Aus der Inneren Medizin V, Pneumologie, Allergologie, Beatmungsmedizin-Klinik,
Universitätsklinikum des Saarlandes, Homburg/Saar
Direktor: Prof. Dr. Dr. Robert Bals
Theoretische Medizin und Biowissenschaften bzw. Klinische Medizin
der Medizinischen Fakultät
der Universität des Saarlandes, Homburg/Saar

Combined exposure to bacterial and cigarette smoke resembles characteristic phenotypes of human COPD in a murine disease model

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vorgelegt von: Gang Han
geb. am: 19.04.1977 in Shandong, PR China

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Gang Han

Innere Medizin V, Pneumologie, Allergologie, Beatmungsmedizin-Klinik,

AG Prof. Dr. Dr. Robert Bals, Gebäude 61.4

Universitätsklinikum des Saarlandes, 66421 Homburg/Saar, Deutschland

E-Mail: Gang.Han@uks.eu

献 给 我 深 爱 的 家 人

To my family

Abstract

Chronic Obstructive Pulmonary Disease (COPD) is predicted by 2020 to be the third leading cause of death. Acute exacerbations of COPD (AECOPDs) are common in the COPD patient and particularly require hospitalization. Cigarette smoke is a major risk factor for the development of COPD. The gram negative bacterium *Haemophilus influnenzae* (*H.i*) plays a major role during exacerbations in patients with COPD.

The aim of this work is to investigate the effects of a combined stimulation with cigarette smoke (CS) and *H.i* on the inflammatory response and the development of pulmonary emphysema in mice. This will help us to better understand the mechanisms that lead to the overwhelming inflammatory response in COPD exacerbations that are believed to be the driving force for lung destruction and overall mortality in human COPD patients.

Mice were exposed to CS in a smoking machine for different time points of 2 weeks up to 6 months at 3 hours per day. After the CS exposure the mice were additionally challenged with nebulized lysate of inactivated *H.i*. We analyzed the inflammatory response using enzyme-linked immunosorbent assay (ELISA), Cytometric Bead Array (CBA), Western-Blot, real-time PCR and immunohistochemistry. To quantify the structural changes in the lungs we performed lung function and analyzed the lungs by stereological methods.

Our data demonstrated that the combination of CS and *H.i* induced a higher release of Matrix metalloproteinase-12 (MMP-12) in lung homogenate, induced Chemokine (C-X-C motif) ligand 1 (CXCL1), also termed KC, Tumor Necrosis Faktor-α (TNF-α), and Interleukin-17α release into the bronchoalveolar lavage fluid (BALF). The immunohistological analysis indicated the enhanced expression of MMP-12 in lung epithelial cells. The combination of CS and *H.i* changed the inflammatory phenotype from a macrophage based inflammation to a neutrophilic inflammation, and induced mucus hypersecretion. We detected changes in lung function and lung architecture, which were dependent on the stimulation with CS and *H.i*. and occurred as early as 3 months after the experiment started.

In summary, our study demonstrated the interaction of CS and *H.i.* in a mouse model. It will broaden our knowledge of the molecular mechanisms that are responsible for the COPD development in humans.

Zusammenfassung

Chronisch obstruktive Lungenerkrankung (COPD) wird im Jahr 2020 voraussichtlich weltweit die dritthäufigste Todesursache sein. Akute Exazerbationen der COPD (AECOPDs) kommen bei COPD-Patienten häufig vor und sind besonders häufig für Krankenhausaufenthalte verantwortlic. Zigarettenrauch ist der wichtige Risikofaktor für die Entwicklung der COPD. Das gramnegative Bakterium *Haemophilus influnenzae (H.i)* spielt eine wichtige Rolle während Exazerbationen bei Patienten mit COPD.

Das Ziel dieser Arbeit ist es, den Einfluss der Stimulation mit Zigarettenrauch (cigarette smoke, CS) und *H.i.* auf die Entzündungsreaktion und die Entstehung des Lungenemphysems zu untersuchen und die Mechanismen, die zu der übermäßigen Entzündungsreaktion im Rahmen einer Infektexazerbation bei COPD führen, genauer zu verstehen.

Die Mäuse wurden in einer Rauchmaschine für verschiedene Zeitintervalle von 2 Wochen bis zu 6 Monaten für 3 Stunden pro Tag mit CS stimuliert. Nach der CS Exposition wurden die Mäuse zusätzlich mit vernebelten inaktivierten *H.i* -Lysat stimuliert. Wir quantifizierten die Abgabe und Expression von Entzündungsmediatoren mit ELISA, Cytometric Bead Array (CBA), Western-Blot, real-time PCR und Immunhistochemie. Um strukturelle Veränderungen zu erkennen, wurden die Lungen mit stereologischen Methoden analysiert.

Unsere Daten zeigen, dass die Kombination von CS und *H.i* eine höhere Freisetzung von MMP-12 im Lungenhomogenisat und CXCL1 oder KC, TNF-α, IL-17α in der BALF verglichen zu den Kontrollen induziert. Die immunhistologische Analyse zeigte die verstärkte Expression von MMP-12 in Lungenepithelzellen. Die Kombination von CS und *H.i* verändert den entzündlichen Phänotyp von einer Makrophagen basierter Entzündung zu neutrophilen Entzündung, induzierte eine Schleim-Hypersekretion, führte zu einer schlechteren Lungenfunktion und einer Veränderung der Lungenstruktur bereits nach 3 Monaten.

Zusammenfassend zeigt unserer Studie das systematische Zusammenspiel von CS und *H.i* im Mausmodell.

Contents

1. Introduction

Chronic obstructive pulmonary disease (COPD) is the most common health problem in the world and projected to be the third leading cause of death worldwide by 2020. COPD ranked as the fourth leading cause of death in 2010 in Germany and the third place in the U.S. The health care costs of COPD in the U.S. were $42.6 billion in 2007 and $2.1 trillion worldwide in 2010 (1-3).

1.1 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is defined as a chronic lung disease with poorly reversible airway obstruction, pulmonary and systemic inflammation, and pulmonary emphysema (4, 5). Airway obstruction is resulted mostly from chronic bronchitis induced mucus hypersecretion and air way fibrosis (6, 7).

Pulmonary emphysema is characterized by irreversible destruction of airway parenchyma (8). Emphysema is a result of the tissue remodeling in air space. Emphysema is an important manifestation of COPD, and induced mostly by long term cigarette smoke. The cause of emphysema are normally accepted as apoptosis/proliferation of type I/II pneumocytes, proteinase/anti-proteinase imbalance and oxidative stress in lung (9). The patients with genetic α1-antitrypsin deficiency develop spontaneous emphysema (10).

COPD patients show systemic manifestation, such as skeletal muscle weakness and cachexia (5). The proinflammatory cytokines TNF-α and IL-1β from the pulmonary and systemic inflammation may play an important role. Some study reported that animal administrated with TNF-α induced cachexia, anemia, and leukocytosis (11).

Furthermore, the pulmonary and systemic inflammation induced inflammatory cytokines into circulation may play an important role for the comorbidities of COPD, such as ischemic heart dysfunction, cerebrovascular disease, arrhythmias, osteoporosis, lung cancer, depression and diabetes. COPD patients with important

comorbidities are in general older and require more medical attention. The comorbidities increased hospitalization, mortality, healthcare costs of COPD patients and worsen life quality (5, 12).

Figure.1.1

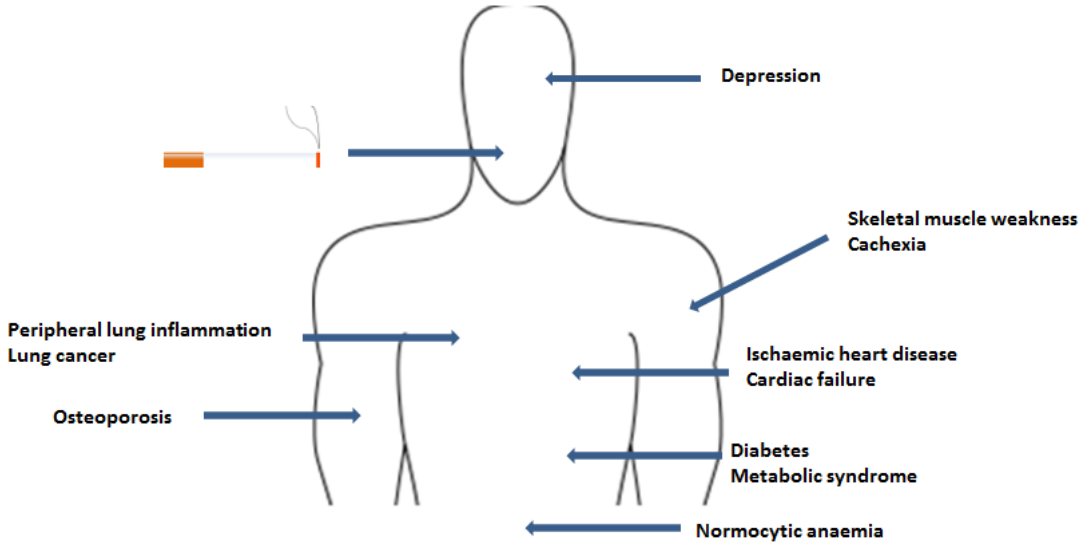


Figure.1.1. the extrapulmonary comorbidities of COPD.

The systemic inflammation induced the release of proinflammatory cytokine such as, IL-6, IL-8, IL-1β and TNF-α into circulation, may worsen COPD and initiate comorbidities, including skeletal muscle weakness, ischemic heart dysfunction, cerebrovascular disease, arrhythmias, osteoporosis, lung cancer, depression and diabetes.

1.1.1 Risk factors of COPD

The risk factors which have been found to induce and increase COPD, are cigarette smoking, severe infections, occupational dust, vapors, and fumes, indoor and outdoor pollutants, ageing, infections and genetic factors. It is now worldwide accepted that 10-20% of smoker are susceptible and develop clinically COPD (13). Severe

infections during childhood have been associated with reduced lung function and increased respiratory symptoms in adulthood (14). Smoking during pregnancy may affect fetal lung growth and therefore increase the risk of COPD. Indoor and outdoor air pollutants are also the important risk factors for the development of COPD. WHO estimates that, in countries of low and middle income, 35% of people with COPD developed the disorder after exposure to indoor smoke from biomass fuels (15). Compared to the indoor air pollutants the contribution of outdoor air pollutants to the development of COPD is much smaller. WHO estimates that urban air pollution causes 1% of COPD cases in high-income countries and 2% in nations of low and middle income (16). Infections have an important role in both development and progression of COPD. Bacterial or viral infections are normally involved in most COPD exacerbations (17). The deficiency of the serine protease α1 antitrypsin is the best known genetic factor related to COPD, which arises in 1–3% of COPD patients. In total, the cause of COPD is a multi-factor event (18).

1.1.2 Cigarette Smoke in COPD

The most important risk factor for COPD in the developed world is cigarette smoking. Active and passive cigarette smoke exposure contributes to increased respiratory diseases such as COPD, asthma, and lung cancer (9). Cigarette smoking induced about 80–90% of COPD cases in the United States (19).

Smoking, mainly refer to Tabaco, has a long history and reported to begin at 5000-3000 BC (20). The first study about biological effect of cigarette smoking on health problem was focus on the lung cancer in the late of 1920s in Germany (21).

Tabaco contained complex chemical substances. The gas phase alone contained over 1000 different reactive specie, and the tar phase contained also abundant reactive substances, such as ROS and RNS**.** People inhaled by puffing 45% of the total biomass in the burning cigarette smoke from mainstream smoke and about 55% of the content from side stream smoke (9).

Passive smoking, also known as side stream smoke, second-hand smoke (SHS) or Environmental Tobacco Smoke (ETS), is more toxic than active smoke and relates with elevated risk in lung cancer of non-smokers or children (19).

Inhaled reactive species by smoking induces pulmonary and circulating inflammation. Cigarette smoke activates immunocells such as macrophage, neutrophil and lymphocytes to release the proinflammatory cytokines such as TNF-α, IL-6, IL-8 and IL-1β in to the lung. The recruited immunocells release more proinflammatory cytokines. The NF-kB signaling and extracellular signal-regulated kinase 1 and 2 (ERK1/2) are believed to involve in this pathological process. It was reported in several paper that tobacco components include nicotine and N-nitrosonornicotine (NNN) activated NF-kB in some cell lines (22, 23). NF-kB signaling plays an important role in regulation of innate, adaptive immunity and inflammation (24-26). Various papers reported that NF-kB is involved in the pathological process of COPD (27, 28). CS-extract activates the NF-kB signaling in mouse lung (29). However, target deletion of IKK, a subunit of NF-kB, shows no effect on the regulation of pulmonary inflammation (30). Taken together, the role of NF-kB in pathogenesis of COPD and emphysema is largely unknown.

Recent studies report that a 7nAChR dependent nicotinic anti-inflammatory pathway modulates the inflammatory response by vagus nerve. Lipopolysaccharide (LPS) stimulation in 7nAChR deficient mice induces more inflammatory response than wild type mice (31). Nicotinic acetylcholine receptors (nAChRs) is mainly studies in neurons and muscle and also associated with macrophage, which mainly regulate the pulmonary inflammation (32-34).These studies provide a beneficial role with cigarette smoking induced inflammation. However, it common accepted currently that cigarette smoking is harmful and the most important risk factor in COPD.

1.1.3 Systemic inflammation in COPD

Recent studies highlight the systemic inflammation including increased

concentrations of circulating inflammatory cytokines, chemokines, and acute phase proteins, or abnormalities in circulating cells during COPD (35). However, the mechanism including the origin of the systemic inflammation in patients with COPD remains poorly understood. Cigarette smoke is the most important risk factor of some important extrapulmonary effects. The systemic inflammation in COPD may contributed by tobacco. Passive smokers and smokers with only few pack-years suffered from systemic oxidative stress and peripheral vascular endothelial dysfunction (36, 37). The other possible role of tobacco smoke in COPD is the local pulmonary inflammatory response in systemic inflammation (38). Whether like IL-6, IL-8, IL-1β and TNF-α are important proinflammatory cytokines which released in the peripheral lung, and related to comorbid disease remains also poorly understood. Systemic inflammation is increased during exacerbations and induced accelerated impaired lung function (39, 40).

1.1.3.1 The proinflammatory cytokines and chemokines in COPD

The proinflammatory cytokines and chemokines such as IL-6, TNF-a, IL-1β, IL-8 and other CXC chemokines are increased in the BALF and systemic circulation of COPD patients and also during exacerbations (41).

The increased level of circulating IL-6 was reported mostly in viral induced acute exacerbation of patients with COPD (42). Stable COPD patients have also increased circulating IL-6, which may be associated with skeletal muscle wasting (43), cachexia, inflammatory bowel diseases (IBD) (44). IL-6 is produced mainly from T lymphocytes, macrophage, smooth muscle cells, and partially in osteoblast. The major function of IL-6 is the induction of fever (45). In air space, IL-6, IL-8 and TNF-α are an important chemoattractant for the monocyte.

IL-1β is an important proinflammatory cytokine, which produced mainly in activated macrophage. IL-1β has various cellular function such as apoptosis and proliferation. IL-1β also affects the central nervous system (CNS). Cachexia patients have increased

level of IL-1β in circulating system (46). IL-1β in airway correlates the severity of COPD (47). IL-1β and TNF-α mainly activate NF-κB signaling (48).

TNF-α is also released mainly from activated macrophage and also from other cell type, such as neutrophil, eosinophils, NK cells and mast cells. The TNF-α is involved in local pulmonary and systemic inflammation (49).The main related signaling of TNF-α is NF-κB, MAPK pathways and death signaling. In mice the TNF-α level is associated with tissue remodeling in air space (50).

CXCL8 (IL-8) is released mainly in macrophage and other cell types, such as epithelial cells, endothelial cells and smooth muscle cells. IL-8 can be produced in all cells which contain the toll-like receptors. The major function of IL-8 is as chemoattractant of neutrophil. Circulating IL-8 concentration is associated with muscle wasting (51).

1.1.3.2 Inflammatory cells in COPD

Smoker has increased amount of pulmonary neutrophils, lymphocytes and macrophages (52). The circulating leukocytes show also abnormal function in COPD patients. The specific roles of each circulating cells in COPD patients remain to be determined.

Neutrophil is the major cell type in innate immunity. Neutrophil releases the neutrophil elastase, which is believed to play an important role in tissue remodeling in lung. The inhibitor of neutrophil elastase is α1-antitrypsin.α1-antitrypsin deficiency induces spontaneous emphysema. Neutrophil release also various proinflammatory cytokines, such as, IL-8, IFNγ, C3a, C5a, and LB4, the chemoattractant of neutrophil is IL-8.

COPD patients have not increased neutrophil in circulation and pulmonary air space. Some studies report that impaired lung function of COPD patients is related with neutrophil (53).

Phagocytosis is the major function of airway macrophages. Alveolar macrophages from smokers show decreased phagocytosis of bacteria, such as *Haemophilus influenzae* and *S. pneumoniae*, which colonize the lower airways of COPD patients. Monocyte derived macrophages from COPD patients showed a decreased phagocytic activity of *S.pneumoniae* and *H.influenzae* when compared to smokers or non-smokers. COPD patients showed a decreased phagocytic activity of *S.pneumoniae* and *H.influenzae* when compared to smokers or non-smokers. Some researchers suspect that it might be due to the defect of phagocytic machinery which is essential for the bacteria uptake. It may also because of the defect of innate immunity against bacteria (54). The reason of the defected innate immunity may be an intrinsic defect in monocytes, which can induce the defect of macrophages.

The MMP12 are mainly released from macrophage, which play an important role tissue remodeling in lung (55).

Some other studies indicate that the amount of B-lymphocytes in COPD patients is increased, whereas no change in total T-cell population is detected (56, 57). Peripheral T lymphocytes from COPD patients have an increased apoptosis level, which accompanies the increased expression level of TNF-α and TGF-b (58). One study reported that CD8+ cells are increased, which might induce the increased apoptosis (59). Normal smokers, but not COPD patients, show increased circulating CD4+ T-cells (60). In the lung of COPD patients the cytotoxic and phagocytic function of natural killer cells are decreased (61, 62).

1.1.4 Molecular and cellular mechanisms of COPD

Recently, the cellular and molecular mechanisms of COPD have been extensively explored. The accumulation of alveolar macrophages, neutrophils and T-lymphocytes in air space of COPD patients was confirmed. Various lipids, cytokines, chemokines, growth factors and other inflammatory mediators are released. The increased oxidative stress and the activation of several elastolytic enzymes, such as serine

proteases, cathepsins and matrix metalloproteinases, amplify the inflammation. Macrophages play an important role in COPD, which are activated, for example, by cigarette smoke. Macrophages are an important source of inflammatory mediators and provide a cellular mechanism which links smoking with inflammation in COPD. Some cellular mechanisms have been intensively studied. A short summary is shown in Figure 1-2. Macrophages release IL-8, GRO-a, leukotriene B4 (LTB4) in order to activate neutrophils via CXCR2 receptor. Alveolar destruction is possible caused by neutrophil secreted serine proteases, including neutrophil elastase (NE), proteinase-3, MMP-8 and MMP-9 (63). These serine proteases stimulate also mucus secretion. It was observed that neutrophil recruited to the airways and parenchyma and adhered to endothelial cells, E-selectin is upregulated from endothelial cells in COPD patients (64). Monocytes will be stimulated through macrophage chemotactic protein-1 (MCP-1), (growth-related oncoprotein alpha (GRO-a) and CXC chemokines via CXCR2 receptor. The chemokines interferon-γ inducible protein 10 (IP-10) are released from macrophages and activate $CD8^+$ cells by CXCR3 receptor.

Figure.1.2

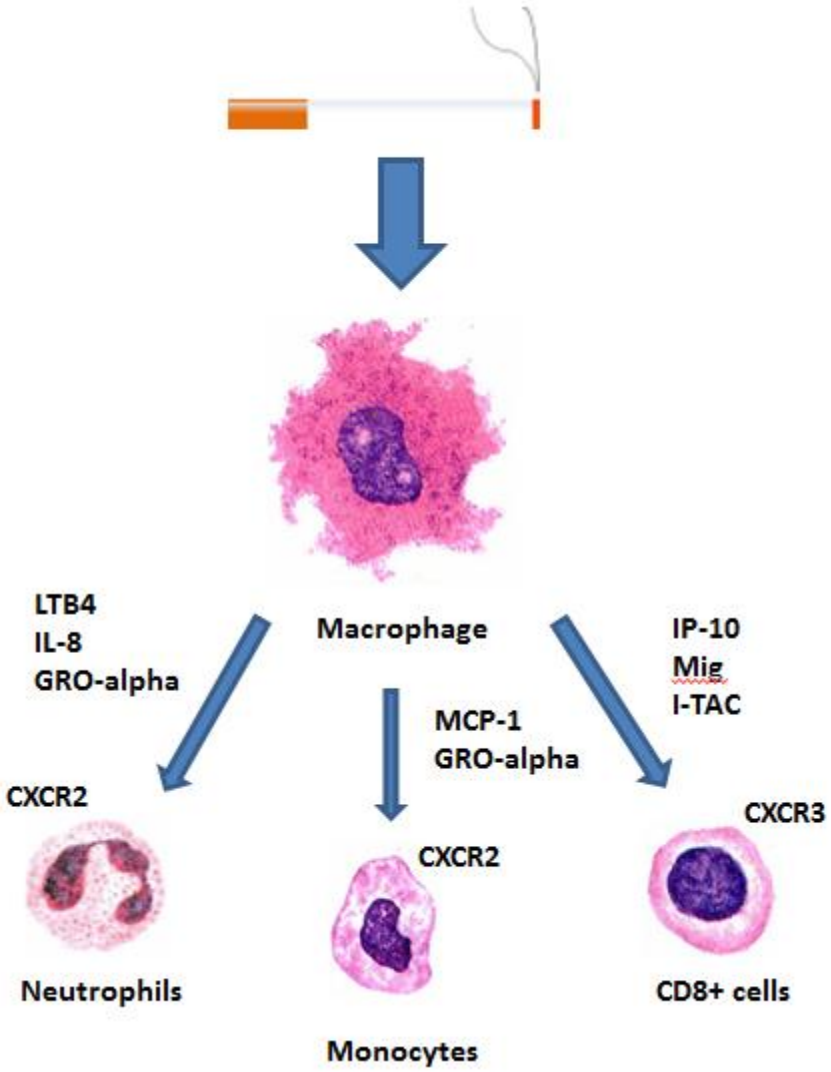


Figure.1.2. Systemic inflammation of chronic obstructive pulmonary disease (COPD) (4).
Cigarette smoke mainly induces inflammatory response in macrophage, neutrophil, and lymphocyte in lung. Macrophages release inflammatory cytokines such as TNF-α, IL-8, growth-related oncogene-α, MCP-1 and IP-10. Neutrophils are attracted by IL-8, GRO-α and leukotriene B4 (LTB4), monocytes attracted by macrophage chemotactic protein-1 (MCP-1), and $CD8^+$ lymphocytes attracted by interferon-c inducible protein (IP-10), monokine-induced interferon-c (Mig) and interferon-inducible T-cell a-chemoattractant (I-TAC). Proteolytic enzymes including matrix metalloproteinases (MMP) and cysteine proteinase such as cathepsins induced ECM breakdown.

Some other cells, such as eosinophils, dendritic cells and epithelial cells may be important sources of inflammatory mediators and proteases in COPD (65-67).

The alveolar macrophages from COPD patients release most of inflammatory mediators. The transcription factor NF-kB is activated in alveolar macrophage, and is believed to regulate the expression of inflammatory mediator from COPD patients, particularly during exacerbation (68, 69).

NF-kB signal pathway plays an important role in COPD. NF-kB acts as a central transcription factor by inflammatory, and also a key molecule involved in cell survival as well as a responsible molecule for apoptosis and oxidative stress (70-72).

Two NF-kB activation pathways are well investigated. Inhibitor of NF-kB (IkB) kinase (IKK)-IkB-p50/pREL-A (p65) is involved in classic NF-kB signal pathway. IKK is composed of two catalytic subunits, namely IKKα and IKKβ, and a regulatory subunit IKKγ. IKKβ can phosphorylate IkB-α and IkB-β induces its proteasomal degradation. NF-kB-inducing kinase (NIK)-IKK-p100/p65-p52/REL-B is the key molecular in NF-kB alternative pathway. The alternative pathway contains the upstream pertain kinase NIK, which phosphorylates IKK-α homodimers, resulting the phosphorylation of p100. P52 is degraded from p100 in proteasome. The degradation of IKK releases the NF-kB dimerization and leads to nuclear translocation of NF-kB to activate gene transcription (24).

IkBα proteosomal degradation and NF-kB DNA binding in the human lung has been well studied. Smoker with stable COPD has decreased level of IkBα than nonsmokers (73). Cigarette smoke induced NF-kB DNA activity in the mice lung *in vivo* (74). NF-kB was mainly activated in bronchial epithelial cells, $CD4^+$ T cells, $CD8^+$ T cells and airway macrophages in COPD patients. The activation of NF-kB was not observed in neutrophils (27). NF-kB regulates the expression of IL-1 and TNF-α in MonoMac6 with stimulation of aqueous cigarette smoke extract (CSE) (28, 75).

1.1.5 The role of MMPs in COPD

COPD is defined as airway and systemic inflammation and airway obstruction (4, 5). The protease/antiprotease imbalance, apoptosis/proliferation and oxidative stress induced cell dysfunction related tissue remodeling are not well understood. Most study of tissue remodeling in lung relate to the protease/antiprotease imbalance theory (76-78).

1.1.5.1 The MMP family

Matrix metalloproteinases (MMPs) are zinc binding enzymes for the degradation of extracellular matrix (ECM). They play an important role in various biological process, such as angiogenesis, tumor invasion, bone development and wound healing (79-82). They are also involved in regulation of inflammation. MMPs comprise at least 24 proteolytic enzymes in human. According to subsrate the MMPs can be divided into collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysin (MMP-7), macrophage metalloelastase (MMP-12), membrane-type MMPs (MMP-14, -15, -16, -17) and other MMPs (55). The major inhibitors of the MMPs are the nonspecific inhibitor α-2 macroglobulin and specific inhibitor, the TIMP family proteins (55).

Figure.1.3

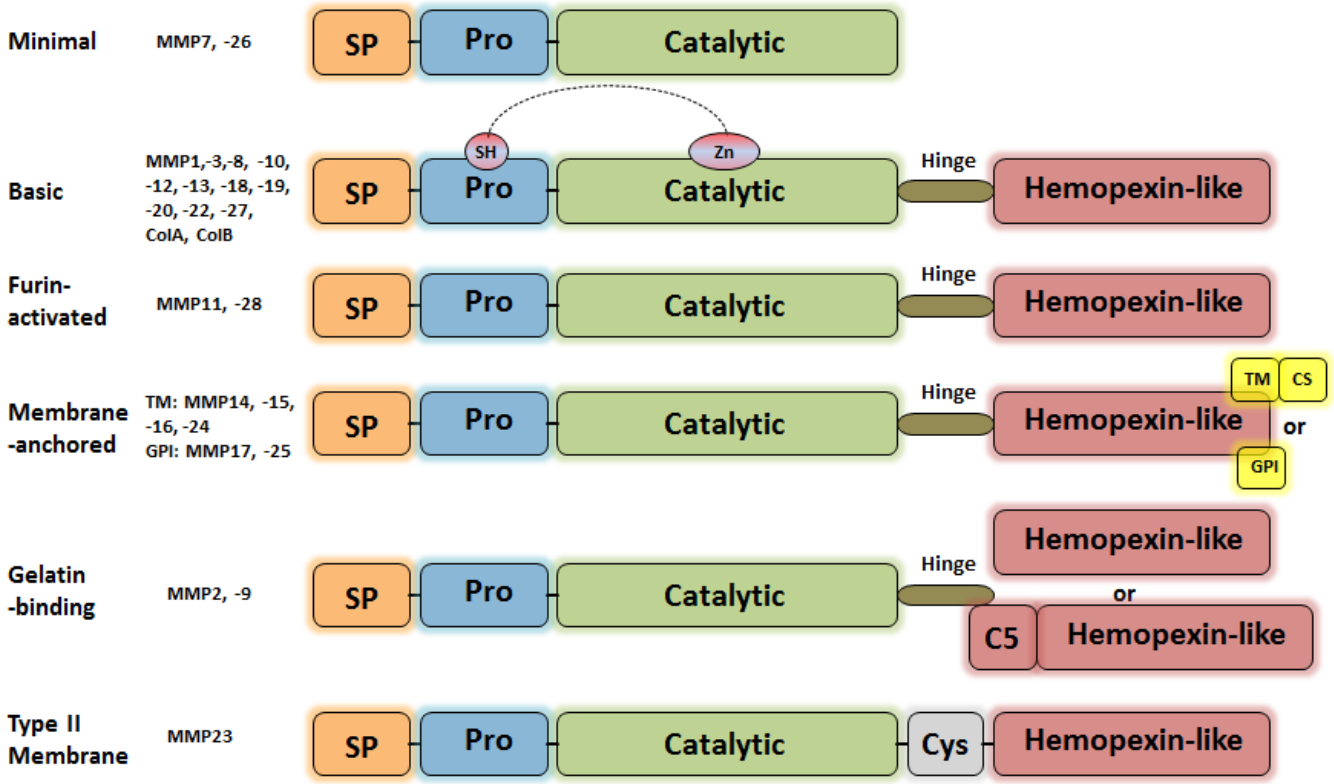


Figure.1.3. Domain structure of the MMP family.

The common domain structure of MMPs are the signal peptide (SP), propeptide (Pro), catalytic domain (Catalytic), hinge region, and mainly include the hemopexin like C-terminal domain. MMP-7 and -26 have the minimal domain structure contained only signal peptide, propeptide and catalytic domain. MMP-1, -3, -8, -10, -12, -13, -18, -19, -20, -22, -27, ColA and ColB have the basic domain with thiol group. MMP-11 and -28 are the furin-activated MMP. MMP-14, -15, -16, -24 are the membrane anchored MMP, and contain the transmanbrane domain, cytosolic domain and glycosylphosphatidylinositol (GPI) anchor domain. MMP-2 and -9 are the gelatinases. MMP-23 is the type II membrane MMP. C5, type-V-collagen-like domain; Col, collagen-like protein; Fn, fibronectin repeat; Cs, cytosolic; Cys, cysteine array; Fr, furin-cleavage site; SH, thiol group; Pro, pro-domain: SP, signal peptide; Zn, zinc (sourced from Parks WC, et al, 2004)

The MMPs contain a common domain structure, the pro-peptide, the catalytic domain, and the hemopexin-like domain. The pro-peptide domain is responsible for the activation of MMPS, since the remove of pro-peptide induces the activation of zymogen. The pro-peptide domain consists of 80 amino acids and with a conserved sequence PRCXXPD, which interacts with zinc ion. The catalytic domain contains the zinc ion, which bind three conserved histidine residues with conserved sequence HEXXHXXGXXH. The catalytic domain links the hemopexin-like domain through hinge region. The hinge region is about 75 amino acids. The hemopexin-like C-terminal domain is similar as the hemopexin. This domain is responsible for the substrate recognition and binding with TIMPs (83).

1.1.5.2 The role of MMPs in the degradation of ECM

As described before, MMPs cleavage the connective-tissue elements. The first MMP, which was discovered by Gross and Lapiere in 1962 (84), was found as an collagenase for neutral pH. The discovery of MMP function is based on identification of its physiological substrates (85). All MMPs have been shown to be able to degrade various protein components of the ECM (86). MMPs induced ECM degradation has been well studied in *in vitro* systems. The co-incubation of purified MMP and purified ECM protein under optimal conditions verified ECM cleavage function of MMPs. various studies indicate that some MMPs show effect on ECM proteins. Under situations, such as an inflamed tissue or tumor, the MMPs is involved in diverse biological process such as angiogenesis, phagocytosis and remodeling. Diverse cells produce a spectrum of MMPs, which have different localization and various functions. MMP secreted by macrophages would probably function different function than which secreted from epithelial cells. The same MMP may have several substrates in same tissue. Recent studies support that multiple functions of MMPs are not always refers to ECM degradation. The using of MMP inhibitors in clinical trials is a new beneficial and prospect MMP function.

1.1.5.3 The role of MMPs in inflammation

Inflammation is a complex biological response to harmful stimuli. The inflammatory process is a complicated physiological process. Inflammation is initiated mainly from leukocyte such as macrophages, dendritic cells, neutrophil and mast cells which already exist in tissues. The upregulation of MMPs is observed in many diseases that are associated with inflammation (87). A hallmark of the inflammatory process is the matrix proteolysis. The function of MMPs as matrix degrading proteinases contributes to their function in the inflammation. MMP inhibitors are used as anti-inflammatory drugs (88) and have been considered as a mediator to inhibit tissue destruction in some inflammatory diseases (89, 90). The exact function of MMPs in inflammatory diseases remains poorly understood. But some mouse studies indicate that MMPs, such as MMP2 and MMP3, are associated with joint destruction and may provide protection (91). MMP-12 is produced mostly by alveolar macrophages, smooth muscle cells, and epithelia in response to cigarette smoke. It is a key molecule in the recruitment of inflammatory cells. MMPs also contribute to some repair processes and leukocyte recruitment, which are central processes to inflammation.

1.1.5.4 The MMPs in COPD

Patients with COPD caused by cigarette smoke showed elevated MMP-2, -9, and -12 in alveolar macrophages (92). MMP-12 is related to acute and chronic pulmonary inflammatory diseases such as COPD and other smoking related injury. COPD patients have elevated MMP-12 than controls, ant it indicates that MMP-12 may play an important role in the pathogenesis of COPD and emphysema (93). MMP-12 knockout (KO) mice exposed to cigarette smoke showed reduced lung injury. MMP-12 KO mice exposed to long-term cigarette smoke showed also macrophage recruitment in lungs (94).

MMP-12 is one of the most important proteinases which are responsible for airway wall destruction. COPD patients produce MMP-12 protein in the sputum, BAL, bronchial biopsies (85, 95, 96), and also in lung tissue. MMP12 expression in mRNA and protein level was also observed in alveolar macrophage and lung tissue of animal COPD model using C57BL/6 mice (97). The expression of TIMP-1 and -2 was not observed (98).

MMP-12 acted in macrophage as TNF-α converting enzyme, induced the release of active TNF-α from macrophages and initiated a cascade of inflammatory responses. TNF-α mostly promotes the recruitment of neutrophils to the lung. These acute pulmonary inflammation were independent on NF-kB pathway. Some studies using MMP-12-null mice showed same rapid increase of NF-kB DNA binding activity as and wild-type mice (99).

Increasing evidence indicate that MMP-12 not only express in macrophages but also in other cells. MMP-12 was also released from dendritic cells, and the dendritic cells expressed increasing MMP-12 after stimulation with aqueous CSE (100).

1.1.6 Toll-like receptors

In 1985, the toll gene was firstly identified in Drosophila in Germany(101). Toll-like receptors (TLRs) belong to the pattern recognition receptor (PRR). To date, 13 principle members comprising TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13 descript (102-105) have been reported in human and mouse. The TLR11, TLR12, and TLR13 are not present in human (106). TLRs are believed to play an important role in both innate and adaptive immunity. The study in Drosophila outlined the role of TLRs in innate immunity, thus Drosophila has only innate immunity system. The presence of TLRs in dendric cells indicated the link of TLRs in both innate and adaptive immunity (107). Our understanding about the function of the TLRs is mostly focus on the recognition of the pathogen and the associated molecule of infectious diseases.

To date, the ligands of TLRs are well studied. TLR3, TLR7, TLR8 and TLR9 are activated by nucleic acid derived from viruses, and induced the release of inflammatory cytokines such as type I interferons. TLR3 is activated by double-stranded viral RNA (108). TLRs 7 and 8 are activated by single-stranded RNAs (109, 110). TLR9 is an important receptor for respiratory disease, thus it activated by bacterial DNA known as Cytosine–guanine Pairs DNA (CpG DNA) (111)and Herpes viruses (112).

TLR2 and TLR4 are activated by the bacterial membrane components known as bacterial peptidoglycans or lipopolysaccharides. TLR4 is also reported to be activated by pneumolysin derived from Streptococcus pneumonia (113). TLR2 is activated by lipoprotein derived from Gram-positive bacteria and mycobacteria (114, 115), and also activated by some lipoproteins from wide range of microorganisms such as Borrelia burgdorferi, Treponema pallidum, Aspergillus fumigatus and Mycoplasma fermentes. TLR2 and TLR4 are also reported to activated by some host prorein such as defensins (116), ROS (117), surfactant protein A (103) and fibrinogen (118).

1.1.7 Toll-like receptor signaling

The TLR signaling is well studied in recent papers (119-121). TLRs have a leucine-rich repeats in extracellular domain, which thought to be important for the ligand recognition. The TLRs have an about 200 amino acids region named TIR (Toll/IL-1R) domain, which shares the same sequence as IL-1R, and thought to be crucial for the signaling. The point mutation of TIR in C3H/HeJ mouse indicated the role in TLR4 signaling 51. The TIR domain serves as a negative regulation of many signaling pathway such as MAPKs (mitogen-activated protein kinases), PI3K (phosphoinositide 3-kinase) and NF-κB (nuclear factor κ B) (120). TLR3 and TLR4 regulate the release of type 1 IFNs by activation of the IRF3 (IFN regulatory factor 3), and induced further IFN-dependent gene expression (122).

The adapter protein of the TLRs included the MyD88, Mal (MyD88 adapter-like)/TIRAP (TIR-domain-containing adapter protein), TRIF/TICAM1 and TRAM/TICAM2 were also well studied recently. Normally accepted is the TLR signaling can be divided into two different signaling: the MyD88-dependent and TRIF-dependent or MyD88-independent pathway (113, 114, 123).

The MyD88-dependent TLR signaling is shared by all TLR but TLR3. The TLR ligand binds the TLR, then recruits the MyD88, and induced the accumulation of IRAK4, IRAK1 and IRAK2. IRAK kinases phosphorylate the TRAF6. The TRAF6 then phosphorylates IKK-β, and activates the NF-κB signaling in order to release the inflammatory cytokines (120).

TLR3 and TLR4 signaling are the typical TRIF-dependent or MyD88-independent pathway. dsRNA and LPS activate the TLR3 and TLR4, induced the recruitment of TRIF. TRIF induced the activation of TBK1 and RIPK1, which activated the RIF3, and the RIF3 then translocated to nucleus, and induced the activation of TAK1 and the transcription of NF-κB, which induced the inflammatory response. Only TLR4 uses all four known adaptor proteins (120).

1.1.8 The expression of TLRs in lung

The lung is the main place for the innate immunity. In lung, the airway epithelial cells, alveolar type II epithelial cells and the immunocells such as macrophage and dendric cell express TLRs (124-128). Subcellularly, TLRs are expressed not only on the cell surface but also within extracellular organism of cells. The expression of TLR1, TLR2 and TLR4 are located on the cell surface by positive staining of TLRs with specific antibodies. However, TLR3, TLR7, TLR8 and TLR9 shown to be located in intracellular compartments such as endosomes (128-131). Macrophages and dendritic

cells can engulf the microbial component by phagocytosis in case of infection. TLRs involved also in phagocytosis. The target deletion of TLR2/TLR4 or MyD88 induced abnormal phagocytosis of bacteria such as Escherichia coli, Salmonella typhimurium and Staphylococcus aureus by abnormal phagosome maturation (132). The mechanism of TLR9 expression can be hypothesized that in case of phagocytosis of microbial organism by macrophage or dendric cells the degradation products such as CpGDNA from bacteria released from phagosomes or endosomes and then induced the expression or recruitment of TLR9 in phagosomes or endosomes. Of viral infection, the virus are degradated in the endosomal compartment results the expression of dsRNA and ssRNA, which known as TLR ligands, then induced the recruitment of on cell surface expressed TLRs like TLR2 (133).

1.1.9 The Toll-like receptor in response of CS

Bacterial components such as lipopeptides of NTHi, LPS of Gram-negative bacteria and flagellin of Pseudomonas aeruginosa are able to activate the TLRs of alveolar macrophage such as TLR2, TLR4 and TLR5 (114, 134-136). The activation of TLRs activates intracellular signaling pathway such as mitogen-activated protein kinases (MAPK) and NF-κB. COPD patients in stable state or in acute exacerbation have bacterial or viral colonized in airway (137-139). Therefore the activation of TLRs in COPD patients in stable or exacerbation state may always exist.

There are studies demonstrate that the release of proinflammatory cytokines such as IL-6 and TNF-α in smoker's alveolar macrophage after stimulation with LPS were significantly decreased compare to controls (140, 141). The reduced inflammatory response of smoker's alveolar macrophage is associated with negative regulation of NF-κB and p38 MAPK pathway. This effect was also confirmed in vitro study. CSE suppressed the gene expression of proinflammatory cytokines in smoker's alveolar macrophage except IL-8, which known as neutrophil chemoattractant (142-144). Furthermore, there are also studies reported that alveolar macrophage from COPD

patients has impaired phagocytosis (145, 146). Accumulated studies indicated that TLRs and p38 MAPK may play a key role in the innate immunity in COPD.

1.2 COPD exacerbation

Acute exacerbations of COPD (AECOPDs) are common in the COPD patient. For example, in the United Kingdom, each COPD patient has a frequency of 0.8–2.5 AECOPD per year (147). Severe AECOPDs particularly require hospitalization. In the UK, 34% of COPD patients of emergency hospital admission were readmitted and 14% patients were dead within 3 month of discharge (148). Furthermore, AECOPDs, resulting a significantly higher health care costs, service and a burden to society (149).

1.2.1 Risk factors of COPD Exacerbation

The main risk factors in acute exacerbations of COPD are thought to be respiratory infection including viral and bacterial infections, as well as air pollutants.

1.2.1.1 Bacterial Infection in COPD exacerbations

Non-typeable Haemophilus influenza (*H.i*), *Moraxella catarrhalis* (gram-negative bacteria) and *Streptococcus pneumoniae* (gram-positive bacteria) are the three most common isolated bacterial pathogens from respiratory tract infections in COPD patients. Some atypical bacteria, especially *Chlamydia pneumonia*, have also been

detected to be able to cause COPD exacerbations (150). 25 to 50% of COPD patients are colonized by bacteria in the lower airways, especially *Non-typeable Haemophilus influenzae, Streptococcus pneumonia*, and *Moraxella catarrhalis (151).* This colonization plays an important role in the severity of COPD and cigarette smoking (152, 153). The bacterium in the lower airways of stable COPD patients stimulates host defense mechanisms that induce increased airway inflammation (154). These bacteria are different in stable COPD patients, and the patients with high exacerbation frequency of bacterial infection exhibit faster impaired lung function (155). A recent study showed that the sputum IL-8 levels and bacterial load are related with FEV1 in COPD patients (156). The exacerbation frequency in COPD patients is dominantly increased with an increased airway bacterial load (157). Antibiotic therapy is useful for the clearance of bacterial load, the clearance of bacteria from the sputum and decreased airway inflammation in airway (158). Taken together, the bacterial infection in the airway during exacerbations of COPD patients led to increased airway inflammation and decreased lung function.

1.2.1.2 Viral Infection in COPD exacerbations

COPD patients infected with virulent strains, for example *H. influenza*, often show severe airway inflammation than bacterial infection. It suggested that virulent strains may be more dangerous than colonizing strains (150, 159). Almost 50% of COPD exacerbations are associated with viral infections, the major viral infection are due to rhinovirus (160). Rhinoviruses are the most frequently identified virus during exacerbations in COPD patients. Rhinovirus can be recovered from sputum more frequently than from nasal aspirates at exacerbations. This study suggested that wild-type rhinovirus is able to infect lower airways and contribute to inflammatory changes at exacerbation. COPD exacerbations associated with the presence of rhinovirus induced higher airway IL-6 levels. It indicated that viruses induced more

severe airway inflammation during COPD exacerbation (63). Respiratory viruses result normally longer and severer air way inflammation than bacteria and have a major impact on health care burden (42, 160). The systemic inflammatory markers

were also increased in serum from viral infected COPD patients (63). The major receptor of rhinoviruses is intercellular adhesion molecule (ICAM)-1. Increased epithelial expression of ICAM-1 indicates the infection of rhinovirus. Some studies indicated the increased ICAM-1 in COPD exacerbation patients (161). Additionally, rhinovirus stimulate mucus secretion from airway epithelium (162).

1.2.1.3 Air Pollution in COPD exacerbations

Epidemiologic studies indicate that air pollution can induce COPD exacerbations (163). Outdoor and indoor air pollution was mostly produced by biomass fuel and volcanic eruption (164). The outdoor pollution has been reported to affect the lung development in teenage of 10-18 years (165). The epidemiologic studies in the US

and Europa significantly support the correlation between outdoor pollution and hospital admission of COPD patients (166). The increased level of sulfur dioxide (SO2), nitrogen dioxide (NO2) and O_3 is strong related to increased mortality of COPD patients (167-169). Some clinical studies indicated that air pollution may account for 6 to 9% of hospitalization in COPD patients (168). In total, air pollution may lead to exacerbations of COPD.

1.2.2 The airway inflammation during COPD exacerbation

Exacerbations are highly related with airway inflammation. An increased airway inflammation is strong associated with the pathogenesis of exacerbations. Airway inflammation could result in airway wall edema and increased mucus secretion. Increased mucus secretion thought to be an important phenotype of acute episodes of COPD. The increased sputum secretion in the airways would reduce the airway diameter and this effect would be enhanced with increased viscosity of the sputum.

On the cellular level, the inflammatory phenotype of the airways is characterized by increasing infiltration of macrophages, neutrophils and $CD8^+$ T lymphocytes in the airway wall and in air space (170). At exacerbations the cellular pattern will be changed. Eosinophils and neutrophils change to be the major component of the inflammatory response (170, 171). Recently, T cell-mediated immunity has been detected in sputum samples at exacerbations. CD4:CD8 ratio is decreased and observed during severe episodes of hospitalization (172). These results indicate that T lymphocyte subpopulations may be correlated with the development of COPD exacerbations.

Currently, soluble mediators are used as indirect markers of airway inflammation. Endothelin-1 is one of the marker of airway inflammation, which is expressed from the bronchial and pulmonary epithelium and alveolar macrophage, may result mucus secretion (173).

1.3 Aim of this work

The aim of this work is to establish a new mouse model for COPD development, in order to investigate the interaction of CS and *H.i* and to preliminary study the cellular and molecular mechanisms of the pathogenesis of COPD exacerbation, and provide the new approach to the treatment of COPD exacerbation.

2. Materials and Methods

2.1 Materials

2.1.1 Laboratory apparatus

Autoclave	Holzner Medizintechnik GmbH, Nußloch Systec GmbH Labor Systemtechnik, Wettenberg
Analytical balance	Kern & Sohn GmbH, Balingen
Agarose gel-apparatus	FB-SB-1316 Electrophoresis Systems FisherBiotech, Wembley, Australien
BD FACSCanto™ II	BD, Heidelberg, Germany
Centrifuges	Centrifuge 5415R, Eppendorf AG (Hamburg) neoLab-mini-centrifuge, neoLab GmbH, Heidelberg Rotanta/TR, Hettich GmbH & Co.KG, Tuttlingen Beckman Coulter GmbH, Krefeld
Fluorescence plate reader	Tecan Ultra384 Tecan Austria GmbH
Ice machine	Scotsman Frimont AF20, HIBU Eismaschinen GmbH & Co. KG, Sprockhövel
Incubators	Nu-5100, Integra Biosciences GmbH, Fernwald Heraeus* Function Line Microbiological Incubators, Heraeus Holding GmbH, Hanau
Microscope	BX51 Olympus, Olympus GmbH, Hamburg
Microwave	Sharp Electronics GmbH, Hamburg

NanoDrop	Thermo Fisher Scientific GmbH, Schwerte
pH-meter	20-SevenEasy^{TM}pH, Mettler-Toledo GmbH, Giessen
Photometer	UItrospec 2100 pro, Amersham Pharmacia, Uppsala, Sweden
Power-Supply	Consort EV231, Consort, Turnhout, Belgien Thermo Electron Corporation, Waltham, USA
Real Time PCR Machine	MyCycler, BioRad
SDS PAGE apparatus	Mini PROTEAN 3 Cell, Bio-Rad Laboratories GmbH, München
Speed vac	Eppendorf concentrator 5301
Water bath	W19, Haake
Western Blot apparatus	Biorad

2.1.2 Chemicals and reagents

β-Mercaptoethanol	Roth, Karlsruhe, Germany
Polyacrylamide (37.5:1)	Roth, Karlsruhe, Germany
APS	Sigma, Steinheim, Germany
BSA	Calbiochem, Darmstadt, Germany
Citric acid monohydrate	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany

Glycerin	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
H_2SO_4	Roth, Karlsruhe, Germany
HCl	Roth, Karlsruhe, Germany
Methanol	Center of chemical storage, University of Saarland
NaCl	Roth, Karlsruhe, Germany
NaH_2PO_4. H_2O	Merck, Darmstadt, Germany
Na_2HPO_4	Fluka, Buchs (CH), Germany
NaOH	AppliChem, Darmstadt, Germany
Paraformaldehyde	Roth, Karlsruhe, Germany
Protein Standard (SeeBlue® Plus2)	Invitrogen, Carlsbad, CA, USA
Skim milk	Roth, Karlsruhe, Germany
SDS	Roth, Karlsruhe, Germany
TEMED	Sigma, Steinheim, Germany
Tris base	Sigma, St. Louis, MO, USA
Tween® 20	AppliChem, Darmstadt, Germany
TRIzol® Reagent	Invitrogen, Carlsbad, CA, USA

2.1.3 Consumable laboratory supplies

All cell culture Materials	Greiner bio-one (Frickenhausen)
Cannula 23/25G	Microlance 3 Needles, Becton Dickinson GmbH, Heidelberg
Cuvette	10x4x45 mm, Sarstedt AG & Co.KG, Nümbrecht
Membrane (Western Blot)	Immobilon-p Transfer Membrane PVDF Pore Size 0,45µl, Millipore
PCR-reaction tubes	Biozym Scientific GmbH, Hess. Oldendorf
1.5 ml and 2.0 ml reaction tubes	SafeSeal Reagiergefäße, Sarstedt AG & Co., Nümbrecht
15 ml and 50 ml sterile tubes	Greiner Bio-One, Frickenhausen

2.1.4 Antibodies

anti p65 (C20)	Rabbit, polyclonal, IgG, Santa Cruz Biotechnology, Heidelberg
anti actin (H-196)	Rabbit, polyclonal, IgG, Santa Cruz Biotechnology, Heidelberg
anti MMP12(EP1261Y)	Rabbit, monoclonal, IgG, Abcam plc, Cambridge
anti CD3	Rabbit, polyclonal, IgG, Abcam plc, Cambridge
anti CD22	Rabbit, polyclonal, IgG, Abcam plc, Cambridge
anti CD68	Rabbit, polyclonal, IgG, Abcam plc, Cambridge

2.1.5 Bacteria

non-typeable Haemophilus influenza	kindly obtained from clinical microbiology of University of Saarland

2.1.6 Primers

The following PCR primers (Metabion, Martinsried, Germany) were used in this work

Name	Sense primer	Antisense primer	T*m*
mouse MMP2	5'-TCTTCTTCAAGGACCGGTTTATTTGG-3'	5'-TGAAGAAGTAGCTATGACCACCACCCT-3'	60 ℃
mouse MMP9	5'-CTTCTGCCCTACCCGAGTGGAC-3'	5'-AGGCTTAGAGCCACGACCATACAGA-3'	60 ℃
mouse MMP12	5'-TGCATTTGGAGCTCACGGAGACTT-3'	5'-GAAGCTTCCACCAGAAGAACCAGTCTT-3'	60 ℃
mouse TIMP1	5'-TCCCCAGAAATCAACGAGAC-3'	5'-CACAGACTTCAGCGAATGGA-3'	60 ℃
mouse TIMP2	5'-CACAGACTTCAGCGAATG-3'	5'-CTTGGGAAGCTTGAGAGTGG-3'	60 ℃
mouse Neutrophil elastase	5'-GGCTTTGACCCATCACAACT-3'	5'-CGGCACATGTTAGTCACCAC-3'	60 ℃
mouse β-actin	5'-AGCCTCGCCTTTGCCGA-3'	5'-CTGGTGCCTGGGGCG-3'	60 ℃
human GAPDH	5'-AGGTCGGAGTCAACGGATTTGGT-3'	5'-GTGCAGGAGGCATTGCTGATGAT-3'	60 ℃
human MMP12	5'-ACACATTTCGCCTCTCTGCT-3'	5'-AAGCAGCTTCAATGCCAGAT-3'	60 ℃

2.2 Methods

2.2.1 Animal Studies

All animal experiments in this study were performed in accordance with and approved by the "Landesamt für Soziales, Gesundheit und Verbraucherschutz" of the State of Saarland following the national guidelines for animal treatment. The animals were housed under pathogen free conditions in the animal facility of the institute for experimental surgery at the Saarland University Hospital, Homburg. The 8 to 10 week old female wild type C57BL/6N mice used for this study were purchased from Charles River (Sulzbach, Germany).

2.2.2 Cigarette smoke extract (CSE) preparation

84mm filtered research cigarettes grade 3R4F (36.8 mg tar and 2.45 mg nicotine) were bought from university of Kentucky in United States. Cigarette was burned with peristaltic pump into 10 ml DMEM medium in 2 min. The CSE extract was sterilizly filtered with a 0.2 µm pore acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI) for removing bacteria and other large particles in medium. This CSE extract was considered as 100% concentration. This CSE extract was aliquoted in 500 µl stock and frozen immediately in -80 °C.

2.2.3 *H.i*-lysate preparation

Clinical isolated *H.i* strain was kindly received from the Institute of Pathology of the University hospital of the Saarland, Homburg and was stored as frozen stock ($1x10^7$

colony-forming units/ml). *H.i* in stock was confirmed by 16S rDNA PCR analysis. *H.i* was grown on chocolate agar with IsoVitaleX at 300 µl per 10-cm plate (BD, Heidelberg, Germany) for 24 hours at 37 ℃, 5% CO_2. After this, the *H.i* is harvest and incubated for 24 hours in 700mL brain-heart infusion broth (BD, Heidelberg, Germany) containing 3.5 mg/ml NAD (Sigma-Aldrich, St. Louis, MO) and 5% Fildes enrichment (BD, Heidelberg, Germany). The culture was centrifuged at 2,500g for 15 minutes at 4 ℃, washed with ice cool PBS for 1 time then resuspended in 20 ml PBS, and heat inactivated at 70 ℃ with shaker for 45min, then sonicated for 3 times of 30 seconds in a 50ml plastic tube. Protein concentration was adjusted to 2.5 mg/ml in PBS with bicinchoninic assay (Pierce, Rockford, IL), and the *H.i* lysate was frozen in 7 ml aliquots at -80 ℃. The inactivation of *H.i* was also confirmed by grown the *H.i* lysate on chocolate agar with IsoVitaleX for 24h at 37 ℃ in 5% CO_2.

2.2.4 Cigarette smoke exposure

Male C57/BL6N mice were exposed to smoke with 3R4F reference cigarettes (College of Agriculture, Reference Cigarette Program, University of Kentucky, Lexington, USA) in a TE-10 smoking machine (Teague Enterprises, Woodland, California, USA) for 3 hours and 5 days per week. The total suspended particles (TSP) concentration was 120 mg/m^3. The TSP was measured by gravimetric methods as advised by the manufacturer and controlled in regular intervals. The total duration of the smoke exposure was from 2 weeks up to 6 months.

2.2.5 *H.i*-lysate challenge

The mice were placed in a plexi glass box connected to a Pari MASTER® nebulizer

(Pari GmbH, Starnberg, Germany) and exposed to the inactivated bacterial lysate for 40 minutes either daily (high dose *H.i.*, hd-*H.i.*) or once per week (low dose *H.i.*, ld-*H.i.*) at a concentration of 2,5 mg of total bacterial protein. The protein concentration was measured by the Pierce BCA-protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.2.6 Animal preparation

At the endpoints of the experiments the mice were anesthetized by an i.p. injection of a ketamine and xylazin mixture. The chest was opened and blood was drawn from the heart for the preparation of serum and the analysis of inflammatory cytokines. The trachea was exposed and cannulated with an 18G cannula. Bronchoalveolar lavage (BAL) was performed by 3 times instillation of 1 ml sterile filtered phosphate buffered saline (PBS) including protease inhibitors (Complete protease inhibitor cocktail, Roche Applied Science, Mannheim, Germany). The lung was removed and the right lobes were homogenized in protein extraction buffer (PBS with protease inhibitor cocktail) and immediately frozen in liquid nitrogen. The left lobe was used for RNA extraction by Trizol.

2.2.7 Lung function

Lung function was performed with a FlexiVent system (EMKA Technologies, Paris, France). The mice were anesthetized by a mixture of ketamine and xylazine. The trachea was exposed and cannulated using the cannula included with the FlexiVent system. Mice were placed on a plastic plate as described previously (174-176), and stabilized in the cages for 5 minutes. In the single compartment model the lung is

recognized as on compartment and Respiratory system resistance (Rrs), Respiratory system elastance (Ers), and Respiratory system compliance (Crs) are calculated based upon SnapShot-150. The maneuver PVs-P calculates Vital capacity (A), Quasi-static compliance (Cst), and Quasi-static elastance (Est) based on the Salazar Knowles equations. In the constant phase model the parameters Central airway resistance (Rn), Tissue damping (G), Tissue Elastance (H), and Inertance (I) are calculated based upon the QuickPrim3 maneuver. All maneuvers are repeated at least in triplicate.

2.2.8 Stereological quantification of emphysema

After lung function measurements the lung was removed completely and fixed with fresh 4% formalin in PBS-buffer of constant hydrostatic pressure of 20 cm H_2O for 20 minutes. Then the trachea was ligated in order to contain constant intrapulmonary pressure for the embedding of lung tissue. The whole lung was through in to 25 ml plastic tube with same formalin-PBS solution for 72 h. The lung volume was determined by fluid displacement. Then the fixed lung was embedded in paraffin and cut to slides of 1µm thickness for stereological and immunohistochemistry analysis. The mean chord length (mean linear intercept, L_m) was measured by random sampling using the Visiopharm Integrator System (Visiopharm, Hoersholm, Denmark) on an Olympus BX51 microscope (177).

2.2.9 Real-time PCR

The mRNA expression was measured using quantitative real-time PCR. We isolated the total DNA using 3 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for the homogenization of 1/4 lung-tissue. We used 1.5 μg RNA for the reverse transcription with First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). Total 25-μL SYBR-Green-PCR mixed with 1:9 diluted cDNA as template. The SensiMix SYBR & Fluorescein Kit (Bioline, Luckenwalde, Germany) was used for the RT-PCR. iCycler machine was bought from Bio-Rad in Munich, Germany. The program of RT-PCR is listed below.

Step	Cycle	Temperature	Time
polymerase activation	1	95° C	15 min
denaturation anneal extension	45	95° C Tm 72° C	30 sec 30 sec 45 sec
final extension	1	72° C	3 min
denaturation	1	95° C	1 min
start-temperature	1	55° C	1 min
melt temperature	80	55° C (+0.5° C /cycle)	10 sec

We analyzed the results using ΔΔCT (cycle threshold) method. GAPDH levels was used for the normalization of each sample.

2.2.10 Immunohistochemical analyses

The lungs were prepared as described before and incubated at 60 ℃ overnight. After

deparaffinization sections were heated at 100 ℃ for 20 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. 3% (v/v) H_2O_2 was used for blocking of the unspecific tissue peroxidases and followed by incubation in blocking solution (2% BSA in TBS-T) for 60 min to prevent nonspecific binding. Slides were incubated with primary antibodies at 4 ℃ overnight. Secondary antibody incubation and staining were performed using the EnVision®+ System–HRP (AEC) kit (Dako, Carpinteria, CA, USA) according to manufacturer's recommendations. The sections were counterstained with hematoxylin. Rabbit anti-MMP12 (1:1000) (for mouse, 1:1000, ABCAM, Cambridge, UK) antibody was used in this work: rabbit anti-MMP12 (for mouse, 1:1000, Abcam, Cambridge, UK), mouse anti-MMP12 (1:1000) (for mouse, 1:1000, ABCAM, Cambridge, UK).

2.2.11 Heidenhain ś Azan stain

Samples were prepared as described before and incubated at 60 ℃ overnight. After deparaffinization sections were rinsed in distilled water for 5 min, and then Incubated in warm azocarmine solution for 30 min at 57 ℃. After rinsing for 5 min in distilled water the slides were incubated in 0.1 % alcoholic aniline solution for 3 min, 1% acetic acid alcohol for 1 min and 5% phosphotungstic acid solution for 20 min under visual inspection. After rinsing briefly in distilled water the slides were incubated in aniline blue-Orange-G acetic acid solution for 20 min. Then the slides were rinsed briefly in distilled water. Slides were differentiated by a few short immersions in 96% ethanol. Then the slides were dehydrated twice in 96% ethanol, cleared in acetic acid-n-butylester EBE® (Roth, Karlsruhe, Germany), and finally mounted using Promountes® RCM2000 (Medite, Burgdorf, Germany).

2.2.12 SDS-PAGE and Western blot

2.2.12.1 SDS-PAGE

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) is a useful method for the separation of different protein according to molecular size.

Running conditions: 10 min 100 V const. and then about 1h 30 min, 150 V const. The glass plates were washed with water and then dried with Kleenex. It was assembled in the apparatus. The buffers and reagents for separating gel (12%), except APS and TEMED were pipetted together. APS and TEMED were added into the solution and it was mixed well by swirling gently. This solution was directly pipetted into space between glass plates. Immediately it was overlaid with isopropanol and polymerised more than half hour at RT. Then the overlay was pour away, the separating gel was washed with water shortly. The prepared stacking gel was pipetted on the separating gel until the space was filled completely. A comb was placed in the stacking gel. After about 10 min polymerisation the comb was taken away. The gel was assembled in the gel apparatus and was immersed in the 1x running buffer.

2.2.12.2 Western Blot analysis

The electro blotting (150 mA overnight or 300 mA for 4 h at 4℃) was performed in order to blot the protein to nitrocellulose membranes. After blotting the nitrocellulose membrane was blocked with with 5% milk in TBST for 30 min. After this, the membrane was incubated with diluted primary antibody at 4℃ over night. After the primary antibody incubation the membrane was wasched 3 time 15 min. The detection using Kodak scientific imaging films (Eastman Kodak, Rochester, N.Y., USA) was performed by enhanced chemiluminescence kit (Cell Signaling Technology, Danvers,

MA, USA).

2.2.13 Enzyme-linked immunoabsorbent assay

To determine the concentration of cytokines in Lavage and Lung-homogenate the Enzyme-linked immunoabsorbent assay was performed in this work.

The 96-well PVC micro titer plate was coated with the capture antibody at a concentration of 1-10 µg/ml in carbonate/bicarbonate buffer (pH9.6) over night at 4 ℃. After removing the coating solution the plates were washed 3 times by filling the wells with 200 µl PBS. And then the plates were blocked with 300 µl / well blocking reagent for 2-3h at 37 ℃. The blocking reagent was removed by washing 3 times with PBS-T. The samples were normalized to same protein concentration by BCA-Assay and were added and incubated over night at 4 ℃. After washing for 3 times with PBS-T the detection antibody at a concentration of 1-10 µg/ml were added and incubated for 2h at room temperature. After washing for 3 times 200 µl/well of Streptavidin-POD conjugate solution were added and incubated at RT for 5-30 min. 100 µl 3N H_2SO_4 was added to each well and incubated for 1 min on the shaker. The absorbance was measured using ELISA reader at 450nm.

2.2.14 Cytometric bead assay (CBA)

Cytometric bead assay (CBA) was performed as descript from BD science for detecting the concentration of cytokines of bronchial alveolar lavage fluid (BALF) and Lung-homogenate. We added respectively 50 µl beads, 50 µl mouse Th1/Th2 cytokine standard and 50 µl sample into the assay tubes. The mixture was incubated at room temperature for 2 h in a dark room. After this, we added 1 ml wash buffer into

the tubes. After centrifugation at 200g for 5 min, we removed the supernatant and resuspended the pellet with 300 μl wash buffer. All samples were measured on flow cytometer. Results were calculated with FCAP Array software.

2.2.15 Statistical analysis

We analyzed the data using GraphPrims 5 (GraphPad Software Inc., La Jolla, CA, USA) software. The two group was comprised using two side t test. P values less than 0.05 was considered as significance.

3. Results

It is the goal of this work is to characterize and develop the COPD and AECOPD mouse model in response of CS and *H.i*, additional the pathological mechanisms of COPD and AECOPD will be investigated. As previously described *H.i* colonized the lower airways of patients with chronic obstructive pulmonary disease. The contribution of *H.i* in CS induced COPD progression is largely unknown. The basic findings of this work in mice provide a new aspect of role of *H.i* in progression of COPD and AECOPD in human.

In order to determine the role of *H.i* in CS induced inflammation wild type C57/BL6 mice were exposed to CS for different time points from 2 weeks up to 6 months and additionally were challenged with heat inactivated *H.i* lysate with different doses at a concentration of 2.5 mg/ml of total protein for 40 minus per day and from 1 time per week to 5 times per week. An increased inflammation, significant changes in lung function and pulmonary remodeling were confirmed in this study.

3.1 CS and *H.i* stimulation did not induce significant loss of bodyweight after 6 month.

The wild type C57/BL6 mice were exposed to CS and *H.i* stimulation from 2 weeks up to 6 months. The body weight of the mice was recorded on every Wednesday before stimulation with CS and *H.i*. There was no loss of body weights observed in all 6 groups at the end of experiment. The health status of mice was also recorded during the experiment. Hair loss and shortness of breath was observed in group hd-*H.i* and CS+hd-*H.i*. The shortness of breath of mice in group CS+hd-*H.i* occurred more frequently than in group hd-*H.i*, whereas no significant difference of the hair loss of mice observed between two groups.

Figure.3.1

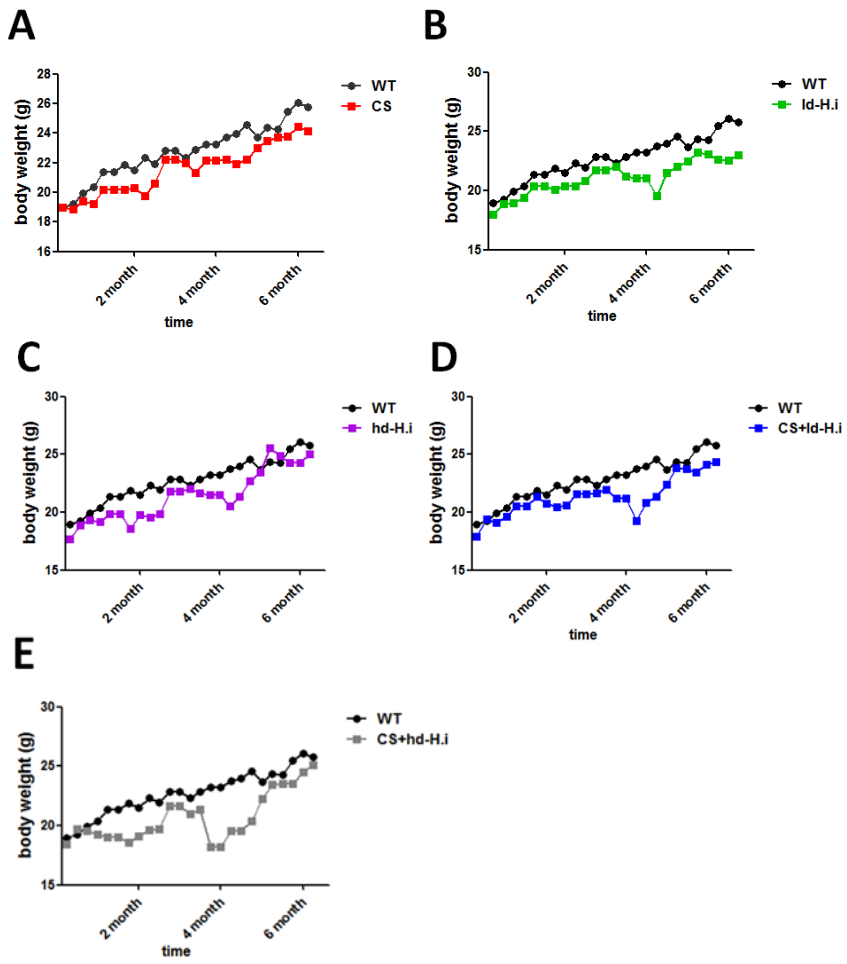


Figure.3.1. Effect of cigarette smoke and *Haemophilus influenzae* exposure on body weight in mice. The body weight of every mouse was recorded at every Wednesday before the stimulation with CS and H.i. The time point of every Wednesday was considered to be the most suitable for the record of body weight, and it thought to be avoided for the influence of the simulation with CS and *H.i*. **A**. CS group compared with air controlled group. **B**. ld-H.i compared with air controlled group. **C**. hd-H.i compared with air controlled group. **D**. CS+ ld-H.i compared with air controlled group. **E**. CS+ hd-H.i compared with air controlled group.

3.2 CS and H.i induced influx of leukocytes in lung.

We analyzed the influx and composition of leukocytes into the lung. Neutrophils and macrophages are an important source of tissue remodeling enzymes and cytokines that in turn are responsible for the regulation of inflammation. The absolute number and differential composition of leukocytes in the BALF was analyzed after 2 weeks, 3 month and 6 month.

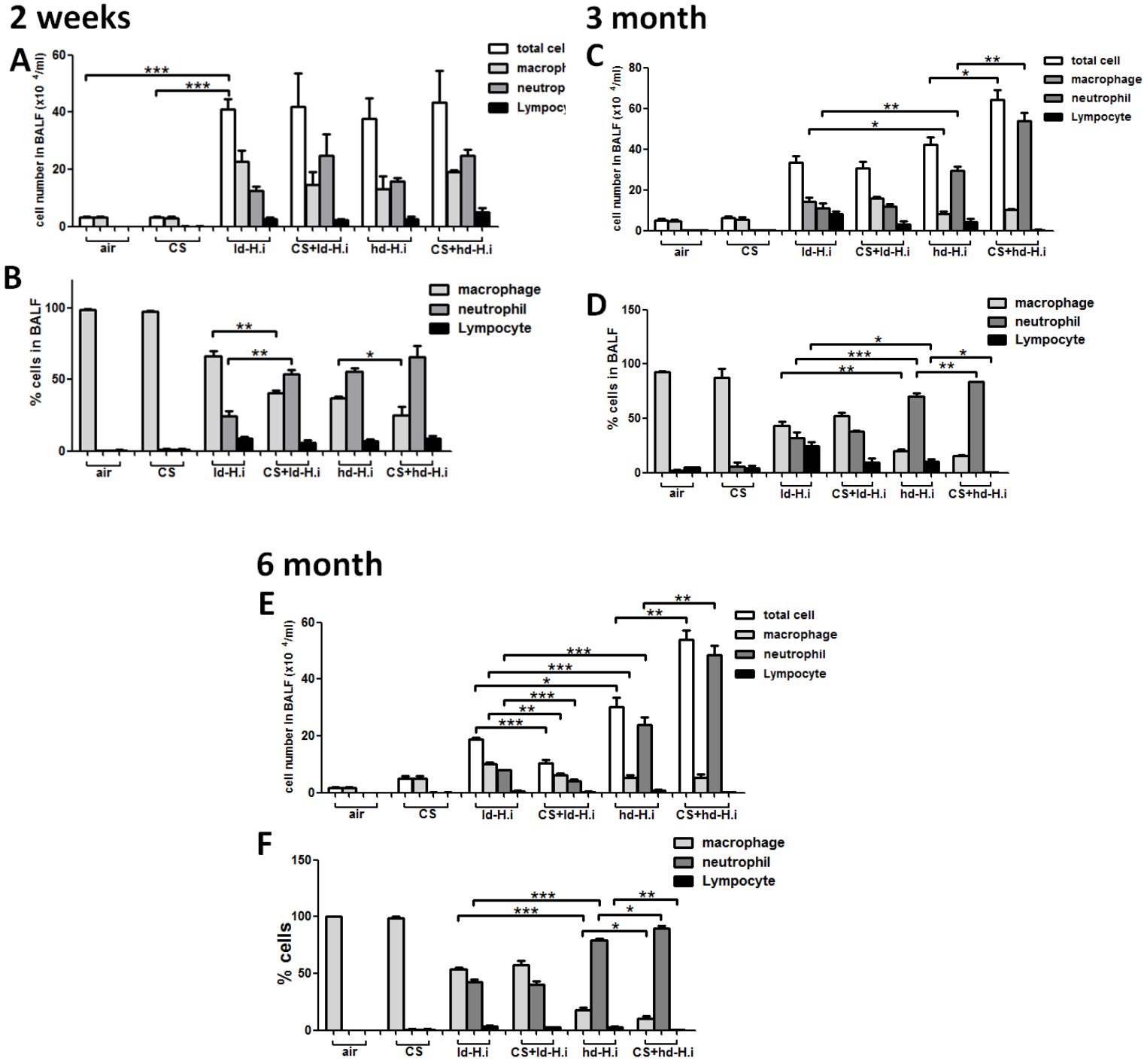
CS stimulation at an average concentration of 120 ± 5 mg/m^3 of total suspended particles (TSP) for 3 hours per day did not induce a marked cellular influx into pulmonary airspaces compared with air controlled mice in 2 weeks, 3 month and 6 month.

At 2 weeks, the stimulation with ld-*H.i.* significantly up regulated the number of total cells in the BALF and induced a macrophage dominant inflammation in the lung. The total cells in BALF increased to 41 ± 2.3 x 10^4 cells/ml in BALF, and the percentage of neutrophils increased from 0.33% at baseline to 24.56% after ld-*H.i* exposure in 2 weeks (Fig. 3.1A).. The *H.i* stimulation in CS exposed mice changed the phenotype of pulmonary inflammation from a macrophage based inflammation to a neutrophilic inflammation. The BALF neutrophil percentage of ld-*H.i* in CS exposed mice was significantly higher and changed from 24.56% of only *H.i* exposed mice to 53.78% (Fig. 3.2B) and the relative number of macrophages was significantly lower. hd-*H.i* induced a neutrophil dominant inflammation in BALF compared to ld-H.i stimulation. hd-*H.i* in CS-exposed mice showed also lesser acute increases of neutrophil percentage than only hd-*H.i* (Fig. 3.2B). H&E staining confirmed the influx of immunocells in the pulmonary airspace (Fig. 3.2C). CS stimulation did not induce an influx of leukocytes in the lung in this model. ld-*H.i* stimulation caused a marked influx of cells the in airspace and around small airways. hd-*H.i* stimulation in CS-exposed mice showed more immunocells influx around airways, blood vessels, in alveoli and lymph-follicle like immunocells deposition around small airway compared to only hd-*H.i* stimulated mice.

CS exposure for 3 months did not induce a marked influx of leukocytes into BALF and pulmonary airspaces. The stimulation with ld-*H.i* lysate significantly increased the number of total cells in the BALF and induced a macrophage dominant inflammation in pulmonary airspace. ld-*H.i* significantly increased lymphocytes influx in BALF up to 24.59% of total cells compared 2 weeks ld-*H.i*. exposed mice. An increase of neutrophil percentage in BALF of ld-H.i in CS exposed mice was not observed. However the numbers and percentage of lymphocytes was significantly decreased in this group (Fig. 3.3B). hd-*H.i* significantly increased total cells in BALF compared ld-*H.i*, and induced neutrophilia in BALF. hd-*H.i* in CS exposed mice induced a significant increase of total cells compared to only hd-*H.i* exposed mice. The percentage of neutrophils was also significantly increased.

CS exposure for 6 months induced a slight influx of macrophages into the BALF and pulmonary airspaces. The exposure with ld-*H.i* significantly increased the number of immunocells in the BALF and induced a macrophage dominant inflammation in the lung such as already observed after 3 months exposure. The percentage of neutrophils in BALF of group ld-*H.i* in CS exposed mice was not increased. The numbers and percentage of lymphocytes compared to other 3 *H.i* exposed groups was significantly decreased in this group such as the data at 3 month (Fig. 3.4B). hd-*H.i* exposure significantly increased total cells in BALF compared to ld-*H.i*, and induced neutrophilia in BALF. hd-*H.i* in CS exposed mice significantly increased total cells compared only hd-*H.i* exposed mice. The percentage of neutrophils was also significantly increased.

Figure.3.2


2 weeks
3 month
6 month
A
B
C
D
E
F
cell number in BALF (x10 4/ml)
% cells in BALF
% cells
total cell
macrophage
neutrophil
Lympocyte
air
CS
ld-H.i
CS+ld-H.i
hd-H.i
CS+hd-H.i

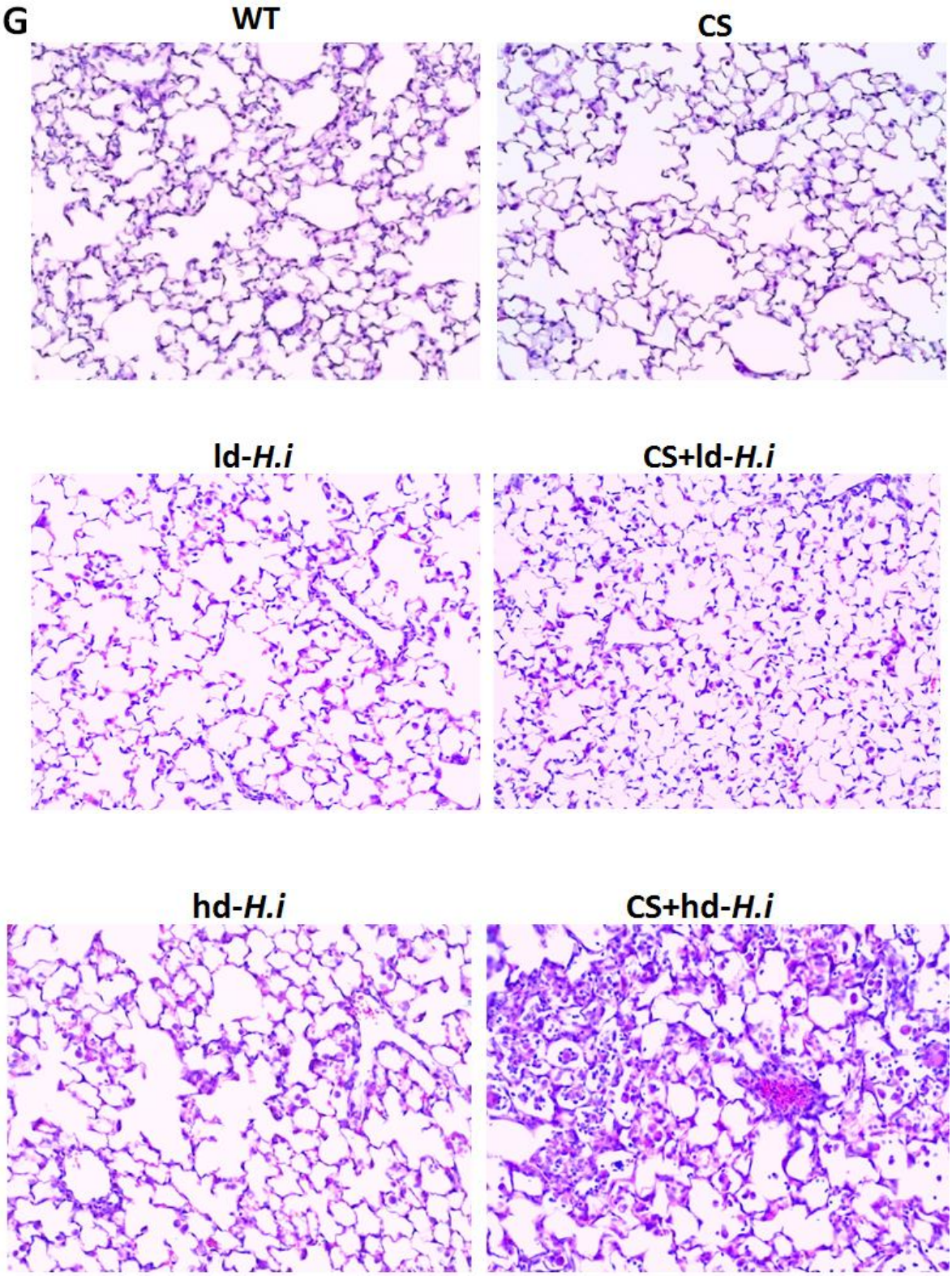

G
WT
CS
ld-*H.i*
CS+ld-*H.i*
hd-*H.i*
CS+hd-*H.i*

Figure.3.2. CS and *H.i* stimulation increased the total cell number and altered inflammatory phenotype in lung.

A. total cell count and differential cell count in BALF for 2 weeks stimulation. **B**. The percentage of different cells in BALF for 2 weeks stimulation. **C**. total cell count and differential cell count in BALF for 3 month stimulation. **D**. The percentage of different cells in BALF for 3 month stimulation. **E**. total cell count and differential cell count in BALF for 6 month stimulation. **F**. The percentage of different cells in BALF for 6 month stimulation. **G**. H&E staining of lung tissue for 2 weeks stimulation. Results are means ± SEM, n=5, ** $p<0.01$; ***$p<0.001$.

3.3 CS and *H.i* stimulation significantly increased parenchymal remodeling in lung from 3 month.

To confirm the emphysema-like changes in mouse lung following CS and *H.i* stimulation the lung volume and the mean chord length (*Lm*) were determined. The mean chord length (*Lm*) is a parameter which describes free distance of air spaces.

3.3.1 CS plus hd-*H.i* stimulation enhanced the parenchymal remodeling after 3 month.

CS or *H.i* stimulation did not significantly enlarge the lung volume at 3 month compared the air exposed control mice. But all groups with *H.i* or CS stimulation showed the trend of enlargement of lung volume. hd-*H.i* and the combination of CS and hd-*H.i* were significantly increased the mean linear intercept. It indicated the emphysema-like changes were induced in these two groups.

The HE staining of paraffin-embedded lung tissue confirmed the destruction of alveoli walls and infiltration of immunocells in hd-*H.i* and CS exposed mice. CS or ld-*H.i* stimulation for 3 month was not induced significant structural changes in lung. hd-*H.i* exposure and combined with CS for 3 month induced significant structural changes in lung parenchyma.

The lung function inclusive vital capacity, tissue elastance and Quasi-static compliance were measured in mice after 3 month. CS and *H.i* stimulation changed the lung functions from 3 month.

Lung function in 3 month stimulation showed significant decrease in vital capacity of ld-*H.i*, ld-*H.i* and hd-*H.i* in CS exposed mice, a significant increase in elastance of hd-*H.i* and a significant decrease in compliance of hd-*H.i* and hd-*H.i* in CS exposed mice.

Figure.3.3.1

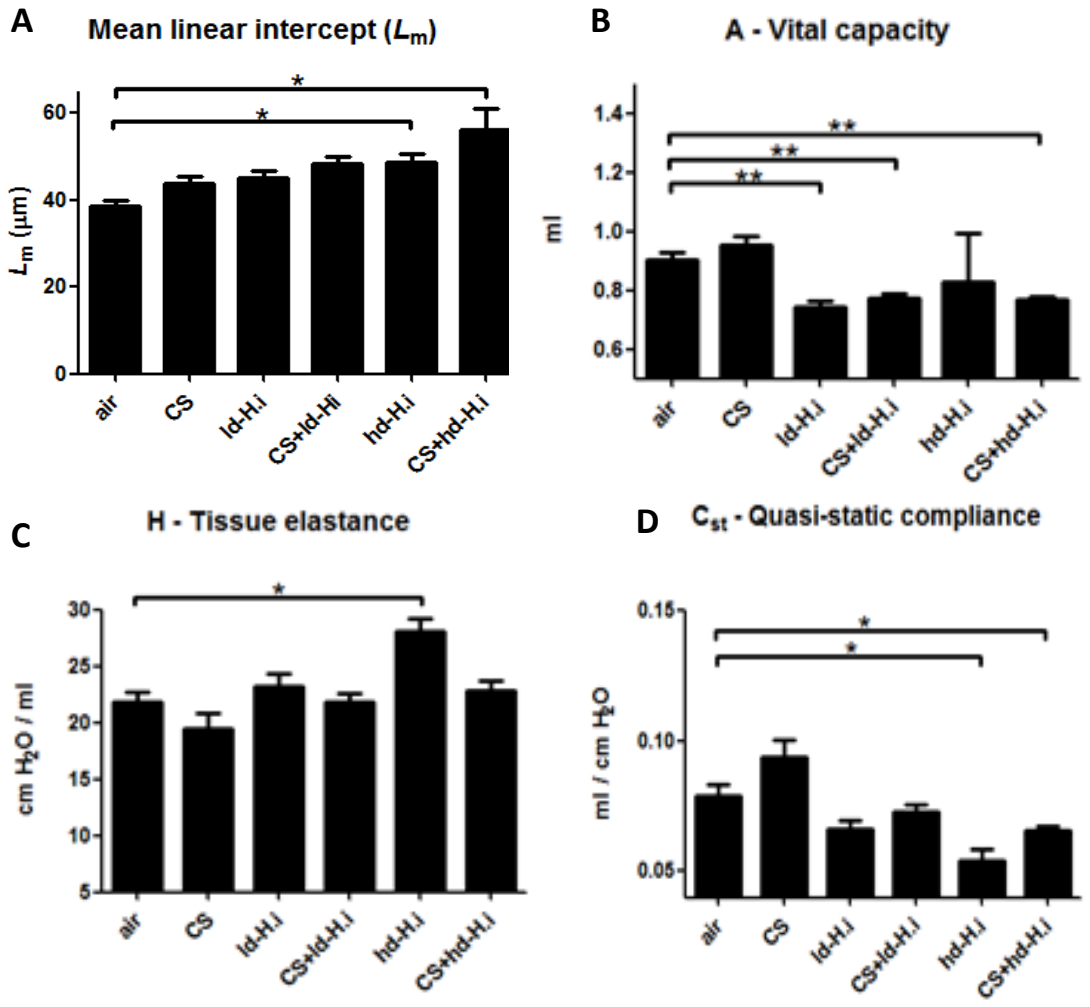


Figure. 3.3.1 CS and *H.i* induced emphysema-like changes in mouse lung after 3 month.

Mice were treated as described before for 3 months. Lung tissues were fixed and stained with HE, a quantitative stereological analysis was undertaken, and then the stereological tests were performed. **A** L_m was analyzed using uniformly random samples. Results are means ± SEM, n=5, *p<0.05; ** p<0.01; ***p<0.001. **B**. **C**. **D**. Mice were treated as described before for 3 and 6 month. Lung function was performed using a FlexiVent system. The measurement of vital capacity, tissue elastance and Quasi-static compliance of CS and *H.i* exposed mice for 3 month. Results are means ± SEM, n=5, *p<0.05; ** p<0.01; ***p<0.001.

3.3.2 CS plus hd-*H.i* stimulation enhanced the parenchyma remodeling after 6 month.

hd-*H.i* and the combination of CS and hd-*H.i* for 6 months significantly enlarged lung volume compared to air controlled mice. CS alone or ld-*H.i* did not induce significant changes in lung volume, but it showed the trend of enlargement of lung volume in this 3 groups.

ld-*H.i*. and hd-*H.i* induced also significant changes in peripheral alveolar size quantified by L_m measurement (Fig. 3.3.2.B). CS alone did not induce significant changes in peripheral alveolar size compared to air controlled mice and ld-*H.i* exposed mice. But hd-*H.i* in CS exposed mice induced significant changes of L_m compared to hd-*H.i* alone. The effect of enlargement of lung volume was also confirmed by L_m measurement in Fig.3.3.2.A.

The peripheral alveolar size quantified by L*m* measurement of 3 time points from 2 week up to 6 months was also figured out in Fig.3.3.2.C. The curve of group CS+ *H.i* hd-*H.i*. was always above all other groups included group of hd-*H.i* from 3 months up to 6 months. It clearly confirmed that hd-*H.i* played an important role in the destruction of lung tissue in CS exposed mice.

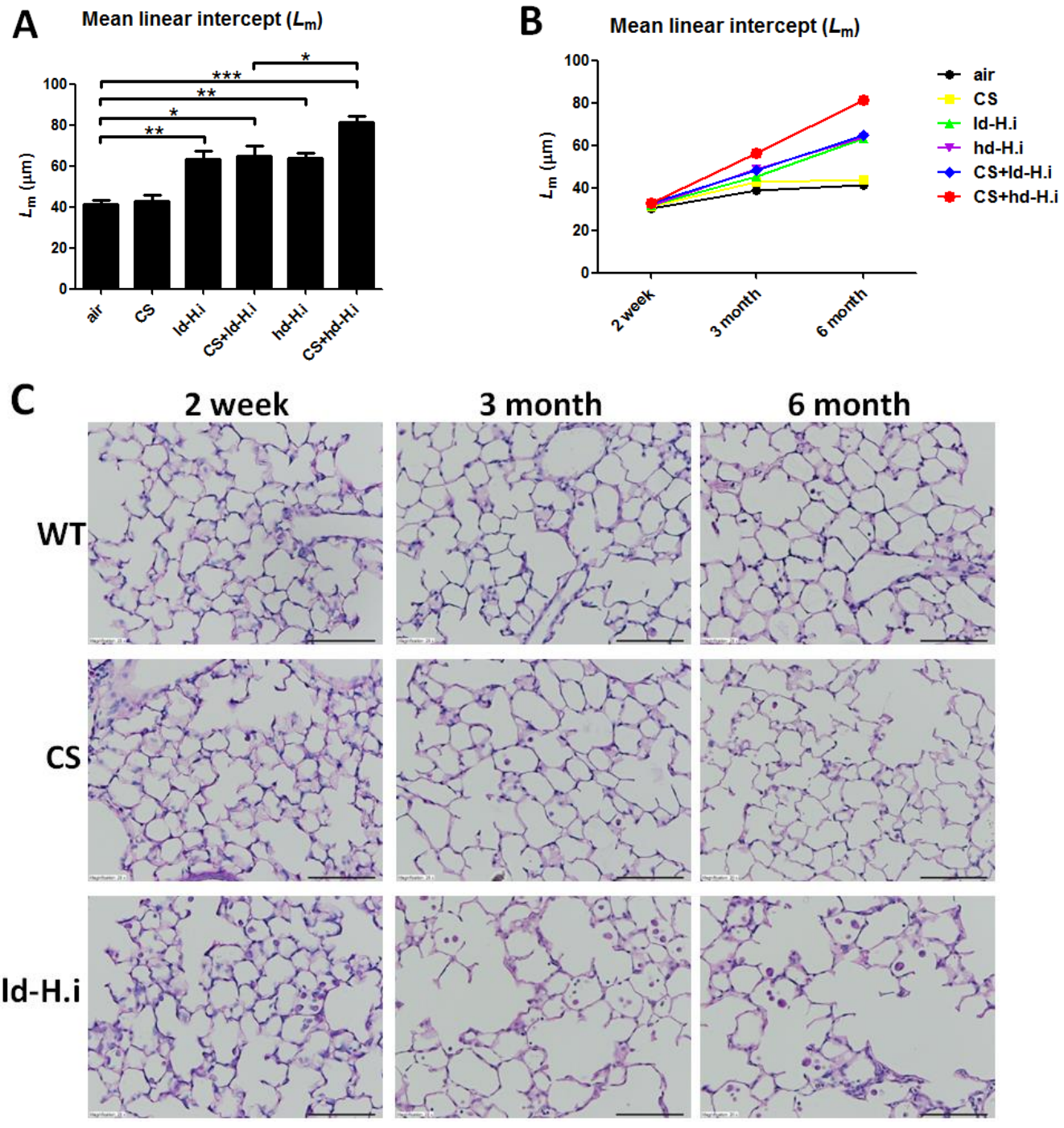
The HE staining of lung tissue at different time points also confirmed the structural changes in CS and *H.i* exposed mice lung. The lung of every mice in different group (n=5) were fixed and stained with hematoxylin and eosin. 2 random selected slices of every lung were microscoped. It showed the peripheral alveolar size of ld-*H.i*, CS+ld-*H.i*, hd-*H.i* and CS+hd-*H.i* were marked enlarged after 3 months and the effect of destruction of alveolar were enhanced in 6 months.

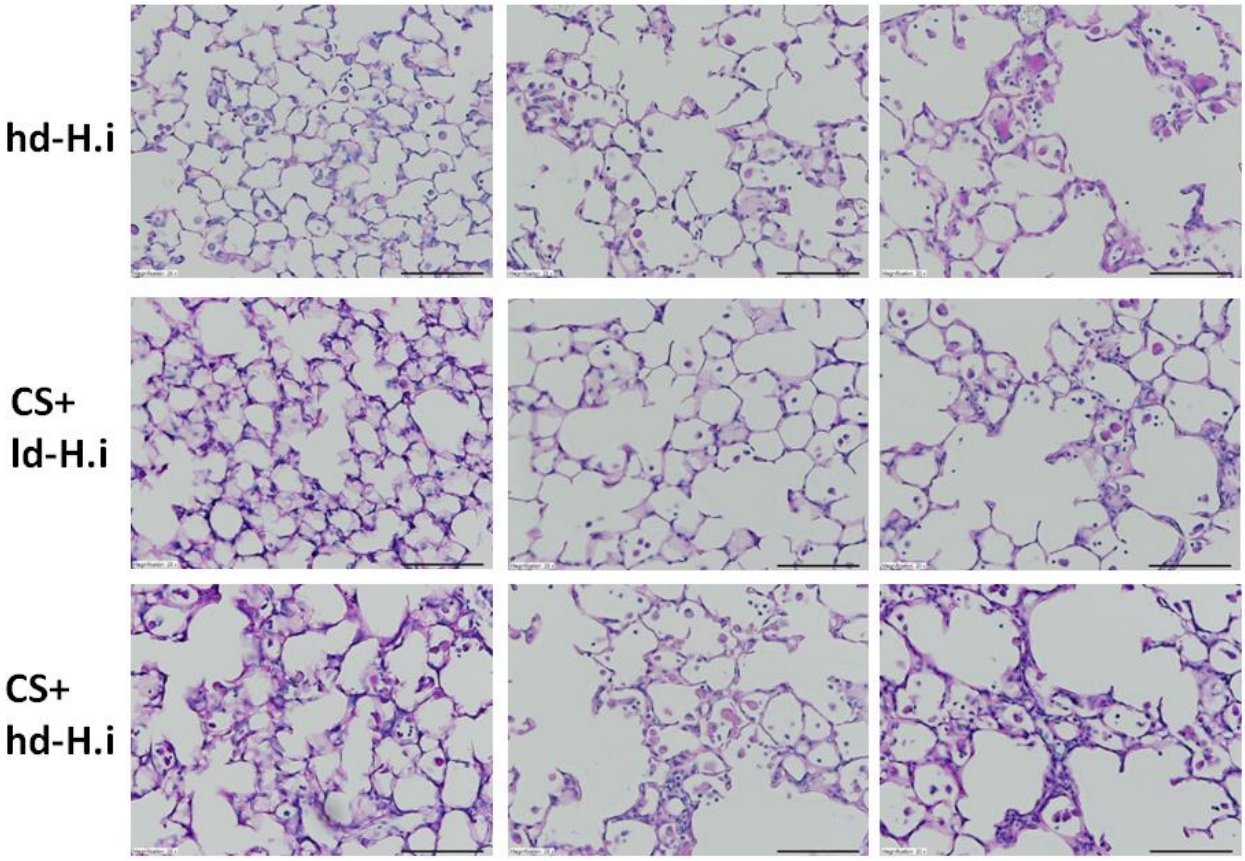
The lung function inclusive vital capacity, tissue elastance and Quasi-static compliance were measured in mice after 6 month. It showed an increase in vital capacity of about 20 %, which correlated with lung volume, a decrease in tissue elastance and an increase in compliance in CS treated mice after 6 months. The different doses of *H.i*. caused a decrease in vital capacity, an increase in elastance and a decrease in compliance. While the combination of ld-*H.i*. and CS showed similar

effect like CS alone, the combination of hd-*H.i.* and CS showed a similar phenotype like hd-*H.i.* alone.

Lung function of 6 month stimulation compared with 3 month stimulation showed enhanced changes in all 3 parameters. The 3 month CS exposure did not significantly increase vital capacity, compliance and decreased elastance but in 6 month have significantly increased vital capacity, compliance and decreased elastance. The combination of hd-*H.i.* and CS in elastance showed no significant changes in elastance in 3 month but significantly increased elastance in 6 month.

Figure.3.3.2


A
Mean linear intercept (L_m)
L_m (μm)
100
80
60
40
20
0
***
**
*
**
*
air
CS
ld-H.i
CS+ld-H.i
hd-H.i
CS+hd-H.i
B
Mean linear intercept (L_m)
L_m (μm)
100
80
60
40
20
0
2 week
3 month
6 month
air
CS
ld-H.i
hd-H.i
CS+ld-H.i
CS+hd-H.i
C
2 week
3 month
6 month
WT
CS
ld-H.i


hd-H.i
CS+
ld-H.i
CS+
hd-H.i

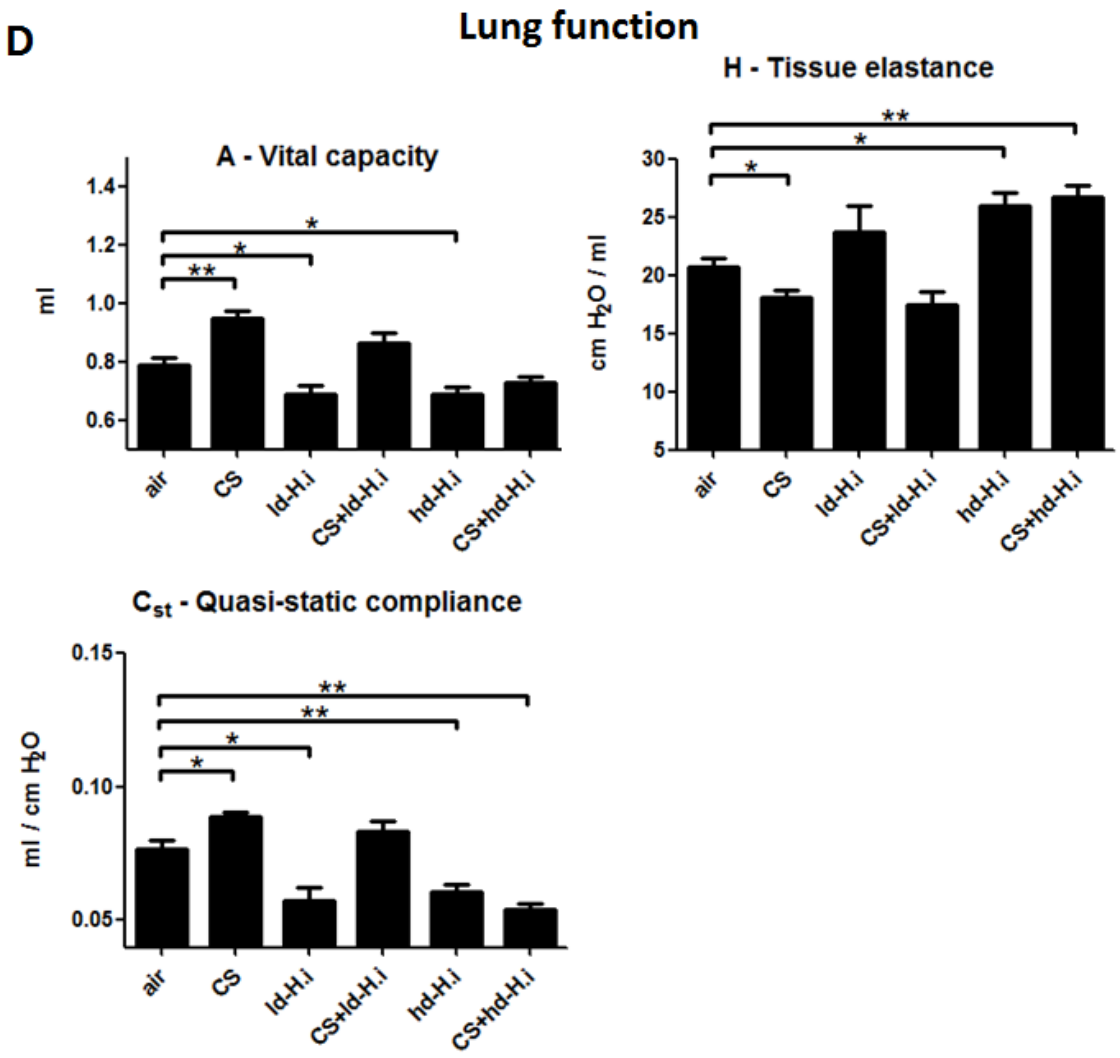


Figure. 3.3.2 The analysis of *L*m from 2 weeks up to 6 month.

A. The lung volume of 6 months treated mice was measured by fluid displacement. **B**. The analysis of L_m of 6 months treated mice. Results are means ± SEM, n=5, *p<0.05; ** p<0.01; ***p<0.001. C. HE staining of lung tissue. **C.** The time curve of L_m from 2 weeks up to 6 months. HE staining of mice lung. Bar = 50 μm. **D**. The measurement of vital capacity, tissue elastance and Quasi-static compliance of CS and *H.i* exposed mice for 6 month. Results are means ± SEM, n=5, *p<0.05; ** p<0.01; ***p<0.001

3.4 CS and *H.i* stimulation induced the expression of proinflammatory cytokines in BALF and lung-homogenate

To determine the inflammatory response of *H.i* in CS exposed mice the Th1, Th2 and Th17 cytokines and chemokins such as TNFα, KC, IL-1, MIP1γ, IFNγ, IL-2, IL-17, IL-10, and IL-17A were analyzed using CBA or ELISA in BALF and lung-homogenate.

3.4.1 CS and *H.i* stimulation significantly increased proinflammtory cytokines in BALF and lung-homogenate at 2 weeks.

The stimulation with ld- and hd-*H.i* increased the release of proinflammatory mediators such as TNFα and KC in BALF and lung-homogenate. The combination of CS and hd-*H.i* significantly increased the release of TNFα and KC in BALF but not in lung-homogenate. CS stimulation caused a significant decrease of IL-1β and MIP1γ concentrations in lung homogenate of the hd-*H.i* treated groups.

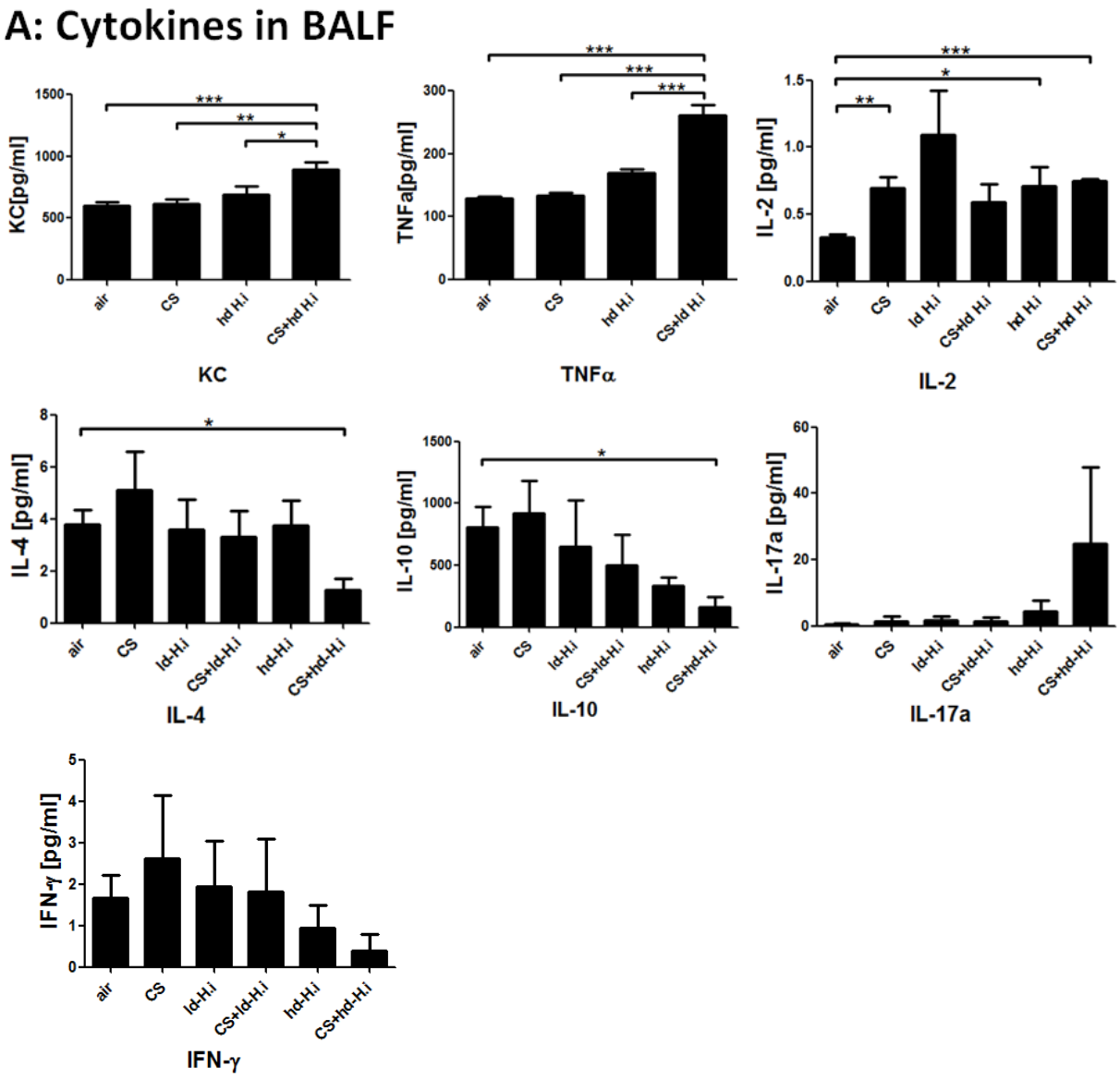
Th2 cytokines include IL-2 and IL-4 play a critical role in skewing inflammation in asthma and also prominent in the inflammation observed in COPD patients. In this study the release of IL-2 was significantly increased in ld-*H.i*, hd-*H.i* and CS stimulated mice, whereas TNFα and KC in BALF of CS exposed mice was not synergistically increased by *H.i* stimulation. In addition, hd-*H.i* significantly decreased the release of IL-4 in lung homogenate of CS exposed mice.

IFNγ is a cytokine which produced from Th1 and Tc1 cells. It usually be found at decreased levels in asthmatic patients. In this study CS stimulation but not *H.i* stimulation increased the release of IFNγ. And the combination of CS with ld-*H.i* decreased IFNγ compared to all groups in BALF.

The role of Th17 cells in both asthma and COPD is largely unknown. However, IL-17A level which released by Th17 cells are reported to be increased in the sputum of asthma patients. Some studies reported that The Th17 cells were also increased in the airways of asthmatic patients (178). IL-17a and IL-17f are related to

neutrophil-mediated inflammation. Airway epithelial cells and airway smooth muscle cells are able to release CXCL1and CXCL8 which are chemo-attractant for neutrophil (179). Therefore, the IL-17A may play a role in the neutrophilic inflammation in severe asthma and COPD (180). In this study, the ld-*H.i* and CS stimulation did not induce the expression of IL-17A in the mice lung. However, the combination of hd-*H.i* with additional CS exposure induced a remarkable increase of IL-17a in lung homogenate.

Figure.3.4.1


A: Cytokines in BALF
KC[pg/ml]
KC
TNFa[pg/ml]
TNFα
IL-2 [pg/ml]
IL-2
IL-4 [pg/ml]
IL-4
IL-10 [pg/ml]
IL-10
IL-17a [pg/ml]
IL-17a
IFN-γ [pg/ml]
IFN-γ
air
CS
hd H.i
CS+hd H.i
ld-H.i
CS+ld-H.i
hd-H.i
CS+hd-H.i

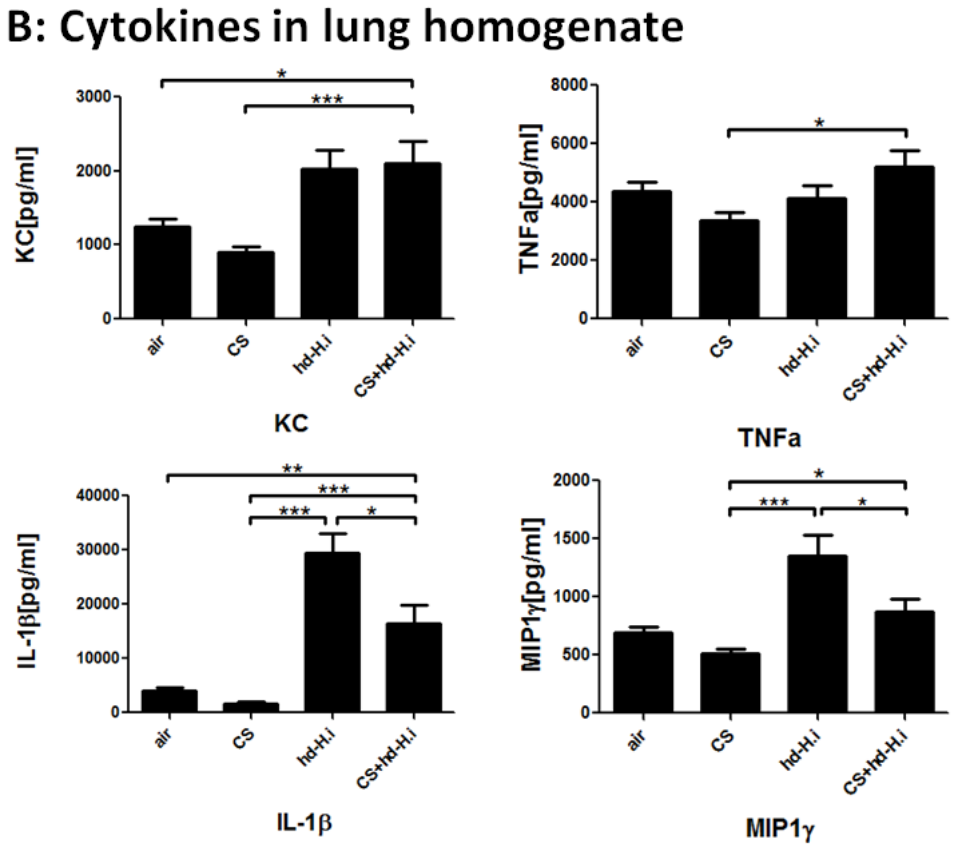


Figure.3.4.1 CS and nebulized *H.i* stimulation increased the release of pro-inflammatory cytokines in BALF.

Mice were lavaged using protease-inhibitor contained ice-cold PBS 24h after last CS exposure. Cytokines were analyzed by CBA or ELISA. **A**. The release of KC, TNFα, IL-2, IL-4, IL-10, IL-17a and IFN-γ in BALF. **B**. The release of KC, TNFα, IL-1β and MIP-1γ in lung-homogenate. Results are means ± SEM, n=5, *p<0,05; ** p<0,01; ***p<0,001.

3.4.2 CS and *H.i* stimulation at 6 month significantly induced the release of IL-17a in BALF.

To understand the inflammatory response of mice after 6 months *H.i* and CS stimulation, the release of TNFα, KC, IFNγ, IL-2, and IL-17a in BALF was analyzed using CBA. The stimulation with CS and *H.i* had no influence on the release of IL-2 in BALF after 6 month. CS alone decreased the concentration of IFNγ in BALF after

6 months compared to 2 weeks, but the combination of hd-*H.i* and CS significantly increased IFNγ in BALF.

ld-*H.i* and hd-*H.i* in CS exposed mice did not significantly change the release of IFNγ in BALF.

The stimulation with ld-*H.i* and hd-*H.i* for 6 months significantly increased the release of IL-17αinto the BALF. hd-*H.i* in CS exposed mice significantly increased IL-17α in BALF compared hd-*H.i* alone.

The release of TNFα in BALF was not increased after 6 months of CS exposure. The stimulation with ld-*H.i*. significantly increased the concentration of TNFα in BALF while the combination with CS slightly reduced TNFα concentrations. The further increase in microbial stimulation in the hd-*H.i*. groups caused only a slight increase in TNFα-release compared to ld-*H.i*. alone.

Figure.3.4.2

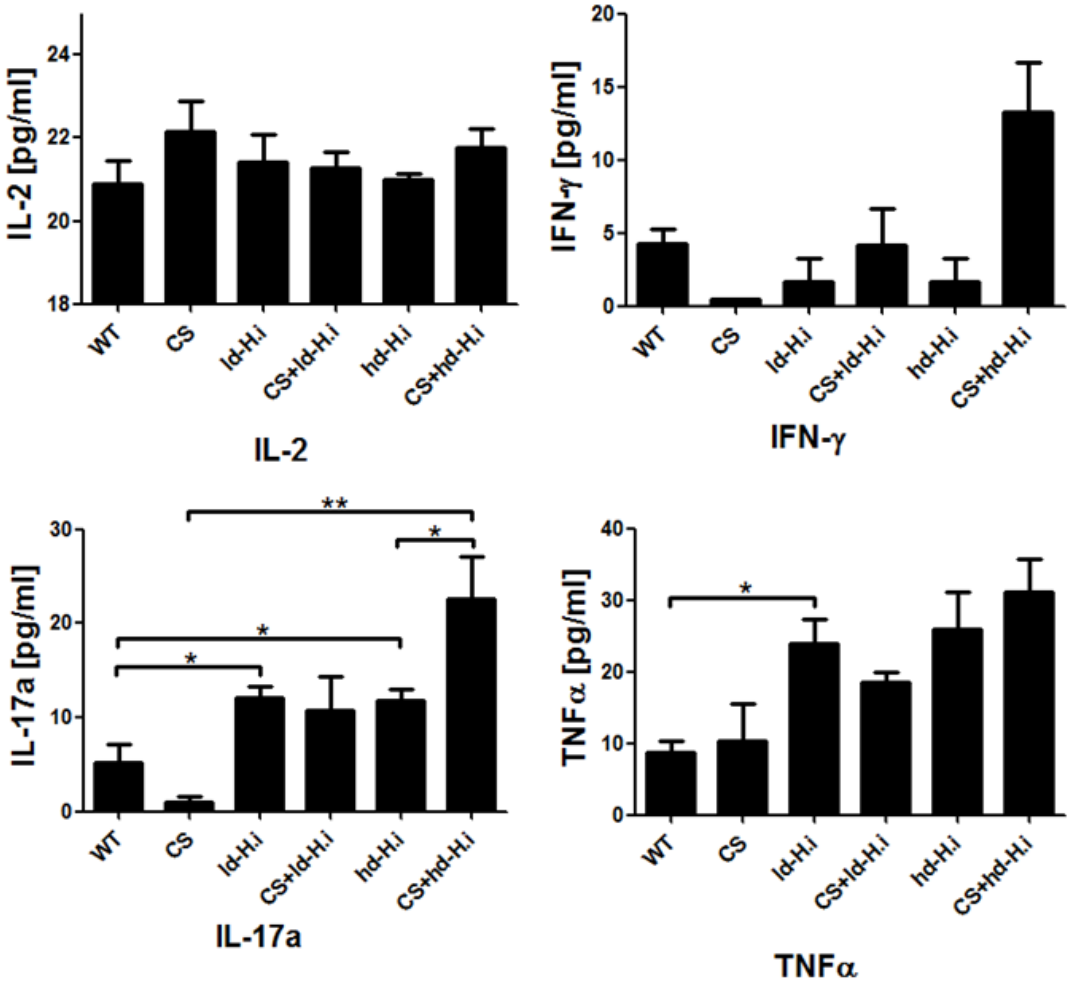


Figure.3.4.2. nebulized *H.i* synergistically stimulated the release of IL-17a in BALF.

Mice were as described in 2 weeks exposed. The release of IL-2, IFN-γ, IL-17a and TNFα in BALF was analyzed by CBA. Results are means ± SEM, n=5, *p<0,05; ** p<0,01; ***p<0,001.

3.5 CS and *H.i* stimulation induced the expression of MMPs and TIMPs in lung-homogenate

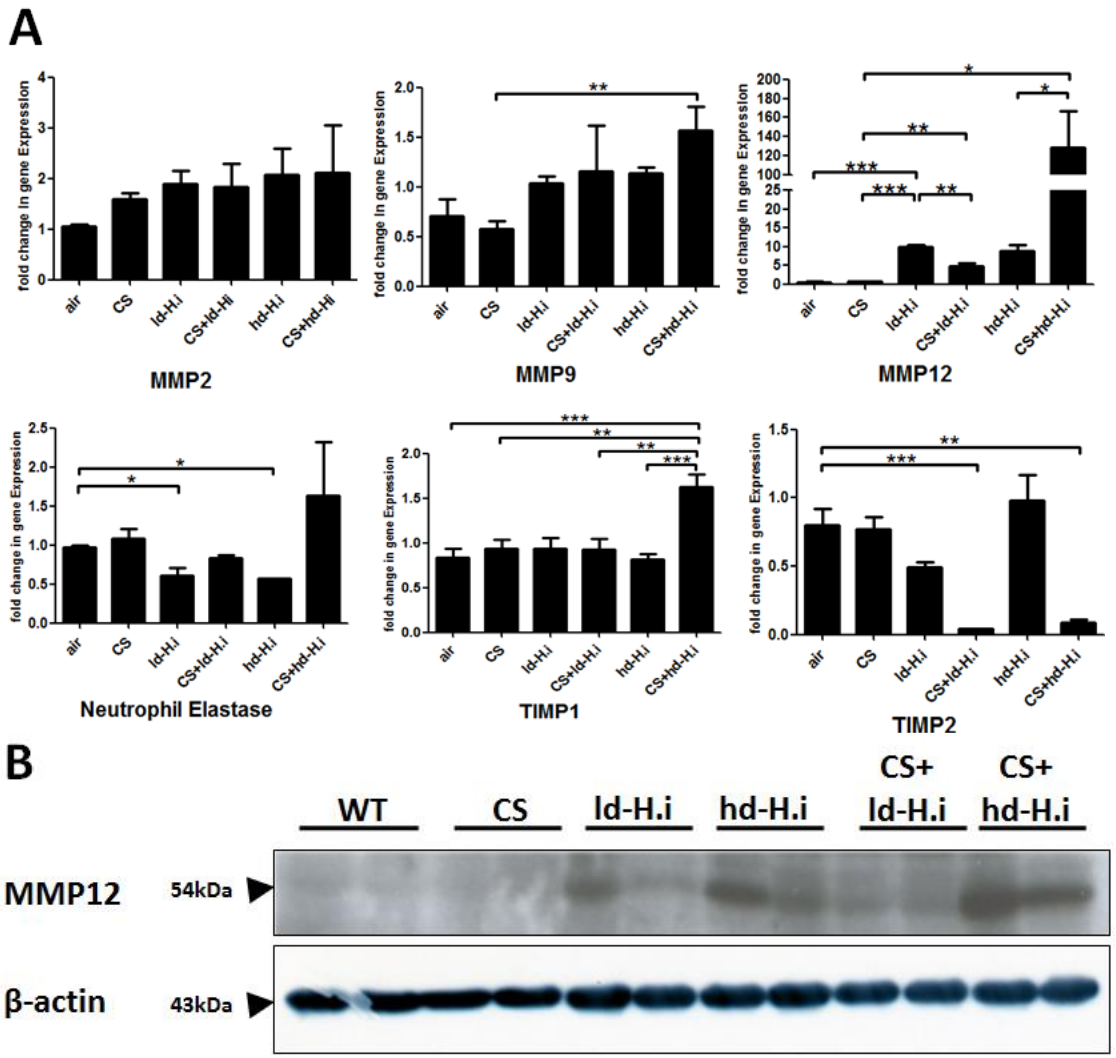
Matrix metalloproteinases (MMPs) are believed to play an important role in the pathogenesis of emphysema and are believed to be responsible for the destruction of alveolar walls and the degradation of connective tissue elements. Therefore we systematically analyzed the expression of various MMPs and their inhibitors (TIMPs) in CS and *H.i* exposed mice. The expression of MMP2, MMP9, MMP12, neutrophil elastase, TIMP1, and TIMP2 in lung homogenate was analyzed using qRT-PCR.

3.5.1 hd-H.i in CS exposed mice synergistically stimulated the expression of MMP12 in lung-homogenate at 2 weeks.

The stimulation with CS and *H.i* did not affect the expression of MMP2. hd-*H.i* stimulation in CS exposed mice induced the expression of MMP9 and TIMP1. ld-*H.i* and the hd-*H.i* stimulation decreased the expression of neutrophil elastase. The ld-*H.i* and the hd-*H.i* in CS exposed mice significantly decreased the expression of TIMP2.

The mRNA level of MMP12 was determined using qRT-PCR. CS had no influence on the expression of MMP12 after 2 weeks exposure. ld-*H.i* significantly induced the expression of MMP12 in lung homogenate compared to air controlled mice and CS exposed mice (Fig. 3.5A). ld-*H.i* stimulation in CS exposed mice decreased the expression of MMP12 in lung homogenate. hd-*H.i* in CS exposed mice increased the MMP12 mRNA level up to 120 folds compared air controlled mice. The expression of MMP12 in protein level was also confirmed through Western Blot analysis using antibody against MMP12. As it is shown in Fig.3-5B hd-*H.i* stimulation induced dominant increase of MMP12 in lung-homogenate. To confirm the localization of MMP12 in lung the IHC analysis was performed on paraffin-embedded lung tissue (Fig.3.5C). The expression of MMP12 in group of hd-*H.i* stimulation was detected both in airway epithelial cells and in alveolar immune-cells.

Figure.3.5.1


A
fold change in gene Expression
air
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ld-H.i
CS+ld-Hi
hd-H.i
CS+hd-Hi
MMP2
MMP9
MMP12
Neutrophil Elastase
TIMP1
TIMP2
B
WT
CS
ld-H.i
hd-H.i
CS+ ld-H.i
CS+ hd-H.i
MMP12
54kDa
β-actin
43kDa

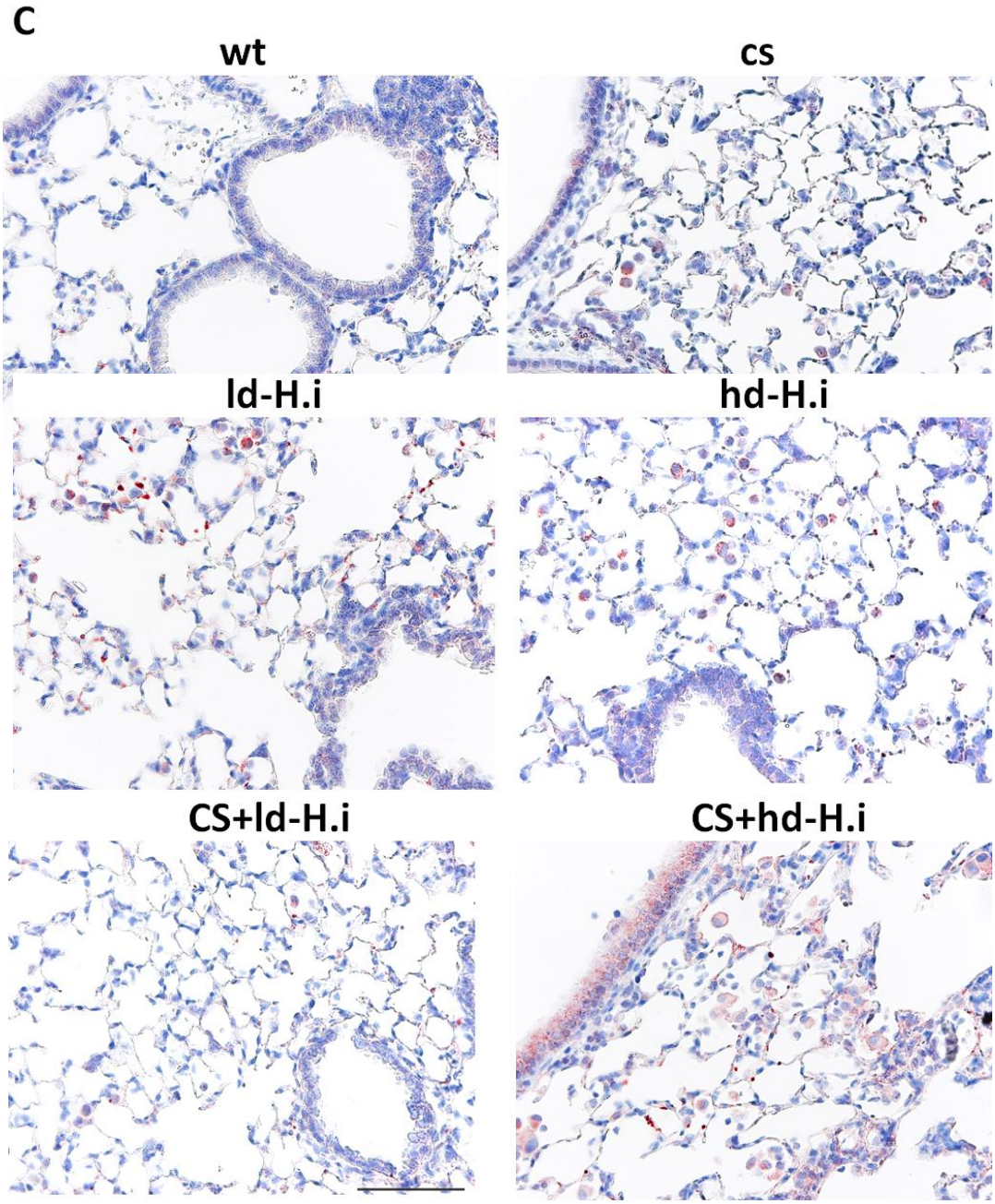


Figure. 3.5.1 CS and *H.i* stimulations induced the expression of MMPs and TIMPs in lung homogenate after 2 weeks.

A. The expression of MMPs and TIMPs in lung-homogenate was determined after 2 weeks CS- and *H.i*. exposure using qRT-PCR. **B**. The expression of MMP12 under various conditions was determined through Western Blot analysis using antibody against MMP12. β-actin was used as loading -control. Results are means ± SEM, n=5, *p<0,05; ** p<0,01; ***p<0,001. **C**. Immunohistological analysis confirmed the expression and the localization of MMP12 in lung tissue. The stimulation of CS, ld-*H.i*, hd-*H.i* and CS+ld.*H.i* increased the expression of MMP12 in immune cells while stimulation with CS+hd-*H.i* leads to the accumulation of MMP12 in immune cells and also in epithelial cells.

3.5.2 CS and *H.i* stimulated the expression of MMP9 and TIMP1 in lung-homogenate at 6 month.

hd-*H.i* in CS exposed mice significantly increased the expression of MMP12 in lung homogenate compared to air controlled mice at 6 month but not in hd-*H.i* alone. ld-*H.i* also reduced the expression of MMP12 in CS exposed mice compared ld-*H.i* alone.

hd-*H.i* in CS exposed mice significantly increased the expression of MMP9 in lung homogenate compared to air controlled mice and hd-*H.i*. exposed mice alone. The expression of MMP9 was lower in hd-*H.i*. exposed mice than in ld-*H.i* treated animals.

CS and ld-*H.i* significantly increased the expression of TIMP1 at 6 month compared to air controlled mice. hd-*H.i* also significantly increased the expression of TIMP1 in CS exposed mice.

Figure.3.5.2

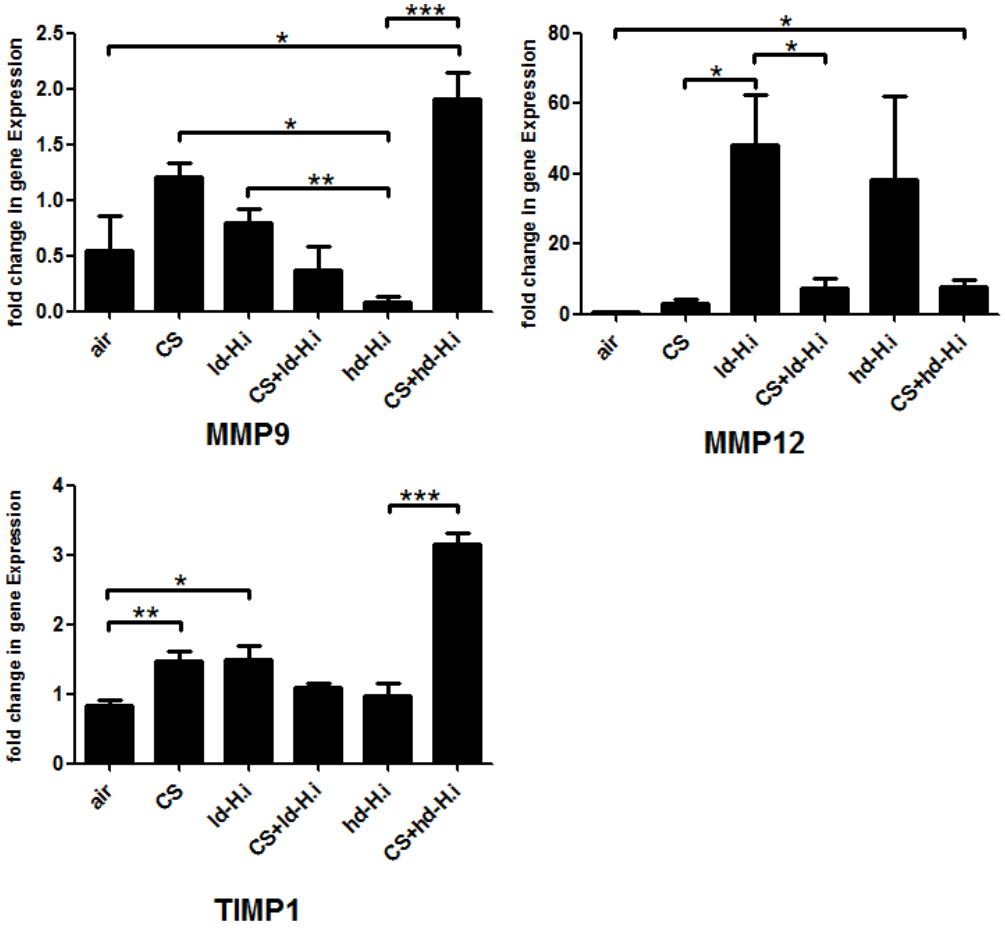


Figure. 3.5.2 CS and *H.i* stimulated the expression of MMPs and TIMPs in lung homogenate after 6 month.

Mice were treated as described before for 6 months. The expression of MMP9, MMP12 and TIMP1 in lung-homogenate was determined by qRT-PCR. Results are means ± SEM, n=5, *p<0,05;** p<0,01; ***p<0,001.

3.6 CS and *H.i* stimulation induced the mucus hypersecretion

To determine whether mucus production was affected following *H.i* and CS stimulation in mice, lung tissues were stained with PAS to analyze mucus-producing cells (Fig.3.6.1 A).

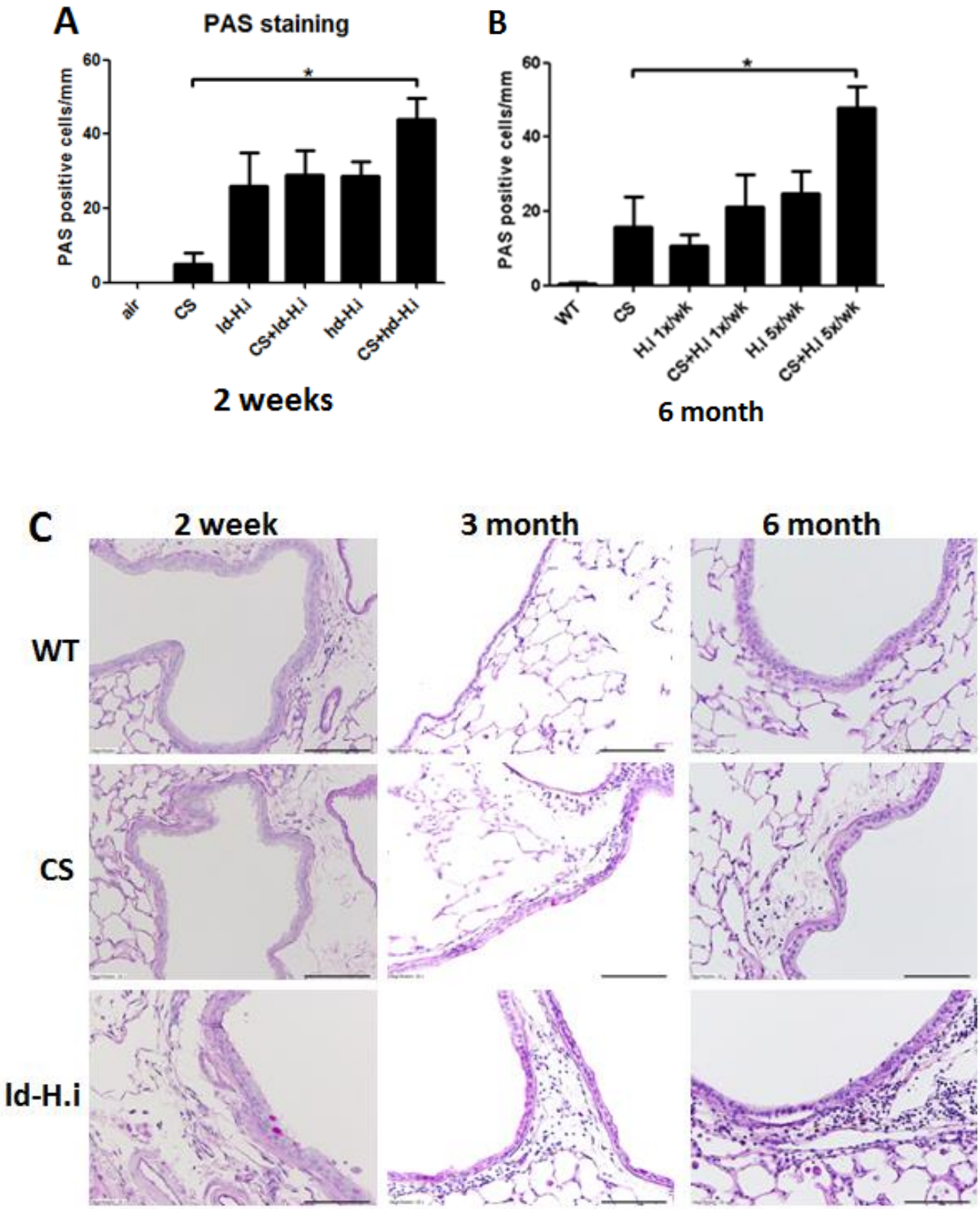
3.6.1 CS plus hd-*H.i* stimulation induced more PAS positive cells in large airways from 2 week up to 6 month.

Increased mucus hypersecretion by goblet cells of hd-*H.i* and CS exposed mice were observed in this study (Fig 3.6.1). We identify the PAS positive cells from 3 blind random selected slices of each mouse in every group. The hd-*H.i* stimulation plus CS exposed mice showed the most dominant effect on the PAS positive cells.

CS and *H.i* stimulation for 6 month also increased mucus production by goblet cells. hd-*H.i* in CS exposed mice significantly increased the number of PAS positive cells in big airways compared with only CS exposed mice. The groups of CS alone, ld-*H.i* and hd-*H.i* also increased PAS positive cells.

The number of PAS positive cells from 2 weeks up to 6 months was quantified and is shown in Fig. 3.6.1 A and B. The curve of group CS+hd-*H.i* was always above all other groups from 2 weeks up to 6 months. It confirmed that the combination of CS and hd-*H.i* induced a marked increase of PAS positive cells in big airways compared to CS alone or hd-*H.i* alone exposed mice. And it showed that the numbers of PAS positive cells following CS or *H.i* stimulation were not marked changed with time. The PAS positive cells in big airway of different groups in different time point were clearly figured out in Fig. 3.6.1C. It showed, that hd-*H.i* in CS exposed mice induced most PAS positive cells in big air way.

Figure.3.6.1


A
PAS staining
PAS positive cells/mm
60
40
20
0
*
air
CS
ld-H.i
CS+ld-H.i
hd-H.i
CS+hd-H.i
2 weeks
B
PAS positive cells/mm
60
40
20
0
*
WT
CS
H.I 1x/wk
CS+H.I 1x/wk
H.I 5x/wk
CS+H.I 5x/wk
6 month
C
2 week
3 month
6 month
WT
CS
ld-H.i

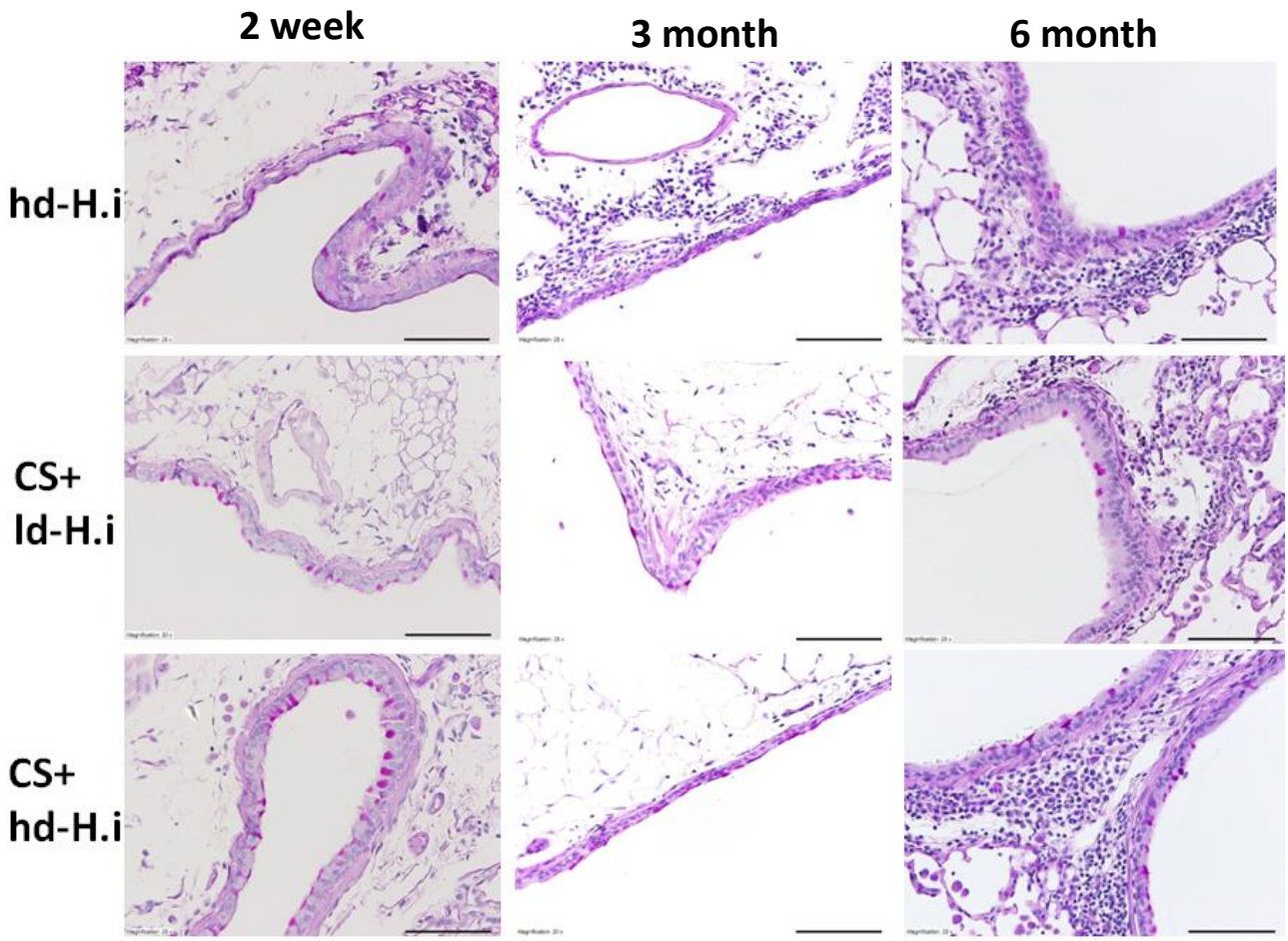


Figure. 3.6.1 The effect of *H.i* and CS on mucus secretion from 2 weeks up to 6 month.
Mice were treated as described before for 2 weeks, 3month and 6 month. Lung tissues were fixed and stained with PAS. **A**. statistical analysis of PAS positive cells in big air way at 2 weeks. **B**. statistical analysis of PAS positive cells in big air way at 6 month. **C**. PAS staining from 2 weeks to 6 month. Results are means ± SEM, n=5, *p<0,05; ** p<0,01; ***p<0,001. C. PAS staining of Lung tissue. Bar = 100 μm.

3.7 *H.i* stimulation induced connective tissue deposition in lungs at 6 month.

AZAN staining for connective tissue was performed on this study. The connective tissue deposition was observed in all groups of *H.i* stimulated mice. The ld-*H.i* and

hd-*H.i* stimulation significantly induced the connective tissue deposition around small airway and alveolar wall. hd-*H.i* in CS exposed mice increased connective tissue deposition compared only hd-*H.i* stimulated mice. But ld-*H.i* in CS exposed mice showed decreased connective tissue deposition in mice lung. This effect was also confirmed in lung function. The ld-*H.i* in CS exposed mice at 6 month showed negative effect of vital capacity and Quasi-static compliance compared only ld-*H.i* stimulated mice.

Figure. 3.7

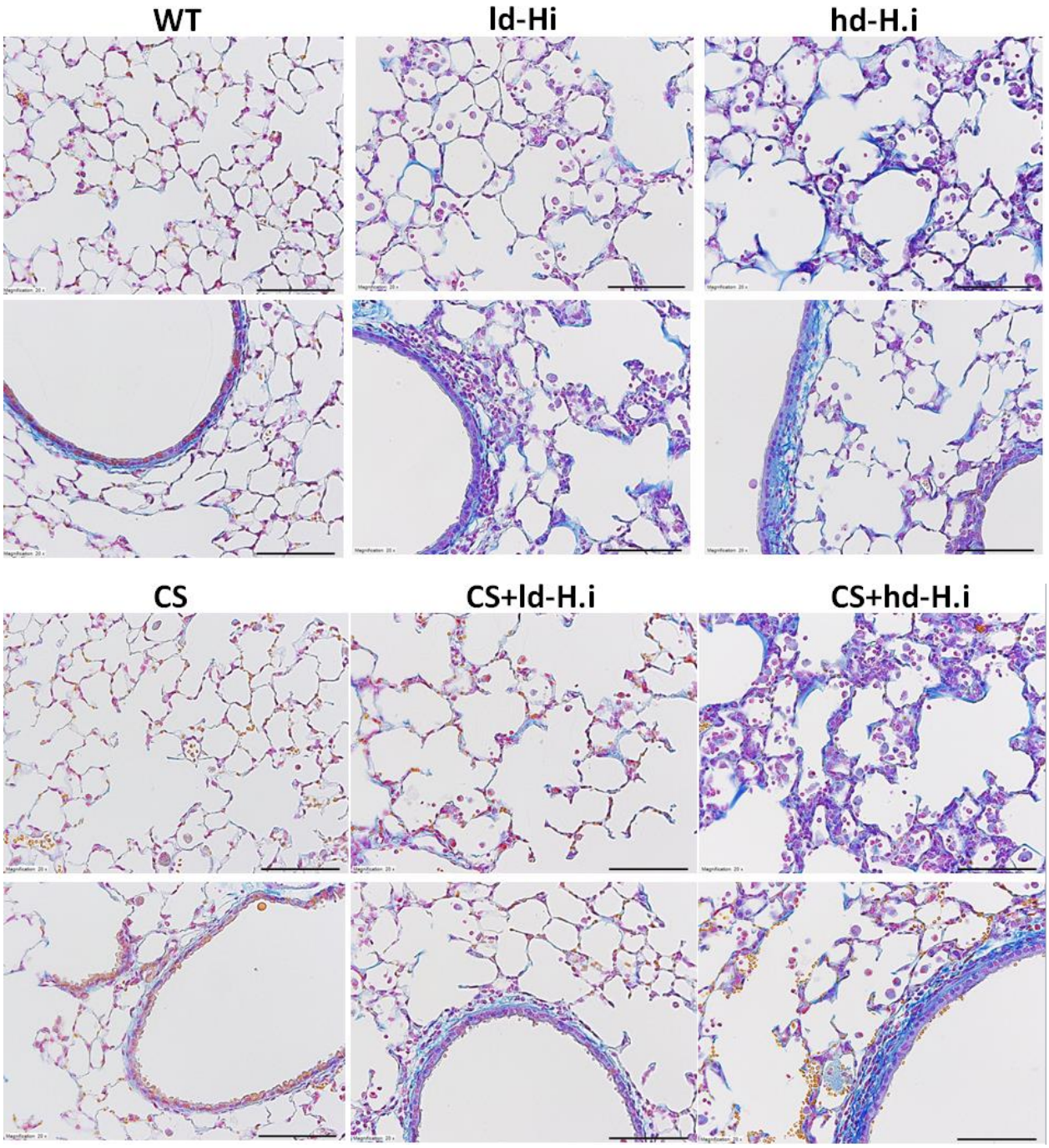


Figure. 3.7 The CS and H.i stimulation induced connective tissue deposition in lung at 6 month.

Mice were treated as descripted before for 6 month. Lung tissues were fixed and stained with AZAN. Bar = 50 μm.

4. Discussion

The main cause of chronic obstructive pulmonary disease in the developed countries is cigarette smoke. COPD include systematic inflammation and emphysema led to airway inflammation, destruction of airway parenchyma, and elevated protease expression (181). Exacerbations of COPD are defined as a periods of acute worsening symptoms, resulting to morbidity and mortality. Increased airway and systemic inflammation and pulmonary parenchymal remodeling are the main manifestation of COPD during exacerbation. It is normally accepted, that COPD exacerbations are triggered by respiratory virus, bacterial infection and/or air pollution, which infected the lower airway and increase airway inflammation (182). However, the interaction of bacterial infection and CS in the exacerbations of COPD, whether frequent exacerbations accelerate the progression of emphysema, and the intracellular signaling mechanisms activated by tobacco smoke and bacteria such as *Haemophilus influenzae* that mediate the protease/antiprotease imbalance and subsequent matrix degradation in the lungs are not well understood.

In this study we focus on the interaction of CS and *H.i* in the mouse model, and examined the effect of CS on different doses of *H.i* including the inflammatory response, changes in lung function, mucus hypersecretion, MMP12 and TIMP1 expression, and tissue remodeling. We demonstrate that CS suppresses the inflammatory response on ld-*H.i* exposed mice and enhances the inflammatory response on hd-*H.i* exposed mice, induces the MMP12, TIMP1 and IL-17α expression, promotes changes in lung function and tissue remodeling. According to our observation of IL-17α expression, MMP12 expression in macrophage and epithelial cells we conclude that the interaction of CS and *H.i* in macrophage and epithelial cells play an important role in the pathogenesis of CS and *H.i* induced inflammation and tissue remodeling in lung.

4.1 The inflammatory response during CS and *H.i* stimulation

Expose to cigarette smoke changes lung immunity and induces impaired pulmonary function and mucus hypersecretion (183). *Haemophilus influenzae (H.i)* as an opportunistic bacterial pathogen is frequently colonized in upper airway in healthy humans. *H.i* was isolated frequently from acute and chronic respiratory infections such as pneumonia, chronic bronchitis and COPD (150, 156). Alveolar macrophages are important for the clearance of bacterial infections. However, the interaction of alveolar cigarette smoke and pathogens with innate immunity in lung at cellular level remains poorly understood.

As described in introduction, the cellular pattern changes during exacerbations, eosinophils and neutrophils are the major components of the inflammatory response (170, 171). Patients suffering from chronic bronchitis showed in bronchial biopsies a dominant airway eosinophilia up to 30-fold increase of eosinophils, and also lightly increased neutrophils, T lymphocytes, and other TNFα–positive cells (184). Other study reported that patients with severe chronic bronchitis showed increased neutrophils and eosinophils in bronchial alveolar lavage fluid, and the neutrophil is more dominant than eosinophils (154). COPD patients during severe exacerbation in bronchial biopsies showed increased numbers of neutrophils compared with stable COPD patients. However, other cell types in bronchial biopsies were not reported (150).

According to our finding in this study, the additional stimulation with CS in 2 weeks ld-*H.i* exposed mice induces the changes of inflammatory phenotype from macrophage based inflammation to neutrophilic inflammation (Figure 3-2B). This finding is similar with the changes of cellular pattern during exacerbations in patients with COPD. This change is not observed in long term stimulation with CS, for example 3 month and 6 month (Figure 3.2, Figure 3.4). And this finding indicates an adaptive process in group of CS plus ld-*H.i* exposed mice. We also observed the upregulation of KC and TNFα in BALF of CS plus ld-*H.i* exposed mice. We hypothesize, that this change of inflammatory phenotype may be caused by the KC and TNFα induced recruitment of neutrophils in BALF.

IL-8 is a chemoattractant for neutrophils and $CD8^+$ T lymphocytes and secreted by diverse cell types, including bronchial epithelial cells, macrophages, and neutrophils. The expression of IL-8 is upregulated in air space in response to cigarette smoke. COPD patients have significantly increased IL-8 level in sputum than healthy smokers (19), and the IL-8 level relates to the decline in FEV_1 (155). Other studies reported that COPD patients during severe exacerbation have increased IL-8 level in both sputum and serum, but there are also studies demonstrated that IL-8 was not significantly increased at exacerbation. COPD patients with frequent exacerbations have also higher sputum IL-8 level (185).

TNF-α is a proinflammatory cytokine. Alveolar macrophages release most TNF-α in lung. TNF-α shows diverse proinflammatory effects, such as neutrophil degranulation, respiratory burst and induction of interleukin IL-8 expression. TNF-α is increased in the sputum of COPD patients compared with healthy smokers (186) and also in COPD patients colonized with *H.i* (187). TNF-α is further increased in sputum from COPD patients during exacerbation compared with stable COPD (183), and also during bacterial exacerbations of chronic bronchitis (154). TNF-α recruits and activates neutrophils in lung. In total, TNF-α may act as a central mediator of neutrophilia in COPD exacerbation.

Our finding in the mouse model is similar with the expression of IL-8 and TNF-α in exacerbation of COPD. Additional stimulation with CS induces significantly expressed KC and TNF-α in BALF in hd-*H.i* exposed mice. But the significant expression of KC and TNF-α in BALF in CS+ld-*H.i* exposed mice is not observed.

The molecular mechanisms whereby inflammatory mediators are upregulated at exacerbation remain undetermined. It has been reported in some study that the transcription factor NF-κB may act as a central role of inflammatory response at exacerbation. The transcript activation of nuclear factor NF-κB and activator protein-1 was also observed in some human bronchial epithelial cell lines which infected with rhinovirus (63, 187).

According to our findings in this study, we also observe the enhanced expression of TNF-α in epithelial cells of group of CS+ hd *H.i* (Data not shown). It suggests that CS stimulation in hd-*H.i* exposed mice induces the enhanced expression of TNF-α in

epithelial cells.

4.2 The expression of MMPs and TIMPs during CS and H.i stimulation.

The MMPs are a family of Zn binding enzymes for the degradation of most extracellular matrix components. We have chosen gelatinase (MMP-2 and MMP-9), metalloelastase (MMP-12) and its inhibitors TIMP1 and TIMP2 for the present study. All these MMPs have been implicated in the genesis of COPD and are reported to be increased in the pulmonary parenchyma of COPD patients (188).

MMP-12 is released mainly in alveolar macrophages. Recent studies reported the detection of MMP-12 in other cell types such as normal human bronchial epithelial cells, smooth muscle cells and in various primary brain neoplasms (189-191). In this study, the results based on IHC using antibody against MMP12 also indicated the expression of MMP12 in both macrophage and epithelial cells in response of CS and *H.i*. We believe that this is the first description of MMP-12 production in lung epithelial cells in response of CS and *H.i.*

In this study, we have analyzed for the first time the changes of expression level of MMP2, MMP9, MMP12, TIMP1 and TIMP2 is related to the exposure of CS and *H.i* in mouse model. ld-*H.i* and hd-*H.i* exposure alone induced the expression of MMP12 at 2 weeks, the combination of hd-*H.i* and CS synergistically induced the expression of MMP12 at 2 weeks but not after 6 month (Fig. 3.8). The MMP12 expression induced by the combination of CS and hd-*H.i* was also confirmed using western blot in protein level. In total, the combination of cigarette smoke and *H.i* induces increases in gene expression and protein levels of MMP12 that are potentially important in parenchyma remodeling of lungs.

TIMP proteins are the specific inhibitors of MMPs. Up to now, there are 4 TIMPs include TIMP1, TIMP2, TIMP3 and TIMP4 discovered in vertebrates. TIMP-1 releases mainly from macrophages and fibroblasts suggesting that TIMP-1 is involved

in tissue remodeling linking to inflammatory process. The present study indicates that MMP-9 and TIMP-1 are elevated in COPD patient during exacerbation. Our findings are similar with these studies.

We were surprised to observe that the TIMP2 was dramatic reduced as a result of exposing to CS and *H.i.* Strong evidence indicates that MMP/TIMP imbalance is an import element of the process of tissue remodeling. With regards to our findings, we cannot make definitive comments on the balance between MMP and its TIMP.

4.3 The possible anti-inflammatory effect of CS in *H.i* exposed mice.

Cigarette smoke suppresses the innate immune system (192). Increased evidence indicated that the cigarette smoke is responsible for many diseases such as heart failure, strokes, COPD and cancer (193). Interestingly, smokers have lower risk for many diseases, including ulcerative colitis, pigeon breeders' disease, and Parkinson's disease (194). As descripted in introduction, the alpha 7 nicotinic receptor pathway may play associate with the anti-inflammatory function of nicotine attenuates inflammation. The harmful effects are commonly accepted of cigarette smoke. The cigarette smoke triggered inflammation is believed as the main risk factor in COPD.

With regards to our findings, CS exposure at an average concentration of 120 ± 5 mg/m^3 of total suspended particles (TSP) for 3 hours per day lasted 6 month was not sufficient to induce a marked inflammatory response in lungs. But the CS in smoke box according to 3 days smoke protocol (30 min smoke and break for 15 min, then repeat for 5 times per day lasted 3 days) of our lab induced significant infiltration of immunocells and increased the neutrophil percentage in mouse lungs (Data not shown). CS increased total immunocells infiltration in hd-*H.i* exposed mice in 2 weeks, 3 month and 6 month. But CS significantly decreased total immunocells infiltration in ld-*H.i* exposed mice at 6 month (Fig. 3.2). CS inhibited the expression of TNFα of ld-*H.i* exposed mice at 6 month (Fig. 3.4.2). It was also shown in Fig. 3.4.2 that the CS decreased the connective tissue deposition in ld-*H.i* exposed mice

after 6 month. It indicated that the CS induced anti-inflammatory effect in 1 x/wk *H.i* exposed mice at 6 month.

Taken together our finding clearly suggests that CS suppresses the inflammatory response in ld-*H.i* exposed mice and enhances the inflammatory response in hd-*H.i* exposed mice. The reason for the effect of CS in different doses of *H.i* exposed mice is unknown. It is possible that CS in ld-*H.i* exposed mice activates the 7 nicotinic receptor pathway suppresses the inflammatory response, then results the decreased connective tissue deposition in lung.

4.4 The expression of IL-17a

Recent studies reported that IL-17RA deficient mice showed anti-inflammatory effects in response to cigarette smoke. And in mice lung was also showed reduced transcription activity of MMP12. IL-17RA deficient mice were protected against cigarette smoke induced emphysema after 6 months smoke exposure. In total, these studies indicated the potential role of TH17 cells in emphysema and COPD.

Some other studies reported that the IL-17A derived germinal center formation of lymphoid follicles in mice and humans lung. Furthermore, the increased TH17 cells were observed in COPD patients. In summary, these studies indicated the possible role of IL-17A in pulmonary development of lymphoid follicle in COPD patients (195).

In our study the combination of CS and hd-*H.i* induced the clear expression of IL17A at 2 weeks and significant expression at 6 month. The lymphoid follicle development is also observed after 3 month and 6 month in CS and *H.i* exposed mice lung. We hypothesize that IL-17a dependent signaling is mainly activated in epithelial cells. The CS and *H.i* exposure activates epithelial cells resulting the lymphoid follicle development in mice lung.

4.5 Airway and alveolar fibrosis

Airway wall fibrosis is believed to be one of the most common tissue remodeling in COPD, and results to the airflow limitation (196) (197). *H.i* is the most frequently isolated pathogen in COPD patients with bacterial exacerbation (159). In this study, repetitive exposure to nebulized lysate of heat inactivated *H.i* induced progressive airway wall fibrosis with a clear increase of collagen deposition around airway wall from 3 month was observed in mice lung (Figure 3.7). Some Studies also reported that COPD patients have coexisting of alveolar fibrosis with parenchyma destruction (198) . This finding indicates that frequent intrapulmonary infection of COPD patients by *H.i* may lead to alveolar fibrosis in lung. For the further treatment, this finding implies the consideration of anti- fibrosis therapy for the OPD patients with bacterial infection might be undertaken.

4.6. Conclusion

Current studies regarding COPD and AECOPD focus on the inflammatory response including the proinflammatory mediator such as IL-8, IL-6, IL-1β and TNF-α with inflammatory signaling pathway including NF-κB, MAP kinases, STATs and IFN regulatory factor 1 (IRF1) (153), which believed to be important for the pathogenesis of COPD and exacerbation. There is also various studies focus on functional abnormalities in leukocyte of COPD patients. However, there are no studies which intensively investigate the interaction of CS and *H.i* in mice lung. We believe that this is the first study to investigate the interaction of CS and *H.i* related immune and structure alteration in mice lung, and firstly demonstrate that CS and *H.i* induced synergetic inflammatory results to impaired lung function.

According to our findings, we demonstrated that the *H.i* stimulation induced the recruitment of macrophage and neutrophil to the lung, mainly induced the release of

inflammatory mediators in immunocells, such as macrophage, neutrophils. Additional CS stimulation induced the activation of epithelial cells, and enhanced the macrophage recruitment, induced the expression of IL-17a in epithelial cells, and the mucus hypersecretion and also induced the expression of MMP12 and TIMP1 not only in macrophage but also in epithelial cells (Figure 4.1). The activation of epithelial cells depends on the doses of *H.i* stimulation: CS inhibited the inflammatory response of low doses *H.i* induced inflammation, CS selective enhanced the release of inflammatory mediators in epithelial cells in high doses *H.i* exposed mice, and resulted impaired lung function and tissue remodeling. Firstly, our studies establish a new mouse model for the study of COPD with bacterial infection. Our findings highlight the targets of the interaction of combined CS and *H.i* simulation in mice lung and provide with a new insight for the therapy of COPD patients with bacterial infection.

Figure.4.1

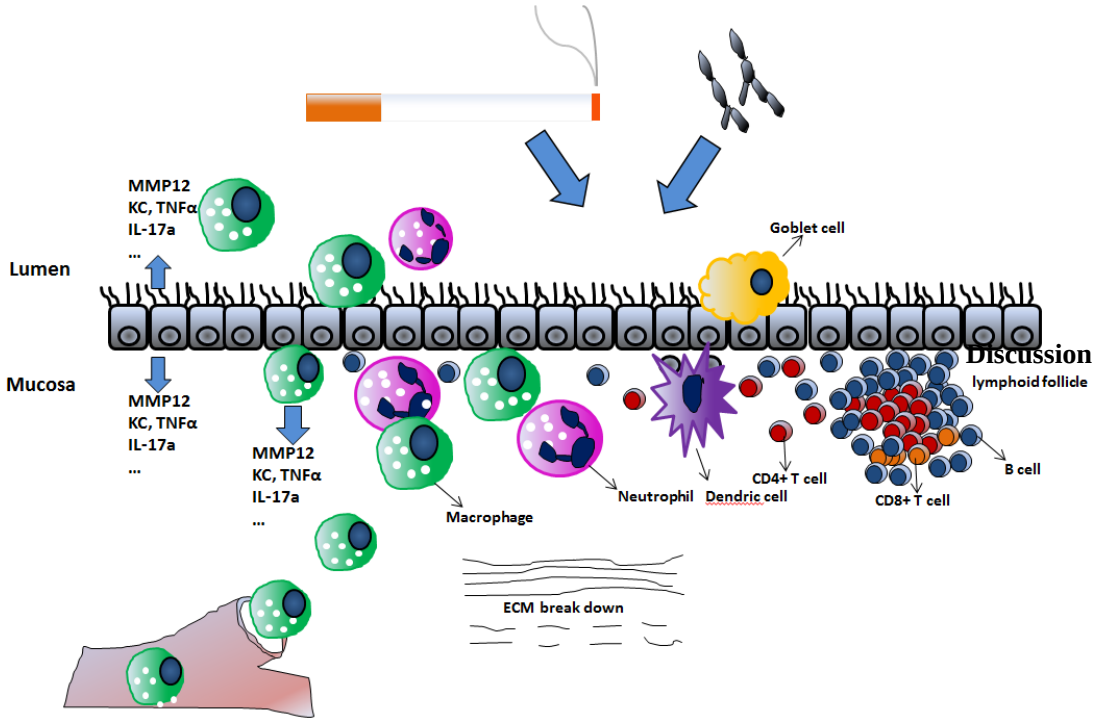


Figure. 4.1. The novel aspect of the interaction of CS and H.i in exacerbation of COPD

H.i induces the immunocells recruitment in lung and induces the release of proinflammatory mediators such as KC, TNF-α. Additional CS stimulation induces the activation of epithelial cells and induces the expression of MMP12 and IL-17A in epithelial cells, enhances the recruitment of immunocells in lung, results the changes in lung function and enhanced tissue remodeling in lung.

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Publications and Presentations

Parts of this work have been revealed in the following publication:

- **G.Han**, C. Herr, D. Li, T.Tschernig, T.Dinh , C.Beißwenger. R. Bals. Combined exposure to bacterial and cigarette smoke resembles characteristic phenotypes of human COPD in a murine disease model. Exp Toxicol Pathol. 2015 Mar;67(3):261-9. doi: 10.1016/j.etp.2015.01.002. Epub 2015 Jan 16.

- D. Li, C. Beisswenger, C. Herr, J. Hellberg, **G. Han**, T. Zakharkina, R. Wiewrodt, R. M. Bohle, H. P. Lenhof, R. Bals. Myeloid cell RelA/p65 promotes cigarette smoke-induced lung cancer proliferation through Wnt/-catenin signaling in tumor cells. Oncogene. 2014 Mar 6;33(10):1239-48. doi: 10.1038/onc.2013.75. Epub 2013 Apr 8.

- D. Li, C. Beisswenger, C. Herr, RM. Schmid, Gallo RL, **G. Han**, T. Zakharkina, R. Bals. Expression of the antimicrobial peptide cathelicidin in myeloid cells is required for lung tumor growth. Oncogene. 2014 May 22;33(21):2709-16. doi: 10.1038/onc.2013.248. Epub 2013 Jul 1.

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1. ATS International Conference (Denver, USA, May, 2011)

2. 52. Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V. (Dresden, Germany, April, 2011)

3. 51. Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V. (Hannover, Germany, March, 2010)

4. COSYCONET 2010 (Frankfurt, Germany, March, 2010)

5. COSYCONET 2011 (Würzburg, Germany, March, 2011)

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Curriculum Vitae

Personal data

Name:	**Gang Han**
Gender:	Male
Date of Birth:	April 19th, 1977
Birth place:	Shandong, China
Nationality:	China
Address:	Kaiser-Wilhelm Str. 69, 67059 Ludwigshafen am Rhein
phone:	+49(0)176 7025 9563
Fax:	+49(0)6841 16-47939
Email:	Gang.Han@uks.eu

Education

07/2009-present:	PhD candidate, AG Prof. Bals, Universitätsklinikum des Saarlandes, Homburg, Germany
04/2004-10/2008:	Diploma, Nutrition Science, University Hohenheim, Stuttgart, Germany
09/1995-07/2000:	Bachlor, Nutrition Science, Medical University in Tianjin, Tianjin, China

Working experience

02/2012-03.2015:	Antibodies-online GmbH, Manager China Operation, Aachen Germany
01/2009-06/2009:	G&G GmbH, Marketing and Sales manager, Düsseldorf, Germany