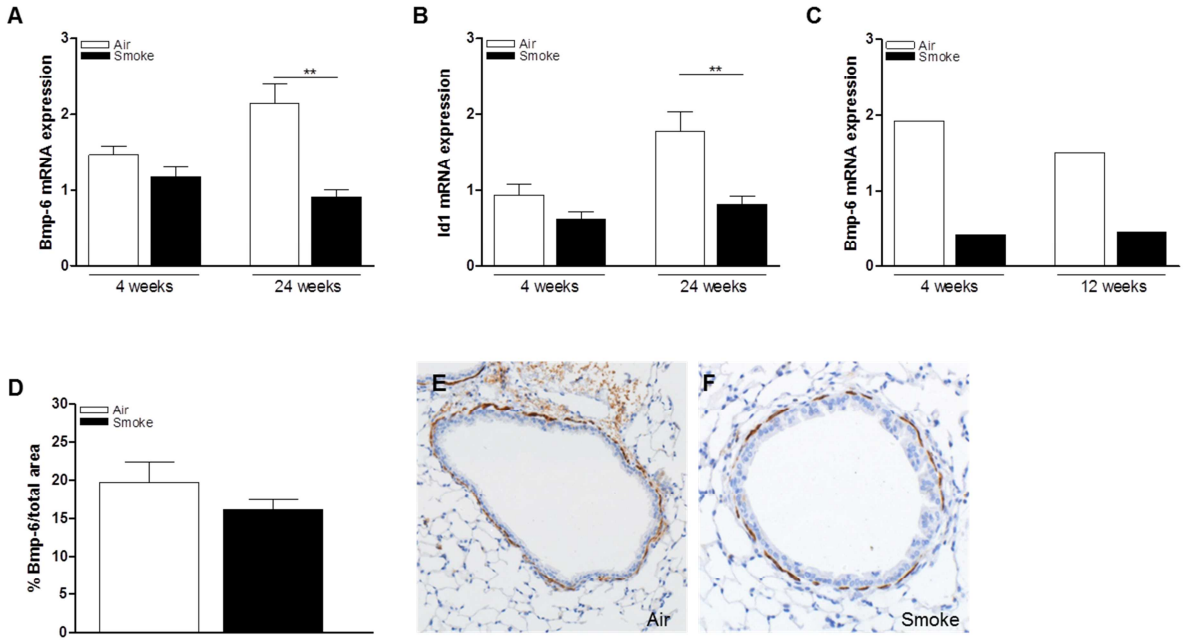


Figure 2. Pulmonary mRNA expression and localization of BMP-6 in cigarette smoke- exposed C57BL/6 mice. mRNA levels of Bmp-6 **(A)** and Id1 **(B)** in lung of C57BL/6 mice exposed to CS for 4 and 24 weeks, as measured by qRT-PCR. mRNA levels of Bmp-6 **(C)** in macrophages isolated from lung tissue of C57BL/6 mice exposed to CS for 4 and 24 weeks. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, TFRC, HPRT-1). Representative images of BMP-6-stained lung sections of **(E)** air- and **(F)** CS-exposed mice. Quantification of BMP-6 protein expression in airway smooth muscle cell layer of mice upon exposure to air or CS for 24 weeks is shown in **(D)**. Results are expressed as the airway smooth muscle cell area positive for BMP-6 normalized to the total airway wall (total area). Data are expressed as mean ± SEM (N = 8-10 animals/group; **P<0.01).

Pulmonary inflammation is increased in BMP-6 KO mice after 4 weeks CS exposure, but not after 24 weeks CS

The role of BMP-6 in CS-induced pulmonary inflammation was evaluated by exposing BMP-6 KO and control WT mice to CS for 4 (subacute) and 24 weeks (chronic exposure). Exposure to CS significantly increased the absolute numbers of total BAL fluid cells, macrophages, neutrophils, monocytes and $CD4^+$ and $CD8^+$ T lymphocytes **(Figure 3A-F)**. After subacute CS exposure, the CS-induced increase in total cells, macrophages, neutrophils, monocytes and $CD4^+$ T cells in the BAL fluid was significantly aggravated in BMP-6 KO mice, compared to control littermates. In contrast, BMP-6 deficiency had no effect on the CS-induced inflammation in BAL after chronic exposure **(Figure 3)**.

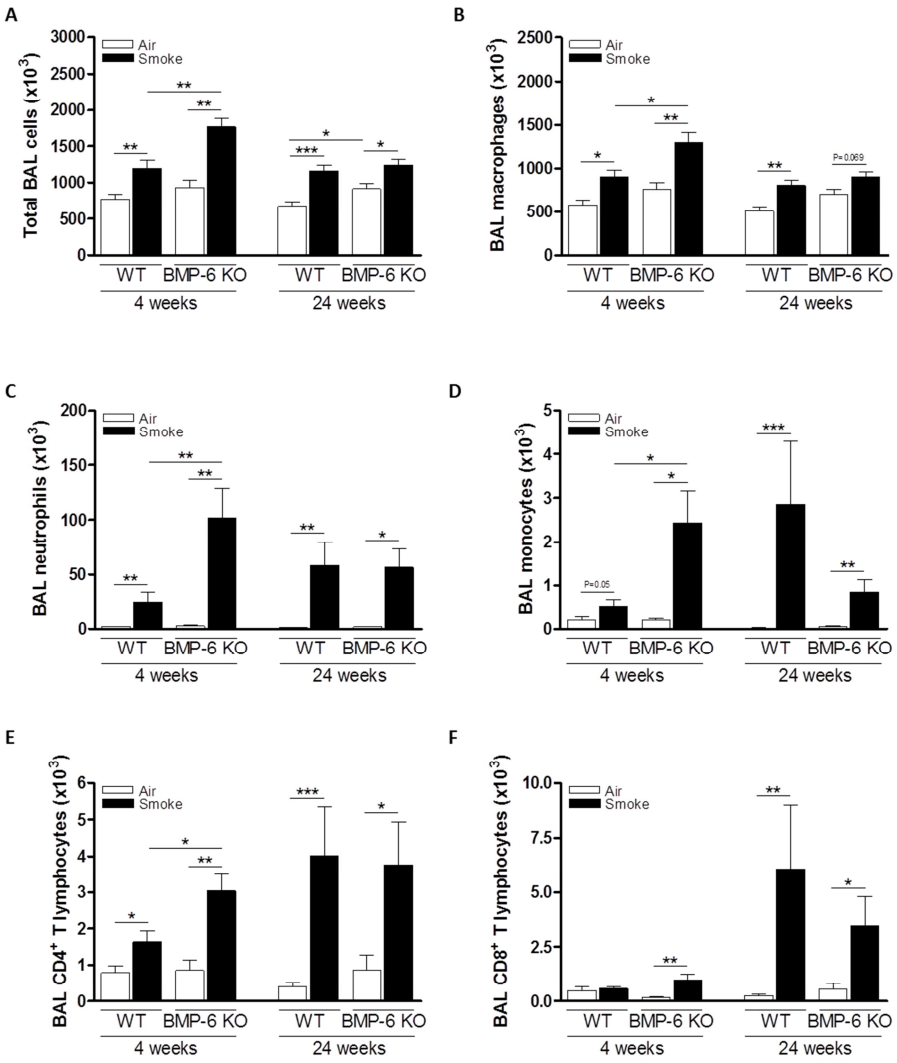


Figure 3. Effect of CS exposure and BMP-6 deficiency on the total number of bronchoalveolar lavage (BAL) cells and cell subsets in BAL fluid. Total BAL cells **(A)**, macrophages **(B)**, neutrophils **(C)**, monocytes **(D)**, $CD4^+$ T lymphocytes **(E)** and $CD8^+$ T lymphocytes **(F)** in bronchoalveolar lavage fluid of WT and BMP-6 deficient mice upon 4 and 24 weeks exposure to air or CS. All cell types were enumerated by flow cytometry. Data are expressed as mean ± SEM (N = 8-12 animals/group; *P<0.05, **P<0.01, ***P<0.001).

In lung homogenates, only macrophages numbers are significantly increased after CS exposure at both timepoints in WT mice **(Figure 4A)**. After 4 weeks of CS, BMP-6 KO mice displayed higher numbers of activated CD4+ T lymphocytes, compared to the control group **(Figure 4B)**, whereas the other cell types were not different between the 2 mouse strains **(Figure 4A and 4C)**. Similar to the BAL fluid, the CS-induced inflammation in lung tissue was not altered by the genotype after 24 weeks exposure **(Figure 4).**

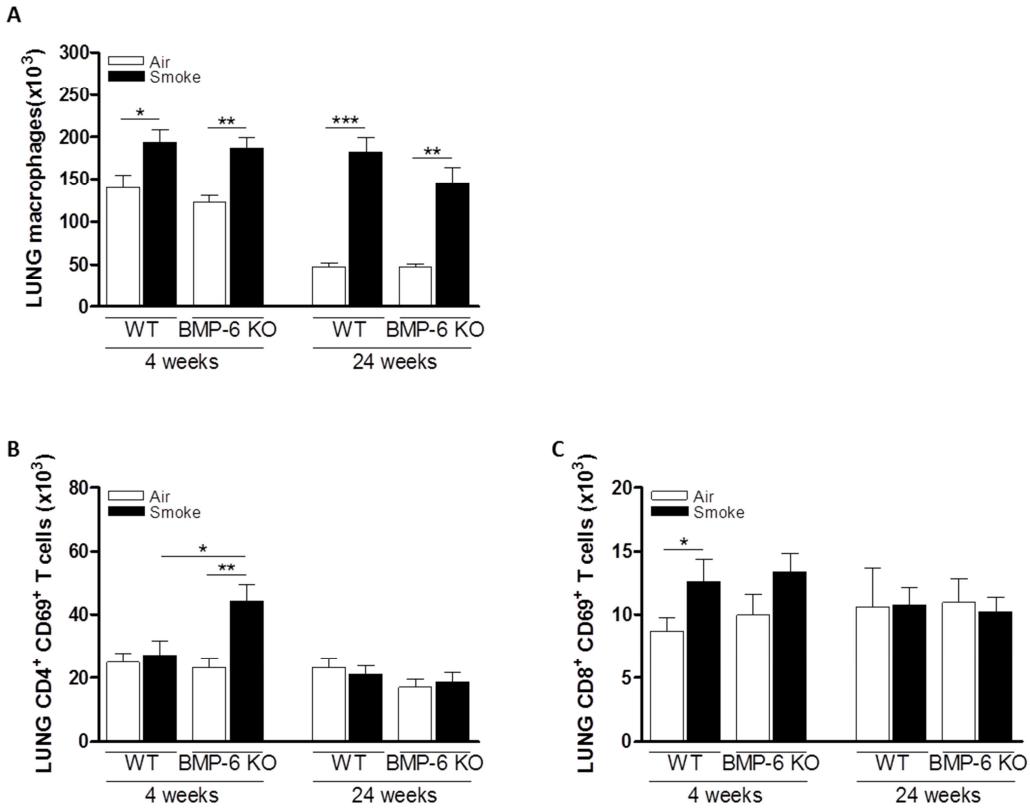


Figure 4. Effect of CS exposure and BMP-6 deficiency on inflammatory cell numbers in lung tissue. Macrophages **(A)**, $CD4^+$ $CD69^+$ T lymphocytes **(B)** and $CD8^+$ $CD69^+$ T lymphocytes **(C)** in lung tissue of WT and BMP-6 deficient mice upon 4 and 24 weeks exposure to air or CS. All cell types were enumerated by flow cytometry. Data are expressed as mean ± SEM (N = 8-12 animals/group; *P<0.05, **P<0.01, ***P<0.001).

Effect of BMP-6 deficiency on cytokine/chemokine levels after 4 weeks of CS exposure

Because we observed an increased inflammatory cell recruitment in BMP-6 KO mice upon 4 weeks CS exposure, we measured the pulmonary mRNA expression of the chemokines CCL2 and CXCL1, which attract monocytes and neutrophils, respectively. While CS exposure had no influence on the expression in WT mice, the mRNA levels of CCL2 and CXCL1 were significantly higher in CS-exposed BMP-6 KO mice, compared to both air-exposed BMP-6 KO mice and CS-exposed WT mice **(Figure 5A-B)**. Furthermore, the CS-induced increase in protein levels of TGF-β1 was significantly aggravated in BMP-6 KO mice **(Figure 5C)**.

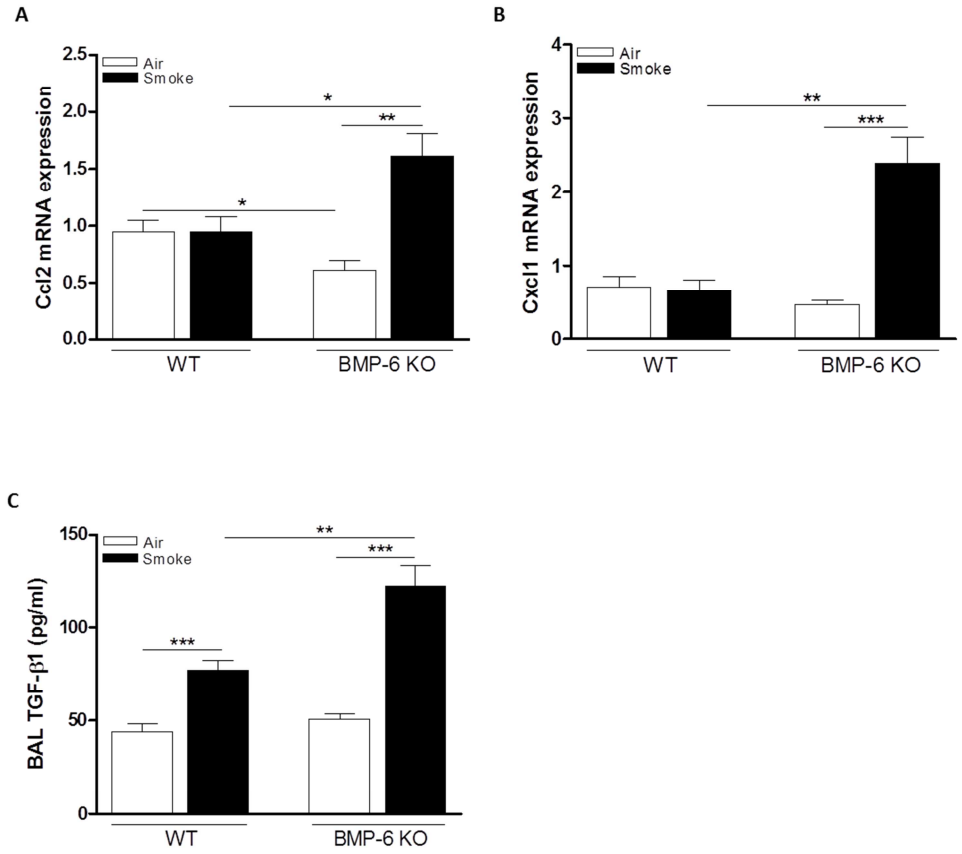


Figure 5. Effect of CS exposure and BMP-6 deficiency on chemokines in lung tissue and TGF-β1 in BAL supernatants. CCL2 **(A)** and CXCL1 **(B)** mRNA expression in lung tissue of WT and BMP-6 deficient mice upon 4 weeks exposure to air or CS. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, TFRC, HPRT-1). Protein levels of TGF-β1 **(C)** in BAL supernatants of WT and BMP-6 deficient mice upon 4 weeks exposure to air or CS, as measured by ELISA. Data are expressed as mean ± SEM (N = 8-12 animals/group; *P<0.05, **P<0.01, ***P<0.001).

CS-induced airway wall remodelling is not affected by CS or BMP-6 deficiency

Airway wall remodelling was investigated by measuring the deposition of fibronectin and collagen in the airway wall of WT and BMP-6 KO mice exposed to CS for 24 weeks. However, deficiency of BMP-6, exposure to CS or the combination of both, did not alter the fibronectin and collagen deposition in the airway wall **(Figure 6A-B)**.

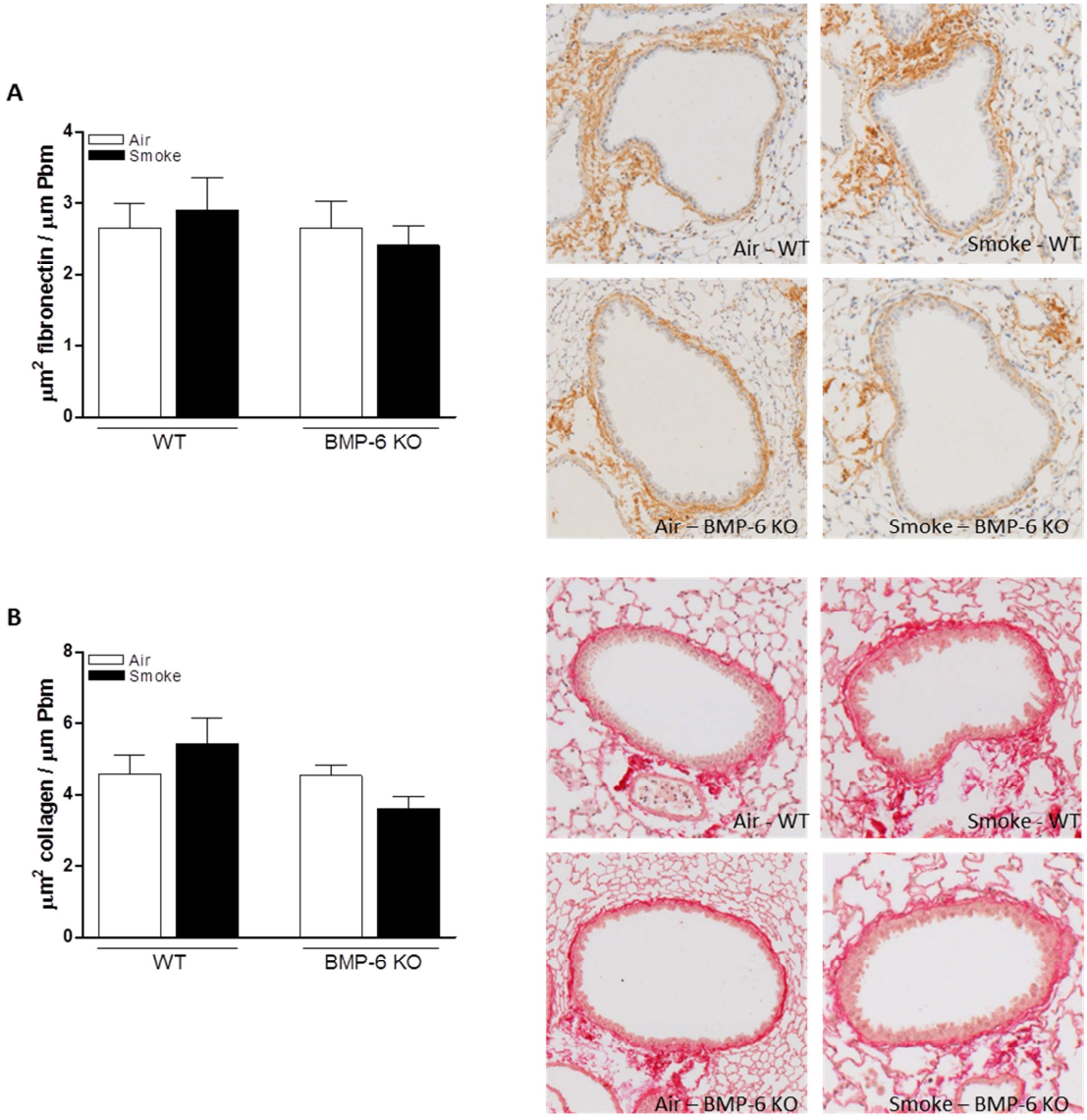


Figure 6. Effect of chronic CS exposure and BMP-6 deficiency on airway wall remodelling. (A) Quantification of fibronectin deposition in the airway wall of WT and BMP-6 deficient mice exposed to air or CS for 24 weeks. Representative images are shown on the right. **(B)** Quantification of collagen deposition in the airway wall of WT and BMP-6 deficient mice exposed to air or CS for 24 weeks. Representative images are shown on the right. Data are expressed as mean ± SEM (N = 8-12 animals/group).

BMP-6 deficiency results in increased pulmonary iron deposition

BMP-6 deficiency was significantly associated with increased iron levels in serum and lung homogenates and increased concentration of ferritin, an iron storage protein, in the BAL supernatant **(Figure 7A-C)**. In the subacute exposure model, CS increased iron concentration in serum in WT mice, but decreased iron levels in BMP-6 KO mice **(Figure 7A)**. Iron levels in the BAL fluid could not be detected. After 4 weeks of CS, CS-exposed BMP-6 KO mice showed a significant increase in ferritin compared to air-exposed BMP-6 KO mice and CS-exposed WT mice **(Figure 7C)**.

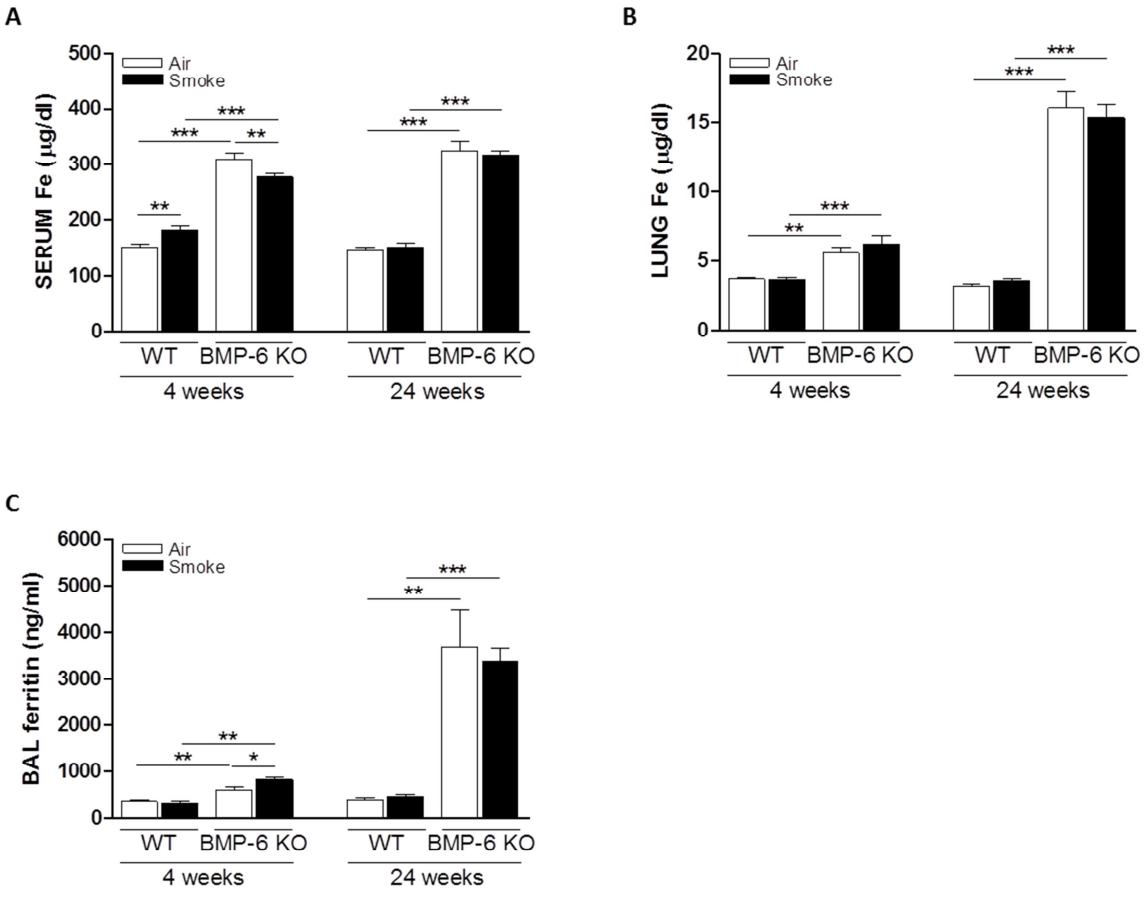


Figure 7. Effect of CS exposure and BMP-6 deficiency on iron and ferritin levels. Quantification of the iron concentration in serum **(A)** and lung homogenates **(B)** of WT and BMP-6 deficient mice exposed to air or CS for 4 and 24 weeks. Protein levels of ferritin **(C)** in BAL supernatants of WT and BMP-6 deficient mice upon 4 and 24 weeks exposure to air or CS, as measured by ELISA. Data are expressed as mean ± SEM (N = 8-12 animals/group; *P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

We reveal decreased mRNA expression of BMP-6 in the lungs of patients with COPD and CS-exposed mice. BMP-6 levels decreases with disease severity. In lung tissue, BMP-6 protein expression is observed in airway smooth muscle cells, endothelial cells and pulmonary macrophages. Using *in vivo* studies with BMP-6 KO and control WT mice, we demonstrate that BMP-6 deficiency aggravates the pulmonary inflammation after a subacute CS exposure of 4 weeks. After chronic CS exposure, both strains show similar inflammation and airway wall remodelling. Moreover, BMP-6 deficient mice develop massive iron overload in the lungs.

We demonstrated a lower BMP-6 mRNA expression in the lungs of smokers without airflow limitation and patients with COPD compared to never smokers. Importantly, patients with severe COPD had the lowest BMP-6 levels, which were also significantly lower than the levels of smokers without airflow limitation. We confirm thus decreased expression of BMP-6 in patients with COPD, as found in a recent gene expression analysis [311]. Also in the CS-driven murine model of COPD, we revealed decreased levels of BMP-6 at the mRNA level in lung tissue after 24 weeks of CS. In experimental models of fibrosis, both increased and decreased BMP-6 levels were reported in respectively liver and kidney fibrosis models [298;299]. BMP-6 is also induced in chronic human skin wounds [354]. Data regarding the expression of BMP-6 in respiratory models are scarce. One study has demonstrated higher BMP-6 levels in an OVA-induced mouse model of allergic asthma [301]. To fully address whether the BMP-6 pathway is up- or downregulated, activation of downstream mediators and/or target genes, such as phosphorylation of Smad1/5/8 should be investigated.

Next, we determined the localization of BMP-6 protein in lung tissue of patients with COPD and CS-exposed mice by immunohistochemical staining. Interestingly, airway smooth muscle cells, endothelial cells and pulmonary macrophages stained positively for BMP-6. This is in agreement with *in vitro* studies demonstrating that both smooth muscle cells and endothelial cells can express BMP-6 [257;355]. The localization of BMP-6 in these specific cell types makes sense, knowing its regulatory role in endothelial activation and macrophage proliferation [283;356]. Next, we looked more into detail to the expression of BMP-6 by macrophages. For this purpose, we isolated macrophages from lung tissue of mice exposed to CS for 4 and 12 weeks. Expression of BMP-6 is reduced in lung macrophages of CS-exposed mice.

Exposure of mice to CS for 4 weeks results in pulmonary inflammation characterized by increased numbers of macrophages, neutrophils, DCs and lymphocytes in BAL fluid [312]. Most of these manifestations were aggravated in BMP-6 KO mice. Elevated levels of the chemokines CCL2 and CXCL1 can explain the increased recruitment of monocytes and neutrophils to the airways of CS-exposed BMP-6 KO mice. The protective effect of BMP-6 appears to involve modulation of chemokine production, as is also shown in kidney epithelial cells, but BMP-6 can also directly affect inflammatory cells [357]. Macrophages and $CD4^+$ T cells express functional type II BMP receptors and type I receptors and BMP-6 is able to inhibit cell proliferation of both cell types [283;285].

In the chronic CS exposure model, loss of BMP-6 had no influence on the levels of pulmonary inflammation. It is possible that functional redundancy occurs and that other BMP proteins can take over, such as BMP-7. BMP-6 and BMP-7 are structurally very similar and BMP-7 also displays anti-inflammatory actions [357]. We assessed the development of airway wall remodelling in the chronic experiment by quantification of collagen and fibronectin in the airway wall. Similar to the inflammation findings, we did not observe a difference between WT and BMP-6 KO mice, nor between air- and CS- exposed mice. Exposure of C57BL/6 mice to CS for 24 weeks normally results in the development of chronic inflammation and airway wall remodelling, as already published by our research group [159]. In our chronic CS experiment, the CS-induced inflammation is mild and no airway wall remodelling is present. We hypothesize that the hybrid C57Bl/6Jx129Sv genetic background of the BMP-6 KO mice is relatively resistant to the effects of CS. Indeed, the development of CS-induced inflammation is strain dependent and the 129SvJ strain has been shown to be resistant to CS-mediated inflammatory and oxidative responses [358]. Therefore, experiments with BMP-6 KO mice on a pure C57BL/6 background should be performed.

We hypothesize that the loss of BMP-6 signalling releases the control break on the expression of pro-growth factor signalling pathways, such as TGF-β1. We have indeed observed elevated TGF-β1 protein levels in BAL fluid of CS-exposed BMP-6 KO mice in the subacute experiment. Similar observations were made in renal fibrosis in mice [299].

Finally, BMP-6 KO mice develop iron accumulation in the lungs. With these data, we expand the list of organs (liver, pancreas, heart and kidney) where massive iron overload is present in BMP-6 KO mice [279]. We speculate that iron accumulation in the lungs contributes to tissue damage and inflammation via oxidative stress. Indeed, increased concentrations of iron and ferritin, an iron storage protein, have been found in healthy smokers and patients with COPD [359]. Furthermore, the percentage of iron containing macrophages was positively correlated with COPD and emphysema severity [352]. This pulmonary iron overload in smokers can have functional implications, considering

the tight link between iron, immunity and oxidative stress [360]. By limiting iron availability for colonizing and/or invading bacteria, the immune system can protect the host from bacterial overgrowth. High iron Fe (Hfe) deficiency, characterized by systemic iron overload quite similar to BMP-6 deficiency, impairs pulmonary neutrophil recruitment in response to intratracheal instillation of LPS [361]. However in our experiments, iron levels are not different between air- and CS-exposed mice, so we cannot conclude whether iron-mediated oxidative stress also contributes to the higher CS-induced accumulation of inflammatory cells in BMP-6 KO mice. Although we revealed a subtle, but significantly higher increase in ferritin levels in CS-exposed BMP-6 KO mice compared to the control group, the functional consequences are not clear.

In conclusion, we have shown that BMP-6 mRNA expression is significantly decreased in the lungs of CS-exposed mice and patients with COPD. Deficiency of BMP-6 renders mice more susceptible to the development of CS-induced pulmonary inflammation in a subacute setting, while in a chronic setting, deficiency of BMP-6 is dispensable for both inflammation and airway wall remodelling. Future studies have to further unravel the role of BMP-6 in airway remodelling and iron-induced oxidative stress after CS exposure.

[CHAPTER 9: TGF-β SUPERFAMILY IN OBSTRUCTIVE LUNG DISEASES: MORE SUSPECTS THAN TGF-β ALONE]

The TGF-β superfamily comprises TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs). TGF-β1 is the most studied ligand in obstructive lung diseases; however considerable evidence has been presented for a role of many of the other ligands of the TGF-β superfamily in lung pathology, including activins, BMPs and GDFs.

Verhamme FM, Bracke KR, Joos GF, Brusselle GG. TGF-β superfamily in obstructive lung diseases: more suspects than TGF-β alone. American Journal of Respiratory Cell and Molecular Biology. 2014 November 14. Epub ahead of print [195].

ABSTRACT

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are respiratory disorders and a major global health problem with increasing incidence and severity. Genes originally associated with lung development could be relevant in the pathogenesis of COPD/asthma, either due to an early life origin of adult complex diseases or due to their dysregulation in adulthood upon environmental stressors (e.g. smoking). The TGF-β superfamily is conserved through evolution and is involved in a range of biological processes both during development and in adult tissue homeostasis. TGF-β1 has emerged as an important regulator of lung and immune system development. However, considerable evidence has been presented for a role of many of the other ligands of the TGF-β superfamily in lung pathology, including activins, bone morphogenetic proteins (BMP), and growth differentiation factors (GDF). In this review, we summarize the current knowledge on the mechanisms by which activin, BMP and GDF signalling contribute to the pathogenesis of obstructive airway diseases.

INTRODUCTION

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are both obstructive airway diseases, that involve chronic inflammation and remodelling of the respiratory tract. The airflow limitation present in asthma is variable and reversible, but progressive and largely irreversible in COPD. Patients with asthma/COPD experience wheezing, breathlessness, abnormal sputum production and cough. Although several similarities exist between COPD and asthma, the inflammatory profile and anatomical sites of involvement are different, resulting in different responses to treatment [1;362].

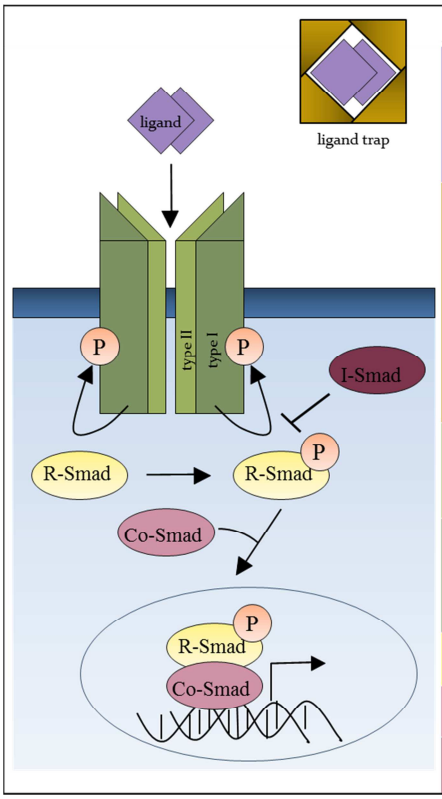
Most patients with asthma are atopic and present with aberrant immune responses towards inhaled allergens. The inflammation in asthma, primarily eosinophilic and Th2 driven, is located in the larger conducting airways and bronchioles. Subepithelial fibrosis, due to collagen deposition at the basement membrane, is present. In COPD, the most important risk factor is cigarette smoking, causing an abnormal inflammatory response, primarily consisting of macrophages, neutrophils and $CD8^+$ T lymphocytes. Collagen is deposited throughout the airway wall (small airway remodelling) and there is destruction of the lung parenchyma, leading to emphysema [363;364].

Accumulated evidence suggests that developmental processes have a substantial effect on long-term lung health, since interference with many key genes of lung development results in early-onset lung disease [365]. One of these essential growth factor pathways is the transforming growth factor (TGF)-β pathway, which is one of the most evolutionarily conserved signal transduction pathways within the animal kingdom [196]. There are more than 30 TGF-β superfamily ligands in mammals, including the TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs).

Proteins of the TGF-β superfamily are pleiotropic mediators, playing key roles in diverse developmental and physiological pathways, owing to ubiquitous expression of their signalling components. It is not surprising that disruptions in this tightly regulated pathway are associated with a wide range of human diseases, including respiratory disorders [201-203]. Both candidate gene and genome-wide association studies demonstrated association of several genes of the TGF-β superfamily, such as TGF-β1/2 and BMP-6 genes, with lung function [3;4;219;366]. Most research has focussed on the TGF-β1 ligand, showing its involvement in chronic sinus disease, pulmonary fibrosis, asthma and COPD [204-206]. In this review, we want to shed light on the other members of the TGF-β superfamily such as activins, BMPs and GDFs, both in the normal development of the lung and in obstructive pulmonary diseases (asthma/COPD).

TGF-β SUPERFAMILY

All ligands of the TGF-β superfamily are synthesized as prepropeptide precursors and then processed and secreted as homodimers or heterodimers. The mature ligands are dominated by the cystine-knot architecture that results from 6-9 conserved cysteine residues forming intra- and intermolecular disulfide bonds. Next to structural features, this protein family also shares a similar signal transduction cascade. For signalling, each ligand requires a set of type I and type II serin/threonine kinase receptors. In humans, 5 type II and 7 type I receptors have been identified. The activated receptor complex propagates the signal into the canonical pathway through phosphorylation of the receptor-regulated Smads (R-Smads), subsequently forming complexes with Co-Smad4 and translocating to the nucleus to regulate gene expression (**Figure 1**). Other pathways can also be activated by the TGF-β family, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), and are referred to as 'non-canonical' signalling [197-200].



	TGF-β	activin	BMP	GDF
Ligands	TGF-β1 TGF-β2 TGF-β3	Activin-A Activin-B Activin-AB	BMP-1 BMP-2/4 BMP-3 BMP-5/6/7/8 BMP-9/10 BMP-15	GDF-1/3 GDF-5/6/7 GDF-8/11 GDF-9/15
Ligand traps	Follistatin and related proteins Hyaluronan Decorin Latency associated peptide α2-macroglobulin	Follistatin and related proteins Inhibin	Dan Twisted gastrulation Chordin/Noggin Others (follistatin and related proteins, …)	Dan Twisted gastrulation Chordin/Noggin Others (follistatin and related proteins, …)
Type II receptors	TGFBR2	ActR2A ActR2B	ActR2 ActR2B BMPR2	TGFBR2 ActR2A ActR2B BMPR2
Type I receptors	ALK1 ALK2 ALK5	ALK1 ALK2 ALK4 ALK7	ALK 1 ALK2 ALK3 ALK6	ALK3 ALK4 ALK5 ALK6 ALK7
R-Smad	Smad2/3	Smad2/3	Smad1/5/8	Smad1/5/8 Smad2/3
Co-Smad	Smad4	Smad4	Smad4	Smad4
I-Smad	Smad7	Smad7	Smad6 Smad7	Smad6 Smad7

Figure 1. Signalling components of the TGF-β superfamily. The canonical signalling pathway through the SMADs is shown for the transforming growth factor-βs (TGF-βs), activins, bone morphogenetic proteins (BMPs) and growth differentation factors (GDFs). ALK (activin receptor-like kinase).

Activins

Originally isolated from ovarian fluid as a stimulator of follicle-stimulating hormone, activin-A is a multi-faceted cytokine regulating many aspects of the immune system. Activin-A can display either anti- or pro-inflammatory properties depending on the cellular and temporal context [235-237]. Activin-A inhibits the processing of IL-1β precursor into the mature cytokine and stimulates the development of regulatory T cells [238;243]. On the other hand, the pro-inflammatory function of activin-A is underlined by its ability to promote M1 macrophage polarization [240]. Activin-A has been implicated in inflammatory and fibrotic diseases of the lung, but also in other organs such as the gut in inflammatory bowel diseases [247].

Activins are dimeric proteins consisting of 2 β subunits, βA and βB, that form the hetero- or homodimers, activin-A (βA:βA), activin-B (βB:βB), or activin-AB (βA:βB). Two other subunits (βC and βE) have also been identified [234]. Activin-A is expressed in alveolar and bronchiolar epithelium, alveolar macrophages and vascular smooth muscle cells [252;253]. The less studied βB, βC and βE subunits have a lower expression, but can be detected in lung tissue [254].

Activins interact with activin type II receptor A and B (ActR2A and ActR2B), which recruits the type I receptor (ALK4, but also ALK7) [255]. The type II receptors are broadly expressed in every structural cell type of the lung [253;254;256]. Lung fibroblasts and bronchial epithelial cells are known to express the type I receptor ALK4 [254].

The activated type I receptor propagates the signal by phosphorylation of R-Smad2 and R-Smad3. Smad2/3 expression has been seen in several cell types in the lung, such as bronchial epithelial cells, fibroblasts, pulmonary artery smooth muscle cells and alveolar type 2 cells; the phosphorylated form of Smad2 is especially detected in bronchial epithelial cells. Also Smad4, a signalling mediator which is shared by different members of the TGF-β superfamily, is abundantly expressed in the lung [222;254;257].

Activin signalling is negatively regulated both extracellularly and intracellularly by diverse mechanisms **(Figure 1)**. Inhibitory Smad6 and 7 function as a negative feedback mechanism in TGF-β signalling [258]. Smad7 has been detected in airway epithelial cells, stromal cells and alveolar type 2 cells [222]. Follistatin is an extracellular glycoprotein that binds and neutralizes activin with high affinity by blocking the receptor binding sites. Follistatin also binds with lower affinity to some other members of the TGF-β superfamily, including GDF-8/9 and BMP-2/5/7/8 [234;258]. Follistatin expression has been found in alveolar macrophages, bronchial and alveolar epithelial cells and vascular smooth

muscle cells [253]. Follistatin-related protein (or follistatin-like 3), which has a high degree of structural homology with follistatin, also binds and inhibits activins and BMPs and is expressed in lung tissue [259].

Bone morphogenetic proteins (BMPs)

Originally identified as proteins that stimulate bone morphogenesis, it is now clear that bone morphogenetic proteins regulate the development of nearly all organs and tissues [276]. Abnormalities in BMP genes are linked to a wide variety of clinical disorders. Genetic studies have shown that patients with familial and sporadic pulmonary arterial hypertension (PAH) exhibit heterozygous, germline mutations in the BMP receptor type 2 gene (BMPR2), which encodes a type II receptor member (BMPR-II) [367-369]. However, the functional contribution of the BMP family in the lung is still not fully understood.

With over 20 mammalian members identified, BMPs constitute the largest subfamily of the TGF-β superfamily **(Figure 1)**. Based on their amino acid sequence similarity, BMPs can be classified into at least 4 subgroups: BMP-2/4, BMP-5/6/7/8, BMP-9/10, BMP-12/13/14 [277]. Most BMP ligands are present in the lung, more specifically in pulmonary artery smooth muscle cells, but also in the airway epithelium, lung fibroblasts and pulmonary endothelium [257;301;304;305;370;371].

Originally, 3 type II receptors (BMPR2, ActR2A and ActR2B) and 3 type I receptors (ALK2, ALK3 and ALK6) were described for the BMPs. However, ligand-receptor promiscuity can occur in the TGF-β superfamily, particularly in the BMP/GDF subfamily, indicating that ligands can bind to several receptors of either subtype and vice versa [198]. Similar to the BMP ligands, also its receptors are abundantly expressed in the pulmonary vasculature, airway epithelium and lung fibroblasts [256;303-305]. BMPs pass the signal on through R-Smad1, R-Smad5 and R-Smad8 (canonical pathway). Phosphorylation of these Smad proteins, hence an active BMP signalling, exists in airway epithelial cells, alveolar macrophages and lung fibroblasts, next to the pulmonary endothelium and vascular smooth muscle [304;306]. In addition, non-canonical signalling through p38, ERK1/2 and JNK has been observed in pulmonary artery smooth muscle cells, lung fibroblasts and airway epithelial cells [304;307;308].

One of the most important targets of BMP signalling are the basic helix-loop-helix inhibitor of differentiation (Id) proteins. There are 4 known members in mammals (Id1 through 4), exhibiting similar, but not identical biological functions [309]. Id4 is barely detectable in the lung, but Id1 and Id3 are expressed in pulmonary vacular endothelial cells and alveolar epithelium, while Id2 is detected in bronchial epithelium and smooth muscle cell compartments of the lung [310].

The activity of BMPs is controlled at different molecular levels. Smad6 is, similar to Smad7, an inhibitory Smad (I-Smad) protein preferentially repressing BMP signalling [372]. Smad6 is expressed in pulmonary artery smooth muscle cells and airway epithelial cells [257;373]. At the extracellular level, BMP antagonists have been classified into 3 subfamilies: the Dan, the Twisted gastrulation and the Chordin/Noggin family [278]. Gremlin is a member of the Dan family and specifically binds to and inhibits BMP-2, BMP-4 and BMP-7. Gremlin expression is reported in the pulmonary endothelium, but also in lung fibroblasts, alveolar macrophages and epithelial cells [374]. BMP-binding endothelial cell precusor-derived regulator (BMPER), belonging to the Chordin family, is found in alveolar and bronchial epithelial cells. BMPER binds directly to BMPs and prohibits BMPs from binding their cognate receptors [375].

Growth differentiation factors (GDFs)

The growth differentiation factor (GDF) proteins form the most heterogeneous subfamily of the TGF-β superfamily. More distant members, such as GDF-9 are categorized in this group, and few studies have explored the expression of these ligands in the lungs [198]. GDF-5 is localized in alveolar type 1 epithelial cells, endothelium and alveolar interstitial fibroblasts of adult rat lung [376]. Expression of GDF-15 is found in endothelial cells of small pulmonary arteries, alveolar macrophages, smooth muscle cells and bronchial ephithelial cells [377;378].

Several receptors exist for the GDF superfamily. Next to the activin and BMP receptors, GDF ligands can also bind the TGF-β type II (TGFBR2) and type I receptors (ALK5). Both receptors are abundantly expressed in lung cells such as pulmonary artery smooth muscle cells, fibroblasts, endothelial cells, bronchial and alveolar epithelial cells[254;256;304]. After ligand binding, the signalling occurs through the canonical pathway, both via R-Smad2/3 or R-Smad1/5/8, or through non-canonical signalling pathways like ERK.

LUNG DEVELOPMENT

During branching morphogenesis, the BMP canonical pathway is mainly active in the vascular network and the airway smooth muscle layer. During later stages, the BMP pathway is activated in developing airway and alveolar epithelial cells, after which BMP signalling declines to minimal levels in adult mice [379]. BMP-5 and BMP-7 are expressed in the mesenchyme and endoderm, respectively, while BMP-4 is found mostly in the distal tips of the epithelium, but also in the adjacent mesenchyme [380;381]. There is still some debate whether BMP-4 promotes or inhibits epithelial cell proliferation [380;382;383].

Conditional epithelium-specific ALK3 knockout mice have abnormal lungs containing large, fluid-filled sacs due to reduced proliferation combined with extensive apoptosis of both epithelium and mesoderm [384]. Another approach with inducible abrogation of ALK3, also specifically in epithelium, resulted in prenatal lung malformation and respiratory failure by disrupting distal airway formation [385]. Further downstream of the pathway, Smad1 protein is found mainly in peripheral airway epithelial cells of early embryonic lung tissue, whereas more Smad1 positive mesenchymal cells are found in late gestation. Similar to the phenotypes described above, knockdown of Smad1 negatively regulates lung branching by inhibiting epithelial cell proliferation and differentiation [386;387].

Finally, BMP antagonist follistatin-like 1 (Fstl1) is also detected in the developing mouse lung both in the mesenchymal and epithelial compartment [388]. The observed respiratory failure of Fstl1 KO mice by two independent studies demonstrated that Fstl1 is essential for tracheal cartilage formation and alveolar maturation [389;390]. Gremlin, another extracellular BMP antagonist is observed in the proximal airway epithelium during development and its overexpression disrupted proximal-distal patterning and BMP-4-induced branching morphogenesis [391;392].

Collectively, these data provide evidence that tight control of BMP signalling activity in every step of the BMP pathway is required for normal lung development, especially for the proximal-distal axis formation in lung epithelial cells. During normal lung development, extensive modelling of the lung takes place. In asthma and COPD, this modelling process is partially recapitulated in response to (persistent) injury/inflammation leading to altered tissue structure and abnormal airway function. It is interesting to speculate whether BMP proteins - originally described in developmental processes - may participate in complex adult lung diseases, including asthma and COPD.

ASTHMA

Activins

In human disease, activin-A was elevated in the lungs, serum and peripheral blood $CD4^+$ T cells of patients with asthma [261;269]. Furthermore, patients with asthma exhibited less inhibitory Smad7 immunoreactivity in bronchial epithelial cells than normal subjects. After allergen challenge of asthma patients, P-Smad2, ALK4 and ActR2A positive cells were upregulated, whereas activin-A and follistatin levels showed no change [270]. In contrast, Semitekolou and coworkers demonstrated, despite increased activin-A levels, declined ALK4 and ActR2A positive cells [269].

In a mouse model of ovalbumin (OVA)-induced allergic airway inflammation, activin-A was strongly upregulated, together with an increase of ALK4 and Smad mediators [254]. Similarly, other studies in this allergic asthma model have shown elevated levels of activin-A in bronchoalveolar lavage fluid, released from airway epithelial cells or inflammatory cells, such as activated mast cells and macrophages [266-268]. Importantly, follistatin concentrations also increased, but not as dramatically as activin-A, suggesting that there is a relative excess of activin-A [266]. Overall, these data suggest an activation of the activin-A pathway in experimental asthma.

Mechanistically, activin-A appears to provide a link between early Th2-driven allergic responses and long-term structural changes in the airways that are characteristic for asthma **(Figure 2)**. Intranasal delivery of follistatin in an acute OVA mouse model inhibited the allergen-specific Th2 immune response and mucus production [266]. IL-13, the key cytokine for mucus production, controlled the activin-A secretion by bronchial epithelial cells [271]. Systemic administration of a neutralizing antibody against activin-A exacerbated OVA-induced allergic airway disease, whereas recombinant activin- A reduced the symptoms, through the modulation of antigen-specific regulatory T cells [269]. Remarkably, it thus seems that activin-A may exert distinct functions depending on the route of administration and type of inhibitor. Finally, a role for activin-A together with TGF-β1 was suggested in Th9-mediated allergic inflammation upon house dust mite instillation [272].

In vitro studies demonstrating that activin-A promotes the proliferation of airway smooth muscle cells and bronchial epithelial cells, pointed towards a possible role of activin-A in the process of airway remodelling [267;270]. Furthermore, activin-A stimulates the proliferation and differentiation of lung fibroblasts into myofibroblasts [261;262]. Activin-A neutralization by intranasal instillation of follistatin in a murine model of chronic allergic asthma confirmed that activin-A is crucial for mucus hypersecretion, subepithelial collagen deposition and thickening of the subepithelial smooth muscle [273]. Mice deficient in Smad3 have significantly decreased airway remodelling, which is associated with

decreased numbers of peribronchial myofibroblasts [268]. Smad2 overexpression in the airway epithelium resulted in enhanced airway hyperreactivity after allergen challenge concomitant with changes in smooth muscle and matrix remodelling. Remodelling was markedly inhibited by injection of an activin-A neutralizing antibody, providing direct evidence for a role of activin-A in remodelling [274].

Bone morphogenetic proteins (BMPs)

In patients with mild asthma, it has been demonstrated that protein levels of ALK2, ALK6 and BMPR2 in epithelial cells are decreased at baseline. Allergen challenge was associated with up-regulation of BMP-7 in the airway epithelium and inflammatory cells (mostly eosinophils) and with increased levels of P-Smad1/5 and ALK6 [305].

In an ovalbumin-induced airway inflammation model, almost all bronchial epithelial cells in the inflamed airways stained positively for phosphorylated Smad1/5, in contrast to the healthy epithelium. This activated Smad signalling is accompanied by an upregulation of BMP type I receptors (ALK2, 3 and 6), mainly in bronchial epithelial and endothelial cells. Furthermore, BMP ligands BMP-2, 4 and 6 were induced, whereas BMP-5 and 7 were decreased in ovalbumin-exposed mice [301]. Another study in a similar mouse model of asthma (also OVA-induced) demonstrated higher BMP-7 expression in an acute setting, whereas in the chronic setting, no difference was observed. Moreover, they found an elevated TGF-β1/BMP-7 ratio, which correlated with the levels of collagen deposition. Treatment of asthmatic mice with recombinant BMP-7 reduced lung inflammation and collagen deposition [297].

Several mechanistic studies pointed towards a role for BMPs in remodelling and epithelial-to-mesenchymal transition (EMT), 2 mechanisms implicated in the pathogenesis of asthma. *In vitro* studies with BMP-7 demonstrated the pro-fibrotic function of BMP-7 in airway epithelial cells, but in cultured lung fibroblasts BMP-7 opposed TGF-β1 dependent fibrogenic activity [294-297]. BMP-4 induced a mesenchymal-like morphology in airway epithelial cells in a wound closure model by altering E-cadherin, vimentin and α-smooth muscle actin expression, thereby facilitating cell migration [295;308;393]. However, BMP-4 inhibited TGF-β1 induced cell proliferation of normal human lung fibroblasts and extracellular matrix protein release [296]. Next to BMP-4, BMP-2 can also stimulate the acquisition of an epithelial-mesenchymal transition phenotype in epithelial cells [295]. Moreover, gremlin, an extracellular antagonist of BMPs, stimulates the promotion of myofibroblast generation, likely through decreased BMP-mediated fibroblast apoptosis and higher susceptibility to TGF-β induced epithelial-to-mesenchymal transition [394].

Growth differentiation factors (GDFs)

Although the TGF-β and activin-A subfamily have been extensively studied in asthma, very little is known about the expression and functionality of GDFs in asthma. One study revealed that GDF-15 could induce MUC5AC expression in human lung NCI-H292 cells, indicating a possible role in mucus hypersecretion [395]. GDF-15 is a divergent member of the TGF-β superfamily, operates during stress responses, and is a promising new biomarker for cardiovascular disease [396]. Expression and functional studies in relevant animal models and patients with asthma should elucidate the role of GDFs in asthma.

COPD

Activins

Recently, our group has demonstrated that epithelial levels of activin-A are higher in patients with COPD compared to never-smokers and smokers without airflow limitation [337]. Several studies reported dysregulated Smad signalling in the lungs of patients with COPD [222;227;275]. Smad3 deficiency in mice resulted in progressive age-related airspace enlargement, associated with a high expression of MMP-9 and MMP-12 [232]. Exposure of these Smad3 null mice to cigarette smoke aggravated air space enlargement and alveolar cell apoptosis [233]. These effects are thought to be primarily mediated by TGF-β1, based on its known immunomodulatory and fibrogenic functions and since TGF-β1 is increased in patients with COPD [48;221]. Activin-A is induced in bronchial epithelial cells upon cigarette smoke exposure. Activin-A levels are increased in the lungs of cigarette smoke-exposed mice, whereas follistatin levels are reduced. Interestingly, systemic administration of follistatin reduced cigarette smoke-induced inflammation in mice [337]. Taking these new observations together with the fact that activin-A has similar functions as TGF-β1, activin-A should be considered as an important mediator in the pathogenesis of COPD. However, its role in the full spectrum of COPD, including airway wall remodelling, emphysema and exacerbations, still needs to be elucidated.

Bone morphogenetic proteins (BMPs)

There is indirect evidence that the BMP signalling pathway is also dysregulated in COPD. Bronchial biopsies of patients with COPD showed reduced expression of Smad6 and Smad7 [373]. The Secreted Frizzled-related proteins (SFRPs) are extracellular antagonists of Wingless/Integrated (Wnt) signalling, but are also involved in the negative regulation of the BMP pathway [397]. SFRP2 is upregulated in bronchial brushings from healthy smokers and smokers with COPD compared to never smokers. Moreover, cigarette smoke extract increased the expression of SFRP2 in airway epithelial cells *in vitro* [398]. In a *Pseudomonas aeruginosa*-induced rat model of COPD, BMP-7 expression was reduced [399].

Since BMPs are involved in remodelling and epithelial-to-mesenchymal transition (EMT), one can hypothesize that BMPs contribute to these processes in COPD. Additional studies are necessary to unravel the functional role of BMPs in the pathogenesis of COPD.

Growth differentiation factors (GDFs)

Plasma levels of GDF-15 were increased in patients with COPD compared to healthy smokers [400]. In this context, exposure to cigarette smoke, the major risk factor for COPD, induced GDF-15 production in human airway epithelial cells, which subsequently promoted MUC5AC expression [395]. However, mechanistic studies in animal models are needed to elucidate whether GDF-15 is merely a biomarker or an active contributor to the pathogenesis of COPD.

COPD is a complex disease characterized by airflow obstruction, but in many patients also by systemic manifestations, such as skeletal muscle wasting, which is associated with exercise limitation and reduced quality of life [21]. The expression of GDF-8 or myostatin, a negative regulator of skeletal muscle growth, was higher in quadriceps muscle biopsies of smokers versus non-smokers and in quadriceps and diaphragm muscle biopsies of patients with COPD compared to controls [401-404]. In addition, GDF-8 serum levels are elevated in patients with COPD as compared with healthy controls and are positively correlated with TNF-α levels [405]. Both cigarette smoke and chronic hypoxia can induce myostatin expression. Together these studies suggest the involvement of GDF-8 in the pathogenesis of skeletal muscle atrophy associated with COPD [402;403]. Interestingly, GDF-8 null mice are partially protected against skeletal muscle loss upon intratracheal instillation of LPS [406].

CONCLUSIONS AND FUTURE DIRECTIONS

Evidence in favour for a role of TGF-β superfamily members in the pathogenesis of obstructive airway diseases (asthma and COPD) has emerged from genetic studies. The candidate gene association approach has identified single-nucleotide polymorphisms of the TGF-β1 gene that were associated with asthma and COPD [219;366]. Through genome-wide association studies, the TGFB2 and BMP-6 locus were found to be associated with pulmonary function which is used to define airflow limitation/obstruction [3;4].

Secondly, expression data from *ex vivo* studies indicate differential expression of TGF-β superfamily ligands between patients with obstructive lung diseases and control subjects. From a mechanistic point of view, *in vitro* studies on cell lines and primary human cells emphasize divergent roles, going from epithelial repair/homeostasis to mucus production and immune regulation. Lastly, experimental *in vivo* studies using animal models provide promising possibilities for treatment by interfering with the TGF-β pathway. However, caution is needed, since the clinical relevance of acute animal models for chronic human diseases is doubtful. Also species differences and difference in outcomes (pathology in animal models vs. lung function in patients) and timing of the intervention (prevention vs treatment) have to be accounted for. The potential actions of the TGF-β ligands in obstructive lung diseases, based on the existing evidence, are summarized in **Figure 2**.

For reasons of simplicity, we have discussed the different subfamilies separately; however, as with every signalling pathway, interaction between the different TGF-β members is possible. For example, BMP-7 counteracts TGF-β1-induced epithelial-to-mesenchymal transition in the kidney [407]. This provides evidence that the balance between TGF-β and BMP signalling is of crucial importance during repair processes. Furthermore, cross-talk between the TGF-β superfamily and other pathways is common. During lung bud morphogenesis, Bmp-4 and fibroblast growth factor 10 (Fgf-10) play opposing roles [408]. In order to get a complete picture of the molecular pathways underlying lung disease, studies using systems biology approaches and bio-informatic tools investigating this interplay, are required.

For TGF-β1, the prototype protein of the TGF-β family, both harmful and protective roles have been postulated, depending on the cell type and stimulation involved. Similarly, a pleiotropic action of activin-A during the course of inflammation is known, where activin-A exerts a pro-inflammatory effect early on in the course of inflammation, but functions as an anti-inflammatory cytokine, once the inflammatory response has been established [234]. Given this complexity, the use of future

therapies targeting the TGF-β family is not straightforward. This highlights the fact that route of administration, timing and dose of agents that modulate the TGF-β signalling will need further investigation.

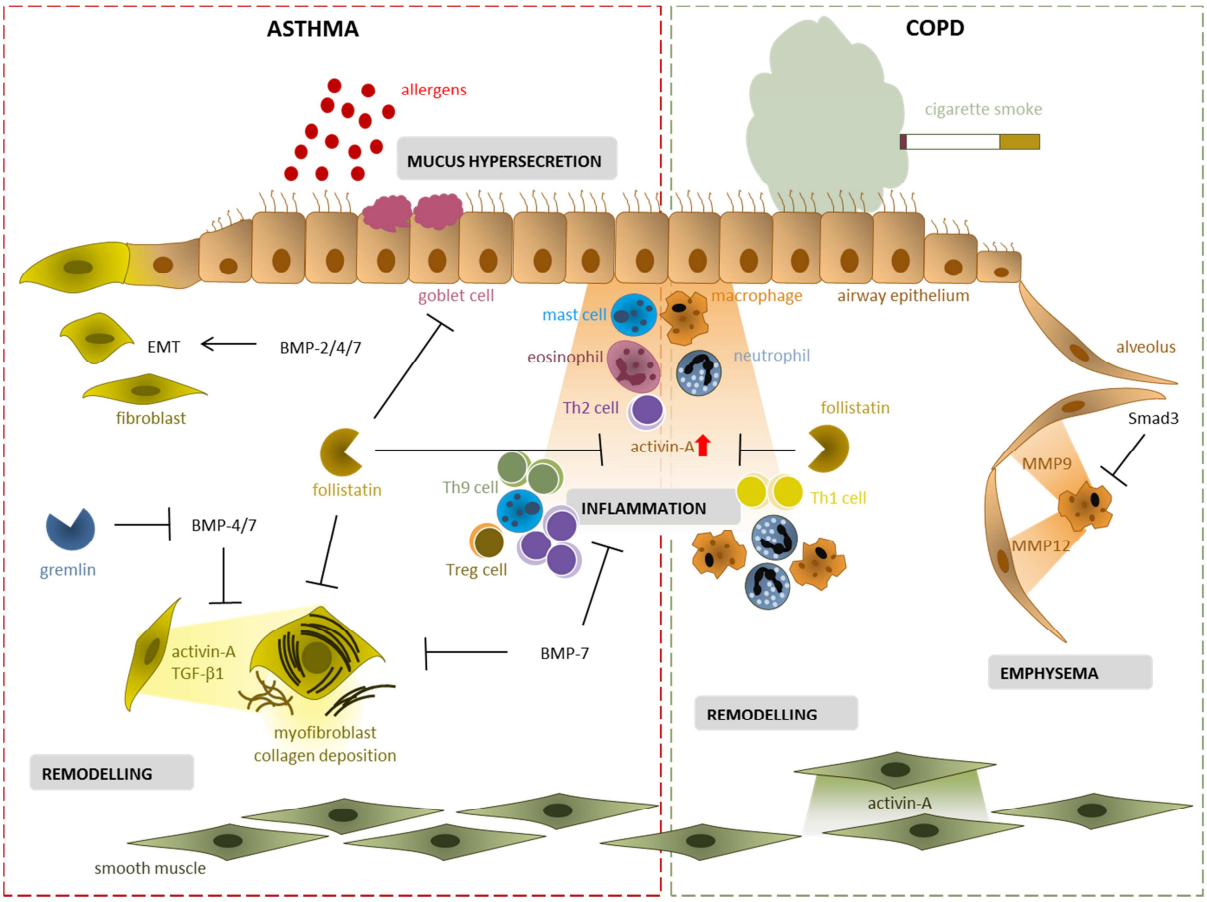


Figure 2. Mechanisms of action of activins and bone morphogenetic proteins (BMP) in obstructive lung diseases. Dysregulation of activins and BMPs can have deleterious effects on normal lung homeostasis, leading to pathogenetic mechanisms in obstructive lung diseases (asthma/COPD), including inflammation, airway remodelling, emphysema and mucus hypersecretion.

[**CHAPTER 10:** DISCUSSION AND FUTURE PERSPECTIVES]

10.1. CXCL13 and lymphoid follicles

Patients with severe COPD develop pulmonary ectopic lymphoid follicles, also termed tertiary lymphoid organs (TLOs). Importantly, the number of lymphoid follicles increased with disease progression [33]. How these lymphoid follicles are formed and whether they are "the good or the bad guys" remain unanswered questions. CXCL13 is a lymphoid chemokine that attracts $CXCR5^+$ B cells and is involved in the development of secondary lymphoid organs (SLOs) [175;409]. The importance of CXCL13 in TLO development has been strengthened by the fact that overexpression of CXCL13 induces TLO formation, while blocking CXCL13 disrupts this process [184;189]. We hypothesized that pulmonary CXCL13 levels are increased in COPD and that neutralization of CXCL13 will eliminate the development of lymphoid follicles and will impact disease progression.

10.1.1. Expression study

First, we have demonstrated that the levels of CXCL13 are increased in the lungs and airways of CS-exposed mice and patients with COPD. This is in line with the observation of increased CXCL13 in other inflammatory airway diseases, such as asthma and chronic rhinosinusitis [194;331]. CXCL13 was found to be elevated in patients with COPD with lymphoid follicles versus those without lymphoid follicles. Moreover, a significant association between total CXCL13 levels and density of pulmonary lymphoid follicles was apparent [329]. While we observed positive staining for CXCL13 in the B cell zone of lymphoid follicles, we have not addressed the exact cellular sources of CXCL13. CXCL13 is mainly produced by non-hematopoietic stromal cells and DCs, but also B cells can produce CXCL13, suggesting that B cells can acquire the function of lymphoid tissue inducer cells [165;179;329]. As is shown for the formation of SLOs, CXCL13 also induces a positive feed-back loop during the formation of TLOs by upregulating lymphotoxin (LT) which further promotes CXCL13 production [329].

10.1.2. Functional study

Administration of anti-CXCL13 antibodies to mice during 6 months of CS exposure completely prevented the formation of lymphoid follicles, accompanied by decreased levels of immunoglobulins IgM, IgG and IgA in BAL fluid. This indicates that CXCL13 is crucial for the formation of lymphoid follicles upon CS exposure. In contrast, our research group has demonstrated that LTα and CCR7 are not essential for the development of CS-induced lymphoid follicles, although it is still possible that LTα and CCR7 are needed for the organization of functional lymphoid follicles[180;181]. Importantly, the CS-induced influx of inflammatory cells into the airways (BAL) was significantly reduced upon CXCL13 neutralization. Also in the ovalbumin (OVA)-induced mouse model of allergic asthma, CXCL13 neutralization abrogated the inflammatory process [194]. While the recruitment of B cells into the BAL

and mediastinal lymph nodes (MLN) was completely prevented, the number of B cells in the lung tissue was not altered. We conclude that CXCL13 is not required for the recruitment of B cells to the lung, but rather important for the organization into lymphoid follicles.

In this setting, we could assess the contribution of CS-induced ectopic lymphoid follicles to other hallmarks of COPD, such as emphysema and airway wall remodelling. Alveolar wall destruction was partially reduced by CXCL13 neutralization, in parallel with lower levels of MMP-12, a metalloelastase implicated in the development of emphysema. On the other hand, antibody treatment had no effect on lung inflammation, airway remodeling and airspace enlargement. These last data support our previous work in which SCID mice, which lack B and T lymphocytes, still develop emphysema [47]. Altogether, we conclude from our study that disruption of lymphoid follicles is partially protective against CS-induced airway inflammation and alveolar wall destruction.

10.1.3. Future directions

Nevertheless, we have to keep in mind that it is possible that lymphoid follicles are initially formed to be protective against infectious agents entering the lower airways. However, the persistent nature of these lymphoid structures may result in tissue destruction, thereby generating (altered) self-antigens and self-reactive lymphocytes, ultimately leading to autoantibodies and potentially more lung damage in a vicious circle [152]. Although presence of autoantibodies has been reported, there is still some debate about their importance [168;171].

In this context, research into the involvement of lymphoid follicles in a combined CS exposure-infection model would be an added value. This is especially interesting since patients with COPD are susceptible to recurrent respiratory infections, which could result in disease exacerbation and hospitalization. The protective role of lymphoid follicles in an influenza model has already been confirmed [164]. But studying the impact of CS on the formation of TLOs upon infection with respiratory pathogens would be clinically more relevant. Several groups have developed animal models of that kind. CS-exposure before influenza infection in mice resulted in exacerbated inflammation, higher viral titers and higher mortality [410;411]. Of interest, mice that were exposed to CS followed by infection with nontypeable *Haemophilus influenzae* developed increased lung inflammation but compromised adaptive immunity with impaired IgG1, IgG2a and IgA class switching [412]. Nevertheless, none of these studies investigated the functional role of pulmonary lymphoid tissue.

Secondly, more research is needed into the reversibility of the neogenesis process of ectopic lymphoid tissue. One can wonder whether the lymphoid aggregates, which are not influenced by the anti-CXCL13 antibody, would organize into lymphoid follicles once the antibody treatment is stopped. Or, from another perspective, whether smoking cessation induces the regression of

lymphoid follicles or whether these structures persist, similar to what is seen in patients with COPD who have stopped smoking. Our study should also be expanded by analyzing other hallmarks of COPD, such as mucus hypersecretion and worsened lung function. Finally, the burning issue remains the identification of the antigen(s) to which the B cell responses are directed.

Although we have demonstrated that CXCL13 is essential for the formation of lymphoid follicles, other molecules and pathways can be targeted to modulate TLO formation, such as adhesion molecules, other lymphoid chemokines or molecules that promote B cell survival and proliferation [151]. B-cell activating factor of tumor necrosis factor family (BAFF) is a crucial survival factor for B cells and is associated with autoimmunity [413]. It has been shown that BAFF is increased in alveolar macrophages and lymphoid follicles of patients with COPD compared with healthy smokers and never smokers [414]. Scavenging BAFF with a soluble protein comprised of a human IgG1-Fc fused with the extracellular domain of the mouse TACI receptor reduced asthma symptoms in an OVA-induced mouse model [415]. Neutralization of BAFF and/or other chemokines/cytokines in a CS-induced mouse model of COPD could be advantageous and provide an additional platform to study lymphoid follicles.

10.2. Activin-A

Activin-A belongs to the transforming growth factor (TGF)-β superfamily, which is one of the most evolutionarily conserved signal transduction pathways within the animal kingdom [196]. There are more than 30 TGF-β superfamily ligands in mammals, including the TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs). Activin-A is originally identified as a stimulator of follicle-stimulating hormone, but during last years, activin-A has emerged as a multi-faceted cytokine. Activin-A has been extensively studied in the context of pulmonary fibrosis and asthma, however not yet in the pathogenesis of COPD. We hypothesized that activin-A levels were increased in patients with COPD and investigated whether neutralization impacts the development of pulmonary inflammation after 4 weeks of CS exposure.

10.2.1. Expression study

We performed immunohistochemical and expression analysis on an extensive study population comprised of subjects who have never smoked, smokers without airflow limitation and patients with COPD. Activin-A expression analysis revealed that activin-A mRNA levels are significantly higher in current smokers, independent of airflow limitation. Because of its multifunctional nature, activin-A can be synthesized by many cells in the body [416;417]. In our immunohistochemical study, we observed strong staining for activin-A in bronchiolar epithelial cells with a minor staining of smooth muscle cells and macrophages. We have demonstrated that the production of activin-A is up-regulated in patients with COPD, especially in the airway epithelium. This suggests that the epithelium is the main source of activin-A in COPD, consistent with previous findings in allergic asthma [269]. Indeed, we have confirmed *in vitro* that exposure of primary human bronchial epithelial cell cultures to CS induced the expression and secretion of activin-A. Not only epithelial cells, but also neutrophils, mast cells and $CD4^+$ T cells have been described as possible sources of activin-A in the lungs[267;270]. We conclude that pulmonary levels of activin-A are increased in patients with COPD. This corresponds with similar observations made in pulmonary fibrosis and asthma [260;261].

We also confirmed increased activin-A signalling by the presence of phosphorylated Smad2 in the airway epithelium. In patients with COPD, different results have been found. Podowski and colleagues found that p-Smad2 levels are elevated in both the airspace and airway compartment of patients with moderate COPD, compared to ex-smoking controls [227]. On the other hand, it has been suggested that p-Smad2 signalling activity is different according to the localisation in lung tissue. p-Smad2 is reduced in emphysematous lung tissue, while in thickened peribronchial areas increased p-

Smad2 has been found [275]. It would be interesting to test whether activin-A levels are also differentially expressed in emphysematous lung tissue versus fibrotic lung tissue.

Follistatin is the endogenous antagonist of activin-A by binding to activin-A and thus preventing activin-A from binding to its receptor. Interestingly, follistatin levels were either unchanged or decreased in response to CS both *in vivo* and *in vitro*. This indicates that there is a relative excess of activin-A, unopposed by follistatin. In our human study population, follistatin mRNA expression was not significantly different between the study groups. However, we have not investigated the protein levels, nor the localization of follistatin in the lung tissue of patients with COPD. Data regarding the localization of follistatin in lung tissue are scarce. Follistatin is present in bronchial epithelium, pneumocytes and macrophages in lung tissue of control mice. Allergen challenge with OVA decreased folllistatin immunoreactiviy in the airway epithelium, in parallel with goblet cell metaplasia [266;273]. There is definitely a need for a better characterization of follistatin in lung tissue of patients with COPD.

10.2.2. Functional study and future directions

To study the functional role of activin-A in the development of CS-induced inflammation, we performed a subacute *in vivo* neutralization experiment where we exposed C57BL/6 mice to CS for 4 weeks and administered follistatin 3 times a week. We observed that mice treated with follistatin were partially protected against CS-induced influx of monocytes and lymphocytes in the airways. Moreover, the CS-induced increase of several chemokines and cytokines was reduced upon activin-A neutralization. Although we can conclude that activin-A is implicated in CS-induced inflammatory responses, the protective effect of follistatin was, albeit statistically significant, rather small. Therefore, it would be advantageous to repeat the experiment with a higher dose and/or higher frequency of follistatin injections.

Another possibility is to use alternative agents to antagonize activin-A, such as a neutralizing anti-activin-A antibody, which is efficiently used in an OVA-induced experimental model of allergic asthma [269]. This would generate a more specific inhibition, since follistatin can bind to other members of the TGF-β superfamily, including several BMPs such as BMP-2, -4, -5, -6, -7, -8. However, it should be kept in mind that the binding affinity of follistatin for activin-A is comparable to the affinity of activin-A for the activin receptor itself and is essentially non-reversible, whereas the affinity of follistatin for BMPs is much lower [342]. Another way of activin-A neutralization is by the administration of a soluble form of the activin type IIB receptor (ActR2B), which is demonstrated to attenuate LPS-induced acute lung injury [344]. Finally, different routes of administration (systemic vs local, intraperitoneal vs intranasal) should be tested, since this can affect outcome [269].

In this study, we have only investigated the development of pulmonary inflammation upon CS exposure, which is only one aspect of the complex pathogenesis of COPD. In future research, activin-A should be neutralized in a chronic CS experiment in mice, where after airway wall remodelling, emphysema, lymphoid follicle formation, mucus production and lung function should be evaluated. It will be interesting to test whether activin-A still functions as a pro-inflammatory cytokine in a chronic setting, similar as we found in our subacute experiment, or whether activin-A shifts towards an anti-inflammatory cytokine once inflammation is established. Finally, activin-A might affect other organs than the lungs and influence systemic manifestations such as muscle weakness and cardiovascular disease. Activin-A is expressed in atherosclerotic lesions and is involved in atherogenesis by inducing differentiation of neointimal smooth muscle cells [418]. Moreover, muscle injury is attenuated by inhibition of activin-A in mice [419]. This highlights that we should also look outside the lungs for a role of activin-A in COPD.

10.3. BMP-6

Bone morphogenetic protein (BMP)-6 is, similar to activin-A, a member of the TGF-β superfamily. The BMP proteins form the largest subfamily within the TGF-β superfamily. BMP proteins were initially discovered to induce bone growth in muscle. Today, it is generally accepted that BMPs regulate processes throughout the entire body and can be called "body morphogenetic proteins" [351]. BMP-6 was of particular interest since its locus is associated with forced vital capacity in a genome wide association meta-analysis of 2 large consortiums [3]. Even more, BMP-6 is identified to control tissue remodeling and iron homeostasis, 2 mechanisms implicated in the pathogenesis of COPD [279;420]. We hypothesized that the BMP-6 pathway is dysregulated in COPD and that this will contribute to the CS-induced development of inflammation and airway wall remodelling.

10.3.1. Expression study

We started by investigating whether the pulmonary expression of BMP-6 was indeed altered in patients with COPD compared to smokers without COPD and never smokers. The results demonstrated that BMP-6 expression is decreased in both patients with COPD and smokers. Importantly, patients with severe COPD (GOLD stage III and IV) had lowest BMP- levels, indicating that BMP-6 levels decrease with disease severity. In line with these observations, exposure of mice to CS for 24 weeks, but not 4 weeks reduced BMP-6 levels at the mRNA level.

Furthermore, we were interested in what kind of cells in the lungs are capable of producing BMP-6. It is known from *in vitro* studies that smooth muscle cells, bronchial epithelial cells and endothelial cells can express BMP-6 [3;257;355]. Our results confirm that airway smooth muscle cells, endothelial cells and macrophages are the main source of BMP-6 in both human and murine lung tissue. Quantification of BMP-6 in the smooth muscle cell layer around the airways in mice revealed no differences between air- and CS-exposed mice. On the other hand, pulmonary macrophages isolated from the lungs of CS-exposed mice expressed less BMP-6 mRNA compared to macrophages derived from air-exposed murine lungs.

10.3.2. Functional study

We aimed to characterize the *in vivo* functional role of BMP-6 in the pathogenesis of COPD by using BMP-6 deficient mice (and the appropriate WT control littermates) in a CS model of COPD. In the context of inflammation, the outcome was different between the subacute 4 weeks experiment and chronic 24 weeks experiment. While BMP-6 deficiency resulted in an aggravated pulmonary inflammation upon 4 weeks of smoke, no effect of BMP-6 deficiency on the development of inflammation was observed in mice exposed to CS for 24 weeks. The enhanced inflammation by loss

of BMP-6 has been reported earlier in models of hepatic and renal fibrosis [298;299]. This suggests that BMP-6 predominantly plays a role in the subacute inflammatory response to CS. The fact that BMP-6 deficiency has no influence on CS-induced inflammation after chronic CS exposure could suggest a redundancy mechanism where other proteins of the BMP family could take over. Especially BMP-7 is a good candidate, since BMP-6 and BMP-7 are structurally very similar and BMP-7 can act as an anti-inflammatory cytokine [357]. Furthermore, we assessed airway wall remodeling by measuring the deposition of collagen and fibronectin after 24 weeks of smoke. Consistent with the inflammation data, BMP-6 deficiency had no influence on airway wall remodeling. However, 24 weeks of CS was not sufficient to induce airway wall remodeling in these mice, so we cannot make any conclusions about the effect of BMP-6 deficiency on CS-induced airway wall remodelling.

On the other hand, 4 weeks of CS exposure was sufficient to induce the pro-fibrotic TGF-β1 in the BAL supernatants. Importantly, the TGF-β1 protein levels were significantly higher in CS-exposed BMP-6 deficient mice compared to the CS-exposed control group. This effect has also been observed in the renal fibrosis model and has been suggested to be derived from the inhibition of the TGF-β1 auto-inductive mechanism by BMP-6 [299]. Although reversing TGF-β1-induced actions was originally described to BMP-7, BMP-6 is equally potent in inhibiting TGF-β1 functions [289;300]. The interaction of BMP-6 and TGF-β1 should be further researched in the context of CS-induced airway wall remodelling.

As a final part of our study, we measured levels of iron and ferritin, an iron storage protein, in CS-exposed BMP-6 deficient mice. Next to increased levels of iron in the serum of BMP-6 deficient mice, we report for the first time iron overload in the lungs, similar to the iron accumulation found in the liver, pancreas, heart and kidney [279]. Iron levels were not influenced by exposure to CS both after 4 and 24 weeks of CS. In contrast, ferritin levels were significantly elevated in BMP-6 deficient mice exposed to CS for 4 weeks compared to the control groups. This could result in more oxidative stress and account for the higher inflammatory response in BMP-6 deficient mice. This hypothesis should be tested further.

10.3.3. Future directions

The BMP family comprises more than 30 members, some of them are structurally and functionally very similar to each other. None of these proteins has been studied in COPD. It would be interesting to build a complete expression profile of the BMP family in patients with COPD, similar to what has been reported for asthma. In an OVA-induced mouse model of allergic airway inflammation, activation of the BMP pathway was evident as indicated by the presence of phosphorylated Smad1/5. This is accompanied by increased levels of the BMP type I receptors ALK2, ALK3, ALK6 and

the BMP ligands BMP-2, BMP-4 and BMP-6, whereas BMP-5 and BMP-7 expression is reduced [301]. In contrary, patients with asthma exhibit reduced BMP receptor expression, while allergen provocation increased expression of BMP-7 and activated BMP signalling [305].

Especially BMP-7 is an excellent candidate for future research. In cultured lung fibroblasts BMP-7 opposed TGF-β1 dependent fibrogenic activity. Moreover, BMP-7 has a protective effect in animal models of asbestos- and silica-induced pulmonary fibrosis [292-294]. In an OVA-induced mouse model of asthma, intranasal treatment with BMP-7 reduced lung inflammation and type 1 collagen deposition [297]. Also BMP-4, involved in lung morphogenesis and epithelial-to-mesenchymal transition, can be targeted [380;393].

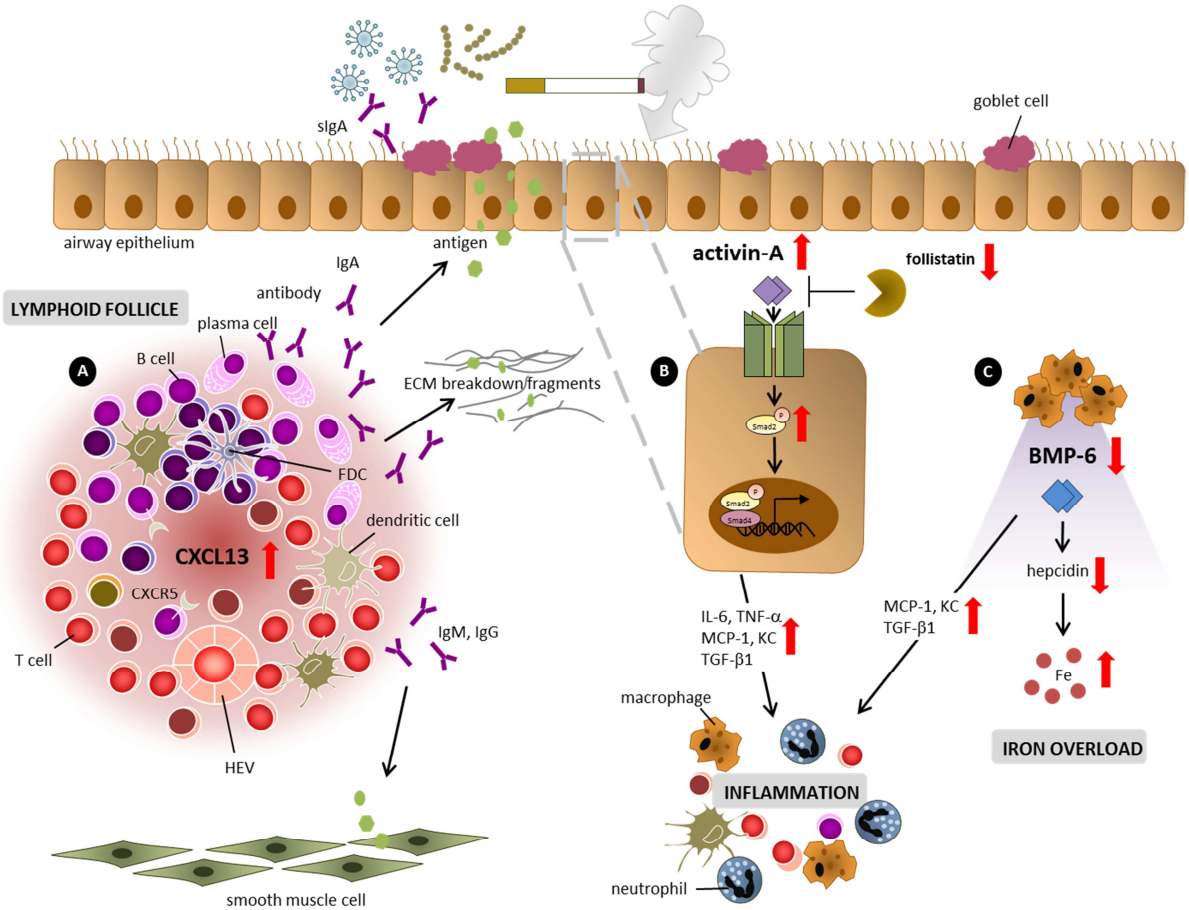
Finally, the BMP signalling pathway is tightly regulated at different levels. Extracellular antagonists negatively regulate signalling by binding to the BMP ligands and inhibiting receptor binding. BMP antagonists have been classified into 3 subfamilies: the Dan, the Twisted gastrulation and the Chordin/Noggin family [278]. Evidence for the functional significance of these antagonists in disease pathology has been extensively documented. For example, overexpression of gremlin, belonging to the Dan family, resulted in lung fibrosis [421]. These endogenous antagonists should be taken into account when studying BMP signalling in COPD.

Figure 25. Schematic overview of our research work. A) CXCL13, a lymphoid chemokine attracting $CXCR5^+$ B cells is increased in the lungs of patients with COPD. Moreover, CXCL13 is found in the B cell zone of lymphoid follicles. Lymphoid follicles may contain high endothelial venules (HEV), follicular dendritic cells (FDC) and germinal centers with the potential to produce plasma cells and antibody responses against infectious agents and/or autoantigens, such as breakdown fragments from the extracellular matrix (ECM). We have demonstrated that neutralization of CXCL13 prevented the formation of lymphoid follicles and attenuated cigarette smoke (CS)-induced inflammation and alveolar wall destruction. **B) Activin-A**, a pleiotropic cytokine of the TGF-β superfamily, and phosphorylated Smad2, a downstream mediator, are increased in the airway epithelium of patients with COPD. In contrast, follistatin, a natural antagonist of activin-A is decreased upon CS exposure. An imbalance between activin-A and follistatin contributes to CS-induced inflammation. **C)** Bone morphogenetic protein-6 (**BMP-6**), also a member of the TGF-β superfamily, is decreased in the lungs of patients with COPD. While we have found that BMP-6 is important for pulmonary inflammation upon sub-acute CS exposure, BMP-6 seems less important in chronic CS-induced inflammation and remodelling. Reduced pulmonary BMP-6 levels result in decreased levels of hepcidin, a hormone that degrades the iron exporter ferroportin. Eventually, iron will accumulate in the lungs, potentially contributing to oxidative stress. (s)Ig: (secretory) immunoglobulin, CXCL: chemokine (c-x-c motif) ligand, CXCR: chemokine (c-x-c motif) receptor, IL: interleukin, TNF-α: tumor necrosis factor-α, TGF-β1: transforming growth factor-β1, MCP-1: monocyte chemotactic protein-1, KC: keratinocyte chemoattractant, Fe: iron. →

10.4. General conclusion

We conclude that CXCL13 is increased in patients with COPD and is crucial for the organization of CS-induced lymphoid follicles in mice. Neutralization of CXCL13 partially protects mice against CS-induced inflammation in bronchoalveolar lavage and alveolar wall destruction (**Figure 25**).

We have demonstrated that activin-A and BMP-6, 2 members of the TGF-β superfamily, are respectively increased and decreased in COPD. While neutralization of activin-A partially attenuated CS-induced inflammation, deficiency of BMP-6 aggravated CS-induced inflammation in a subacute setting. In contrast, BMP-6 is less important in CS-induced inflammation and airway wall remodelling after chronic exposure to CS (**Figure 25**). With these data, we underline that TGF-β1 is not the only member of the TGF-β superfamily that is involved in COPD and more research is needed into this interesting protein family.



[CHAPTER 11: SUMMARY / SAMENVATTING]

SUMMARY

Chronic Obstructive Pulmonary Disease (COPD) represents a major cause of chronic morbidity and mortality throughout the world. COPD is characterized by an abnormal inflammatory reaction in the airways and lung parenchyma to cigarette smoke (CS), resulting in destruction of lung parenchyma (= emphysema) and structural changes in and around the airways. Therapies or drugs that slow down the accelerated decline in lung function in patients with COPD are still lacking. Therefore, it is essential to unravel the mechanistic processes that underlie the inflammatory reaction and subsequent structural changes in COPD. The main objective of this study was to explore the functional role of lymphoid follicles and the TGF-β superfamily in the pathogenesis of COPD.

Patients with severe COPD develop pulmonary **lymphoid follicles**. It was unclear whether these highly specialized lymphoid structures were harmful or beneficial in COPD. CXCL13 is a chemokine that attracts B lymphocytes and is crucial for lymphoid neogenesis. We studied the role of lymphoid follicles by neutralization of CXCL13. First, we have demonstrated that CXCL13 is increased in COPD and is produced within the B cell zone of lymphoid follicles of patients with severe COPD. Secondly, preventing the formation of lymphoid follicles by pharmacological neutralization of CXCL13 partially inhibited airway inflammation and destruction of the lung parenchyma, but had no influence on the development of airway wall remodelling.

The transforming growth factor (TGF)-β superfamily comprises TGF-βs, activins and bone morphogenetic proteins (BMPs). Dysregulation of TGF-β1, the most studied ligand of the superfamily, is associated with numerous fibrotic and immune-mediated diseases, including COPD. However, little is known about the other ligands of this superfamily in the pathogenesis of COPD**.** Since **activin-A** signaling is activated in pulmonary fibrosis and asthma, we reasoned that activin-A is involved in CS-induced inflammation and COPD. First, we have found that activin-A is increased in the airway epithelium of patients with COPD. Moreover, activin-A is induced upon CS exposure both *in vivo* in a murine model, and *in vitro* in epithelial cell cultures. By administration of follistatin, an endogenous inhibitor of activin-A, the recruitment of inflammatory cells and mediators was partially inhibited after subacute CS exposure.

BMPs are the largest subfamily of the TGF-β superfamily with more than 20 members. We have investigated the role of **BMP-6** in the pathogenesis of COPD, since its locus is associated with lung function. Moreover, BMP-6 is important for tissue remodelling, which indicates a clear rationale to study the role of BMP-6 in CS-induced inflammation and airway wall remodelling. We have documented that BMP-6 mRNA expression is decreased in the lung tissue of patients with COPD and

of CS-exposed mice. BMP-6 deficiency renders mice more susceptible to the development of pulmonary inflammation after 4 weeks of smoke. In a chronic setting however, BMP-6 is not important for pulmonary inflammation, nor for the development of airway wall remodelling. Finally, we observed iron accumulation in the lungs of BMP-6 deficient mice which could contribute to oxidative stress. With these results, we underline that future research on the role of the TGF-β superfamily in COPD, should be expanded beyond the classical TGF-β1 ligand.

SAMENVATTING

Chronisch obstructief longlijden (COPD) is wereldwijd een belangrijke oorzaak van chronische morbiditeit en mortaliteit. COPD wordt gekarakteriseerd door een abnormale inflammatoire reactie in de luchtwegen en long parenchym op sigarettenrook (SR), wat resulteert in de destructie van het long parenchym (=emfyseem) en structurele verandering in en rond de luchtwegen. Therapieën of medicatie die de versnelde daling in longfunctie in patiënten met COPD vertragen, ontbreken nog steeds. Daarom is het essentieel om de mechanismen die aan de basis liggen van de inflammatoire reactie en de daaropvolgende structurele veranderingen in COPD te ontrafelen. Het belangrijkste doel van deze studie is het onderzoeken van de functionele rol van lymfoïde follikels en de TGF-β superfamilie in de pathogenese van COPD.

Patiënten met ernstig COPD ontwikkelen **lymfoïde follikels** in het longweefsel. Het was niet duidelijk of deze zeer gespecialiseerde lymfoïde structuren schadelijk zijn of beschermend werken in COPD. CXCL13 is een chemokine dat B lymfocyten aantrekt en is cruciaal voor lymfoïde neogenese. We hebben de rol van lymfoïde follikels bestudeerd door neutralisatie van CXCL13. Ten eerste hebben we aangetoond dat CXCL13 is toegenomen in COPD en wordt geproduceerd binnen de B cel zone van lymfoïde follikels van patiënten met ernstig COPD. Ten tweede wordt de luchtweg inflammatie en destructie van het long parenchym partieel geïnhibeerd door het verhinderen van de vorming van lymfoïde follikels door farmacologische neutralisatie van CXCL13.

De transforming growth factor (TGF)-β superfamilie bestaat uit TGF-β's, activines en bone morphogenetic proteins (BMP's). Dysregulatie van TGF-β1, het meest bestudeerde ligand van de superfamilie, is geassocieerd met verschillende fibrotische en immuun-gemedieerde ziektes, waaronder COPD. Er is echter weinig gekend over de andere liganden van deze superfamilie in de pathogenese van COPD. Aangezien **activine-A** signalisatie geactiveerd is in pulmonaire fibrose en astma, redeneerden we dat activine-A betrokken is in SR-geïnduceerde inflammatie en COPD. Allereerst hebben we gevonden dat activine-A verhoogd is in het luchtweg epitheel van patiënten met COPD. Bovendien wordt activine-A geïnduceerd na blootstelling aan SR, zowel *in vivo* in een muismodel, als *in vitro* in epitheliale celculturen. Door administratie van follistatine, een endogene inhibitor van activine-A, is de rekrutering van inflammatoire cellen en mediatoren gedeeltelijk geïnhibeerd na subacute SR blootstelling.

BMP's zijn de grootste subfamilie van de TGF-β superfamilie met meer dan 20 leden. We hebben de rol van **BMP-6** onderzocht in de pathogenese van COPD, aangezien zijn locus geassocieerd is met longfunctie. Bovendien is BMP-6 belangrijk voor weefsel remodellering, wat een duidelijk rationale

aantoont om de rol van BMP-6 te bestuderen in SR-geïnduceerde inflammatie en luchtweg wand remodellering. We hebben aangetoond dat de mRNA expressie van BMP-6 verminderd is in het longweefsel van patiënten met COPD en van SR-geïnduceerde muizen. BMP-6 deficiëntie zorgt ervoor dat muizen meer gevoelig zijn voor de ontwikkeling van pulmonaire inflammatie na 4 weken rook. In een chronische setting daarentegen, is BMP-6 niet belangrijk voor pulmonaire inflammatie, noch voor de ontwikkeling van de luchtweg wand remodellering. Tenslotte, hebben we accumulatie van ijzer waargenomen in de longen van BMP-6 deficiënte muizen, wat mogelijk kan bijdragen tot oxidatieve stress. Met deze resultaten, benadrukken we dat toekomstig onderzoek naar de rol van de TGF-β superfamilie in COPD, moet uitgebreid worden zich verder moet focussen op deze eiwit familie, verder dan het typische TGF-β1 ligand.

[PART III: ADDENDUM]

ABBREVIATIONS

REFERENCES

CURRICULUM VITAE

DANKWOORD

ABBREVIATIONS

BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BMP	Bone morphogenetic protein
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
DC	Dendritic cell
DI	Destructive index
ELISA	Enzyme-linked immunosorbent assay
FDC	Follicular dendritic cell
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GDF	Growth differentiation factor
GOLD	Global Initiative for chronic obstructive lung disease
GWAS	Genome wide association study
HBEC	Human bronchial epithelial cell
HEV	High endothelial venule
iBALT	Inducible bronchus-associated lymphoid tissue
Ig	Immunoglobulin
IL	Interleukin
KO	Knockout
LF	Lymphoid follicle
L_m	Mean linear intercept
LPS	Lipopolysaccharide
LT	lymphotoxin
MLN	Mediastinal lymph node
MMP	Matrix metalloproteinase
Pbm	Length of the basement membrane
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time polymerase chain reaction
ROS	Reactive oxygen species
SLO	Secondary lymphoid organ
SNP	Single nucleotide polymorphism
TGF-β	Transforming growth factor-beta
TLO	Tertiary lymphoid organ

REFERENCES

1. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, Stockley RA, Sin DD, and Rodriguez-Roisin R. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease GOLD Executive Summary. *Am J Respir Crit Care Med* 2013;187:347-365.

2. Mannino DM and Buist AS. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet* 2007;370:765-773.

3. Loth DW, Artigas MS, Gharib SA, Wain LV, Franceschini N, Koch B, Pottinger TD, Smith AV, Duan Q, Oldmeadow C, Lee MK, Strachan DP, James AL, Huffman JE, Vitart V, Ramasamy A, Wareham NJ, Kaprio J, Wang XQ, Trochet H, Kahonen M, Flexeder C, Albrecht E, Lopez LM, de Jong K, Thyagarajan B, Alves AC, Enroth S, Omenaas E, Joshi PK, Fall T, Vinuela A, Launer LJ, Loehr LR, Fornage M, Li G, Wilk JB, Tang W, Manichaikul A, Lahousse L, Harris TB, North KE, Rudnicka AR, Hui J, Gu X, Lumley T, Wright AF, Hastie ND, Campbell S, Kumar R, Pin I, Scott RA, Pietilainen KH, Surakka I, Liu Y, Holliday EG, Schulz H, Heinrich J, Davies G, Vonk JM, Wojczynski M, Pouta A, Johansson A, Wild SH, Ingelsson E, Rivadeneira F, Volzke H, Hysi PG, Eiriksdottir G, Morrison AC, Rotter JI, Gao W, Postma DS, White WB, Rich SS, Hofman A, Aspelund T, Couper D, Smith LJ, Psaty BM, Lohman K, Burchard EG, Uitterlinden AG, Garcia M, Joubert BR, McArdle WL, Musk AB, Hansel N, Heckbert SR, Zgaga L, van Meurs JBJ, Navarro P, Rudan I, Oh YM, Redline S, Jarvis DL, Zhao JH, Rantanen T, O'Connor GT, Ripatti S, Scott RJ, Karrasch S, Grallert H, Gaddis NC, Starr JM, Wijmenga C, Minster RL, Lederer DJ, Pekkanen J, Gyllensten U, Campbell H, Morris AP, Glaser S, Hammond CJ, Burkart KM, Beilby J, Kritchevsky SB, Gudnason V, Hancock DB, Williams OD, Polasek O, Zemunik T, Kolcic I, Petrini MF, Wjst M, Kim WJ, Porteous DJ, Scotland G, Smith BH, Viljanen A, Heliovaara M, Attia JR, Sayers I, Hampel R, Gieger C, Deary IJ, Boezen HM, Newman A, Jarvelin MR, Wilson JF, Lind L, Stricker BH, Teumer A, Spector TD, Melen E, Peters MJ, Lange LA, Barr RG, Bracke KR, Verhamme FM, Sung J, Hiemstra PS, Cassano PA, Sood A, Hayward C, Dupuis J, Hall IP, Brusselle GG, Tobin MD, and London SJ. Genome-wide association analysis identifies six new loci associated with forced vital capacity. *Nat Genet* 2014;46:669-677.

4. Soler Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang WB, Zhai GJ, Zhao JH, Smith AV, Huffman JE, Albrecht E, Jackson CM, Evans DM, Cadby G, Fornage M, Manichaikul A, Lopez LM, Johnson T, Aldrich MC, Aspelund T, Barroso I, Campbell H, Cassano PA, Couper DJ, Eiriksdottir G, Franceschini N, Garcia M, Gieger C, Gislason GK, Grkovic I, Hammond CJ, Hancock DB, Harris TB, Ramasamy A, Heckbert SR, Heliovaara M, Homuth G, Hysi PG, Alan L, Jankovic S, Joubert BR, Karrasch S, Klopp N, Koch B, Kritchevsky SB, Launer LJ, Liu YM, Loehr LR, Lohman K, Loos RJF, Lumley T, Al Balushi KA, Ang WQ, Barr RG, Beilby J, Blakey JD, Boban M, Boraska V, Brisman J, Britton JR, Brusselle GG, Cooper C, Curjuric I, Dahgam S, Deary IJ, Ebrahim S, Eijgelsheim M, Francks C, Gaysina D, Granell R, Gu XJ, Hankinson JL, Hardy R, Harris SE, Henderson J, Henry A, Hingorani AD, Hofman A, Holt PG, Hui JN, Hunter ML, Imboden M, Jameson KA, Kerr SM, Kolcic I, Kronenberg F, Liu JZ, Marchini J, McKeever T, Morris AD, Olin AC, Porteous DJ, Postma DS, Rich SS, Ring SM, Rivadeneira F, Rochat T, Sayer AA, Sayers I, Sly PD, Smith GD, Sood A, Starr JM, Uitterlinden AG, Vonk JM, Wannamethee SG, Whincup PH, Wijmenga C, Williams OD, Wong A, Mangino M, Marciante KD, McArdle WL, Meibohm B, Morrison AC, North KE, Omenaas E, Palmer LJ, Pietilainen KH, Pin I, Polasek O, Pouta A, Psaty BM, Hartikainen AL, Rantanen T, Ripatti S, Rotter JI, Rudan I, Rudnicka AR, Schulz H, Shin SY, Spector TD, Surakka I, Vitart V, Volzke H, Wareham NJ, Warrington NM, Wichmann HE, Wild SH, Wilk JB, Wjst M, Wright AF, Zgaga L, Zemunik T, Pennell CE, Nyberg

F, Kuh D, Holloway JW, Boezen HM, Lawlor DA, Morris RW, Probst-Hensch N, Kaprio J, Wilson JF, Hayward C, Kahonen M, Heinrich J, Musk AW, Jarvis DL, Glaser S, Jarvelin MR, Stricker BHC, Elliott P, O'Connor GT, Strachan DP, London SJ, Hall IP, Gudnason V, and Tobin MD. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nat Genet* 2011;43:1082-1090.

5. Salvi SS and Barnes PJ. Chronic obstructive pulmonary disease in non-smokers. *Lancet* 1929;374:733-743.

6. Scanlon PD, Connett John E., Waller Lance E., Altose Murray D., Bailey William C., Buist Sonja A., Tashkin Donald P., and for the lung health study research group. Smoking Cessation and Lung Function in Mild-to-Moderate Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2000;161:381-390.

7. Barnes PJ. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nat Rev Drug Discov* 2013;12:543-559.

8. European Lung White Book - Chapter 1: the burden of lung disease. 2013.

9. Menezes AMB, Perez-Padilla R, Jardim JRB, Muino A, Lopez MV, Valdivia G, de Oca MM, Talamo C, Hallal PC, and Victoria CG. Chronic obstructive pulmonary disease in five Latin American cities (the PLATINO study): a prevalence study. *Lancet* 2005;366:1875-1881.

10. Buist AS, McBurnie MA, Vollmer WM, Gillespie S, Burney P, Mannino DM, Menezes AM, Sullivan SD, Lee TA, Weiss KB, Jensen RL, Marks GB, Gulsvik A, and Nizankowska-Mogilnicka E. International variation in the prevalence of COPD (The BOLD Study): a population-based prevalence study. *Lancet* 2001;370:741-750.

11. van Durme YMTA, Verhamme KMC, Stijnen T, van Rooij FJA, Van Pottelberge GR, Hofman A, Joos GF, Stricker BHC, and Brusselle GG. Prevalence, incidence, and lifetime risk for the development of copd in the elderly: The rotterdam study. *Chest* 2009;135:368-377.

12. Pauwels RA and Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet* 2004;364:613-620.

13. Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, Schmid V, and Buist S. Chronic obstructive pulmonary disease: current burden and future projections. *Eur Respir J* 2006;27:397-412.

14. Murray CJ and Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet* 1997;349:1498-1504.

15. European Lung White Book - Chapter 2: the economic burden of lung disease. 2013.

16. Global Initiative for Chronic Obstructive Lung Disease - Global Strategy for the diagnosis, management and prevention of COPD. 2014.

17. Medical Research Council. Definition and classification of chronic bronchitis for clinical and epidemiological purposes: a report to the Medical Research Council by their Committee on the Aetiology of Chronic Bronchitis. *Lancet* 1965;1:775-780.

18. Schols AM, Soeters PB, Dingemans AM, Mostert R, Frantzen PJ, and Wouters EF. Prevalence and characteristics of nutritional depletion in patients with stable COPD eligible for pulmonary rehabilitation. *Am Rev Respir Dis* 1993;147:1151-1156.

19. Rodriguez-Roisin R. Toward a consensus definition for copd exacerbations. *Chest* 2000;117:398S-401S.

20. Celli BR and Barnes PJ. Exacerbations of chronic obstructive pulmonary disease. *Eur Respir J* 2007;29:1224-1238.

21. Barnes PJ and Celli BR. Systemic manifestations and comorbidities of COPD. *Eur Respir J* 2009;33:1165-1185.

22. Agusti A, Calverley PM, Celli B, Coxson HO, Edwards LD, Lomas DA, MacNee W, Miller BE, Rennard S, and Silverman EK. Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respir Res* 2010;11:122.

23. Soriano JB, Visick GT, Muellerova H, Payvandi N, and Hansell AL. Patterns of comorbidities in newly diagnosed copd and asthma in primary care. *Chest* 2005;128:2099-2107.

24. McGarvey LP, John M, Anderson JA, Zvarich M, and Wise RA. Ascertainment of cause-specific mortality in COPD: operations of the TORCH Clinical Endpoint Committee. *Thorax* 2007;62:411-415.

25. Fletcher C and Peto R. The natural history of chronic airflow obstruction. Brit Med J 1, 1645-1648. 1977.

26. Viegi G, Pistelli F, Sherrill DL, Maio S, Baldacci S, and Carrozzi L. Definition, epidemiology and natural history of COPD. *Eur Respir J* 2007;30:993-1013.

27. Brusselle GG. Matrix Metalloproteinase 12, Asthma, and COPD. *N Engl J Med* 2009;361:2664-2665.

28. Reid L. Pathology of chronic bronchitis. *Lancet* 1954;263:275-278.

29. Niewoehner DE, Kleinerman J, and Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. *N Engl J Med* 1974;291:755-758.

30. O'Shaughnessy TC, Ansari TW, Barnes NC, and Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med* 1997;155:852-857.

31. Saetta M, Turato G, Facchini FM, Corbino L, Lucchini RE, Casoni G, Maestrelli P, Mapp CE, Ciaccia A, and Fabbri LM. Inflammatory Cells in the Bronchial Glands of Smokers with Chronic Bronchitis. *Am J Respir Crit Care Med* 1997;156:1633-1639.

32. Hogg JC, Macklem PT, and Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 1968;278:1355-1360.

33. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, and Paré PD. The Nature of Small-Airway Obstruction in Chronic Obstructive Pulmonary Disease. *N Engl J Med* 2004;350:2645-2653.

34. Wright JL, Lawson LM, Pare PD, Kennedy S, Wiggs B, and Hogg JC. The detection of small airways disease. *Am Rev Respir Dis* 1984;129:989-994.

35. Cosio MG, Hale KA, and Niewoehner DE. Morphologic and morphometric effects of prolonged cigarette smoking on the small airways. *Am Rev Respir Dis* 1980;122:265-221.

36. Bosken CH, Hards J, Gatter K, and Hogg JC. Characterization of the Inflammatory Reaction in the Peripheral Airways of Cigarette Smokers Using Immunocytochemistry. *Am Rev Respir Dis* 1992;145:911-917.

37. Kim V, Rogers TJ, and Criner GJ. New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2008;5:478-485.

38. Kim WD, Eidelman DH, Izquierdo JL, Ghezzo H, Saetta MP, and Cosio MG. Centrilobular and Panlobular Emphysema in Smokers: Two Distinct Morphologic and Functional Entities. *Am Rev Respir Dis* 1991;144:1385-1390.

39. Saetta M, Ghezzo H, Kim WD, King M, Angus GE, Wang NS, and Cosio MG. Loss of alveolar attachments in smokers. A morphometric correlate of lung function impairment. *Am Rev Respir Dis* 1985;132:894-900.

40. Magee F, Wright JL, Wiggs BR, Pare PD, and Hogg JC. Pulmonary vascular structure and function in chronic obstructive pulmonary disease. *Thorax* 1988;43:183-189.

41. Smith P, Rodgers B, Heath D, and Yacoub M. The ultrastructure of pulmonary arteries and arterioles in emphysema. *J Pathol* 1992;167:69-75.

42. Dinh-Xuan AT, Higenbottam TW, Clelland CA, Pepke-Zaba J, Cremona G, Butt AY, Large SR, Wells FC, and Wallwork J. Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N Engl J Med* 1991;324:1539-1547.

43. Peinado V, Barberà JA, Ramírez J, Gómez FP, Roca J, Jover L, Gimferrer JM, and Rodriguez-Roisin R. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *Am J Physiol Lung Cell Mol Physiol* 1998;274:L908-L913.

44. Peinado V, Barberà JA, Abate P, Ramírez J, Roca J, Santos S, and Rodriguez-Roisin R. Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:1605-1611.

45. Opitz B, van Laak V, Eitel J, and Suttorp N. Innate Immune Recognition in Infectious and Noninfectious Diseases of the Lung. *Am J Respir Crit Care Med* 2010;181:1294-1309.

46. Botelho FM, Gaschler GJ, Kianpour S, Zavitz CC, Trimble NJ, Nikota JK, Bauer CM, and Stämpfli MR. Innate immune processes are sufficient for driving cigarette smoke–induced inflammation in mice. *Am J Respir Cell Mol Biol* 2010;42:394-403.

47. D'hulst AI, Maes T, Bracke KR, Demedts IK, Tournoy KG, Joos GF, and Brusselle GG. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir Res* 2005;6.

48. Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Nakahara K, and Umeda A. Increased expression of transforming growth factor-beta 1 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.

49. Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, and Rennard SI. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* 1997;155:1770-1776.

50. Masubuchi T, Koyama S, Sato E, Takamizawa A, Kubo K, Sekiguchi M, Nagai S, and Izumi T. Smoke extract stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activity. *Am J Pathol* 1998;153:1903-1912.

51. Mortaz E, Henricks PAJ, Kraneveld AD, Givi ME, Garssen J, and Folkerts G. Cigarette smoke induces the release of CXCL-8 from human bronchial epithelial cells via TLRs and induction of the inflammasome. *Biochim Biophysic Act - Molecular Basis of Disease* 2011;1812:1104-1110.

52. Ferhani N, Letuve S, Kozhich A, Thibaudeau O, Grandsaigne M, Maret M, Dombret MC, Sims GP, Kolbeck R, Coyle AJ, Aubier M, and Pretolani M. Expression of High-Mobility Group Box 1 and of Receptor for Advanced Glycation End Products in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2010;181:917-927.

53. Lommatzsch M, Cicko S, Müller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Dürk T, Zissel G, and Ferrari D. Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:928-934.

54. Brusselle GG, Joos GF, and Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011;378:1015-1026.

55. Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, and Saetta M. Severity of Airflow Limitation Is Associated with Severity of Airway Inflammation in Smokers. *Am J Respir Crit Care Med* 1998;158:1277-1285.

56. Saetta M, Di Stefano A, Maestrelli P, Ferraresso A, Drigo R, Potena A, Ciaccia A, and Fabbri LM. Activated T-Lymphocytes and Macrophages in Bronchial Mucosa of Subjects with Chronic Bronchitis. *Am Rev Respir Dis* 1993;147:301-306.

57. Traves SL, Smith SJ, Barnes PJ, and Donnelly LE. Specific CXC but not CC chemokines cause elevated monocyte migration in COPD: a role for CXCR2. *J Leukocyte Biol* 2004;76:441-450.

58. Tomita K, Caramori G, Lim S, Ito K, Hanazawa T, Oates T, Chiselita I, Jazrawi E, Chung KF, Barnes PJ, and Adcock IM. Increased p21CIP1/WAF1 and B Cell Lymphoma Leukemia-xL Expression and Reduced Apoptosis in Alveolar Macrophages from Smokers. *Am J Respir Crit Care Med* 2002;166:724-731.

59. Kent L, Smyth L, Clayton C, Scott L, Cook T, Stephens R, Fox S, Hext P, Farrow S, and Singh D. Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. *Cytokine* 2008;42:205-216.

60. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, and Chung KF. Balance of Matrix Metalloprotease-9 and Tissue Inhibitor of Metalloprotease-1 from Alveolar Macrophages in Cigarette Smokers. *Am J Respir Crit Care Med* 2000;162:1355-1360.

61. Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA, and Brusselle GG. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax* 2006;61:196-201.

62. Morrison D, Rahman I, Lannan S, and MacNee W. Epithelial Permeability, Inflammation, and Oxidant Stress in the Air Spaces of Smokers. *Am J Respir Crit Care Med* 1999;159:473-479.

63. Wallace AM, Sandford AJ, English JC, Burkett KM, Li H, Finley RJ, Müller NL, Coxson HO, Paré PD, and Abboud RT. Matrix metalloproteinase expression by human alveolar macrophages in relation to emphysema. *COPD* 2008;5:13-23.

64. Hautamaki RD, Kobayashi DK, Senior RM, and Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002-2004.

65. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, and Reynolds PN. Smoking Alters Alveolar Macrophage Recognition and Phagocytic Ability. *Am J Respir Cell Mol Biol* 2007;37:748-755.

66. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.

67. Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, Calabrese F, Casoni G, Maestrelli P, and Papi A. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* 2004;59:308-312.

68. O'Donnell RA, Peebles C, Ward JA, Daraker A, Angco G, Broberg P, Pierrou S, Lund J, Holgate ST, Davies DE, Delany DJ, Wilson SJ, and Djukanovic R. Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD. *Thorax* 2004;59:837-842.

69. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, and Belaaouaj A. Neutrophil Elastase Contributes to Cigarette Smoke-Induced Emphysema in Mice. *Am J Pathol* 2003;163:2329-2335.

70. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, and Zychlinsky A. Neutrophil Extracellular Traps Kill Bacteria. *Science* 2004;303:1532-1535.

71. Obermayer A, Stoiber W, Krautgartner WD, Klappacher M, Kofler B, Steinbacher P, Vitkov L, Grabcanovic-Musija F, and Studnicka M. New Aspects on the Structure of Neutrophil Extracellular Traps from Chronic Obstructive Pulmonary Disease and *In Vitro* Generation. *Plos One* 2014;9:e97784.

72. Sommerhoff CP, Nadel JA, Basbaum CB, and Caughey GH. Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Invest* 1990;85:682.

73. Stringer KAT. Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1572-L1579.

74. Lacoste JY, Bousquet J, Chanez P, Van Vyve T, Simony-Lafontaine J, Lequeu N, Vic P, Enander I, Godard P, and Michel FoB. Eosinophilic and neutrophilic inflammation in asthma, chronic

bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immun* 1993;92:537-548.

75. Fujimoto K, Kubo K, Yamamoto H, Yamaguchi S, and Matsuzawa Y. Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. *Chest* 1999;115:697-702.

76. Saetta M, Di Stefano A, Maestrelli P, Turato G, Ruggieri MP, Roggeri A, Calcagni P, Mapp CE, Ciaccia A, and Fabbri LM. Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med* 1994;150:1646-1652.

77. Andersson CK, Mori M, Bjermer L, Lofdahl CG, and Erjefalt JS. Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:206-217.

78. Grashoff WF, Sont JK, Sterk PJ, Hiemstra PS, de Boer WI, Stolk J, Han J, and Van Krieken JM. Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages. *Am J Pathol* 1997;151:1785.

79. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, and Ugolini S. Innate or adaptive immunity? The example of natural killer cells. *Science* 2011;331:44-49.

80. Motz GT, Eppert BL, Wortham BW, Amos-Kroohs RM, Flury JL, Wesselkamper SC, and Borchers MT. Chronic Cigarette Smoke Exposure Primes NK Cell Activation in a Mouse Model of Chronic Obstructive Pulmonary Disease. *J Immunol* 2010;184:4460-4469.

81. Urbanowicz RA, Lamb JR, Todd I, Corne JM, and Fairclough LC. Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients. *Respir Res* 2010;11:76.

82. Prieto A, Reyes E, Bernstein ED, Martinez B, Monserrat J, Izquierdo JL, Callol L, de Lucas P, Alvarez-Sala R, Alvarez-Sala José Luis, Villarrubia VG, and Alvarez-Mon Melchor. Defective natural killer and phagocytic activities in chronic obstructive pulmonary disease are restored by glycophosphopeptical (inmunoferon). *Am J Respir Crit Care Med* 2001;163:1578-1583.

83. Demedts IK, Bracke KR, Van Pottelberge G, Testelmans D, Verleden GM, Vermassen FE, Joos GF, and Brusselle GG. Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;175:998-1005.

84. Van Pottelberge GR, Bracke KR, Demedts IK, De Rijck K, Reinartz SM, Van Drunen CM, Verleden GM, Vermassen FE, Joos GF, and Brusselle GG. Selective accumulation of langerhans-type dendritic cells in small airways of patients with COPD. *Respir Res* 2010;11:35.

85. Heath WR and Carbone FR. Dendritic cell subsets in primary and secondary T cell responses at body surfaces. *Nat Immunol* 2009;10:1237-1244.

86. Rogers AV, Ädelroth E, Hattotuwa K, Dewar A, and Jeffery PK. Bronchial mucosal dendritic cells in smokers and ex-smokers with COPD: an electron microscopic study. *Thorax* 2008;63:108-114.

87. Freeman CM, Martinez FJ, Han MK, Ames TM, Chensue SW, Todt JC, Arenberg DA, Meldrum CA, Getty C, McCloskey L, and Curtis JL. Lung Dendritic Cell Expression of Maturation

Molecules Increases with Worsening Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2009;180:1179-1188.

88. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, and 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.

89. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallesco G, ropeano G, Mapp CE, and Maestrelli P. CD8+ ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:711-717.

90. Saetta M, Mariani M, Panina-Bordignon P, Turato G, Buonsanti C, Baraldo S, Bellettato CM, Papi A, Corbetta L, and Zuin R. Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;165:1404-1409.

91. Chrysofakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, Klimathianaki M, and Siafakas NM. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest* 2004;125:71-76.

92. Hodge S, Hodge G, Nairn J, Holmes M, and Reynolds PN. Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. *COPD* 2006;3:179-187.

93. Majo J, Ghezzo H, and Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J* 2001;17:946-953.

94. Taraseviciene-Stewart L, Scerbavicius R, Choe KH, Moore M, Sullivan A, Nicolls MR, Fontenot AP, Tuder RM, and Voelkel NF. An Animal Model of Autoimmune Emphysema. *Am J Respir Crit Care Med* 2005;171:734-742.

95. Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, Hacken J, Espada R, Bag R, Lewis DE, and Kheradmand F. An Immune Basis for Lung Parenchymal Destruction in Chronic Obstructive Pulmonary Disease and Emphysema. *PLos Med* 2004;1:e8.

96. Knobloch J, Schild K, Jungck D, Urban K, Müller K, Schweda EKH, Rupp J, and Koch A. The T-Helper Cell Type 1 Immune Response to Gram-Negative Bacterial Infections Is Impaired in COPD. *Am J Respir Crit Care Med* 2011;183:204-214.

97. Miossec P, Korn T, and Kuchroo VK. Interleukin-17 and Type 17 Helper T Cells. *N Engl J Med* 2009;361:888-898.

98. Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, McCormick M, Woods J, May R, and Sleeman MA. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest* 2010;138:1140-1147.

99. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, Magno F, D'Anna SE, Zanini A, and Brun P. T helper type 17–related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol* 2009;157:316-324.

100. Vargas-Rojas MI, Ramírez-Venegas A, Limón-Camacho L, Ochoa L, Hernández-Zenteno R, and Sansores RH. Increase of Th17 cells in peripheral blood of patients with chronic obstructive pulmonary disease. *Resp Med* 2011;105:1648-1654.

101. Chen K, Pociask DA, McAleer JP, Chan YR, Alcorn JF, Kreindler JL, Keyser MR, Shapiro SD, Houghton AM, Kolls JK, and Zheng M. IL-17RA Is Required for CCL2 Expression, Macrophage Recruitment, and Emphysema in Response to Cigarette Smoke. *Plos One* 2011;6:e20333.

102. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, and Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 2009;180:720-730.

103. Tang Q and Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008;9:239-244.

104. Barcelo B, Pons J, Ferrer JM, Sauleda J, Fuster A, and Agusti AGN. Phenotypic characterisation of T-lymphocytes in COPD: abnormal CD4+ CD25+ regulatory T-lymphocyte response to tobacco smoking. *Eur Respir J* 2008;31:555-562.

105. Smyth LJ, Starkey C, Vestbo J, and Singh D. CD4-regulatory cells in COPD patients. *Chest* 2007;132:156-163.

106. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, and Bakaeen F. Antielastin autoimmunity in tobacco smoking–induced emphysema. *Nat Med* 2007;13:567-569.

107. Plumb J, Smyth LJC, Adams HR, Vestbo J, Bentley A, and Singh SD. Increased T-regulatory cells within lymphocyte follicles in moderate COPD. *Eur Respir J* 2009;34:89-94.

108. Isajevs S, Taivans I, Strazda G, Kopeika U, Bukovskis M, Gordjusina V, and Kratovska A. Decreased FOXP3 expression in small airways of smokers with COPD. *Eur Respir J* 2009;33:61-67.

109. Gosman MM, Willemse BW, Jansen DF, Lapperre TS, van Schadewijk A, Hiemstra PS, Postma DS, Timens W, and Kerstjens HAM. Increased number of B-cells in bronchial biopsies in COPD. *Eur Respir J* 2006;27:60-64.

110. MacNee W. Pulmonary and Systemic Oxidant/Antioxidant Imbalance in Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc* 2005;2:50-60.

111. Corradi M, Montuschi P, Donnelly LE, Pesci A, Kharitonov SA, and Barnes PJ. Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. *Am J Respir Crit Care Med* 2001;163:854-858.

112. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, Van Herwaarden CL, and Bast AALT. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996;154:813-816.

113. Montuschi P, Collins J, Ciabattoni G, Lazerri N, Corradi M, Kharitonov SA, and Barnes PJ. Exhaled 8-Isoprostane as an In Vivo Biomarker of Lung Oxidative Stress in Patients with COPD and Healthy Smokers. *Am J Respir Crit Care Med* 2000;162:1175-1177.

114. Rahman I, Morrison D, Donaldson K, and MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med* 1996;154:1055-1060.

115. Malhotra D, Thimmulappa R, Navas-Acien A, Sandford A, Elliott M, Singh A, Chen L, Zhuang X, Hogg J, Pare P, Tuder RM, and Biswal S. Decline in NRF2-regulated Antioxidants in Chronic

Obstructive Pulmonary Disease Lungs Due to Loss of Its Positive Regulator, DJ-1. *Am J Respir Crit Care Med* 2008;178:592-604.

116. Agler AH, Kurth T, Gaziano JM, Buring JE, and Cassano PA. Randomised vitamin E supplementation and risk of chronic lung disease in the Women's Health Study. *Thorax* 2011;66:320-325.

117. Britton JR, Pavord ID, Richards KA, Knox AJ, Wisniewski AF, Lewis SA, Tattersfield AE, and Weiss ST. Dietary antioxidant vitamin intake and lung function in the general population. *Am J Respir Crit Care Med* 1995;151:1383-1387.

118. Louhelainen N, Rytila P, Haahtela T, Kinnula V, and Djukanovic R. Persistence of oxidant and protease burden in the airways after smoking cessation. *BMC Pulm Med* 2009;9:25.

119. Barnes PJ, Shapiro SD, and Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J* 2003;22:672-688.

120. Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, Ioli F, Chung KF, Donner CF, and Barnes PJ. Increased expression of nuclear factor-κB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J* 2002;20:556-563.

121. Gilmour PS, Rahman I, Donaldson K, and MacNee W. Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L533-L540.

122. Nishikawa M, Kakemizu N, Ito T, Kudo M, Kaneko T, Suzuki M, Udaka N, Ikeda H, and Okubo T. Superoxide Mediates Cigarette Smoke-Induced Infiltration of Neutrophils into the Airways through Nuclear Factor-κB Activation and IL-8 mRNA Expression in Guinea Pigs In Vivo. *Am J Respir Cell Mol Biol* 1999;20:189-198.

123. Adler KB, Holden-Stauffer WJ, and Repine JE. Oxygen metabolites stimulate release of high-molecular-weight glycoconjugates by cell and organ cultures of rodent respiratory epithelium via an arachidonic acid-dependent mechanism. *J Clin Invest* 1990;85:75.

124. Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, and Levine RL. Oxidation of either methionine 351 or methionine 358 in α1-antitrypsin causes loss of anti-neutrophil elastase activity. *J Biol Chem* 2000;275:27258-27265.

125. Tuder RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, and Flores SC. Oxidative Stress and Apoptosis Interact and Cause Emphysema Due to Vascular Endothelial Growth Factor Receptor Blockade. *Am J Respir Cell Mol Biol* 2003;29:88-97.

126. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, and Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke–induced emphysema in mice. *J Clin Invest* 2004;114:1248-1259.

127. Ito K, Lim S, Caramori G, Chung KF, Barnes PJ, and Adcock IM. Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *FASEB J* 2001;15:1110-1112.

128. Laurell CB and Eriksson S. The Electrophoretic α1-Globulin Pattern of Serum in α1-Antitrypsin Deficiency. *Scand J Clin Lab Invest* 1963;15:132-140.

129. Lafuma C, Frisdal E, Harf A, Robert L, and Hornebeck W. Prevention of leucocyte elastase-induced emphysema in mice by heparin fragments. *Eur Respir J* 1991;4:1004-1009.

130. Dhami R, Gilks B, Xie C, Zay K, Wright JL, and Churg A. Acute Cigarette Smoke–Induced Connective Tissue Breakdown Is Mediated by Neutrophils and Prevented by α1-Antitrypsin. *Am J Respir Cell Mol Biol* 2000;22:244-252.

131. Fischer BM and Voynow JA. Neutrophil Elastase Induces MUC 5AC Gene Expression in Airway Epithelium via a Pathway Involving Reactive Oxygen Species. *Am J Respir Cell Mol Biol* 2002;26:447-452.

132. Nakamura H, Yoshimura K, McElvaney NG, and 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.

133. Eidelman D, Saetta MP, Ghezzo H, Wang NS, Hoidal JR, King M, and Cosio MG. Cellularity of the Alveolar Walls in Smokers and Its Relation to Alveolar Destruction: Functional Implications. *Am Rev Respir Dis* 1990;141:1547-1552.

134. Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, Fitzgerald MX, and O'Connor CM. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med* 1997;156:240-247.

135. Ohnishi K, Takagi M, Kurokawa Y, Satomi S, and Konttinen YT. Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Lab Invest* 1998;78:1077-1087.

136. Churg A, Zay K, Shay S, Xie C, Shapiro SD, Hendricks R, and Wright JL. Acute Cigarette Smoke induced Connective Tissue Breakdown Requires both Neutrophils and Macrophage Metalloelastase in Mice. *Am J Respir Cell Mol Biol* 2002;27:368-374.

137. Taggart CC, Lowe GJ, Greene CM, Mulgrew AT, O'Neill SJ, Levine RL, and McElvaney NG. Cathepsin B, L, and S Cleave and Inactivate Secretory Leucoprotease Inhibitor. *J Biol Chem* 2001;276:33345-33352.

138. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, and Barnes PJ. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2002;26:602-609.

139. Leco KJ, Waterhouse P, Sanchez OH, Gowing KLM, Poole AR, Wakeham A, Mak TW, and Khokha R. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest* 2001;108:817-829.

140. Hirano K, Sakamoto T, Uchida Y, Morishima Y, Masuyama K, Ishii Y, Nomura A, Ohtsuka M, and Sekizawa K. Tissue inhibitor of metalloproteinases-2 gene polymorphisms in chronic obstructive pulmonary disease. *Eur Respir J* 2001;18:748-752.

141. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, and Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;106:1311-1319.

142. Aoshiba K, Yokohori N, and Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* 2003;28:555-562.

143. D'hulst AI, Bracke KR, Maes T, De Bleecker JL, Pauwels RA, Joos GF, and Brusselle GG. Role of tumour necrosis factor-α receptor p75 in cigarette smoke-induced pulmonary inflammation and emphysema. *Eur Respir J* 2006;28:102-112.

144. Hodge S, Hodge G, Holmes M, and Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Respir J* 2005;25:447-454.

145. Imai K, Mercer BA, Schulman LL, Sonett JR, and D'Armiento JM. Correlation of lung surface area to apoptosis and proliferation in human emphysema. *Eur Respir J* 2005;25:250-258.

146. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, and Selman M. Upregulation of gelatinases a and b, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest* 2000;117:684-694.

147. Calabrese F, Giacometti C, Beghe B, Rea F, Loy M, Zuin R, Marulli G, Baraldo S, Saetta M, and Valente M. Marked alveolar apoptosis/proliferation imbalance in end-stage emphysema. *Respir Res* 2005;6:14.

148. Yokohori N, Aoshiba K, and Nagai A. Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. *Chest* 2004;125:626-632.

149. Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, and Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med* 2001;163:737-744.

150. Hodge S, Hodge G, Scicchitano R, Reynolds PN, and 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.

151. Aloisi F and Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. *Nat Rev Immunol* 2006;6:205-217.

152. Brusselle GG, Demoor T, Bracke KR, Brandsma CA, and Timens W. Lymphoid follicles in (very) severe COPD: beneficial or harmful? *Eur Respir J* 2009;34:219-230.

153. Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, and Lambrecht BN. Tertiary lymphoid organs in infection and autoimmunity. *Trends Immunol* 2012;33:297-305.

154. Drayton DL, Liao S, Mounzer RH, and Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol* 2006;7:344-353.

155. Pabst R and Gehrke I. Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? *Am J Respir Cell Mol Biol* 1990;3:131.

156. Tschernig T and Pabst R. Bronchus-Associated Lymphoid Tissue (BALT) Is Not Present in the Normal Adult Lung but in Different Diseases. *Pathobiology* 2000;68:1-8.

157. Richmond I, Pritchard GE, Ashcroft T, Avery A, Corris PA, and Walters EH. Bronchus associated lymphoid tissue (BALT) in human lung: its distribution in smokers and non-smokers. *Thorax* 1993;48:1130-1134.

158. van der Strate BWA, Postma DS, Brandsma CA, Melgert BN, Luinge MA, Geerlings M, Hylkema MN, van den Berg A, Timens W, and Kerstjens HAM. Cigarette Smoke-induced Emphysema: A Role for the B Cell? *Am J Respir Crit Care Med* 2006;173:751-758.

159. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, and Brusselle GG. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *J Immunol* 2006;177:4350-4359.

160. Brandsma CA, Kerstjens HA, van Geffen WH, Geerlings M, Postma DS, Hylkema MN, and Timens W. Differential switching to IgG and IgA in active smoking COPD patients and healthy controls. *Eur Respir J* 2012;40:313-321.

161. Olloquequi J, Montes JF, Prats A, Rodríguez E, Montero MA, García-Valero J, and Ferrer J. Significant increase of CD57+ cells in pulmonary lymphoid follicles of COPD patients. *Eur Respir J* 2011;37:289-298.

162. Van Pottelberge GR, Bracke KR, Van den Broeck S, Reinartz SM, Van Drunen CM, Wouters EF, Verleden GM, Vermassen FE, Joos GF, and Brusselle GG. Plasmacytoid dendritic cells in pulmonary lymphoid follicles of patients with COPD. *Eur Respir J* 2010;36:781-791.

163. Sethi S and Murphy TF. Infection in the Pathogenesis and Course of Chronic Obstructive Pulmonary Disease. *N Engl J Med* 2008;359:2355-2365.

164. Moyron-Quiroz JE, Rangel-Moreno J, Kusser KR, Hartson L, Sprague F, Goodrich S, Woodland DL, Lund FE, and Randall TD. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med* 2004;10:927-934.

165. GeurtsvanKessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, Osterhaus AD, Hendriks R, Rimmelzwaan GF, and Lambrecht BN. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus–infected mice. *J Exp Med* 2009;206:2339-2349.

166. Hogg JC, Chu FS, Tan WC, Sin DD, Patel SA, Pare PD, Martinez FJ, Rogers RM, Make BJ, and Criner GJ. Survival after lung volume reduction in chronic obstructive pulmonary disease: insights from small airway pathology. *Am J Respir Crit Care Med* 2007;176:454-459.

167. Ernst P, Gonzalez AV, Brassard P, and Suissa S. Inhaled Corticosteroid Use in Chronic Obstructive Pulmonary Disease and the Risk of Hospitalization for Pneumonia. *Am J Respir Crit Care Med* 2007;176:162-166.

168. Feghali-Bostwick CA, Gadgil AS, Otterbein LE, Pilewski JM, Stoner MW, Csizmadia E, Zhang Y, Sciurba FC, and Duncan SR. Autoantibodies in Patients with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2008;177:156-163.

169. Karayama M, Inui N, Suda T, Nakamura Y, Nakamura H, and Chida K. Antiendothelial cell antibodies in patients with copd. *Chest* 2010;138:1303-1308.

170. Taraseviciene-Stewart L, Scerbavicius R, Choe KH, Moore M, Sullivan A, Nicolls MR, Fontenot AP, Tuder RM, and Voelkel NF. An animal model of autoimmune emphysema. *Am J Respir Crit Care Med* 2005;171:734-742.

171. Brandsma CA, Kerstjens HAM, Geerlings M, Kerkhof M, Hylkema MN, Postma DS, and Timens W. The search for autoantibodies against elastin, collagen and decorin in COPD. *Eur Respir J* 2011;37:1289-1292.

172. Mebius RE. Organogenesis of lymphoid tissues. *Nat Rev Immunol* 2003;3:292-303.

173. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, Smith SC, Carlson R, Shornick LP, Strauss-Schoenberger J, Russell JH, Karr R, and Chaplin DD. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 1994;264:703-707.

174. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, and Lipp M. CCR7 Coordinates the Primary Immune Response by Establishing Functional Microenvironments in Secondary Lymphoid Organs. *Cell* 1999;99:23-33.

175. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, and Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 1996;87:1037-1047.

176. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, Browning JL, Lipp M, and Cyster JG. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000;406:309-314.

177. GeurtsvanKessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, Osterhaus AD, Hendriks R, Rimmelzwaan GF, and Lambrecht BN. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus–infected mice. *J Exp Med* 2009;206:2339-2349.

178. Rangel-Moreno J, Carragher DM, de la Luz Garcia-Hernandez M, Hwang JY, Kusser K, Hartson L, Kolls JK, Khader SA, and Randall TD. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol* 2011;12:639-646.

179. Rangel-Moreno J, Moyron-Quiroz JE, Hartson L, Kusser K, and Randall TD. Pulmonary expression of CXC chemokine ligand 13, CC chemokine ligand 19, and CC chemokine ligand 21 is essential for local immunity to influenza. *Proc Natl Acad Sci USA* 2007;104:10577-10582.

180. Demoor T, Bracke KR, Maes T, Vandooren B, Elewaut D, Pilette C, Joos GF, and Brusselle GG. Role of lymphotoxin-α in cigarette smoke-induced inflammation and lymphoid neogenesis. *Eur Respir J* 2009;34:405-416.

181. Demoor T, Bracke KR, Vermaelen KY, Dupont L, Joos GF, and Brusselle GG. CCR7 Modulates Pulmonary and Lymph Node Inflammatory Responses in Cigarette Smoke-Exposed Mice. *J Immunol* 2009;183:8186-8194.

182. Salomonsson S, Jonsson MV, Skarstein K, Brokstad KA, Hjelmström P, Wahren–Herlenius M, and Jonsson R. Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. *Arthritis Rheum* 2003;48:3187-3201.

183. Manzo A, Paoletti S, Carulli M, Blades MC, Barone F, Yanni G, FitzGerald O, Bresnihan B, Caporali R, and Montecucco C. Systematic microanatomical analysis of CXCL13 and CCL21 in

situ production and progressive lymphoid organization in rheumatoid synovitis. *Eur J Immunol* 2005;35:1347-1359.

184. Luther SA, Lopez T, Bai W, Hanahan D, and Cyster JG. BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. *Immunity* 2000;12:471-481.

185. Luther SA, Bidgol A, Hargreaves DC, Schmidt A, Xu Y, Paniyadi J, Matloubian M, and Cyster JG. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol* 2002;169:424-433.

186. Kratz A, Campos-Neto A, Hanson MS, and Ruddle NH. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J Exp Med* 1996;183:1461-1472.

187. Kelsen SG, Aksoy MO, Georgy M, Hershman R, Ji R, Li X, Hurford M, Solomides C, Chatila W, and Kim V. Lymphoid Follicle Cells in Chronic Obstructive Pulmonary Disease Overexpress the Chemokine Receptor CXCR3. *Am J Respir Crit Care Med* 2009;179:799-805.

188. Bagaeva LV, Rao P, Powers JM, and Segal BM. CXC Chemokine Ligand 13 Plays a Role in Experimental Autoimmune Encephalomyelitis. *J Immunol* 2006;176:7676-7685.

189. Henry RA and Kendall PL. CXCL13 Blockade Disrupts B Lymphocyte Organization in Tertiary Lymphoid Structures without Altering B Cell Receptor Bias or Preventing Diabetes in Nonobese Diabetic Mice. *J Immunol* 2010;185:1460-1465.

190. Winter S, Loddenkemper C, Aebischer A, Rabel K, Hoffmann K, Meyer TF, Lipp M, and Hopken UE. The chemokine receptor CXCR5 is pivotal for ectopic mucosa-associated lymphoid tissue neogenesis in chronic Helicobacter pylori-induced inflammation. *J Mol Med* 2010;88:1169-1180.

191. Wengner AM, Höpken UE, Petrow PK, Hartmann S, Schurigt U, Bräuer R, and Lipp M. CXCR5–and CCR7–dependent lymphoid neogenesis in a murine model of chronic antigen–induced arthritis. *Arthritis Rheum* 2007;56:3271-3283.

192. Zheng B, Ozen ZY, Zhang XJ, De Silva S, Marinova E, Guo LJ, Wansley D, Huston DP, West MR, and Han SH. CXCL1.3 neutralization reduces the severity of collagen-induced arthritis. *Arthritis Rheum* 2005;52:620-626.

193. Finch DK, Ettinger R, Karnell JL, Herbst R, and Sleeman MA. Effects of CXCL13 inhibition on lymphoid follicles in models of autoimmune disease. *Eur J Clin Invest* 2013;43:501-509.

194. Baay-Guzman GJ, Huerta-Yepez S, Vega MI, Guilar-Leon D, Campillos M, Blake J, Benes V, Hernandez-Pando R, and Teran LM. Role of CXCL13 in Asthma: Novel Therapeutic Target. *Chest* 2012;141:886-894.

195. Verhamme FM, Bracke KR, Joos GF, and Brusselle GG. TGF-β Superfamily in Obstructive Lung Diseases: More Suspects than TGF-β Alone. *Am J Respir Cell Mol Biol* 2014 November 14. Epub ahead of print.

196. Huminiecki L, Goldovsky L, Freilich S, Moustakas A, Ouzounis C, and Heldin CH. Emergence, development and diversification of the TGF-beta signalling pathway within the animal kingdom. *BMC Evol Biol* 2009;9:28.

197. Shi YG and Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685-700.

198. Mueller TD and Nickel J. Promiscuity and specificity in BMP receptor activation. *Febs Lett* 2012;586:1846-1859.

199. Massague J. TGF beta signalling in context. *Nat Rev Mol Cell Bio* 2012;13:616-630.

200. Chang H, Brown CW, and Matzuk MM. Genetic Analysis of the Mammalian Transforming Growth Factor-β Superfamily. *Endocr Rev* 2002;23:787-823.

201. Blobe GC, Schiemann WP, and Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350-1358.

202. Massagué J, Blain SW, and Lo RS. TGFβ Signaling in Growth Control, Cancer, and Heritable Disorders. *Cell* 2000;103:295-309.

203. Groneberg DA, Wift H, Adcock IM, Hansen G, and Springer J. Smads as intracellular mediators of airway inflammation. *Exp Lung Res* 2004;30:223-250.

204. Halwani R, Al-Muhsen S, Al-Jahdali H, and Hamid Q. Role of Transforming Growth Factor–β in Airway Remodeling in Asthma. *Am J Respir Cell Mol Biol* 2011;44:127-133.

205. Morty RE, Königshoff M, and Eickelberg O. Transforming Growth Factor-β Signaling across Ages. *Proc Am Thorac Soc* 2009;6:607-613.

206. Yang YC, Zhang N, Van Crombruggen K, Hu GH, Hong SL, and Bachert C. Transforming growth factor-beta1 in inflammatory airway disease: a key for understanding inflammation and remodeling. *Allergy* 2012.

207. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, and Karlsson S. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770-774.

208. Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, and Doetschman T. Transforming growth factor-β3 is required for secondary palate fusion. *Nat Genet* 1995;11.

209. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, and Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124:2659-2670.

210. Annes JP, Munger JS, and Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci* 2003;116:217-224.

211. Li MO, Wan YY, Sanjabi S, Robertson AK, and Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 2006;24:99-146.

212. Marie JC, Letterio JJ, Gavin M, and Rudensky AY. TGF-β1 maintains suppressor function and Foxp3 expression in CD4+ CD25+ regulatory T cells. *J Exp Med* 2005;201:1061-1067.

213. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, and Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235-238.

214. Epstein FH, Border WA, and Noble NA. Transforming growth factor β in tissue fibrosis. *N Engl J Med* 1994;331:1286-1292.

215. Desmoulière A, Geinoz A, Gabbiani F, and Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103-111.

216. Willis BC and Borok Z. TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L525-L534.

217. Celedón JC, Lange C, Raby BA, Litonjua AA, Palmer LJ, DeMeo DL, Reilly JJ, Kwiatkowski DJ, Chapman HA, and Laird N. The transforming growth factor-β1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD). *Hum Mol Genet* 2004;13:1649-1656.

218. van Diemen C, Postma D, Vonk J, Bruinenberg M, Nolte I, and Boezen HM. Decorin and TGF-beta1 polymorphisms and development of COPD in a general population. *Respir Res* 2006;7:89.

219. Wu L, Chau J, Young RP, Pokorny V, Mills GD, Hopkins R, McLean L, and Black PN. Transforming growth factor-β1 genotype and susceptibility to chronic obstructive pulmonary disease. *J Med Genet* 2004;41:285.

220. Hersh CP, DeMeo DL, Lazarus R, Celedón JC, Raby BA, Benditt JO, Criner G, Make B, Martinez FJ, and Scanlon PD. Genetic association analysis of functional impairment in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;173:977-984.

221. de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, and van Krieken JH. Transforming growth factor beta-1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:1951-1957.

222. Zandvoort A, Postma DS, Jonker MR, Noordhoek JA, Vos JTWM, van der Geld YM, and Timens W. Altered expression of the Smad signalling pathway: implications for COPD pathogenesis. *Eur Respir J* 2006;28:533-541.

223. Pons AR, Sauleda J, Noguera A, Pons J, Barceló B, Fuster A, and Agustí AGN. Decreased macrophage release of TGF-β and TIMP-1 in chronic obstructive pulmonary disease. *Eur Respir J* 2005;26:60-66.

224. Wang RD, Wright JL, and Churg A. Transforming growth factor-β1 drives airway remodeling in cigarette smoke–exposed tracheal explants. *Am J Respir Cell Mol Biol* 2005;33:387-393.

225. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, and 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.

226. Churg A, Tai H, Coulthard T, Wang R, and 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.

227. Podowski M, Calvi C, Metzger S, Misono K, Poonyagariyagorn H, Lopez-Mercado A, Ku T, Lauer T, Grath-Morrow S, Berger A, Cheadle C, Tuder R, Dietz HC, Mitzner W, Wise R, and Neptune E. Angiotensin receptor blockade attenuates cigarette smoke-induced lung injury and rescues lung architecture in mice. *J Clin Invest* 2012;122:229-240.

228. Churg A, Zhou S, Preobrazhenska O, Tai H, Wang R, and Wright JL. Expression of Profibrotic Mediators in Small Airways versus Parenchyma after Cigarette Smoke Exposure. *Am J Respir Cell Mol Biol* 2009;40:268-276.

229. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bon Signore G, and Bousquet J. Transforming Growth Factor-β Expression in Mucosal Biopsies in Asthma and Chronic Bronchitis. *Am J Respir Crit Care Med* 1997;156:591-599.

230. Milara J, Peiró T, Serrano A, and Cortijo J. Epithelial to mesenchymal transition is increased in patients with COPD and induced by cigarette smoke. *Thorax* 2013;68:410-420.

231. Morris DG, Huang X, Kaminski N, Wang Y, Shapiro SD, Dolganov G, Glick A, and Sheppard D. Loss of integrin αvβ6-mediated TGF-β activation causes Mmp12-dependent emphysema. *Nature* 2003;422:169-173.

232. Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M, Lavery C, Margetts PJ, Roberts AB, and Gauldie J. Smad3 Null Mice Develop Airspace Enlargement and Are Resistant to TGF-β-Mediated Pulmonary Fibrosis. *J Immunol* 2004;173:2099-2108.

233. Farkas L, Farkas D, Warburton D, Gauldie J, Shi W, Stampfli MR, Voelkel NF, and Kolb M. Cigarette smoke exposure aggravates air space enlargement and alveolar cell apoptosis in Smad3 knockout mice. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L391-L401.

234. Hedger MP, Winnall WR, Phillips DJ, and de Kretser DM. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm* 2011;85:255-297.

235. Phillips DJ, de Kretser DM, and Hedger MP. Activin and related proteins in inflammation: Not just interested bystanders. *Cytokine Growth F R* 2009;20:153-164.

236. Aleman-Muench GR and Soldevila G. When versatility matters: activins/inhibins as key regulators of immunity. *Immunol Cell Biol* 2011.

237. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, and Spiess J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 1986;321:776-779.

238. Ohguchi M, Yamato K, Ishihara Y, Koide M, Ueda N, Okahashi N, Noguchi T, Kizaki M, Ikeda Y, Sugino H, and Nisihara T. Activin a regulates the production of mature interleukin-1 beta and interleukin-1 receptor antagonist in human monocytic cells. *J Interferon Cytok Res* 1998;18:491-498.

239. Zhang XJ, Li Y, Tai GX, Xu GY, Zhang PY, Yang Y, Lao FX, and Liu ZH. Effects of activin A on the activities of the mouse peritoneal macrophages. *Cell Mol Immunol* 2005;2:63-67.

240. Sierra-Filardi E, Puig-Kröger A, Blanco FJ, Nieto C, Bragado R, Palomero MI, Bernabéu C, Vega MA, and Corbí AL. Activin A skews macrophage polarization by promoting a proinflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers. *Blood* 2011;117:5092-5101.

241. Chen Y, Wu H, Winnall WR, Loveland KL, Makanji Y, Phillips DJ, Smith JA, and Hedger MP. Tumour necrosis factor-α stimulates human neutrophils to release preformed activin A. *Immunol Cell Biol* 2011;89:889-896.

242. Ogawa K, Funaba M, and Tsujimoto M. A dual role of activin A in regulating immunoglobulin production of B cells. *J Leukocyte Biol* 2008;83:1451-1458.

243. Huber S, Stahl FR, Schrader J, Lüth S, Presser K, Carambia A, Flavell RA, Werner S, Blessing M, Herkel J, and Schramm C. Activin A promotes the TGF-β-induced conversion of CD4+CD25– T Cells into Foxp3+ induced regulatory T cells. *J Immunol* 2009;182:4633-4640.

244. Hubner G. Activin A: A novel player and inflammatory marker in inflammatory bowel disease? *Lab Invest* 1997;77:311-318.

245. Dignass AU, Jung S, Harder-d'Heureuse J, and Wiedenmann B. Functional relevance of activin A in the intestinal epithelium. *Scand J Gastroenterol* 2002;37:936-943.

246. Zhang YQ, Resta S, Jung B, Barrett KE, and Sarvetnick N. Upregulation of activin signaling in experimental colitis. *Am J Physiol Gastrointest Liver Physiol* 2009;297:G768-G780.

247. Dohi T, Ejima C, Kato R, Kawamura YI, Kawashima R, Mizutani N, Tabuchi Y, and Kojima I. Therapeutic potential of follistatin for colonic inflammation in mice. *Gastroenterology* 2005;128:411-423.

248. EL-Gendi SS, Moniem AE, Tawfik NM, Ashmawy MM, Mohammed OA, Mostafa AK, Zakhari MM, and Herdan OM. Value of serum and synovial fluid activin A and inhibin A in some rheumatic diseases. *Int J Rheum Dis* 2010;13:273-279.

249. Yu EW, Dolter KE, Shao LE, and Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. *Clin Exp Immunol* 1998;112:126-132.

250. Gribi R, Tanaka T, Harper-Summers R, and Yu J. Expression of activin A in inflammatory arthropathies. *Mol Cell Endocrinol* 2001;180:163-167.

251. Ota F. Activin a induces cell proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2003;48:2442-2449.

252. Matsuse T, Fukuchi Y, Eto Y, Matsui H, Hosoi T, Oka T, Ohga E, Nagase T, and Orimo H. Expression of immunoreactive and bioactive activin A protein in adult murine lung after bleomycin treatment. *Am J Respir Cell Mol Biol* 1995;13:17-24.

253. Aoki F, Kurabayashi M, Hasegawa Y, and Kojima I. Attenuation of bleomycin-induced pulmonary fibrosis by follistatin. *Am J Respir Crit Care Med* 2005;172:713-720.

254. Rosendahl A, Checchin D, Fehniger TE, ten Dijke P, Heldin CH, and Sideras P. Activation of the TGF-beta/activin-Smad2 pathway during allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001;25:60-68.

255. Walton KL, Makanji Y, and Harrison CA. New insights into the mechanisms of activin action and inhibition. *Mol Cell Endocrinol* 2012;359:2-12.

256. Morrell NW, Yang XD, Upton PD, Jourdan KB, Morgan N, Sheares KK, and Trembath RC. Altered growth responses of muscle cells from patients pulmonary artery smooth with

primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* 2001;104:790-795.

257. Zhang S, Fantozzi I, Tigno DD, Yi ES, Platoshyn O, Thistlethwaite PA, Kriett JM, Yung G, Rubin LJ, and Yuan JXJ. Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L740-L754.

258. Choi SC and Han JK . Chapter Five - Negative Regulation of Activin Signal Transduction. In L. Gerald. *Vit Horm* 2011;85:79-104.

259. Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, and Sugino H. Identification and Characterization of a Novel Follistatin-like Protein as a Binding Protein for the TGF-β Family. *J Biol Chem* 2000;275:40788-40796.

260. Matsuse T, Ikegami A, Ohga E, Hosoi T, Oka T, Kida K, Fukayama M, Inoue S, Nagase T, Ouchi Y, and Fukuchi Y. Expression of immunoreactive activin a protein in remodeling lesions associated with interstitial pulmonary fibrosis. *Am J Pathol* 1996;148:707-713.

261. Karagiannidis C, Hense G, Martin C, Epstein M, Ruckert B, Mantel PY, Menz G, Uhlig S, Blaser K, and Schmidt-Weber CB. Activin A is an acute allergen-responsive cytokine and provides a link to TGF-beta-mediated airway remodeling in asthma. *J Allergy Clin Immunol* 2006;117:111-118.

262. Ohga E, Matsuse T, Teramoto S, Katayama H, Nagase T, Fukuchi Y, and Ouchi Y. Effects of activin A on proliferation and differentiation of human lung fibroblasts. *Biochem Biophys Res Commun* 1996;228:391-396.

263. Ohga E, Matsuse T, Teramoto S, and Ouchi Y. Activin receptors are expressed on human lung fibroblast and activin a facilitates fibroblast-mediated collagen gel contraction. *Life Sci* 2000;66:1603-1613.

264. Zhao J, Shi W, Wang YL, Chen H, Bringas P, Datto MB, Frederick JP, Wang XF, and Warburton D. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L585-L593.

265. Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, and Iwamoto I. Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* 1999;104:5-11.

266. Hardy CL, O'Connor AE, Yao J, Sebire K, de Kretser DM, Rolland JM, Anderson GP, Phillips DJ, and O'Hehir RE. Follistatin is a candidate endogenous negative regulator of activin A in experimental allergic asthma. *Clin Exp Allergy* 2006;36:941-950.

267. Cho SH, Yao Z, Wang SW, Alban RF, Barbers RG, French SW, and Oh CK. Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells. *J Immunol* 2003;170:4045-4052.

268. Le AV, Cho JY, Miller M, McElwain S, Golgotiu K, and Broide DH. Inhibition of Allergen-Induced Airway Remodeling in Smad 3-Deficient Mice. *J Immunol* 2007;178:7310-7316.

269. Semitekolou M, Alissafi T, Aggelakopoulou M, Kourepini E, Kariyawasam HH, Kay AB, Robinson DS, Lloyd CM, Panoutsakopoulou V, and Xanthou G. Activin-A induces regulatory T

cells that suppress T helper cell immune responses and protect from allergic airway disease. *J Exp Med* 2009;206:1769-1785.

270. Kariyawasam HH, Pegorier S, Barkans J, Xanthou G, Aizen M, Ying S, Kay AB, Lloyd CM, and Robinson DS. Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. *J Allergy Clin Immunol* 2009;124:454-462.

271. Hardy CL, LeMasurier JS, Olsson F, Dang T, Yao J, Yang M, Plebanski M, Phillips DJ, Mollard R, Rolland JM, and O'Hehir RE. Interleukin-13 Regulates Secretion of the Tumor Growth Factor-beta Superfamily Cytokine Activin A in Allergic Airway Inflammation. *Am J Respir Cell Mol Biol* 2010;42:667-675.

272. Jones CP, Gregory LG, Causton B, Campbell GA, and Lloyd CM. Activin A and TGF-β promote TH9 cell–mediated pulmonary allergic pathology. *J Allergy Clin Immunol* 2012;129:1000-1010.

273. Hardy CL, Nguyen HA, Mohamud R, Yao J, Oh DY, Plebanski M, Loveland KL, Harrison CA, Rolland JM, and O'Hehir RE. The activin A antagonist follistatin inhibits asthmatic airway remodelling. *Thorax* 2013;68:9-18.

274. Gregory LG, Mathie SA, Walker SA, Pegorier S, Jones CP, and Lloyd CM. Overexpression of smad2 drives house dust mite-mediated airway remodeling and airway hyperresponsiveness via activin and IL-25. *Am J Respir Crit Care Med* 2010;182:143-154.

275. Leppäranta O, Myllärniemi M, Salmenkivi K, Kinnula VL, Keski-Oja J, and Koli K. Reduced Phosphorylation of the TGF-β Signal Transducer Smad2 in Emphysematous Human Lung. *COPD* 2009;6:234-241.

276. Hogan BLM. Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Gene Dev* 1996;10:1580-1594.

277. Kawabata M, Imamura T, and Miyazono K. Signal transduction by bone morphogenetic proteins. *Cytokine Growth F R* 1998;9:49-61.

278. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, and Nohe A. Bone Morphogenetic Proteins: A critical review. *Cell Signal* 2011;23:609-620.

279. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, and Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet* 2009;41:478-481.

280. Andriopoulos Jr B, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, and Babitt JL. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet* 2009;41:482-487.

281. Fleming RE and Ponka P. Iron Overload in Human Disease. *N Engl J Med* 2012;366:348-359.

282. Wang L, Trebicka E, Fu Y, Ellenbogen S, Hong CC, Babitt JL, Lin HY, and Cherayil BJ. The bone morphogenetic protein-hepcidin axis as a therapeutic target in inflammatory bowel disease. *Inflamm Bowel Dis* 2012;18:112-119.

283. Hong JH, Lee GT, Lee JH, Kwon SJ, Park SH, Kim SJ, and Kim IY. Effect of bone morphogenetic protein-6 on macrophages. *Immunology* 2009;128:e442-e450.

284. Lee GT, Jung YS, Lee JH, Kim WJ, and Kim IY. Bone morphogenetic protein 6-induced interleukin-1 beta expression in macrophages requires PU.1/Smad1 interaction. *Mol Immunol* 2011;48:1540-1547.

285. Sivertsen EA, Huse K, Hystad ME, Kersten C, Smeland EB, and Myklebust JH. Inhibitory effects and target genes of bone morphogenetic protein 6 in Jurkat TAg cells. *Eur J Immunol* 2007;37:2937-2948.

286. Kersten C, Sivertsen EA, Hystad ME, Forfang L, Smeland EB, and Myklebust JH. BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. *BMC Immunol* 2005;6.

287. Huse K, Bakkebø M, Oksvold MP, Forfang L, Hilden VI, Stokke T, Smeland EB, and Myklebust JH. Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: Differential effects of BMP-6 and BMP-7. *Eur J Immunol* 2011;41:3135-3145.

288. Lories RJU, Derese I, Ceuppens JL, and Luyten FP. Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis. *Arthritis Rheum* 2003;48:2807-2818.

289. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, and Kalluri R. BMP-7 counteracts TGF-β1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003;9:964-968.

290. Kinoshita K, Iimuro Y, Otogawa K, Saika S, Inagaki Y, Nakajima Y, Kawada N, Fujimoto J, Friedman SL, and Ikeda K. Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. *Gut* 2007;56:706-714.

291. Murray LA, Hackett TL, Warner SM, Shaheen F, Argentieri RL, Dudas P, Farrell FX, and Knight DA. BMP-7 Does Not Protect against Bleomycin-Induced Lung or Skin Fibrosis. *Plos One* 2008;3.

292. Myllärniemi M, Lindholm P, Ryynanen MJ, Kliment CR, Salmenkivi K, Keski-Oja J, Kinnula VL, Oury TD, and Koli K. Gremlin-mediated decrease in bone morphogenetic protein signaling promotes pulmonary fibrosis. *Am J Respir Crit Care Med* 2008;177:321-329.

293. Yang G, Zhu Z, Wang Y, Gao A, Niu P, and Tian L. Bone morphogenetic protein-7 inhibits silica-induced pulmonary fibrosis in rats. *Toxicol Lett* 2013;220:103-108.

294. Izumi N, Mizuguchi S, Inagaki Y, Saika S, Kawada N, Nakajima Y, Inoue K, Suehiro S, Friedman SL, and Ikeda K. BMP-7 opposes TGF-beta 1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L120-L126.

295. McCormack N, Molloy EL, and O'Dea S. Bone morphogenetic proteins enhance an epithelial-mesenchymal transition in normal airway epithelial cells during restitution of a disrupted epithelium. *Respir Res* 2013;14.

296. Pegorier S, Campbell GA, Kay AB, and Lloyd CM. Bone Morphogenetic Protein (BMP)-4 and BMP-7 regulate differentially Transforming Growth Factor (TGF)-beta 1 in normal human lung fibroblasts (NHLF). *Respir Res* 2010;11.

297. Stumm CL, Halcsik E, Landgraf RG, Camara NOS, Sogayar MC, and Jancar S. Lung Remodeling in a Mouse Model of Asthma Involves a Balance between TGF-beta 1 and BMP-7. *Plos One* 2014;9.

298. Arndt S, Wacker E, Dorn C, Koch A, Saugspier M, Thasler WE, Hartmann A, Bosserhoff AK, and Hellerbrand C. Enhanced expression of BMP6 inhibits hepatic fibrosis in non-alcoholic fatty liver disease. *Gut* 2014.

299. Dendooven A, van Oostrom O, van der Giezen DM, Willem Leeuwis J, Snijckers C, Joles JA, Robertson EJ, Verhaar MC, Nguyen TQ, and Goldschmeding R. Loss of Endogenous Bone Morphogenetic Protein-6 Aggravates Renal Fibrosis. *Am J Pathol* 2011;178:1069-1079.

300. Nguyen TQ, Chon H, Van Nieuwenhoven FA, Braam B, Verhaar MC, and Goldschmeding R. Myofibroblast progenitor cells are increased in number in patients with type 1 diabetes and express less bone morphogenetic protein 6: a novel clue to adverse tissue remodelling? *Diabetologia* 2006;49:1039-1048.

301. Rosendahl A, Pardali E, Speletas M, ten Dijke P, Heldin CH, and Sideras P. Activation of Bone Morphogenetic Protein/Smad Signaling in Bronchial Epithelial Cells during Airway Inflammation. *Am J Respir Cell Mol Biol* 2002;27:160-169.

302. Wall NA, Blessing M, Wright CV, and Hogan BL. Biosynthesis and in vivo localization of the decapentaplegic-Vg-related protein, DVR-6 (bone morphogenetic protein-6). *J Cell Biol* 1993;120:493-502.

303. Du L, Sullivan CC, Chu D, Cho AJ, Kido M, Wolf PL, Yuan JXJ, Deutsch R, Jamieson SW, and Thistlethwaite PA. Signaling Molecules in Nonfamilial Pulmonary Hypertension. *N Engl J Med* 2003;348:500-509.

304. Jeffery TK, Upton PD, Trembath RC, and Morrell NW. BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L370-L378.

305. Kariyawasam HH, Xanthou G, Barkans J, Aizen M, Kay AB, and Robinson DS. Basal Expression of Bone Morphogenetic Protein Receptor Is Reduced in Mild Asthma. *Am J Respir Crit Care Med* 2008;177:1074-1081.

306. Richter A, Yeager ME, Zaiman A, Cool CD, Voelkel NF, and Tuder RM. Impaired transforming growth factor-beta signaling in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2004;170:1340-1348.

307. Morty RE, Nejman B, Kwapiszewska G, Hecker M, Zakrzewicz A, Kouri FM, Peters DM, Dumitrascu R, Seeger W, Knaus P, Schermuly RT, and Eickelberg O. Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. *Arterioscl Throm Vas* 2007;27:1072-1078.

308. Masterson JC, Molloy EL, Gilbert JL, McCormack N, Adams A, and O'Dea S. Bone morphogenetic protein signalling in airway epithelial cells during regeneration. *Cell Signal* 2011;23:398-406.

309. Miyazono K, Maeda S, and Imamura T. BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth F R* 2005;16:251-263.

310. Lowery JW, Frump AL, Anderson L, DiCarlo GE, Jones MT, and de Caestecker MP. ID family protein expression and regulation in hypoxic pulmonary hypertension. *Am J Physiol Regul Integr Comp Physiol* 2010;299:R1463-R1477.

311. Ezzie ME, Crawford M, Cho JH, Orellana R, Zhang SL, Gelinas R, Batte K, Yu LB, Nuovo G, Galas D, Diaz P, Wang K, and Nana-Sinkam SP. Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax* 2012;67:122-131.

312. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, and Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005;26:204-213.

313. van Wetering S, Zuyderduyn S, Ninaber DK, van Sterkenburg MAJA, Rabe KF, and Hiemstra PS. Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13. *Mol Immunol* 2007;44:803-811.

314. Macdonald G, Kondor N, Yousefi V, Green A, Wong F, and Aquino-Parsons C. Reduction of carboxyhaemoglobin levels in the venous blood of cigarette smokers following the administration of carbogen. *Radiother Oncol* 2004;73:367-371.

315. Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzo H, Triantafillopoulos A, Whittaker K, Hoidal JR, and Cosio MG. The Development of Emphysema in Cigarette Smoke-exposed Mice Is Strain Dependent. *Am J Respir Crit Care Med* 2004;170:974-980.

316. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, and Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int Arch Allergy Imm* 2006;141:354-368.

317. Saetta M, Shiner RJ, Angus GE, Kim WD, Wang NS, King M, Ghezzo H, and Cosio MG. Destructive index: a measurement of lung parenchymal destruction in smokers. *Am Rev Respir Dis* 1985;131:764-769.

318. Thurlbec WM. Measurement of Pulmonary Emphysema. *Am Rev Respir Dis* 1967;95:752-764.

319. Palmans ELS, Kips JC, and Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 2000;161:627-635.

320. van Wetering S, van der Linden AC, van Sterkenburg MAJA, Rabe KF, Schalkwijk J, and Hiemstra PS. Regulation of secretory leukocyte proteinase inhibitor (SLPI) production by human bronchial epithelial cells: Increase of cell-associated SLPI by neutrophil elastase. *J Investig Med* 2000;48:359-366.

321. Beisswenger C, Platz J, Seifart C, Vogelmeier C, and Bals R. Exposure of differentiated airway epithelial cells to volatile smoke in vitro. *Respiration* 2004;71:402-409.

322. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3.

323. Bracke KR, Verhamme FM, Seys LJ, Bantsimba-Malanda C, Cunoosamy DM, Herbst R, Hammad H, Lambrecht BN, Joos GF, and Brusselle GG. Role of CXCL13 in Cigarette Smoke–induced Lymphoid Follicle Formation and Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2013;188:343-355.

324. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* 2004;364:709-721.

325. Perros F, Dorfmüller P, Montani D, Hammad H, Waelput W, Girerd B, Raymond N, Mercier O, Mussot S, and Cohen-Kaminsky S. Pulmonary lymphoid neogenesis in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2012;185:311-321.

326. Demoor T, Bracke KR, Joos GF, and Brusselle GG. Increased T-regulatory cells in lungs and draining lymph nodes in a murine model of COPD. *Eur Respir J* 2010;35:688-689.

327. Vermaelen K and Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry Part A* 2004;61:170-177.

328. Vermaelen KY, Carro-Muino I, Lambrecht BN, and Pauwels RA. Specific Migratory Dendritic Cells Rapidly Transport Antigen from the Airways to the Thoracic Lymph Nodes. *J Exp Med* 2001;193:51-60.

329. Litsiou E, Semitekolou M, Galani IE, Morianos I, Tsoutsa A, Kara P, Rontogianni D, Bellenis I, Konstantinou M, Potaris K, Andreakos E, Sideras P, Zakynthinos S, and Tsoumakidou M. CXCL13 Production in B Cells via Toll-like Receptor/Lymphotoxin Receptor Signaling Is Involved in Lymphoid Neogenesis in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2013;187:1194-1202.

330. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, and Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med* 2004;199:91-98.

331. Patadia M, Dixon J, Conley D, Chandra R, Peters A, Suh LA, Kato A, Carter R, Harris K, and Grammer L. Evaluation of the presence of B-cell attractant chemokines in chronic rhinosinusitis. *Am J Rhinol Allergy* 2010;24:11.

332. Peperzak V, Vikström I, Walker J, Glaser SP, LePage M, Coquery CM, Erickson LD, Fairfax K, Mackay F, and Strasser A. Mcl-1 is essential for the survival of plasma cells. *Nat Immunol* 2013;14:290-297.

333. Moghaddam SJ, Clement CG, De la Garza MM, Zou X, Travis EL, Young HW, Evans CM, Tuvim MJ, and Dickey BF. Haemophilus influenzae Lysate Induces Aspects of the Chronic Obstructive Pulmonary Disease Phenotype. *Am J Respir Cell Mol Biol* 2008;38:629-638.

334. Vernooy JH, Dentener MA, Van Suylen RJ, Buurman WA, and 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.

335. Leon B, Ballesteros-Tato A, Browning JL, Dunn R, Randall TD, and Lund FE. Regulation of TH2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. *Nat Immunol* 2012;13:681-690.

336. Bracke K, Cataldo D, Maes T, GUEDERS M, FOIDART AJ-M, Brusselle GG, and Pauwels RA. Matrix metalloproteinase-12 and cathepsin D expression in pulmonary macrophages and dendritic cells of cigarette smoke-exposed mice. *Int Arch Allergy Imm* 2005;138:169-179.

337. Verhamme FM, Bracke KR, Amatngalim GD, Verleden GM, Van Pottelberge GR, Hiemstra PS, Joos GF, and Brusselle GG. Role of activin-A in cigarette smoke-induced inflammation and COPD. *Eur Respir J* 2014;43:1028-1041.

338. Demedts I, Demoor T, Bracke KR, Joos GF, and Brusselle GG. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. *Respir Res* 2006;7:53.

339. Abe Y, Minegishi T, and Leung PCK. Activin receptor signaling. *Growth Factors* 2004;22:105-110.

340. ten Dijke P and Hill CS. New insights into TGF-β-Smad signalling. *Trends Biochem Sci* 2004;29:265-273.

341. Harrison CA, Gray PC, Vale WW, and Robertson DM. Antagonists of activin signaling: mechanisms and potential biological applications. *Trends Endocrinol Metab* 2005;16:73-78.

342. Glister C, Kemp CF, and Knight PG. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4,-6 and-7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* 2004;127:239-254.

343. Nogai H, Rosowski M, Grun J, Rietz A, Debus N, Schmidt G, Lauster C, Janitz M, Vortkamp A, and Lauster R. Follistatin antagonizes transforming growth factor-beta 3-induced epithelial-mesenchymal transition in vitro: implications for murine palatal development supported by microarray analysis. *Differentiation* 2008;76:404-416.

344. Apostolou E, Stavropoulos A, Sountoulidis A, Xirakia C, Giaglis S, Protopapadakis E, Ritis K, Mentzelopoulos S, Pasternack A, Foster M, Ritvos O, Tzelepis GE, Andreakos E, and Sideras P. Activin-A overexpression in the murine lung causes pathology that simulates acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2012;185:382-391.

345. Zuyderduyn S, Ninaber D, Schrumpf J, van Sterkenburg M, Verhoosel R, Prins F, van Wetering S, Rabe KF, and Hiemstra PS. IL-4 and IL-13 exposure during mucociliary differentiation of bronchial epithelial cells increases antimicrobial activity and expression of antimicrobial peptides. *Respir Res* 2011;12:59.

346. DiChiara MR, Kiely JM, Gimbrone MA, Lee ME, Perrella MA, and Topper JN. Inhibition of E-selectin gene expression by transforming growth factor beta in endothelial cells involves coactivator integration of Smad and nuclear factor kappa B-mediated signals. *J Exp Med* 2000;192:695-704.

347. Maunders H, Patwardhan S, Phillips J, Clack A, and Richter A. Human bronchial epithelial cell transcriptome: gene expression changes following acute exposure to whole cigarette smoke in vitro. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1248-L1256.

348. Leigh R, Oyelusi W, Wiehler S, Koetzler R, Zaheer RS, Newton R, and Proud D. Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling. *J Allergy Clin Immunol* 2008;121:1238-1245.

349. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Gene Dev* 1994;8:133-146.

350. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, and Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science* 1988;242:1528-1534.

351. Obradovic Wagner D, Sieber C, Bhushan R, Borgermann JH, Graf D, and Knaus P. BMPs: from bone to body morphogenetic proteins. *Sci Signal* 2010;3:mr1.

352. Philippot Q, Deslée G, Adair-Kirk TL, Woods JC, Byers D, Conradi S, Dury S, Perotin JM, Lebargy F, and Cassan C. Increased Iron Sequestration in Alveolar Macrophages in Chronic Obtructive Pulmonary Disease. *Plos One* 2014;9:e96285.

353. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BLM, and Robertson EJ. Mice lacking Bmp6 function. *Dev Genet* 1998;22:321-339.

354. Kaiser S, Schirmacher P, Philipp A, Protschka M, Moll I, Nicol K, and Blessing M. Induction of Bone Morphogenetic Protein-6 in Skin Wounds. Delayed Reepitheliazation and Scar Formation in BMP-6 Overexpressing Transgenic Mice. *J Invest Dermatol* 1998;111:1145-1152.

355. Glienke J, Schmitt AO, Pilarsky C, Hinzmann B, Weiβ B, Rosenthal A, and Thierauch KH. Differential gene expression by endothelial cells in distinct angiogenic states. *Eur J Biochem* 2000;267:2820-2830.

356. Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F, Sideras P, and ten Dijke P. Stimulation of Id1 Expression by Bone Morphogenetic Protein Is Sufficient and Necessary for Bone Morphogenetic Protein–Induced Activation of Endothelial Cells. *Circulation* 2002;106:2263-2270.

357. Gould SE, Day M, Jones SS, and Dorai H. BMP-7 regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells1. *Kidney Int* 2002;61:51-60.

358. Yao H, Edirisinghe I, Rajendrasozhan S, Yang SR, Caito S, Adenuga D, and Rahman I. Cigarette smoke-mediated inflammatory and oxidative responses are strain-dependent in mice. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1174-L1186.

359. Ghio AJ, Hilborn ED, Stonehuerner JG, Dailey LA, Carter JD, Richards JH, Crissman KM, Foronjy RF, Uyeminami DL, and Pinkerton KE. Particulate Matter in Cigarette Smoke Alters Iron Homeostasis to Produce a Biological Effect. *Am J Respir Crit Care Med* 2008;178:1130-1138.

360. Cherayil BJ. Iron and Immunity: Immunological Consequences of Iron Deficiency and Overload. *Arch Immunol Ther Ex* 2010;58:407-415.

361. Benesova K, Spasic MV, Schaefer SM, Stolte J, Baehr-Ivacevic T, Waldow K, Zhou Z, Klingmueller U, Benes V, Mall MA, and Muckenthaler MU. Hfe Deficiency Impairs Pulmonary Neutrophil Recruitment in Response to Inflammation. *Plos One* 2012;7.

362. Global Initiative for Asthma. Global Strategy for asthma management and prevention: updated 2014. www.ginasthma.org .

363. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;8:183-192.

364. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:S28-S38.

365. Shi W, Chen F, and Cardoso WV. Mechanisms of lung development: contribution to adult lung disease and relevance to chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2009;6:558.

366. Silverman ES, Palmer LJ, Subramaniam V, Hallock A, Mathew S, Vallone J, Faffe DS, Shikanai T, Raby BA, and Weiss ST. Transforming growth factor-β1 promoter polymorphism C–509T is associated with asthma. *Am J Respir Crit Care Med* 2004;169:214-219.

367. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JSR, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, and Nichols WC. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet* 2000;37:741-745.

368. Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, Loyd JE, Nichols WC, and Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet* 2000;26:81-84.

369. Deng ZM, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, and Knowles JA. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;67:737-744.

370. Frank DB, Abtahi A, Yamaguchi DJ, Manning S, Shyr Y, Pozzi A, Baldwin HS, Johnson JE, and de Caestecker MP. Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. *Circ Res* 2005;97:496-504.

371. Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, and Fukuchi Y. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L450-L458.

372. Miyazono K, Kamiya Y, and Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem* 2010;147:35-51.

373. Springer J, Scholz FR, Peiser C, Groneberg DA, and Fischer A. SMAD-signaling in chronic obstructive pulmonary disease: transcriptional down-regulation of inhibitory SMAD 6 and 7 by cigarette smoke. *Biol Chem* 2004;385:649-653.

374. Costello CM, Cahill E, Martin F, Gaine S, and McLoughlin P. Role of Gremlin in the Lung Development and Disease. *Am J Respir Cell Mol Biol* 2010;42:517-523.

375. Helbing T, Herold EM, Hornstein A, Wintrich S, Heinke J, Grundmann S, Patterson C, Bode C, and Moser M. Inhibition of BMP activity protects epithelial barrier function in lung injury. *J Pathol* 2013;231:105-116.

376. Sannes PL, Meuten T, Grossi B, Zhang H, and Newman D. Growth And Differentiation Factor 5 (GDF5) In Human And Rodent Lung. *Growth* 2010;1:2.

377. Böttner M, Suter-Crazzolara C, Schober A, and Unsicker K. Expression of a novel member of the TGF-beta superfamily, growth/differentiation factor-15/macrophage-inhibiting cytokine-1 (GDF-15/MIC-1) in adult rat tissues. *Cell Tissue Res* 1999;297:103-110.

378. Nickel N, Jonigk D, Kempf T, Bockmeyer CL, Maegel L, Rische J, Laenger F, Lehmann U, Sauer C, Greer M, Welte T, Hoeper MM, and Golpon HA. GDF-15 is abundantly expressed in plexiform lesions in patients with pulmonary arterial hypertension and affects proliferation and apoptosis of pulmonary endothelial cells. *Respir Res* 2011;12.

379. Sountoulidis A, Stavropoulos A, Giaglis S, Apostolou E, Monteiro R, Lopes SMCD, Chen HY, Stripp BR, Mummery C, Andreakos E, and Sideras P. Activation of the Canonical Bone Morphogenetic Protein (BMP) Pathway during Lung Morphogenesis and Adult Lung Tissue Repair. *Plos One* 2012;7.

380. Bellusci S, Henderson R, Winnier G, Oikawa T, and Hogan BLM. Evidence from normal expression and targeted misexpression that bone morphogenetic protein-4 (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* 1996;122:1693-1702.

381. King JA, Marker PC, Seung KJ, and Kingsley DM. Bmp5 and the Molecular, Skeletal, and Soft-Tissue Alterations in Short Ear Mice. *Dev Biol* 1994;166:112-122.

382. Weaver M, Yingling JM, Dunn NR, Bellusci S, and Hogan BLM. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* 1999;126:4005-4015.

383. Bragg AD, Moses HL, and Serra R. Signaling to the epithelium is not sufficient to mediate all of the effects of transforming growth factor β and bone morphogenetic protein 4 on murine embryonic lung development. *Mech Develop* 2001;109:13-26.

384. Eblaghie MC, Reedy M, Oliver T, Mishina Y, and Hogan BLM. Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. *Dev Biol* 2006;291:67-82.

385. Sun JP, Chen H, Chen C, Whitsett JA, Mishina Y, Bringas P, Ma JC, Warburton D, and Shi W. Prenatal lung epithelial cell-specific abrogation of Alk3-bone morphogenetic protein signaling causes neonatal respiratory distress by disrupting distal airway formation. *Am J Pathol* 2008;172:571-582.

386. Chen C, Chen H, Sun JP, Bringas P, Chen YH, Warburton D, and Shi W. Smad1 expression and function during mouse embryonic lung branching morphogenesis. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L1033-L1039.

387. Xu B, Chen C, Chen H, Zheng SG, Bringas P, Xu M, Zhou XH, Chen D, Umans L, Zwijsen A, and Shi W. Smad1 and its target gene Wif1 coordinate BMP and Wnt signaling activities to regulate fetal lung development. *Development* 2011;138:925-935.

388. Adams D, Larman B, and Oxburgh L. Developmental expression of mouse Follistatin-like 1 (Fstl1): Dynamic regulation during organogenesis of the kidney and lung. *Gene Expr Patterns* 2007;7:491-500.

389. Geng Y, Dong YY, Yu MY, Zhang L, Yan XH, Sun JX, Qiao L, Geng HX, Nakajima M, Furuichi T, Ikegawa S, Gao X, Chen YG, Jiang DH, and Ning W. Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development. *Proc Natl Acad Sci USA* 2011;108:7058-7063.

390. Sylva M, Li VSW, Buffing AAA, van Es JH, van den Born M, van der Velden S, Gunst Q, Koolstra JH, Moorman AFM, Clevers H, and van den Hoff MJB. The BMP Antagonist Follistatin-Like 1 Is Required for Skeletal and Lung Organogenesis. *Plos One* 2011;6.

391. Lu MM, Yang HH, Zhang LL, Shu WG, Blair DG, and Morrisey EE. The bone morphogenic protein antagonist gremlin regulates proximal-distal patterning of the lung. *Dev Dynam* 2001;222:667-680.

392. Shi W, Zhao JS, Anderson KD, and Warburton D. Gremlin negatively modulates BMP-4 induction of embryonic mouse lung branching morphogenesis. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1030-L1039.

393. Molloy EL, Adams A, Moore JB, Masterson JC, Madrigal-Estebas L, Mahon BP, and O'Dea S. BMP4 induces an epithelial-mesenchymal transition-like response in adult airway epithelial cells. *Growth Factors* 2008;26:12-22.

394. Koli K, Myllärniemi M, Vuorinen K, Salmenkivi K, Ryynänen MJ, Kinnula VL, and Keski-Oja J. Bone Morphogenetic Protein-4 Inhibitor Gremlin Is Overexpressed in Idiopathic Pulmonary Fibrosis. *Am J Pathol* 2006;169:61-71.

395. Wu Q, Jiang D, and Chu HW. Cigarette smoke induces growth differentiation factor 15 production in human lung epithelial cells: Implication in mucin over-expression. *J Innate Immun* 2012;18:617-626.

396. Unsicker K, Spittau B, and Krieglstein K. The multiple facets of the TGF-β family cytokine growth/differentiation factor-15/macrophage inhibitory cytokine-1. *Cytokine Growth F R* 2013;24:373-384.

397. Bovolenta P, Esteve P, Ruiz JM, Cisneros E, and Lopez-Rios J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J Cell Sci* 2008;121:737-746.

398. Wang R, Ahmed J, Wang G, Hassan I, Strulovici-Barel Y, Hackett NR, and Crystal RG. Down-regulation of the canonical Wnt β-catenin pathway in the airway epithelium of healthy smokers and smokers with COPD. *Plos One* 2011;6:e14793.

399. Wen W, Wang AM, Liu DL, Zhang YB, Yao LQ, and Lai GX. Expression of Connective Tissue Growth Factor and Bone Morphogenetic Protein-7 in Pseudomonas aeruginosa-induced Chronic Obstructive Pulmonary Disease in Rats. *COPD* 2013;10:657-666.

400. Judith, C. M., L. Jimmy, C. Y. Sze, M. Moira, and S. Mary. Elevated Plasma Levels Of Growth Differentiation Factor (GDF)-15 In Stable Chronic Obstructive Pulmonary Disease (COPD). *Am J Respir Crit Care Med* 2013;187:A1526 [abstract].

401. Plant PJ, Brooks D, Faughnan M, Bayley T, Bain J, Singer L, Correa J, Pearce D, Binnie M, and Batt J. Cellular Markers of Muscle Atrophy in Chronic Obstructive Pulmonary Disease. *Am J Respir Cell Mol Biol* 2010;42:461-471.

402. Petersen AMW, Magkos F, Atherton P, Selby A, Smith K, Rennie MJ, Pedersen BK, and Mittendorfer B. Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. *Am J Physiol Endoc M* 2007;293:E843-E848.

403. Hayot M, Rodriguez J, Vernus B, Carnac G, Jean E, Allen D, Goret L, Obert P, Candau R, and Bonnieu A. Myostatin up-regulation is associated with the skeletal muscle response to hypoxic stimuli. *Mol Cell Endocrinol* 2011;332:38-47.

404. Testelmans D, Crul T, Maes K, Agten A, Crombach M, Decramer M, and Gayan-Ramirez G. Atrophy and hypertrophy signalling in the diaphragm of patients with COPD. *Eur Respir J* 2010;35:549-556.

405. Ju CR and Chen RC. Serum myostatin levels and skeletal muscle wasting in chronic obstructive pulmonary disease. *Respir Med* 2012;106:102-108.

406. Johnston L, Aggarwal NR, Garibaldi BT, Sidhaye V, Chau E, Cohn R, D'Alessio FR, King LS, Crow MT, and Files D. Skeletal Muscle Wasting In Acute Lung Injury Is Suppressed In The Myostatin Null Mouse. *Am J Respir Crit Care Med* 2010;181:A6610 [abstract].

407. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, and Kalluri R. BMP-7 counteracts TGF-beta 1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003;9:964-968.

408. Weaver M, Dunn NR, and Hogan BLM. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* 2000;127:2695-2704.

409. Legler DF, Loetscher M, Roos RS, Clark-Lewis I, Baggiolini M, and Moser B. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med* 1998;187:655-660.

410. Gualano RC, Hansen MJ, Vlahos R, Jones JE, Park-Jones RA, Deliyannis G, Turner SJ, Duca KA, and Anderson GP. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir Res* 2008;9.

411. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, Xu H, Vivier E, Sellars M, Pierre P, Sharp FR, Chan S, Kastner P, and Dalod M. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* 2008;9.

412. Lugade AA, Bogner PN, Thatcher TH, Sime PJ, Phipps RP, and Thanavala Y. Cigarette Smoke Exposure Exacerbates Lung Inflammation and Compromises Immunity to Bacterial Infection. *J Immunol* 2014;192:5226-5235.

413. Mackay F and Schneider P. Cracking the BAFF code. *Nat Rev Immunol* 2009;9:491-502.

414. Polverino F, Baraldo S, Bazzan E, Agostini S, Turato G, Lunardi F, Balestro E, Damin M, Papi A, and Maestrelli P. A novel insight into adaptive immunity in chronic obstructive pulmonary disease: B cell activating factor belonging to the tumor necrosis factor family. *Am J Respir Crit Care Med* 2010;182:1011-1019.

415. Moon EY and Ryu SK. TACI:Fc scavenging B cell activating factor (BAFF) alleviates ovalbumin-induced bronchial asthma in mice. *Exp Mol Med* 2007;39:343-352.

416. Wu H, Chen Y, Winnall WR, Phillips DJ, and Hedger MP. Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice. *Am J Physiol Reg I* 2012;303:R665-R675.

417. Xia Y and Schneyer AL. The biology of activin: recent advances in structure, regulation and function. *J Endocrinol* 2009;202:1-12.

418. Engelse MA, Neele JM, van Achterberg TA, van Aken BE, van Schaik RH, Pannekoek H, and de Vries CJ. Human activin-A is expressed in the atherosclerotic lesion and promotes the contractile phenotype of smooth muscle cells. *Circ Res* 1999;85:931-939.

419. Yaden BC, Wang YX, Wilson JM, Culver AE, Milner A, tta-Mannan A, Shetler P, Croy JE, Dai G, and Krishnan V. Inhibition of Activin A Ameliorates Skeletal Muscle Injury and Rescues Contractile Properties by Inducing Efficient Remodeling in Female Mice. *Am J Pathol* 2014;184:1152-1166.

420. Yan Jd, Yang S, Zhang J, and Zhu Th. BMP6 reverses TGF-β1-induced changes in HK-2 cells: implications for the treatment of renal fibrosis. *Acta Pharmacol Sin* 2009;30:994-1000.

421. Farkas L, Farkas D, Gauldie J, Warburton D, Shi W, and Kolb M. Transient Overexpression of Gremlin Results in Epithelial Activation and Reversible Fibrosis in Rat Lungs. *Am J Respir Cell Mol Biol* 2011;44:870-878.

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Additional courses

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Awards/grants

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2014	Belgian Thoracic Society – GSK Basic Science Award in Pneumology 2014
2014	Abstract Scholarship from the National Emphysema Foundation honouring Claude Lenfant, ATS International Conference, San Diego, 2014
2012	European Respiratory Society: Short-Term Research Training Fellowship

International peer-reviewed publications

Verhamme FM, Bracke KR, Joos GF, Brusselle GG.
TGF-β superfamily in obstructive lung diseases: more suspects than TGF-β alone.
American Journal of Respiratory Cell and Molecular Biology. 2014 November 14. Epub ahead of print.
IF: 4.109 – ranking in respiratory system: 7/54

Eurlings IMJ, Reynaert NL, van den Beucken T, Gosker HR, de Theije CC, **Verhamme FM**, Bracke KR, Wouters EFM, Dentener MA.
Cigarette smoke extract induces a phenotypic shift in epithelial cells; involvement of HIF1α in mesenchymal transition.
Plos One. 2014; 9 (10): e107757.
IF: 3.534 – ranking in multidisciplinary sciences: 8/55

Loth DW, Artigas MS, Gharib SA, Wain LV, Franceschini N, Koch B, Pottinger TD, Smith AV, Duan Q, Oldmeadow C, Lee MK, Strachan DP, James AL, Huffman JE, Vitart V, Ramasamy A, Wareham NJ, Kaprio J, Wang XQ, Trochet H, Kahonen M, Flexeder C, Albrecht E, Lopez LM, de Jong K, Thyagarajan B, Alves AC, Enroth S, Omenaas E, Joshi PK, Fall T, Vinuela A, Launer LJ, Loehr LR, Fornage M, Li G, Wilk JB, Tang W, Manichaikul A, Lahousse L, Harris TB, North KE, Rudnicka AR, Hui J, Gu X, Lumley T, Wright AF, Hastie ND, Campbell S, Kumar R, Pin I, Scott RA, Pietilainen KH, Surakka I, Liu Y, Holliday EG, Schulz H, Heinrich J, Davies G, Vonk JM, Wojczynski M, Pouta A, Johansson A, Wild SH, Ingelsson E, Rivadeneira F, Volzke H, Hysi PG, Eiriksdottir G, Morrison AC, Rotter JI, Gao W, Postma DS, White WB, Rich SS, Hofman A, Aspelund T, Couper D, Smith LJ, Psaty BM, Lohman K, Burchard EG, Uitterlinden AG, Garcia M, Joubert BR, McArdle WL, Musk AB, Hansel N, Heckbert SR, Zgaga L, van Meurs JBJ, Navarro P, Rudan I, Oh YM, Redline S, Jarvis DL, Zhao JH, Rantanen T, O'Connor GT, Ripatti S, Scott RJ, Karrasch S, Grallert H, Gaddis NC, Starr JM, Wijmenga C, Minster RL, Lederer DJ, Pekkanen J, Gyllensten U, Campbell H, Morris AP, Glaser S, Hammond CJ, Burkart KM, Beilby J, Kritchevsky SB, Gudnason V, Hancock DB, Williams OD, Polasek O, Zemunik T, Kolcic I, Petrini MF, Wjst M, Kim WJ, Porteous DJ, Scotland G, Smith BH, Viljanen A, Heliovaara M, Attia JR, Sayers I, Hampel R, Gieger C, Deary IJ, Boezen HM, Newman A, Jarvelin MR, Wilson JF, Lind L, Stricker BH, Teumer A, Spector TD, Melen E, Peters MJ, Lange LA, Barr RG, Bracke KR, **Verhamme FM**, Sung J, Hiemstra PS, Cassano PA, Sood A, Hayward C, Dupuis J, Hall IP, Brusselle GG, Tobin MD, London SJ.
Genome-wide association analysis identifies six new loci associated with forced vital capacity.
Nature Genetics. 2014; 46 (7): 669-677.
IF: 29.648 – ranking in genetics & heredity: 2/165

Verhamme FM, Bracke KR, Amatngalim GD, Verleden GM, Van Pottelberge GR, Hiemstra PS, Joos GF, Brusselle GG.
Role of activin-A in cigarette smoke-induced inflammation and COPD.
European Respiratory Journal. 2014; 43: 1028-1041.
IF: 7.125 – ranking in respiratory system: 4/54

Bracke KR*, **Verhamme FM***, Seys LJM, Bantsimba-Malanda C, Mootoosamy Cunoosamy D, Herbst R, Hammad H, Lambrecht BN, Joos GF, Brusselle GG. (* equal contribution)
Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and chronic obstructive pulmonary disease.
American Journal of Respiratory and Critical Care Medicine. 2013; 188 (3): 343-355.
IF: 11.986 – ranking in respiratory system: 1/54

National and international presentations

COST Conference – A bird's eye view on the ageing lung, Groningen, The Netherlands (26-27 June 2014).
Verhamme FM, Bracke KR, Joos GF, Brusselle GG.
Cigarette smoke-induced inflammation is aggravated in BMP-6 deficient mice (oral presentation).

Belgian Thoracic Society, GSK Awards in Pneumology, Brussels, Belgium (11 June 2014).
Verhamme FM, Bracke KR, Seys LJM, Bantsimba-Malanda C, Mootoosamy Cunoosamy D, Herbst R, Hammad H, Lambrecht BN, Joos GF, Brusselle GG.
Rol van CXCL13 in sigarettenrook-geïnduceerde lymfoïde follikel vorming en COPD (oral presentation).

American Thoracic Society, International Conference, San Diego, CA, USA (16-21 May 2014).
Verhamme FM, Bracke KR, Joos GF, Brusselle GG.
Expression of transforming growth factor-β superfamily ligands in cigarette smoke-exposed mice.
American Journal of Respiratory and Critical Care Medicine 2017; 189: A3008 (poster presentation).

COST Conference – Early origins of chronic lung disease, Munich, Germany (12-15 June 2013).
Invited lecture: Re-activation of activin/BMP signaling pathways in COPD.

American Thoracic Society, International Conference, Philadelphia, PA, USA (17-22 May 2013).
Verhamme FM, Bracke KR, Amatngalim GD, Verleden GM, Van Pottelberge GR, Hiemstra PS, Joos GF, Brusselle GG.
Role of activin-A in cigarette smoke-induced inflammation and chronic obstructive pulmonary disease.
American Journal of Respiratory and Critical Care Medicine 2013; 187: A1094 (poster presentation).

Belgian Thoracic Society, GSK Awards in Pneumology, Brussels, Belgium (27 March 2013).
Verhamme FM, Bracke KR, Amatngalim GD, Verleden GM, Van Pottelberge GR, Hiemstra PS, Joos GF, Brusselle GG.
Rol van activine-A in sigarettenrook-geïnduceerde inflammatie en COPD (oral presentation).

American Thoracic Society, International Conference, San Francisco, CA, USA (18-23 May 2012).
Verhamme FM, Bracke KR, Van Pottelberge G, Joos GF, Brusselle GG.
Expression of Interferon Regulatory Factor 8 in human lung dendritic cell subsets.
American Journal of Respiratory and Critical Care Medicine 2012; 185: A1074 (poster presentation).

American Thoracic Society, International Conference, Denver, CO, USA (13-18 May 2011).
Verhamme FM, Pauwels NS, Demoor T, Vanden Berghe T, Dupont LL, Bracke KR, Joos GF, Vandenabeele P, Brusselle GG.
Role of Caspase-7 in Cigarette Smoke-Induced Inflammation in Mice.
American Journal of Respiratory and Critical Care Medicine 2011; 183: A4101(poster presentation).

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