**HYPOXIA-INDUCED PULMONARY HYPERTENSION AND
CARDIAC DYSFUNCTION; THE ROLE OF
INFLAMMASOMES AND RELATED CYTOKINES**

FADILA TELAREVIC CERO

DISSERTATION FOR THE DEGREE OF PHILOSOPHIAE DOCTOR

DEPARTMENT OF PULMONARY MEDICINE AND

INSTITUTE FOR EXPERIMENTAL MEDICAL RESEARCH

OSLO UNIVERSITY HOSPITAL ULLEVÅL

AND UNIVERSITY OF OSLO

© **Fadila Telarevic Cero, 2019**

*Series of dissertations submitted to the
Faculty of Medicine, University of Oslo*

ISBN 978-82-8377-524-2

All rights reserved. No part of this publication may be
reproduced or transmitted, in any form or by any means, without permission.

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

ACKNOWLEDGEMENTS

I would like to thank my main supervisor, Professor Ole Henning Skjønberg, for seven years of training and cooperation. Under his supervision I have learned to combine knowledge and creativity when making decisions in research projects and I have learned that behind high-quality research lies hard work and dedication. During these years he has showed me that it is important to be structured and organized to lead a project group. I especially thank him for always being available for questions and advice and for giving me comprehensive feedback on each study and carefully considered criticism in the writing process.

Furthermore, I would like to thank my co-supervisor Professor Geir Christensen for his important contribution in planning of our research projects, his carefully considered feedback and inspiring cooperation. I have learned a lot from our work together.

Fredrik Borchsenius, Harald Mellem, Trond Bjørge and Department of Pulmonary Medicine are responsible for giving me the research time needed to complete my work and financing it. This has been a great opportunity and I am very thankful for it. Through Department of Pulmonary Medicine I have also had the chance to visit and to present my work at both national and international research conferences. I also received funding from Trelasthandler A. Delphin og hustrus legat.

I would also like to thank Ole Sejersted, Ivar Sjaastad, Lisbeth Hagen Winer and the Institute for Experimental Medical Research (IEMF) for allowing me perform my research in a professional environment and for letting me use their facilities. Skilful co-workers and well equipped labs make IEMF an inspiring place to work.

Finally, I am grateful to all my co-workers who have participated in the collection and interpretation of data as well as writing of manuscripts. I have had a particular close cooperation with Karl-Otto Larsen, Vigdis Hillestad and Camilla Udjus. Karl-Otto Larsen has been involved in all the projects and has provided help with experimental procedures, planning experiments and giving me helpful advice. Vigdis Hillestad and I have been working together on three of the Papers included in this thesis, while I have had a close cooperation on the Paper III with Camilla Udjus. Ivar Sjaastad has done all the echocardiographic recordings and has provided useful advice about image analysis and interpretations. Skilful technical help was provided by Dina Behmen, Almira Hasic, Solveig Sirnes, Hilde Dischington, Ulla Helene Enger and Ingeborg Goverud. Else Marit Løberg deserves a special gratitude for interpretation

of all histopathological preparations. Arne Yndestad, Bente Halvorsen and Pål Aukrust, from Institute for Clinical Research at Oslo University Hospital, Rikshospitalet, have been important collaborators in Paper II and III. You have all been important contributors and I am very lucky to have had the opportunity to work with all of you.

Finally, I give my warmest thanks to my dearest husband, Senad, father to our daughter Sara. You have been supportive during this process and you have motivated me all the time to do my best. You have been responsible for keeping me fed and happy, so I have been able to focus on work. Thank you so much for always being there for me.

TABLE OF CONTENTS

LIST OF PAPERS	8
SELECTED ABBREVIATIONS	9
INTRODUCTION	12
Inflammatory processes in the lung	12
Alveolar hypoxia	14
Innate immunity and inflammasomes	15
Activation of the inflammasome and innate immunity in lung disease (IL-18, IL-1 β and IL-12)	18
Pulmonary hypertension.....	20
Innate immunity in pulmonary hypertension and cardiac diastolic dysfunction.....	23
AIMS OF THE STUDY	25
METHODOLOGICAL CONSIDERATIONS	26
Animal models	26
Hemodynamic measurements	27
Magnetic resonance imaging of the heart.....	28
Histology	29
Immunohistochemistry	29
Western blotting	30
Quantitative real-time PCR (qRT-PCR)	30
Enzyme-linked immunosorbent assay (ELISA).....	31
Cell and tissue experiments	31
SUMMARY OF RESULTS	32
DISCUSSION	36
Innate immunity in hypoxia-induced inflammation and pulmonary hypertension	36
Innate immunity in cardiovascular disease	40

Anti-inflammatory treatment in hypoxia-induced pulmonary hypertension.....	42
Anti-inflammatory treatment in hypoxia-induced right heart remodeling and left ventricular diastolic dysfunction	43
MAIN FINDINGS AND CONCLUSIONS	45
REFERENCES	47

LIST OF PAPERS

PAPER I

Cero FT, Hillestad V, Løberg EM, Christensen G, Larsen KO, Skjønberg OH
IL-18 and IL-12 synergy induces matrix degrading enzymes in the lung.
Exp Lung Res, 2012;38(8):406-419.

PAPER II

Cero FT, Hillestad, Sjaastad I, Yndestad A, Aukrust P, Ranheim T, Gjervold IL, Olsen MB, Lien E, Zhang L, Haugstad SB, Løberg EM, Christensen G, Larsen KO, Skjønberg OH
Absence of the inflammasome adaptor ASC reduces hypoxia-induced pulmonary hypertension in mice.
Am J Physiol Lung Cell Mol Physiol, 2015;309:L378–L38.

PAPER III

Udjus C, **Cero FT**, Halvorsen B, Behmen D, Carlson CR, Bendiksen BA, Espe EK, Sjaastad I, Løberg EM, Yndestad A, Aukrust P, Christensen G, Skjønberg OH, Larsen KO
Caspase-1 induces smooth muscle cell growth in hypoxia-induced pulmonary hypertension.
In manuscript form.

PAPER IV

Hillestad V, Espe EK, **Cero FT**, Larsen KO, Sjaastad I, Nygard S, Skjønberg OH, Christensen G
IL-18 neutralization during alveolar hypoxia improves left ventricular diastolic function in mice.
Acta Physiol, 2015;213:492–504.

SELECTED ABBREVIATIONS

AECOPD: acute exacerbations of COPD

AH: alveolar hypoxia

AMS: acute mountain sickness

ASC: apoptosis-associated speck like protein containing a caspase recruitment domain

BALF: bronchoalveolar lavage fluid

CF: cystic fibrosis

COPD: chronic obstructive pulmonary disease

CTL: C-type lectins

DAMPs: danger-associated molecular patterns

ECM: extracellular matrix

FCAS: familial cold autoinflammatory syndrome

FEV1: forced expiratory volume in the first second

GOLD: Global Initiative for Chronic Obstructive Lung Disease

HACE: high-altitude cerebral edema

HAPE: high altitude pulmonary edema

HFNEF: heart failure with normal left ventricular ejection fraction

HIF: hypoxia-inducible factor

HPA-SMC: human pulmonary artery smooth muscle cells

HPV: hypoxic pulmonary vasoconstriction

IFN: interferon

IPF: idiopathic pulmonary fibrosis

IL: interleukin

IL-18BP: interleukin-18 binding protein

ILD: interstitial lung disease

LV: left ventricle

MMP: matrix metalloproteinases

mPAP: mean pulmonary artery pressure

NLR: nucleotide-binding oligomerization domain (NOD)-like receptor

NO: nitric oxide

PAAT: pulmonary artery acceleration time

PAMPs: pathogen-associated molecular patterns

PLB: phospholamban

PO₂: partial pressure of oxygen

pSTAT3: phosphorylated signal transducer and activator of transcription 3

ROS: reactive oxygen species

PRR: pattern recognition receptor

RV: right ventricle

RVOT: right ventricular outflow tract

RVSP: right ventricular systolic pressure

SERCA2: sarcoplasmic reticulum Ca²⁺ ATPase

SMC: smooth muscle cell

α -SMA: smooth muscle α -actin

SSc: systemic sclerosis

tau: time constant of isovolumic relaxation

TLR: toll-like receptor

TNF: tumor necrosis factor

TGF- β : transforming growth factor β

INTRODUCTION

Inflammatory processes in the lung

Inflammation is an essential component of lung diseases of various etiologies. Changes to the airway structure have been described in patients with asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). It has been proposed that airway inflammation drives these structural changes, and there has been a focus on investigating the involved mechanisms in these inflammatory processes [1, 2]. Obstructive lung diseases, such as asthma and COPD, are connected to both acute and chronic inflammation. Asthma is a disorder of the airways involving various inflammatory mediators and cells and is characterized by bronchial hyperresponsiveness and chronic inflammation [3]. Symptomatic attacks of asthma may be caused by several factors, such as allergens, viruses and pollutants, which may induce an additional, acute inflammatory response in the airways [4] and are characterized by sudden worsening of respiratory symptoms. Acute exacerbations of COPD (AECOPD) are also characterized by acute decline in airway function and worsening of the airway symptoms [5]. The common causes of AECOPD are viral and bacterial infections, but environmental stresses are also involved [5, 6]. A hallmark of COPD is complex underlying chronic inflammation that affects peripheral airways and lung parenchyma. This inflammatory response is thought to be triggered by inhaled irritants such as smoke and air pollutants [7]. These inhaled irritants can activate pattern recognition receptors such as Toll-like receptors (TLRs) on CD8⁺ T cells, an important cell type which is increased in the central and peripheral airways in COPD patients [8]. TLRs are key components of the innate immune system and acts by sensing danger signals such as pathogen-associated molecular patterns on bacteria and viruses or endogenous danger signals initiating inflammatory responses [9]. They are also a part of adaptive immune responses involving T- and B lymphocytes [10, 11]. Chronic inhalation of irritants initially activates TLRs in epithelial cells and macrophages. This leads to release of chemotactic factors that attract neutrophils and monocytes in COPD patients [7]. In later stages of the disease the adaptive immune responses are activated leading to increased number and activation of T- and B lymphocytes, as well as dendritic cells [7]. Thus, the inflammatory response in COPD is multifactorial involving both innate and adaptive immunity. Long-acting bronchodilators are the basis of COPD therapy, and there are currently no effective anti-inflammatory treatments. Inhaled corticosteroids have been shown to have limited clinical benefits in COPD patients, and there is a need for new anti-inflammatory agents [12].

Interstitial lung diseases (ILDs) are characterized by inflammation and fibrosis of the interstitium of the lungs. As the disease progresses, worsening hypoxemia and respiratory failure may develop [13]. Previous research has shown that neutrophils and macrophages may be important in driving the fibrotic processes in the lung, thus implicating a role for innate immunity in the development of lung fibrosis [14]. ILDs are a large group of diffuse parenchymal lung disorders, of which some, like idiopathic pulmonary fibrosis (IPF), one of the idiopathic interstitial pneumonias, is associated with high morbidity and mortality [15]. Many IPF treatment trials have had negative outcome. During the last years, however, some new compounds have shown beneficial effects on the progression of the disease [16]. Pirfenidone is a pyridone analogue which inhibits cytokines that play a key role in fibrosis and inflammation. Nintedanib is a tyrosine kinase inhibitor targeting several growth factor receptors resulting in reduced proliferation of human endothelial cells, vascular smooth muscle cells and myofibroblasts, and also reduced collagen secretion [17]. However, currently available therapies for IPF have limited effect, and the prognosis associated with this condition remains poor [18]. Systemic sclerosis (SSc) has the highest fatality rate among connective tissue diseases, and pulmonary involvement is considered to be the main cause of mortality [19]. There is also evidence suggesting that morbidity and mortality rates are raising for patients suffering from sarcoidosis in the USA, supporting the need to develop more effective anti-inflammatory therapies for this condition, as well [20]. Altogether, additional research is needed to develop more specific and effective therapies that could potentially improve outcome of patients with inflammatory diseases related to lungs and airways [14].

The outcome of patients with chronic lung diseases such as ILD and COPD are dependent on the severity of the disease and the related complications. Pulmonary hypertension is a serious complication with a high prevalence (30-40%) in ILD patients, leading to reduced exercise capacity and poor prognosis of these patients [21]. The prevalence of pulmonary hypertension in stable COPD varies from 20 to 91% depending on the definition of pulmonary hypertension (mean pulmonary artery pressure (mPAP) > 20 versus >25 mm Hg), the method of measuring the PAP (echocardiography versus right heart catheterization), and the severity of COPD (forced expiratory volume in the first second: FEV1) [22]. Pulmonary hypertension in COPD patients also adversely affects survival and exercise capacity and is associated with an increased risk of acute exacerbations [22]. Patients suffering from pulmonary hypertension have increased resistance in their pulmonary vessels leading to increased work load on the right ventricle (RV), a condition that may result in RV hypertrophy, and eventually dilatation

and failure [23-25]. During recent years, inflammatory processes have been claimed to be important in the pathogenesis of pulmonary hypertension due to lung diseases and/or hypoxia [26]. Furthermore it is shown that inflammatory processes are prominent features of various forms of pulmonary hypertension, and inflammation is claimed to be of major importance during the early phase of pulmonary hypertension [27]. The inflammatory cells produce chemokines and cytokines leading to vascular remodeling by regulating growth, migration, differentiation and metabolism of the different vascular cell types [27, 28]. Thus, better understanding of these inflammatory processes in pulmonary hypertension may identify innovative therapeutic strategies for the treatment of this devastating disease [27].

Alveolar hypoxia

Reduction of the alveolar partial pressure of oxygen (PO_2) (alveolar hypoxia, AH) may occur in chronic lung diseases of various etiologies, such as COPD and ILDs. Ventilation/perfusion mismatch, resulting from progressive airflow limitation found in COPD patients, contributes to the development of AH. It is well known that AH leads to hypoxic vasoconstriction in pulmonary arteries, resulting in increased pulmonary artery pressure [29]. Hypoxic constriction of the pulmonary arteries is a protective mechanism to reduce ventilation/perfusion mismatch by directing the blood flow to better ventilated alveoli. When AH is diffuse, such it is in severe COPD, it causes generalized pulmonary vasoconstriction and consequently higher pulmonary artery pressure [22]. Chronic hypoxia leads to complex pulmonary vascular remodeling [30], which further contributes to increased pulmonary vascular resistance [22]. Elevated pulmonary artery pressure is also a consequence of destruction of the pulmonary vascular bed by emphysema and hypoxic pulmonary vasoconstriction caused by polycythemia seen in COPD patients [22]. Polycythemia causes a local deficiency of nitric oxide (NO) and thus augments hypoxic pulmonary vasoconstriction [22]. Severe emphysema with air-trapping and hyperinflation gives positive alveolar pressure and contributes also to increased pulmonary artery pressure [22].

In addition to pulmonary arterial vasoconstriction, alveolar hypoxia leads to low oxygen levels in the blood (hypoxemia), and uncorrected chronic hypoxemia is associated with systemic inflammation and skeletal muscle dysfunction in COPD patients [29]. When ascending to high altitudes, the barometric pressure falls with increasing altitude in a logarithmic fashion. Thus, the partial pressure of oxygen (21% of barometric pressure) will

therefore also decrease and result in reduced alveolar PO_2 [31]. Acute hypoxic exposure for the first few days gives an increase in heart rate, myocardial contractility and cardiac output, while acclimatization results in decreased left ventricular work and cardiac output and increased right ventricular work [32].

Furthermore alveolar hypoxia causes hypoxic pulmonary vasoconstriction (HPV); this increases the pulmonary arterial pressure, causing pulmonary hypertension. Pulmonary hypertension together with “stress failure” of the pulmonary capillaries, causing leakage of proteins and white blood cells and disturbed alveolar fluid clearance, are thought to be responsible for high altitude pulmonary edema (HAPE) [31]. HAPE is a part of acute clinical syndromes that are a consequence of hypobaric hypoxia due to rapid ascent to high altitude. Acute mountain sickness (AMS) and high-altitude cerebral edema (HACE), both affecting the brain, are also parts of this cluster of hypoxia related acute clinical syndromes [31].

Systemic effects are also observed in other diseases with alveolar hypoxia, such as cardiovascular and metabolic dysfunctions of sleep-apnea, where inflammation is proposed to influence the development and outcome of these conditions [33]. Our research group has previously shown that alveolar hypoxia leads to increased circulating levels of the pro-inflammatory cytokine IL-18 [34], implicating this cytokine in the inflammatory process caused by alveolar hypoxia. Altogether it seems likely that inflammatory mediators may contribute to the pathogenesis of various systemic pathological processes during alveolar hypoxia [33].

Innate immunity and inflammasomes

There are two types of immunity used to protect the host from infections in vertebrates; innate and adaptive immunity. The innate immunity is conserved across evolution and is present in all multicellular organisms. It is the first line of defense and is able to trigger and guide the slower but more specific adaptive immune response, which is evolved through time and of newer evolutionary origin [35]. The innate immune system recognizes antigens that are common to a wide range of pathogens, while the adaptive immune system has more diverse recognition mechanisms where T-cell receptors and immunoglobulins are involved. This results in immunological memory, which provides immunity to pathogens that our body has previously been exposed to. Adaptive immune responses are mainly mediated through T- and

B-cell activation, while neutrophils, monocytes/macrophages, mast cells, natural killer cells and dendritic cells are effector cells in the innate immune response. The complement system and various soluble mediators secreted by these cells are also a part of innate immunity, and together they make up the first line defense against microorganisms and xenobiotics [36].

Furthermore it is suggested that the innate responses are not only the first line of defense in an immunological reaction against microorganisms, but also serves as a sophisticated system for sensing danger signals from host-derived signals of cellular stress and DNA-damage [37]. These danger signals are detected through various cellular receptors called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), membrane bound Toll-like receptors (TLRs) and C-type lectins (CTLs) [37, 38]. These receptors are also known as pattern recognition receptors (PRRs), and several cytoplasmic PRRs are known to function in inflammasome-based innate immunity. The NLR family constitutes the majority of PRRs that function in inflammasome assembly. NLRs can be divided into four subfamilies; NLRA with a transcriptional activation domain, NLRB with a baculovirus inhibitor of apoptosis repeat (BIR) domain, NLRC with a caspase recruitment domain (CARD), and the largest NLRP subfamily with a PYRIN (PYD) domain [39]. The PYRIN-CARD protein is also called apoptosis-associated speck like protein containing a caspase-recruitment domain (ASC) and functions as an adaptor which associates with procaspase-1 [40]. There are 23 NLR genes in the human genome and 34 NLR genes are found present in the mouse genome [41]. There are different activation patterns within the different inflammasomes and NLRs are involved in diverse signaling pathways [37, 41].

Inflammasomes that are well characterized are NLRP1, NLRP3, NLRC4, NLRP6, NLRP7 and AIM2. They activate caspase-1 through the interaction between the sensor and the adaptor protein called ASC [38, 42, 43]. The best characterized inflammasome is the NLRP3 inflammasome, and it consists of a sensor molecule NLR3, the adaptor protein ASC and caspase-1 [37, 38]. During activation, the inflammasome assembles and triggers the activation of caspase-1, which in turn cleaves pro-interleukin (IL)-18 and pro-IL-1 β in the cytosol and converts them to their mature forms. Mature IL-18 and IL-1 β are then released to the extracellular milieu where they can exert their effects. The NLRP3-inflammasome can be activated upon exposure to a wide range of stimuli like whole pathogens, such as fungi (e.g. *Candida albicans*), bacteria that produce toxins (e.g. *Listeria monocytogenes* and *Staphylococcus aureus*), and various viruses (e.g. sendai virus, influenza virus and

adenovirus) [44-47]. These bacterial and fungal cell-wall components and viral nucleic acids that activate the inflammasomes are named pathogen-associated molecular patterns (PAMPs) [48]. The NLRP3 inflammasome is also activated by danger-associated molecular patterns (DAMPs), host-derived molecules, which rise during cellular damage or stress, and include extracellular ATP, hyaluronan and uric acid [45, 49, 50]. Environmental irritants like silica, asbestos and ultraviolet irradiation, have also the ability to interact with the inflammasome and activate the cascade leading to activation of IL-18 and IL-1 β [51, 52], thus demonstrating that the inflammasome is activated upon a wide range of various stimuli, both non-sterile and sterile. There are different mechanisms proposed to trigger inflammasome activation, including potassium influx, mitochondrial dysfunction, lysosomal rupture and reactive oxygen species (ROS) production. However, the role of these events in inflammasome activation still remain unclear [53]. The regulation of the inflammasome activity takes place at transcriptional and posttranscriptional levels. Differential splicing of ASC can generate different ASC isoforms with even inhibitory functions, which may potentially be utilized to regulate inflammasome activity during the inflammatory response [54]. Further studies are needed to explore the exact mechanisms of activation and regulation of the inflammasome during different conditions.

Figure 1 The various activation mechanisms proposed for the NLRP3 inflammasome. Hosseinian N et al, Ther Adv Respir Dis. 2015. Reprinted with permission from SAGE publishing.

Activation of the inflammasome and innate immunity in lung disease (IL-18, IL-1 β and IL-12)

Familial cold autoinflammatory syndrome (FCAS) and Muckle-Well syndrome are inflammatory systemic diseases characterized by episodes of rash, arthralgia, fever and conjunctivitis. The differences are that FCAS symptoms are precipitated by cold exposure, while Muckle-Well syndrome is often associated with sensorineural hearing loss. These two diseases were the first to be linked to mutations in the inflammasome, and mutations in NLR genes were thought to be the cause of the diseases [55]. The role of the inflammasome is also important in lung diseases of other etiology. Several bacteria that can cause pneumonia have been shown to activate the NLRP3 inflammasome, like *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Staphylococcus aureus*, by their secretion of toxins [45, 56, 57].

Mycobacterium tuberculosis also has the ability to activate the NLRP3 inflammasome [58], as well as different types of viruses like influenza A virus [59], demonstrating that infections of various etiologies affecting the respiratory system activate the innate immune system through activation of the inflammasome.

A role for the inflammasome is also proposed in chronic airway inflammation where IL-1 β and IL-18 have been suggested to be involved in the development of COPD, and caspase-1 levels are found to be increased in lung tissue from these patients [60-62]. Increased levels of IL-1 β have been found in COPD patients and correlates with the severity of COPD [63].

Induction of IL-1 β production in the lungs of adult mice caused pulmonary inflammation, enlargement of distal airspaces, mucous cell metaplasia and airway fibrosis, a phenotype that resembles many of the features of COPD [64].

IL-18 protein has been shown to be strongly expressed in alveolar macrophages, CD8⁺ T-cells, and in both the bronchiolar and alveolar epithelia in the lungs of COPD patients [61].

Furthermore, this cytokine was significantly higher in the serum of patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III and IV, compared to smokers and nonsmokers without COPD, and a negative correlation between serum IL-18 levels and the forced expiratory volume in one second has been found, indicating that the level of IL-18 is related to the severity of COPD [61]. Transgenic IL-18 mice that constitutively overproduced mature IL-18 in the lungs showed chronic pulmonary lung inflammation with increased appearance of CD8⁺ T-cells, neutrophils, macrophages and eosinophils, in addition to increased production of interferon (IFN)- γ , IL-5, and IL-13.

Furthermore IL-18 overproduction led to severe emphysematous changes, dilatation of the right ventricle, and mild pulmonary hypertension [65]. These findings indicate that IL-18 may be an important mediator in pulmonary inflammation and features characteristic for COPD, raising the question whether IL-18 inhibition may be a feasible treatment in COPD [65]. Mice exposed to cigarette smoke have increased caspase-1 activity in their lung tissue, and also increased IL-18 and IL-1 β in bronchoalveolar lavage fluid (BALF), compared to mice breathing normal air [62]. Caspase-1 activity is also found to be higher in lungs of COPD patients and smokers compared to non-smokers, further indicating the inflammasome to be involved in the inflammatory process of this disease [62].

Asthma is another chronic airway inflammatory disease where inflammasome activation is proposed to play a role in the pathogenesis. Elevated levels of the IL-1 β protein have been shown to be present in the airways of patients with asthma [66], and there is also evidence supporting a role for IL-1 β in modulating airway constriction and relaxation via effects on airway smooth muscle [67]. Furthermore it has been observed that IL-18 and its receptor are strongly expressed in the lungs of patients with fatal asthma [68]. Circulating IL-18 levels are found to be significantly higher in patients with moderate or severe asthma compared to healthy controls [69]. These findings support a role of IL-1 β and IL-18 in the pathophysiology of asthma [69, 70].

In interstitial lung diseases, excessive accumulation of collagen and other extracellular matrix components in the lung interstitium and basement membranes are responsible for the impaired ventilatory function, which may lead to respiratory failure and death [71]. There are indications that the inflammasome is involved in the pathogenesis of fibrosis since particulates of asbestos, silica, bleomycin and statins, agents known to be able to initiate the fibrotic process, can activate the inflammasome and the production of active IL-1 β [72, 73]. Overexpression of IL-1 β in rat lung stimulates the production of transforming growth factor (TGF)- β , which is associated with progressive fibrosis in the lung [74]. The NLRP3 inflammasome is further claimed to be implicated in the pathogenesis of lung fibrosis in IPF and rheumatoid arthritis with histopathological pattern of usual interstitial pneumonia [75].

IL-12 is proposed to be an important regulator of both innate and adaptive immunity [76]. IL-12 is produced by macrophages and activated dendritic cells within hours after encounter with pathogens. IL-12 drives production of IFN- γ , and is capable of regulating T-cell development and natural killer cell function together with the function of antigen-presenting

cells [76], involving this cytokine in both innate and adaptive immune responses. Previous studies have shown that inflammatory effects of IL-18 are potentiated by IL-12 [77, 78], and with regard to lung disease, both IL-12 and IL-18 are increased in COPD patients, suggesting these two cytokines to be involved in the inflammatory pathways leading to COPD [61]. Furthermore, IL-12 may be involved in conditions involving hypoxia, since it has been shown that murine macrophages exposed to hypoxic condition produced higher levels of this cytokine [79].

Pulmonary hypertension

Pulmonary hypertension is defined as an increase in the mean pulmonary arterial pressure (PAP) ≥ 25 mmHg at rest, as assessed by right heart catheterization [80]. The underlying pathophysiological mechanisms are multifactorial, and the first clinical classification of pulmonary hypertension was established in 1998 when it was divided in two categories, primary pulmonary hypertension and secondary pulmonary hypertension according to the presence of identified causes or risk factors. Five categories of pulmonary hypertension exist currently, based on the underlying pathophysiology [80]. Current classification is presented in Table 1 (next page).

Table 1. Classification of pulmonary hypertension

1. Pulmonary arterial hypertension (PAH)

- 1.1. Idiopathic (IPAH)
- 1.2. Heritable
- 1.3. Drugs and toxins
- 1.4. Associated with:
 - 1.4.1. Connective tissue disease
 - 1.4.2. HIV infection
 - 1.4.3. Portal hypertension
 - 1.4.4. Congenital systemic-to-pulmonary shunts
 - 1.4.5. Schistosomiasis
- 1'. Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
- 1'' Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension due to left heart disease

- 2.1. Left ventricular systolic dysfunction
- 2.2. Left ventricular diastolic dysfunction
- 2.3. Left-sided valvular heart disease
- 2.4. Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung diseases and/or hypoxia

- 3.1. Chronic obstructive pulmonary disease
- 3.2. Interstitial lung disease
- 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4. Sleep-disordered breathing
- 3.5. Alveolar hypoventilation disorders
- 3.6. Chronic exposure to high altitude
- 3.7. Developmental abnormalities

4. Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions

5. Pulmonary hypertension with unclear multifactorial mechanisms

- 5.1. Hematological disorders: chronic hemolytic anemias, sickle cell, thalassaemia, myeloproliferative disorders, post-splenectomy
- 5.2. Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
- 5.3. Metabolic disorders: thyroid disorders, glycogen storage diseases, Gaucher's disease
- 5.4. Others: chronic renal failure, fibrosing mediastinitis, tumoral obstruction, segmental PH

Pulmonary hypertension might develop as a complication of COPD and other pulmonary diseases associated with hypoxia, such as interstitial lung disease, sleep disordered breathing, alveolar hypoventilation disorders and chronic exposure to high altitudes [81]. COPD and

other pulmonary diseases mentioned are often followed by low oxygen tension in the blood. It is known that acute alveolar hypoxia leads to a vasoconstrictor response in the pulmonary vascular bed, redirecting the blood to the best ventilated areas of the lungs [82]. Pulmonary arteries constrict to moderate to severe (2,7-8 kPa) hypoxia, whereas systemic arteries vasodilate. As previously mentioned, this phenomenon is called hypoxic pulmonary vasoconstriction (HPV), and is responsible for optimizing the ventilation–perfusion ratio during localized alveolar hypoxia. However, more widespread alveolar hypoxia results in generalized constriction of pulmonary arteries, resulting in increased load on the right side of the heart [83].

Chronic hypoxic exposure induces alterations in the structure of pulmonary arteries, in both biochemical and functional phenotypes of each of the cell types in the vascular wall [30]. Structural changes related to hypoxic exposure in mammals include the appearance of smooth muscle cells in previously non-muscularized vessels of the alveolar wall. There is also medial and adventitial thickening of the muscular and elastic vessels present [30]. It is believed that medial thickening is due to hypertrophy and increased accumulation of smooth muscle cells (SMCs) and increased deposition of extracellular matrix (ECM) components, mainly collagen and elastin. The increased deposition of ECM proteins, together with accumulation of fibroblasts and myofibroblasts, are responsible for the thickening of adventitial layer of pulmonary arteries. Collagen and elastin are also the main ECM components deposited in adventitia, together with fibronectin. In the larger pulmonary arteries, the media and adventitia also increase in thickness as response to chronic hypoxia [30]. The intimal layer undergoes the least amount of remodeling, but may include endothelial cell hypertrophy, subendothelial edema and fibrosis, and in some humans a longitudinal muscle layer can occur [84].

Figure 2 representing the different layers of an artery: intima, media and adventitia. Free illustration from Servier Medical Art. <https://creativecommons.org/licenses/by/3.0>

There is increasing evidence that both acute and chronic hypoxic exposure results in increased expression of inflammatory cytokines, chemokines, adhesion molecules, and accumulation of leukocytes within the lung and in particular around pulmonary vessels [30, 84], thus probably playing an important role in driving the vascular remodeling observed.

Innate immunity in pulmonary hypertension and cardiac diastolic dysfunction

Hypoxia is a common feature of chronic pulmonary diseases, and our research group has previously linked alveolar hypoxia to inflammasome activation through discovery of increased levels of mature IL-18 during hypoxic exposure [34]. In addition, Villegas et al. suggested ROS to be involved in the pathogenesis of hypoxia induced pulmonary hypertension through the NLRP3 inflammasome [85]. Clinically, increased levels of IL-1 β and IL-18 have been observed in patients with pulmonary arterial hypertension [86, 87]. IL-18 transgenic mice which have overproduction of IL-18 in their lungs, develop severe emphysema, mild pulmonary hypertension and dilatation of the right ventricle [65], further implicating IL-18 in the pathological processes involving the lungs. These studies support a possible role of the inflammasomes in the pathogenesis of pulmonary hypertension, but this mechanism has not been properly documented.

IL-18 has been suggested as an important mediator in diastolic dysfunction of the heart related to alveolar hypoxia [34]. Heart failure may be due to either systolic or diastolic dysfunction, or a combination. Diastolic heart failure is also referred to as heart failure with normal left ventricular ejection fraction (HFNEF) [88]. Diastolic dysfunction may have two underlying mechanisms, an abnormal active relaxation of the cardiomyocytes or increased ventricular stiffness [89]. Parameters used to diagnose diastolic left ventricular (LV) dysfunction can be obtained either invasively during cardiac catheterization or non-invasively by echocardiographic techniques. LV end-diastolic pressure, time constant of isovolumic relaxation (*tau*) and pulmonary capillary wedge pressure are invasive measures used to diagnose diastolic dysfunction [88]. Another parameter derived from pressure curves, the maximum negative rate of change of left ventricular pressure ($-dP/dt_{max}$), describes diastolic function, but is more dependent on the prevailing load whereas *tau* is relatively independent of both load and heart rate [90]. There are also several non-invasive measures that can be used to assess diastolic function, such as mitral valve flow velocity and tissue velocities [88].

The diastolic dysfunction related to alveolar hypoxia, as described in an experimental study by Larsen et al, was related to impaired relaxation of the myocardium due to reduced phosphorylation of the calcium handling protein phospholamban [91]. COPD and pulmonary hypertension, both conditions in which increased levels of IL-18 have been found, can lead to diastolic dysfunction, worsening the outcome of these patients [25, 92]. Daily administration of IL-18 to healthy mice induced interstitial fibrosis in the heart and myocyte hypertrophy resulting in increased ventricular stiffness. These results implicate IL-18 in the pathogenesis of left ventricular diastolic dysfunction [93].

In the present thesis, we have explored the role of the innate immune system in pulmonary inflammation, pulmonary hypertension and diastolic dysfunction. We have specifically examined the role of the inflammasome components NLRP3, ASC and caspase-1 in development of pulmonary hypertension and right ventricular remodeling. By administering IL-18 and IL-12, effects on mediators related to lung inflammation and emphysema were studied, as well as apoptosis. We have further focused on inhibition of IL-18 using a natural occurring antagonist and examined the effects on cardiac function.

AIMS OF THE STUDY

The main aim of the thesis was to explore mechanisms of pulmonary inflammation, pulmonary hypertension, and cardiac function and morphology related to hypoxia and innate immunity.

The specific aims were:

Paper I

To examine the effect of the cytokines IL-18 and IL-12 on inflammatory processes in the lungs.

Paper II

To investigate whether the inflammasome is activated during alveolar hypoxia and involved in the development of hypoxia induced pulmonary hypertension.

Paper III

To study the role of the inflammasome component caspase-1 in hypoxia-induced pulmonary hypertension and explore mechanisms for increased pulmonary artery pressure.

Paper IV

To examine whether inhibition of IL-18, a product of inflammasome activation, during alveolar hypoxia would prevent development of pulmonary hypertension and improve left ventricular (LV) diastolic function.

METHODOLOGICAL CONSIDERATIONS

Animal models

Mouse models are used extensively in the field of pulmonary and cardiac research to increase the knowledge on molecular mechanisms underlying various pulmonary and cardiac conditions. The aim is to achieve new understanding and often to develop new therapeutic principles for treating these diseases in humans. There are several advantages when using mouse models. Mice are small in size, which makes them easy to handle in animal facilities. It is also possible to acquire tissue for examination, which would not be as easily acquired from human subjects. Another important reason for using mice is the possibility to create genetically modified mouse models, where function of one particular gene can be either removed (knocked-out, KO) or added (overexpressed) to the mouse genome. In this way, we can study the phenotype of the respective genes.

In paper II and III we utilized mice lacking the gene for either NLRP3, ASC or caspase-1 to study the function of these components of the inflammasome. The details on how these knock-outs were created are described previously [94, 95]. They were made on C57Bl/6 background and we used C57Bl/6 mice as controls. This is the most widely used inbred strain which features low genetic variability and thus highly reproducible results in experimental studies. Here we used a model of chronic hypoxia to study the role of IL-18 and inflammsome components in the development of pulmonary hypertension, right ventricular hypertrophy and diastolic dysfunction of the heart. In our experiments, C57Bl/6 wild type mice and NLRP3, ASC and caspase-1 KO mice were 8 weeks old when placed in a tightly sealed chamber and exposed to 10% oxygen for 3 days and up to 3 months (Figure 3). The carbon dioxide concentration was monitored and kept under 0.4%, and humidity was measured daily. The control groups were breathing room air.

Figure 3 Mice in hypoxia chambers breathing 10% O₂ and mice breathing room air.

In paper I C57Bl/6 mice were utilized, and they received intraperitoneal (i.p.) injections with IL-18, IL-12 or both of these cytokines combined. Control mice received i.p. injections with phosphate-buffered saline (PBS). The purpose was to evaluate the inflammatory response in the lungs induced by these two cytokines.

A model of chronic alveolar hypoxia was also used in paper IV, where C57Bl/6 mice were placed in a hypoxia chamber, as previously described, for 2 weeks. They were treated with either IL-18 binding protein (IL-18BP) or vehicle (i.p.). Since IL-18 BP is a natural inhibitor of IL-18, the aim was to investigate whether inhibition of IL-18 during alveolar hypoxia would prevent development of pulmonary hypertension and improve LV diastolic function.

During all invasive procedures anesthesia by inhalation of isoflurane was used. Blood was drawn from inferior vena cava, and the heart and lungs were rapidly excised. The atria, right ventricular free wall, left ventricle and lungs were weighed and immediately snap frozen in liquid nitrogen and stored at -70°C. The blood samples were centrifuged to obtain serum or plasma. The blood and organs were used for ELISA, Western blot, PCR, histology and immunohistochemical analyses.

There are many advantages in using mice models, as mentioned previously, but there are also limitations. One of the most important questions is whether our results and findings are transferable to humans. There are genetically differences between the two species, but at the same time 99% of the mouse genes have a human homolog gene [96]. The biological processes are often similar, but may differ between species. Therefore, it is important to evaluate the findings in animal experiments thoroughly and underline that these findings are not necessarily relevant for humans.

Hemodynamic measurements

Our institute has access to specialized equipment used to investigate cardiac function in small animals such as high-frequency probes in echocardiography and pressure catheters of small size, which can be used to access the ventricles through the vessels on the neck. Cardiac catheterization is the gold standard for pressure measurements in the heart. Pressure measurements in the RV were performed with intact chest by catheterization of the right external jugular vein at 30 seconds, 2 minutes, 5 minutes, 2 weeks, 3 weeks, 1 month and 3 months of hypoxia with a 1.1 Fr Samba Preclin 420 LP micro pressure transducer (Samba

Sensors, Sweden) (papers II, III and IV). Pressure measurements were also performed in the LV (Paper IV), using the same Samba catheter for catheterization of the right carotid artery. Left ventricular systolic pressure, end-diastolic pressure and positive/minimum derivative of the pressure curve (dP/dt_{min}) were registered. The time constant of isovolumic relaxation (τ) was calculated using a custom made script fitting the pressure curve during relaxation phase. Echocardiography allows non-invasive, relatively fast and repeated examinations in each animal. Echocardiography was performed with VEVO 21000 (Visual Sonics, Toronto, Canada, Figure 4) to examine pulmonary artery acceleration time (PAAT), which is an indirect measurement of PAP. Mitral flow and tissue velocities were studied to assess LV diastolic function in paper IV.

Figure 4 Echocardiography at our institute.

Magnetic resonance imaging of the heart

Magnetic resonance imaging (MRI) was used to measure RV wall thickness in papers II, III and IV, right ventricular outflow tract (RVOT) flow in paper III, and RV volume in paper II. MRI experiments were performed using a 9.4T preclinical MR system (Agilent Technologies, Inc., Santa Clara, CA) with high-performance gradient and RF coils dedicated to mouse imaging (Figure 5).

Figure 5 MRI machine used to perform our experiments.

Histology

To study the pathological processes within the lungs, histological evaluation is very valuable. In our experiments, the lungs were sectioned transversely and stained with hematoxylin and eosin (HE). To assess the amount of collagen deposition around pulmonary arteries the sections were stained with acid fuchsin orange G-stain (AFOG) and Sirius Red. The amount of collagen deposition in the arterial wall was quantified by measuring the area of small arteries stained with AFOG by subtracting the area of the lesser curvature from the greater curvature and dividing by the lesser curvature x 100.

Immunohistochemistry

To study pulmonary leukocyte infiltration during hypoxic exposure, formalin-fixed paraffin-embedded serial sections of lungs were incubated with primary antibodies against myeloperoxidase (MPO) and CD3. To measure the number of alveolar macrophages and their functional status, lung sections were incubated with primary antibodies against F4/80, inducible nitric oxide synthase and CD206. This was to evaluate the cell influx in the lungs seen during hypoxia. To evaluate the expression of NLRP3 and ASC protein in the lungs and in the infiltrating cells, sections were incubated with primary antibodies against NLRP3 and ASC. We further examined the muscularization of arteries. First, the total number of peripheral arteries at alveolar duct and wall level was counted, as the number of arteries positive for von Willebrand factor per 100 alveoli. Five fields were assessed for each animal. Then immunostaining with smooth muscle α -actin (α -SMA) was used to quantify muscularization of arteries, which were categorized as fully or partially muscularized. Muscularization was measured as the percentage of fully or partially muscularized arteries of the total number of peripheral arteries. To assess presence of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) tyrosine 705 positive cells in pulmonary vessels of hypoxic WT and caspase-1^{-/-} animals, the number of positive nuclei were counted per vessel in the arterial wall. Six images were quantified for each animal.

In paper I, the lung sections were also examined with regard to presence of leukocytes and also incubated with primary antibodies against CD3, CD45R, FoxP3 and F4/80. The presence of IL-18 and IL-12 receptors was evaluated by using antibodies against these receptors. Antibody against active caspase-3 was used to quantify apoptosis following IL-18 and IL-12 injections.

Western blotting

Western blotting is a method used to examine the amount of a specific protein in the tissue, for example the lungs or the heart. The method is semi-quantitative and is based on the size of the different proteins within a tissue. The mixture of proteins isolated from the lungs or heart are separated by gel electrophoresis based on molecular weight. Subsequently they are transferred to a membrane, producing a band for each protein. The membrane is then incubated with antibodies specific to the protein of interest. Excessive antibodies are washed off, leaving the antibodies bound to the protein. The thickness and intensity of the band indicate the amount of the protein present within the tissue. In Paper I antibodies detecting the IL-18 and IL-12 receptors were used, as well as antibodies detecting both pro-forms and mature forms of matrix metalloproteinases (MMP)-9 and MMP-12. In papers II and III protein levels of caspase-1, IL-18 and IL-1 β were measured. Since the inflammasome activity is estimated by activity of these mediators, it was antibodies recognizing the mature forms that were used. In paper IV we were interested also in the relative amount of the phosphorylated form of the protein phospholamban (PLB) and not solely the total amount. Therefore it was important to avoid post-mortem changes, which was prevented by snap freezing the tissue in liquid nitrogen as soon as possible after the tissue was removed from the body of the animal. Levels of phosphorylated STAT3 (pSTAT3) at the tyrosine 705 and serine 727 residues and total STAT3 protein were measured by western blotting after snap freezing the tissue in paper III, to assess whether STAT3 could be important for promoting vascular smooth SMC proliferation.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR is a highly sensitive method used to study gene expression. The first step in the method is to extract RNA from the tissue. RNA is quite unstable and can easily be degraded by enzymes. Thus, it is important to snap freeze the tissue immediately after excision. When isolating RNA, it is important to do it in a lab constructed to avoid contamination with RNases and cDNA. The quality of the RNA isolated is evaluated before it is further reverse transcribed into cDNA. In the qRT-PCR, a probe recognizing the gene of interest is added to the samples together with a buffer needed for the reaction to take place, and the cDNA is amplified. A signal will be recorded, which indicates the amount of cDNA amplified. The

measured mRNA levels were normalized against an endogenous control which is not regulated.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is an assay used to detect and measure the presence of a substance in a liquid sample. In our experiments, we used ELISA to measure the levels of cytokines in the blood and in the media surrounding SMC outgrowth from pulmonary arteries. The method uses two antibodies. First, wells are pre-coated with detection antibodies which recognize the protein of interest. Then, secondary antibodies linked to an enzyme recognize the detection antibodies. In the final step, a substrate for the enzyme is added. The subsequent reaction produces a signal, a color change in the substrate. The intensity of the signal is measured and compared to a standard curve, which enables quantification of the protein. In our work, ELISA was used to measure the concentration of IL-18, IL-1 β and IL-6. In paper IV, ELISA was also used to detect the recombinant human IL-18BP which was injected in the animals.

Cell and tissue experiments

Human pulmonary artery smooth muscle cells (HPA-SMCs) were incubated with a caspase-1 inhibitor (Ac-YVAD-cmk). Cells were placed in a chamber under either hypoxic (1% O₂) or normoxic conditions for 4 hours, before they were harvested for RNA analysis.

Pulmonary arteries were harvested from caspase-1^{-/-} and WT mice and placed into culture dishes. After 3 weeks, the cell viability and number were measured by trypan blue staining and an automated Countess Cell counter. To study whether IL-18 is an important driver of SMC proliferation, IL-18 was added to caspase-1^{-/-} arteries before measuring proliferation. Furthermore, an IL-6 antibody and a pSTAT3 (tyr705) inhibitor was applied to caspase-1^{-/-} arteries treated with IL-18 to investigate their role in SMC proliferation. The caspase-1 inhibitor (Ac-YVAD) was applied on WT arteries to assess pharmacological inhibition of caspase-1 on SMC proliferation and to examine if similar effects occur as by knocking-out the caspase-1 gene.

SUMMARY OF RESULTS

Paper I: IL-18 and IL-12 synergy induces matrix degrading enzymes in the lung

In the first paper, we studied the presence of IL-18 and IL-12 receptors (IL-18R, IL-12R) in the lungs and whether IL-18 and IL-12, alone or in combination given i.p., have the ability to initiate mediators and pathological changes related to inflammatory processes in the lungs.

We found that:

- The expression of the IL-18R mRNA and IL-18R protein levels were abundant in the lungs compared to other organs (heart, liver, and spleen), and that IL-12R was also expressed in lung tissue.
- Mice treated with i.p. injection of recombinant murine IL-18 or IL-12 expressed significantly higher pulmonary mRNA levels of MMP-12 and cathepsin S, in addition to IFN- γ , tumor necrosis factor (TNF)- α , and CXC chemokine ligand 9 (CXCL9) than controls which had received PBS. A combination of IL-18 and IL-12 showed an even more pronounced induction of these mediators, as well as a significant increase in MMP-9, IL-6, IL-1 β , and TGF- β .
- Cellular apoptosis assessed by caspase-3 positive cells in lung tissue was increased in the group receiving IL-18 and IL-12 in combination.
- T-cell infiltration in pulmonary vessels following co-stimulation with IL-18 and IL-12 was prominent.

Paper II: Absence of the inflammasome adaptor ASC reduces hypoxia-induced pulmonary hypertension in mice

The aim of this study was to investigate the role of the inflammasome in hypoxia-induced pulmonary hypertension. Inflammasomes are part of the innate immune system and consist of an enzyme caspase-1, a receptor, where NLRP3 is the best characterized, and the adaptor protein ASC. We utilized mice deficient of NLRP3 and ASC and exposed them to 10% oxygen for three days, one and three months to investigate whether these components played a role in development of pulmonary hypertension. Control mice were breathing room air.

We showed that:

- Right ventricular systolic pressure (RVSP) of ASC^{-/-} mice was significantly lower than WT mice exposed to chronic hypoxia, indicating attenuation of pulmonary hypertension in mice lacking ASC. Furthermore, ASC^{-/-} mice displayed less remodeling of the pulmonary vasculature, as shown by reduced degree of muscularization and less fibrosis of the pulmonary arteries. RVSP of NLRP3^{-/-} mice exposed to hypoxia was not significantly altered compared to WT hypoxia.
- Magnetic resonance imaging supported these findings by demonstrating reduced right ventricular remodeling in ASC^{-/-} mice.
- Three days of hypoxic exposure demonstrated infiltration of leukocytes containing NLRP3 and ASC components around pulmonary vessels.
- Hypoxic exposure increased protein levels of caspase-1, IL-18 and IL-1 β in WT and NLRP3^{-/-} mice after three days and one month, showing inflammasome activation, while this response was absent in ASC^{-/-} mice.

Paper III: Caspase-1 deficiency reduces pulmonary hypertension

The aim of this study was to investigate the role of the inflammasome component caspase-1 in hypoxia-induced pulmonary hypertension. Here we utilized mice deficient of caspase-1 and subjected them to 10% oxygen to examine whether this enzyme influenced the development of pulmonary hypertension and right ventricle remodeling. In addition, mechanisms leading to hypoxia-induced pulmonary hypertension were explored.

We found that:

- Development of pulmonary hypertension in caspase-1 deficient mice was attenuated compared to WT mice after 2 weeks of hypoxic exposure.
- Right ventricular weight and magnetic resonance imaging showed reduced right ventricular remodeling in caspase-1^{-/-} compared to WT mice in hypoxia, which is in concordance with reduced pulmonary hypertension.
- Caspase-1^{-/-} mice displayed less remodeling of the pulmonary vasculature, as shown by reduced degree of muscularization of the pulmonary arteries.
- IL-18 and IL-1 β levels did not increase significantly in caspase-1^{-/-} mice after hypoxic exposure, in contrast to WT mice. Furthermore, there was less perivascular infiltration of leukocytes in caspase-1 deficient mice compared to WT, showing reduced inflammatory response in these animals.
- Upregulation of the IL-18/IL-6/STAT3 pathway observed in hypoxic WT mice was attenuated in caspase-1^{-/-} mice.
- Isolated arteries from caspase-1^{-/-} mice showed impaired smooth muscle cell proliferation compared to WT. Levels of IL-18, IL-1 β and IL-6 were also reduced in the surrounding media of WT arteries treated with caspase-1 inhibitor and in caspase-1 deficient arteries.
- IL-18 induced vascular smooth muscle cell proliferation in caspase-1 deficient pulmonary arteries, while IL-6 inhibition impaired proliferation significantly. pSTAT3 inhibition also showed a reduction in proliferation, although not reaching significant level. These data indicate that caspase-1 mediates smooth muscle cell proliferation through IL-18/IL-6/STAT3 signaling.
- Caspase-1 does not seem to be involved in acute hypoxic pulmonary vasoconstriction.

Paper IV: IL-18 neutralization during alveolar hypoxia improves left ventricular diastolic function in mice.

In this paper, we examined whether inhibition of IL-18, a product of inflammasome activation, would hamper the development of hypoxia-induced LV diastolic dysfunction. In addition, the influence of IL-18 inhibition on calcium handling in the LV was studied. First, we investigated whether IL-18BP given i.p. could inhibit the functional consequence of circulating IL-18 produced in vivo using LPS as a stimulus.

We showed that:

- LPS induced production of IL-18, which in turn induced release of IFN γ , as previously known from other studies. IL-18BP efficiently inhibited production of IFN- γ , indicating IL-18 inhibition.
- Exposure to hypoxia for two weeks induced LV diastolic dysfunction in mice, shown by prolonged time constant of isovolumic relaxation (*tau*).
- Mice treated with IL-18BP during hypoxia had a decrease in *tau* compared to the hypoxia vehicle group, indicating improved diastolic function.
- Hypoxic exposure induced decreased levels of serine16phosphorylated PLB, which were normalized by IL-18BP treatment, indicating a role for IL-18 in regulation of calcium-handling proteins in diastolic dysfunction.
- Mice exposed to hypoxia developed pulmonary hypertension and RV hypertrophy. MRI showed less increase in RV wall thickness in IL-18BP treated animals, showing a reduction in RV remodeling. RV systolic pressure was not significantly reduced by IL-18BP.

DISCUSSION

Chronic lung diseases of various etiologies are often associated with alveolar hypoxia, either regionally or globally. Sleep apnea, obesity hypoventilation syndrome, COPD, asthma, bronchial tumors, ILDs and neuromuscular and skeletal disorders affecting the chest wall are examples of conditions where alveolar hypoxia may occur [30]. Alveolar hypoxia triggers the development of pulmonary hypertension, which is a serious complication to the above mentioned diseases. Our research group has previously reported increased levels of IL-18 during hypoxic exposure of mice, indicating that hypoxia may be linked to inflammasome activation. While it is known that various danger signals, such as bacteria, virus and also sterile stimuli activate the innate immune system through activation of the inflammasome, the results of the current thesis have shown that hypoxia also seems to be perceived as a danger signal activating the immune system through this pathway. Articles included in this thesis support an important role for the innate immunity in development of hypoxia-induced pulmonary inflammation, pulmonary hypertension and cardiac dysfunction.

Innate immunity in hypoxia-induced inflammation and pulmonary hypertension

Localized acute hypoxic exposure leads to regional vasoconstriction of the pulmonary arteries to redirect blood to the better ventilated areas of the lungs, which is beneficial. More widespread alveolar hypoxia leads to generalized vasoconstriction, increased pressure in the pulmonary arteries and increased right ventricular afterload [83]. The link between alveolar hypoxia and development of pulmonary hypertension has not been fully elucidated [83]. Our previous finding of increased levels of IL-18 during alveolar hypoxia, prompted us to explore the role of innate immunity in this process by studying whether the inflammasome is activated during acute and chronic hypoxic exposure and how inflammasome activation may contribute to the development of pulmonary hypertension. In paper II, we exposed WT mice to 3 days, 1 month and 3 months of hypoxia. We found that acute hypoxic exposure for 3 days induced infiltration of leukocytes containing NLRP3 and ASC components around pulmonary vessels and bronchi, implicating involvement of inflammatory cells with the inflammasome. Activation of the inflammasome was shown by increased levels of active caspase-1, mature IL-18 and IL-1 β . This response was still present after 1 month, showing inflammasome activation both during acute and chronic hypoxic exposure. Having in mind that we could document high levels of IL-18 receptors in the lungs, indicating the lung as a target organ for

this cytokine, we wanted to study the effect of inflammasome activation on the development of hypoxia-induced pulmonary hypertension further, by utilizing mice deficient of the inflammasome components caspase-1, NLRP3 and ASC. We found decreased inflammasome activation in hypoxic mice lacking caspase-1 and ASC, corresponding with a significantly decreased RVSP compared to WT mice, both after 2 weeks and 3 months of hypoxia. Our results show that hypoxia acts as a danger signal, activating the innate immune system by inducing leukocyte infiltration and activation of the inflammasome. Furthermore, this thesis suggests that activation of the inflammasome is of importance for development of pulmonary hypertension, probably through caspase-1 mediated activation of the inflammatory cytokines IL-18 and IL-1 β .

Chronic hypoxia causes mainly medial and adventitial thickening of the pulmonary arteries due to hypertrophy and increased number of smooth muscle cells and increased deposition of ECM components, which lead to vascular remodeling, as previously mentioned. The mechanisms behind these changes are not fully known, but in all cases of hypoxia-related vascular remodeling and pulmonary hypertension, inflammation, and particularly persistent inflammation, is thought to play an important role [84]. It is suggested that a combination of hypoxia and local tissue factors/cytokines interact with SMCs, fibroblasts and macrophages and promote proremodeling and a proinflammatory phenotype, which in turn promotes transition to chronic nonresolving inflammation and vascular remodeling, leading to pulmonary hypertension [84]. In the current thesis, it is shown that the innate immune system, through activation of inflammasomes, seems to play an important role in inducing and maintaining this inflammation.

Our articles confirm vascular remodeling of pulmonary arteries after hypoxic exposure. Two weeks of hypoxia induced increased muscularization of pulmonary arteries, and after 3 months collagen deposition and fibrosis was also observed. Caspase-1^{-/-} mice showed reduced muscularization after 2 weeks of hypoxia, indicating a role for inflammasome activation in hypoxia-induced proliferation of SMCs. This finding was supported by decreased proliferation of hypoxia-exposed human SMCs in vitro after treatment with a caspase-1 inhibitor. Attachment of isolated pulmonary arteries to culture dishes seemed to trigger inflammasome activation, as documented by increased levels of IL-1 β in the medium surrounding WT arteries compared to caspase-1^{-/-} arteries. Moreover, the level of IL-18 was significantly reduced in the medium surrounding WT pulmonary arteries inhibited by a

selective caspase-1 inhibitor. Outgrowth of SMCs from arteries deficient in caspase-1 was significantly decreased compared to WT. Decreased outgrowth was also observed in WT pulmonary arteries treated with a caspase-1 inhibitor. Thus, hypoxia-induced proliferation of vascular SMCs seems to be linked to inflammasome activation. In addition to SMC proliferation, hypoxic exposure resulted in collagen deposition in the pulmonary artery wall of WT mice. This process was attenuated in mice lacking the ASC gene after 3 months of hypoxic exposure, while there were no significant differences in collagen deposition after 2 weeks of hypoxia between WT and mice lacking the caspase-1 gene. Our results indicate that the fibrotic process is connected to activation of the innate immune system through the inflammasome when exposed to long-term hypoxia.

After having substantiated that alveolar hypoxia activates the inflammasome, we tried to establish a link between this component of the immune system and the remodeling of pulmonary arteries leading to pulmonary hypertension. The proteolytic cleavage of the pro-forms of IL-18 and IL-1 β seems to be of major importance, leading to increased levels of the biologically active form of these inflammatory cytokines. IL-18 has been most thoroughly studied in the current thesis. Both of these cytokines are shown to be able to induce vascular smooth muscle cell proliferation and migration [97-99], as well as increased collagen production and proliferation of fibroblasts from various origins [100-102]. Therefore the observed reduction of muscularization and fibrosis in ASC^{-/-} and reduced muscularization in caspase-1^{-/-} mice may be due to the impaired activation of IL-18 and IL-1 β . In our experiments, IL-18 induced robust smooth muscle cell outgrowth from pulmonary arteries lacking caspase-1, indicating IL-18 to be an important stimulator of smooth muscle cell proliferation in pulmonary arteries. Our results may be relevant for pulmonary hypertension of various etiologies since different types of pulmonary hypertension have increased IL-18 and IL-1 β [86, 87, 103].

We further wished to explore possible downstream mediators of IL-18 and IL-1 β and analyzed a panel of proinflammatory mediators. IL-6 expression was significantly reduced in mice lacking caspase-1 compared to WT exposed to hypoxia. IL-1 β has previously been shown to induce IL-6 production [104]. The role of IL-6 was further examined by adding an IL-6 inhibitor to caspase-1 deficient arteries replenished with IL-18. In this experiment, we observed that IL-6 inhibition impaired SMC proliferation. IL-6 can induce tyrosine and serine phosphorylation of STAT3 [105], and STAT3 activation has been shown to be responsible for

modulation of the expression of several proteins already established in the pathogenesis of pulmonary hypertension [106]. Our experiments confirmed activation of pSTAT3 in WT animals during hypoxia, while this activation was absent in caspase-1 deficient mice. Furthermore, when a STAT3 inhibitor was added to IL-18 replenished caspase-1 deficient arteries, SMC proliferation was impaired. Taken together our results suggest the IL-6/STAT3 axis to be involved in vascular smooth muscle cell proliferation. Impaired IL-6 expression was also demonstrated in ASC^{-/-} mice exposed to hypoxia, strengthening the link between IL-6 and the inflammasome in the development of pulmonary hypertension. IL-6 has been found to be elevated in patients with pulmonary hypertension [86, 103, 107]. In addition, transgenic mice with lung specific IL-6 overexpression exhibit elevated right ventricular systolic pressure, right ventricular hypertrophy and increased muscularization of the pulmonary arteries, all of which were exacerbated by chronic hypoxia [108]. A pathogenic role for IL-6 in certain types of pulmonary hypertension is further supported by observations that IL-6^{-/-} mice are protected from pulmonary hypertension [109]. Previous studies, together with our findings, support a role of IL-6 in the pathogenesis of pulmonary hypertension.

It has been shown that hypoxia and changes in wall stress induces generation of ROS, activation of nuclear factor (NF)- κ B signaling, production of IL-6 and activation of hypoxia-inducible factor (HIF), which further activate downstream pathways leading to vascular remodeling in all three layers [84]. Matrix metalloproteinases are also involved in vascular remodeling [110], and the level of MMP-9 is positively correlated with the severity of experimental pulmonary hypertension [111, 112]. Our research revealed reduced MMP-9 and HIF-1 α in ASC deficient mice during hypoxic exposure, indicating that these two mediators may be involved in reduced pulmonary hypertension in mice depleted of ASC together with IL-6. Furthermore, in paper III we found reduced pSTAT3 protein levels in caspase-1^{-/-} mice exposed to hypoxia, supporting a role for the IL-6/STAT3 pathway in hypoxic pulmonary hypertension. Toll-like receptors (TLRs) and NOD-like receptors (NLRs) involved in inflammasome formation can activate a series of signaling pathways, resulting in upregulation of genes encoding pro-inflammatory cytokines, such as TNF α and IL-6 [113]. However, we did not find changes in TNF α expression in WT mice exposed to hypoxia. Our work indicates that inflammation and inflammasome activation is important in the development of hypoxic pulmonary hypertension through changes in medial and adventitial layers of the arteries, possibly through actions of IL-6/STAT3, in addition to MMP-9 and HIF-1 α .

In our first study, mice received IL-18 i.p. We discovered that IL-18 had the ability to induce inflammatory mediators and proteolytic enzymes known to participate in the pathogenesis of pulmonary emphysema. This effect was enhanced by co-stimulation with IL-12. Moreover, high levels of the IL-18R were present in the lungs compared to other organs, indicating the lungs to be an important target organ for IL-18. Since both IL-18 and IL-12 are found in COPD patients [61], it is possible that the effects of IL-18 and IL-12 are relevant for development of pulmonary inflammation and emphysema seen in these patients. Furthermore, pulmonary hypertension is a complication to COPD [81], and it is possible that the observed effects are relevant for COPD associated pulmonary hypertension, as well. Co-stimulation induced IL-6 expression, a cytokine shown to be involved in vascular remodeling in pulmonary hypertension both by others and by our group. Previously, it has been shown that pulmonary arterial hypertension patients have significantly higher levels of IL-12 compared with healthy control subjects [103]. Therefore, we cannot rule out that IL-12 is involved in vascular remodeling together with IL-18 through the actions of IL-6 in COPD patients.

Innate immunity in cardiovascular disease

IL-18 has previously been implicated in atherosclerotic plaque instability and is associated with cardiovascular disease risk [114]. The first studies that suggested IL-18 to be involved in human heart failure found increased circulating levels of IL-18 in patients with chronic heart failure. The IL-18 levels were higher in patients with severe heart failure and in those who died during follow-up compared to survivors [115, 116]. IL-1 β is also implicated in both patients and animal models of cardiovascular disease [117], and a significant correlation between declining functional class of heart failure and increasing levels of IL-1 β has been found [118]. These studies show a link between inflammasome activity and cardiovascular diseases of different etiology.

Patients suffering from pulmonary hypertension develop increased resistance in the pulmonary vascular bed and increased work load on the RV. These changes can result in RV dilatation and failure [25], a process relevant for chronic lung diseases associated with hypoxia. In our studies, we showed that healthy mice exposed to chronic hypoxia develop RV hypertrophy after 2 weeks and that they develop RV dilatation after 3 months. In Paper III caspase-1^{-/-} mice had reduced RV remodeling, as demonstrated by reduced RV weight and

RV free wall thickness, compared to WT mice after 2 weeks of hypoxic exposure. ASC^{-/-} mice showed no dilatation of the RV ventricle after 3 months of hypoxia, in contrast to WT mice (Paper II). The right ventricle is exposed to increased afterload by chronic pulmonary hypertension, and the initial adaptive response of myocardial hypertrophy is followed by a progressive contractile dysfunction and dilatation of the ventricle [25]. Since both caspase-1^{-/-} and ASC^{-/-} mice had reduced RV remodeling, it seems likely that these changes are due to reduced right ventricular systolic pressure (RVSP) and hence reduced workload on the RV. However, since both IL-18 and IL-1 β may promote myocardial changes like hypertrophy and myocardial dysfunction [117, 119], in the absence of increased afterload, a more direct impact of these cytokines in the process of cardiac remodeling is plausible, as well.

Diastolic dysfunction of the LV is a frequent echocardiographic finding in COPD patients, and is associated with disease severity [92]. A role for IL-18 in left ventricular diastolic dysfunction caused by alveolar hypoxia was proposed by our group in a study where mice exposed to hypoxia developed LV diastolic dysfunction in parallel with elevated levels of circulating IL-18 [34]. Larsen et al. showed that IL-18 plays a role in dephosphorylation of PLB, a protein that regulates the activity of sarcoplasmic reticulum (SR) Ca²⁺ ATPase (SERCA2). SERCA2 is located in the SR and is responsible for pumping Ca²⁺ into the SR and regulating cardiac muscle relaxation and cardiac contractility. When PLB is phosphorylated it dissociates from SERCA2, activating this Ca²⁺ pump, but in the dephosphorylated state PLB inhibits SERCA2 activity [120].

Based on these previous findings, we wished to investigate whether diastolic dysfunction induced by alveolar hypoxia could be improved by IL-18 inhibition, and if this inhibition was related to the phosphorylation status of PLB. In Paper IV mice developed LV diastolic dysfunction after 2 weeks of hypoxic exposure. This was shown by prolonged *tau*. When mice received IL-18BP, the relaxation of the myocardium improved, as shown by significantly reduced *tau* values. Furthermore, we found decreased levels of phosphorylated PLB in hypoxia, which were normalized by IL-18BP treatment. The restored levels of phosphorylated PLB can be a possible mechanism for the observed improvement in diastolic function by decreased inhibition of SERCA2 activity. However, we did not find a significant reduction in RVSP, although a trend towards both reduced RVSP and RV weight was found. This is in contrast to ASC^{-/-} and caspase-1^{-/-} mice, which both had reduced RVSP and RV

hypertrophy, and ASC^{-/-} mice did not develop RV dilatation after 3 months of hypoxia. Both ASC^{-/-} and caspase-1^{-/-} mice lacked the increase in circulating IL-18, which suggests a role for IL-18 in RV hypertrophy and remodeling. IL-18 and IL-18BP have been shown to play a role in cardiac hypertrophy [97, 121]. Our results, together with these previous studies, indicate that IL-18 may directly affect RV remodeling. However, we did not find a significant reduction in RVSP and RV weight by inhibiting IL-18 with IL-18BP, even though we found a trend. This may be due to incomplete inhibition of IL-18 activity by IL-18BP or that inhibition of both IL-18 and IL-1 β , as in mice lacking ASC and caspase-1, has synergistic effects on the development of pulmonary hypertension.

Anti-inflammatory treatment in hypoxia-induced pulmonary hypertension

Our studies show an important role for innate immunity in pulmonary inflammation and hypoxia-induced pulmonary hypertension. In addition, injection of IL-18 together with IL-12 in mice promoted expression of mediators involved in development of emphysema. Interestingly, this treatment also induced IL-6, a cytokine that previously has been shown to be involved in the development of pulmonary hypertension. Furthermore, by using mice lacking ASC and caspase-1, we demonstrated inhibited activation of IL-18 and IL-1 β during chronic hypoxia and a beneficial effect on hypoxia-induced pulmonary hypertension. This was shown by reduced RVSP, RV remodeling and reduced remodeling of the pulmonary arteries in ASC^{-/-} and caspase-1^{-/-} mice. These results suggest that innate immunity, and particularly components and mediators related to the inflammasome, could be pharmacological targets in treatment of pulmonary hypertension. Inhibitors of caspase-1, IL-18 and IL-1 β already exist and could be potential candidates for treatment of pulmonary hypertension in the future.

There are several therapies shown to be beneficial and approved for treating pulmonary arterial hypertension, such as endothelin-1 receptor antagonists, phosphodiesterase-5 inhibitors, guanylate cyclase stimulators and prostacyclin analogues [122]. However, currently there is no specific therapy for pulmonary hypertension associated with lung diseases. Drugs used for treating pulmonary arterial hypertension have so far not improved symptoms or outcome for this group of patients and are therefore not recommended for patients with pulmonary hypertension due to lung disease [122, 123]. Long-term

O₂-administration is shown to improve survival in stable COPD patients with resting hypoxemia (PaO₂< 7,3kPa) [124, 125], and long-term O₂-administration is the only therapy which has been shown to reduce progression of pulmonary hypertension in COPD patients and which is recommended in hypoxic COPD patients with pulmonary hypertension [122]. Thus, more effective medical treatment of pulmonary hypertension associated with lung disease is warranted [126].

Inflammation has been shown to play a role in hypoxia-related vascular remodeling and pulmonary hypertension, and persistent inflammation is thought to be particularly important [84]. Our findings support the theory that inflammation, and particularly inflammation due to innate immunity, is important in hypoxia-induced pulmonary hypertension.

Anti-inflammatory treatment in hypoxia-induced right heart remodeling and left ventricular diastolic dysfunction

Pulmonary hypertension is a common cause of right heart failure [25]. With regard to medical treatment, regression of RV hypertrophy has been demonstrated after 1 year of treatment with high-dose calcium channel blockers [127]. Prostacyclin treatment also gave a modest RV reverse remodeling with less dilatation and improved RV stroke volume in patients with pulmonary arterial hypertension [128, 129]. Riociguat, a guanylate cyclase stimulator, has decreased RV hypertrophy in rats which developed pulmonary vascular pathology similar to pulmonary arterial hypertension when exposed to vascular endothelial growth receptor antagonist and hypoxia [130]. However, since drugs used for treating pulmonary arterial hypertension seem not to improve symptoms or outcome for patients with pulmonary hypertension due to lung disease [122, 123], these treatment options are not available for this group of patients. Therapy for RV dysfunction associated with chronic lung disease would have to reduce both the increased pulmonary vascular resistance caused by hypoxic vasoconstriction and pulmonary vascular remodeling. Therefore, current recommendations only support long term oxygen therapy and the use of medication targeted at the underlying pulmonary disease [131]. We showed reduced RV hypertrophy and improved LV diastolic function by inhibiting circulating IL-18 (study IV). In study II and III circulating levels of IL-18 were reduced in ASC^{-/-} and caspase-1^{-/-} mice, concomitant with diminished RV

remodeling. To our knowledge, we are the first to show that inhibition of inflammasome components has a beneficial effect on RV remodeling. We are also the first to show improved diastolic function in hypoxic mice treated with an IL-18 inhibitor. Currently, there is no specific therapy to improve LV diastolic function directly, and treatment of underlying disease is therefore the most important therapeutic approach [132]. Our findings in hypoxia exposed mice suggest that inhibition of the inflammasome and related mediators could be an interesting approach to improve RV remodeling and diastolic function in patients with lung disease.

MAIN FINDINGS AND CONCLUSIONS

Paper I

Our research has shown a high concentration of receptors for the inflammatory cytokine IL-18 in mouse lungs, suggesting the lung as a target organ for this cytokine. Administration of IL-18 together with IL-12, cytokines found to be increased in COPD patients, leads to an inflammatory response demonstrated by increased proinflammatory cytokines, as well as T-cell infiltration in the lungs. Stimulation with IL-18 or IL-12 induced MMP-12 and cathepsin S, mediators known to be involved in the development of emphysema. These two cytokines seemed to have a synergistic effect, inducing an additional increase in MMP-9 and cellular apoptosis, implicating a role for IL-18 and IL-12 in emphysema development.

Paper II

In this study, we showed that mice lacking the inflammasome component ASC are partially protected from development of pulmonary hypertension and right ventricle remodeling in response to hypoxia. Hypoxia increased protein levels of caspase-1, IL-18 and IL-1 β in WT and NLRP3^{-/-} mice, showing inflammasome activation. Inflammasome activation was absent in ASC^{-/-} mice. ASC^{-/-} mice also displayed reduced muscularization and collagen deposition around pulmonary arteries. These data suggest an important role for the inflammasome in regulating vascular remodeling. NLRP3^{-/-} mice did not show attenuated development of pulmonary hypertension and they did not have reduced vascular remodeling of pulmonary arteries, indicating NLRP3 to be less important for the development of hypoxia-induced pulmonary hypertension. One possible explanation is that ASC and caspase-1 in combination with other inflammasome receptors than NLRP3 are implicated in this process.

Paper III

Caspase-1 deficient mice subjected to hypoxic exposure demonstrated reduced pulmonary hypertension and right ventricle remodeling compared to WT mice. They also displayed less remodeling of pulmonary vasculature, as shown by reduced degree of muscularization. Inflammasome activation was absent in caspase-1^{-/-} mice. These data further support the inflammasome to be important in hypoxia-induced pulmonary hypertension and cardiac remodeling. We also showed a down regulation of IL-6 and pSTAT3 in caspase-1^{-/-} mice when exposed to hypoxia, suggesting the IL-18/IL-6/STAT3 pathway to be involved in the development of hypoxia induced pulmonary hypertension.

Paper IV

In addition to pulmonary hypertension, alveolar hypoxia induces increased circulating levels of IL-18 and left ventricular diastolic dysfunction. The results in paper IV show that neutralization of IL-18 by IL-18BP leads to improved diastolic function of the left ventricle, possibly mediated through increased phosphorylation of phospholamban. IL-18 neutralization also attenuated hypoxia induced right ventricular remodeling. These data suggest that IL-18, a product of inflammasome activation, is of importance for cardiac function and remodeling.

REFERENCES

1. Aoshiba K, Nagai A: **Differences in airway remodeling between asthma and chronic obstructive pulmonary disease.** *Clin Rev Allergy Immunol* 2004, **27**(1):35-43.
2. Cantin AM, Hartl D, Konstan MW, Chmiel JF: **Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy.** *J Cyst Fibros* 2015, **14**(4):419-430.
3. Samitas K, Delimpoura V, Zervas E, Gaga M: **Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: current knowledge and future perspectives.** *Eur Respir Rev* 2015, **24**(138):594-601.
4. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM: **Asthma. From bronchoconstriction to airways inflammation and remodeling.** *Am J Respir Crit Care Med* 2000, **161**(5):1720-1745.
5. MacIntyre N, Huang YC: **Acute exacerbations and respiratory failure in chronic obstructive pulmonary disease.** *Proc Am Thorac Soc* 2008, **5**(4):530-535.
6. Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, McCormick M, Haldar K, Keadze T, Duvoix A *et al*: **Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers.** *Am J Respir Crit Care Med* 2011, **184**(6):662-671.
7. Barnes PJ: **New anti-inflammatory targets for chronic obstructive pulmonary disease.** *Nat Rev Drug Discov* 2013, **12**(7):543-559.
8. Nadigel J, Prefontaine D, Baglole CJ, Maltais F, Bourbeau J, Eidelman DH, Hamid Q: **Cigarette smoke increases TLR4 and TLR9 expression and induces cytokine production from CD8(+) T cells in chronic obstructive pulmonary disease.** *Respir Res* 2011, **12**:149.
9. Grishman EK, White PC, Savani RC: **Toll-like receptors, the NLRP3 inflammasome, and interleukin-1 β in the development and progression of type 1 diabetes.** *Pediatr Res* 2012, **71**(6):626-632.
10. Kabelitz D: **Expression and function of Toll-like receptors in T lymphocytes.** *Curr Opin Immunol* 2007, **19**(1):39-45.
11. Bernasconi NL, Onai N, Lanzavecchia A: **A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells.** *Blood* 2003, **101**(11):4500-4504.
12. Charron CE, Russell P, Ito K, Lea S, Kizawa Y, Brindley C, Singh D: **RV568, a narrow-spectrum kinase inhibitor with p38 MAPK- α and - γ selectivity, suppresses COPD inflammation.** *Eur Respir J* 2017, **50**(4).
13. Troy L, Corte T: **Interstitial lung disease in 2015: where are we now?** *Aust Fam Physician* 2015, **44**(8):546-552.
14. O'Dwyer DN, Ashley SL, Moore BB: **Influences of innate immunity, autophagy, and fibroblast activation in the pathogenesis of lung fibrosis.** *Am J Physiol Lung Cell Mol Physiol* 2016, **311**(3):L590-601.
15. Antoniou KM, Margaritopoulos GA, Tomassetti S, Bonella F, Costabel U, Poletti V: **Interstitial lung disease.** *Eur Respir Rev* 2014, **23**(131):40-54.
16. Bendstrup E, Hyldgaard C, Altraja A, Sjaheim T, Myllarniemi M, Gudmundsson G, Skold M, Hilberg O: **Organisation of diagnosis and treatment of idiopathic pulmonary fibrosis and other interstitial lung diseases in the Nordic countries.** *Eur Clin Respir J* 2015, **2**.
17. Adegunsoye A, Strek ME: **Therapeutic Approach to Adult Fibrotic Lung Diseases.** *Chest* 2016, **150**(6):1371-1386.
18. Daccord C, Maher TM: **Recent advances in understanding idiopathic pulmonary fibrosis.** *F1000Res* 2016, **5**.
19. Giacomelli R, Liakouli V, Berardicurti O, Ruscitti P, Di Benedetto P, Carubbi F, Guggino G, Di Bartolomeo S, Ciccio F, Triolo G *et al*: **Interstitial lung disease in systemic sclerosis: current and future treatment.** *Rheumatol Int* 2017, **37**(6):853-863.

20. Chiarchiaro J, Chen BB, Gibson KF: **New molecular targets for the treatment of sarcoidosis.** *Curr Opin Pulm Med* 2016, **22**(5):515-521.
21. Behr J, Ryu JH: **Pulmonary hypertension in interstitial lung disease.** *Eur Respir J* 2008, **31**(6):1357-1367.
22. Shujaat A, Bajwa AA, Cury JD: **Pulmonary Hypertension Secondary to COPD.** *Pulm Med* 2012, **2012**:203952.
23. Archer SL, Weir EK, Wilkins MR: **Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies.** *Circulation* 2010, **121**(18):2045-2066.
24. Naeije R: **Pulmonary hypertension and right heart failure in chronic obstructive pulmonary disease.** *Proc Am Thorac Soc* 2005, **2**(1):20-22.
25. Voelkel NF, Quaife RA, Leinwand LA, Barst RJ, McGoon MD, Meldrum DR, Dupuis J, Long CS, Rubin LJ, Smart FW *et al*: **Right ventricular function and failure: report of a National Heart, Lung, and Blood Institute working group on cellular and molecular mechanisms of right heart failure.** *Circulation* 2006, **114**(17):1883-1891.
26. Chen T, Yang C, Li M, Tan X: **Alveolar Hypoxia-Induced Pulmonary Inflammation: From Local Initiation to Secondary Promotion by Activated Systemic Inflammation.** *J Vasc Res* 2016, **53**(5-6):317-329.
27. Pullamsetti SS, Savai R, Janssen W, Dahal BK, Seeger W, Grimminger F, Ghofrani HA, Weissmann N, Schermuly RT: **Inflammation, immunological reaction and role of infection in pulmonary hypertension.** *Clin Microbiol Infect* 2011, **17**(1):7-14.
28. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR: **Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension.** *Circ Res* 2014, **115**(1):165-175.
29. Kent BD, Mitchell PD, McNicholas WT: **Hypoxemia in patients with COPD: cause, effects, and disease progression.** *International journal of chronic obstructive pulmonary disease* 2011, **6**:199-208.
30. Stenmark KR, Fagan KA, Frid MG: **Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms.** *Circ Res* 2006, **99**(7):675-691.
31. Paralikar SJ, Paralikar JH: **High-altitude medicine.** *Indian J Occup Environ Med* 2010, **14**(1):6-12.
32. Bartsch P, Gibbs JS: **Effect of altitude on the heart and the lungs.** *Circulation* 2007, **116**(19):2191-2202.
33. Chao J, Wood JG, Gonzalez NC: **Alveolar hypoxia, alveolar macrophages, and systemic inflammation.** *Respir Res* 2009, **10**:54.
34. Larsen KO, Lygren B, Sjaastad I, Krobert KA, Arnkværn K, Florholmen G, Larsen AK, Levy FO, Taskèn K, Skjøsberg OH *et al*: **Diastolic dysfunction in alveolar hypoxia: a role for interleukin-18-mediated increase in protein phosphatase 2A.** *Cardiovasc Res* 2008, **80**(1):47-54.
35. Raberg L, Vestberg M, Hasselquist D, Holmdahl R, Svensson E, Nilsson JA: **Basal metabolic rate and the evolution of the adaptive immune system.** *Proc Biol Sci* 2002, **269**(1493):817-821.
36. Kumar V, Sharma A: **Neutrophils: Cinderella of innate immune system.** *Int Immunopharmacol* 2010, **10**(11):1325-1334.
37. Schroder K, Tschopp J: **The inflammasomes.** *Cell* 2010, **140**(6):821-832.
38. Tschopp J, Schroder K: **NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production?** *Nat Rev Immunol* 2010, **10**(3):210-215.
39. Zhao Y, Shao F: **The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus.** *Immunol Rev* 2015, **265**(1):85-102.
40. Srinivasula SM, Poyet JL, Razmara M, Datta P, Zhang Z, Alnemri ES: **The PYRIN-CARD protein ASC is an activating adaptor for caspase-1.** *J Biol Chem* 2002, **277**(24):21119-21122.
41. Kanneganti TD, Lamkanfi M, Núñez G: **Intracellular NOD-like receptors in host defense and disease.** *Immunity* 2007, **27**(4):549-559.

42. Khare S, Dorfleutner A, Bryan NB, Yun C, Radian AD, de Almeida L, Rojanasakul Y, Stehlik C: **An NLRP7-containing inflammasome mediates recognition of microbial lipopeptides in human macrophages.** *Immunity* 2012, **36**(3):464-476.
43. Rathinam VA, Vanaja SK, Fitzgerald KA: **Regulation of inflammasome signaling.** *Nat Immunol* 2012, **13**(4):333-342.
44. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N *et al*: **Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA.** *J Biol Chem* 2006, **281**(48):36560-36568.
45. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM: **Cryopyrin activates the inflammasome in response to toxins and ATP.** *Nature* 2006, **440**(7081):228-232.
46. Muruve DA, Pètrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, Tschopp J: **The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response.** *Nature* 2008, **452**(7183):103-107.
47. Gross O, Poeck H, Bscheider M, Dostert C, Hanneschläger N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V *et al*: **Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence.** *Nature* 2009, **459**(7245):433-436.
48. Iwasaki A, Medzhitov R: **Control of adaptive immunity by the innate immune system.** *Nat Immunol* 2015, **16**(4):343-353.
49. Gasse P, Riteau N, Charron S, Girre S, Fick L, Pètrilli V, Tschopp J, Lagente V, Quesniaux VF, Ryffel B *et al*: **Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis.** *Am J Respir Crit Care Med* 2009, **179**(10):903-913.
50. Yamasaki K, Muto J, Taylor KR, Cogen AL, Audish D, Bertin J, Grant EP, Coyle AJ, Misaghi A, Hoffman HM *et al*: **NLRP3/cryopyrin is necessary for interleukin-1 β release in response to hyaluronan, an endogenous trigger of inflammation in response to injury.** *J Biol Chem* 2009, **284**(19):12762-12771.
51. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS: **The Nalp3 inflammasome is essential for the development of silicosis.** *Proc Natl Acad Sci U S A* 2008, **105**(26):9035-9040.
52. Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD: **The inflammasome mediates UVB-induced activation and secretion of interleukin-1 β by keratinocytes.** *Curr Biol* 2007, **17**(13):1140-1145.
53. He Y, Hara H, Núñez G: **Mechanism and Regulation of NLRP3 Inflammasome Activation.** *Trends Biochem Sci* 2016, **41**(12):1012-1021.
54. Bryan NB, Dorfleutner A, Kramer SJ, Yun C, Rojanasakul Y, Stehlik C: **Differential splicing of the apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) regulates inflammasomes.** *J Inflamm (Lond)* 2010, **7**:23.
55. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD: **Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome.** *Nat Genet* 2001, **29**(3):301-305.
56. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, McElvania-Tekippe E, Ting JP, Duncan JA: **Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells.** *PLoS One* 2009, **4**(10):e7446.
57. Witzentrath M, Pache F, Lorenz D, Koppe U, Gutbier B, Tabeling C, Reppe K, Meixenberger K, Dorhoi A, Ma J *et al*: **The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia.** *J Immunol* 2011, **187**(1):434-440.
58. Dorhoi A, Nouailles G, Jorg S, Hagens K, Heinemann E, Pradl L, Oberbeck-Muller D, Duque-Correa MA, Reece ST, Ruland J *et al*: **Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis.** *Eur J Immunol* 2012, **42**(2):374-384.

59. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JP: **The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA.** *Immunity* 2009, **30**(4):556-565.
60. Chung KF: **Cytokines in chronic obstructive pulmonary disease.** *Eur Respir J Suppl* 2001, **34**:50s-59s.
61. Imaoka H, Hoshino T, Takei S, Kinoshita T, Okamoto M, Kawayama T, Kato S, Iwasaki H, Watanabe K, Aizawa H: **Interleukin-18 production and pulmonary function in COPD.** *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2008, **31**(2):287-297.
62. Eltom S, Stevenson CS, Rastrick J, Dale N, Raemdonck K, Wong S, Catley MC, Belvisi MG, Birrell MA: **P2X7 receptor and caspase 1 activation are central to airway inflammation observed after exposure to tobacco smoke.** *PLoS One* 2011, **6**(9):e24097.
63. Hammad DR, Elgazzar AG, Essawy TS, Abd El Sameie SA: **Evaluation of serum interleukin-1 beta as an inflammatory marker in COPD patients.** *Egypt J Chest Dis Tu* 2015, **64**(2):347-352.
64. Lappalainen U, Whitsett JA, Wert SE, Tichelaar JW, Bry K: **Interleukin-1 β causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung.** *Am J Respir Cell Mol Biol* 2005, **32**(4):311-318.
65. Hoshino T, Kato S, Oka N, Imaoka H, Kinoshita T, Takei S, Kitasato Y, Kawayama T, Imaizumi T, Yamada K *et al*: **Pulmonary inflammation and emphysema: role of the cytokines IL-18 and IL-13.** *American journal of respiratory and critical care medicine* 2007, **176**(1):49-62.
66. Borish L, Mascali JJ, Dishuck J, Beam WR, Martin RJ, Rosenwasser LJ: **Detection of alveolar macrophage-derived IL-1 beta in asthma. Inhibition with corticosteroids.** *J Immunol* 1992, **149**(9):3078-3082.
67. Shore SA, Moore PE: **Effects of cytokines on contractile and dilator responses of airway smooth muscle.** *Clin Exp Pharmacol Physiol* 2002, **29**(10):859-866.
68. Oda H, Kawayama T, Imaoka H, Sakazaki Y, Kaku Y, Okamoto M, Kitasato Y, Edakuni N, Takenaka S, Yoshida M *et al*: **Interleukin-18 expression, CD8+ T cells, and eosinophils in lungs of nonsmokers with fatal asthma.** *Ann Allergy Asthma Immunol* 2014, **112**(1):23-28 e21.
69. Wang J, Zhang H, Zheng W, Xie H, Yan H, Lin X, He S: **Correlation of IL-18 with Tryptase in Atopic Asthma and Induction of Mast Cell Accumulation by IL-18.** *Mediators Inflamm* 2016, **2016**:4743176.
70. Whelan R, Kim C, Chen M, Leiter J, Grunstein MM, Hakonarson H: **Role and regulation of interleukin-1 molecules in pro-asthmatic sensitised airway smooth muscle.** *Eur Respir J* 2004, **24**(4):559-567.
71. dos Santos G, Kutuzov MA, Ridge KM: **The inflammasome in lung diseases.** *Am J Physiol Lung Cell Mol Physiol* 2012, **303**(8):L627-633.
72. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J: **Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.** *Science* 2008, **320**(5876):674-677.
73. Xu JF, Washko GR, Nakahira K, Hatabu H, Patel AS, Fernandez IE, Nishino M, Okajima Y, Yamashiro T, Ross JC *et al*: **Statins and pulmonary fibrosis: the potential role of NLRP3 inflammasome activation.** *Am J Respir Crit Care Med* 2012, **185**(5):547-556.
74. Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J: **Transient expression of IL-1 β induces acute lung injury and chronic repair leading to pulmonary fibrosis.** *J Clin Invest* 2001, **107**(12):1529-1536.
75. Lasithiotaki I, Giannarakis I, Tsitoura E, Samara KD, Margaritopoulos GA, Choulaki C, Vasarmidi E, Tzanakis N, Voloudaki A, Sidiropoulos P *et al*: **NLRP3 inflammasome expression in idiopathic pulmonary fibrosis and rheumatoid lung.** *Eur Respir J* 2016, **47**(3):910-918.
76. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ: **IL-12 and IL-23: master regulators of innate and adaptive immunity.** *Immunol Rev* 2004, **202**:96-105.

77. Carson WE, Dierksheide JE, Jabbour S, Anghelina M, Bouchard P, Ku G, Yu H, Baumann H, Shah MH, Cooper MA *et al*: **Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon- γ production and STAT-mediated signal transduction.** *Blood* 2000, **96**(4):1465-1473.
78. Munk RB, Sugiyama K, Ghosh P, Sasaki CY, Rezanka L, Banerjee K, Takahashi H, Sen R, Longo DL: **Antigen-independent IFN- γ production by human naive CD4⁺ T cells activated by IL-12 plus IL-18.** *PloS one* 2011, **6**(5):e18553.
79. Acosta-Iborra B, Elorza A, Olazabal IM, Martin-Cofreces NB, Martin-Puig S, Miro M, Calzada MJ, Aragonés J, Sanchez-Madrid F, Landazuri MO: **Macrophage oxygen sensing modulates antigen presentation and phagocytic functions involving IFN- γ production through the HIF-1 α transcription factor.** *J Immunol* 2009, **182**(5):3155-3164.
80. Galiè N, Humbert M, Vachieri JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M *et al*: **[2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension].** *Kardiol Pol* 2015, **73**(12):1127-1206.
81. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF *et al*: **Updated clinical classification of pulmonary hypertension.** *J Am Coll Cardiol* 2013, **62**(25 Suppl):D34-41.
82. Weir EK, Archer SL: **The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels.** *FASEB J* 1995, **9**(2):183-189.
83. Aaronson PI, Robertson TP, Knock GA, Becker S, Lewis TH, Snetkov V, Ward JP: **Hypoxic pulmonary vasoconstriction: mechanisms and controversies.** *J Physiol* 2006, **570**(Pt 1):53-58.
84. Pugliese SC, Poth JM, Fini MA, Olschewski A, El Kasmi KC, Stenmark KR: **The role of inflammation in hypoxic pulmonary hypertension: from cellular mechanisms to clinical phenotypes.** *Am J Physiol Lung Cell Mol Physiol* 2015, **308**(3):L229-252.
85. Villegas LR, Kluck D, Field C, Oberley-Deegan RE, Woods C, Yeager ME, El Kasmi KC, Savani RC, Bowler RP, Nozik-Grayck E: **Superoxide dismutase mimetic, MnTE-2-PyP, attenuates chronic hypoxia-induced pulmonary hypertension, pulmonary vascular remodeling, and activation of the NALP3 inflammasome.** *Antioxid Redox Signal* 2013, **18**(14):1753-1764.
86. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D: **Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension.** *Am J Respir Crit Care Med* 1995, **151**(5):1628-1631.
87. Ross DJ, Strieter RM, Fishbein MC, Ardehali A, Belperio JA: **Type I immune response cytokine-chemokine cascade is associated with pulmonary arterial hypertension.** *J Heart Lung Transplant* 2012, **31**(8):865-873.
88. Paulus WJ, Tschope C, Sanderson JE, Rusconi C, Flachskampf FA, Rademakers FE, Marino P, Smiseth OA, De Keulenaer G, Leite-Moreira AF *et al*: **How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology.** *Eur Heart J* 2007, **28**(20):2539-2550.
89. Zile MR, Baicu CF, Gaasch WH: **Diastolic heart failure-abnormalities in active relaxation and passive stiffness of the left ventricle.** *N Engl J Med* 2004, **350**(19):1953-1959.
90. Weiss JL, Frederiksen JW, Weisfeldt ML: **Hemodynamic determinants of the time-course of fall in canine left ventricular pressure.** *J Clin Invest* 1976, **58**(3):751-760.
91. Larsen KO, Sjaastad I, Svindland A, Krobert KA, Skjønberg OH, Christensen G: **Alveolar hypoxia induces left ventricular diastolic dysfunction and reduces phosphorylation of phospholamban in mice.** *Am J Physiol Heart Circ Physiol* 2006, **291**(2):H507-516.
92. Caram LM, Ferrari R, Naves CR, Tanni SE, Coelho LS, Zanati SG, Minicucci MF, Godoy I: **Association between left ventricular diastolic dysfunction and severity of chronic obstructive pulmonary disease.** *Clinics (Sao Paulo)* 2013, **68**(6):772-776.
93. Platis A, Yu Q, Moore D, Khojeini E, Tsau P, Larson D: **The effect of daily administration of IL-18 on cardiac structure and function.** *Perfusion* 2008, **23**(4):237-242.

94. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, Whitfield J, Barchet W, Colonna M, Vandenabeele P *et al*: **Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3**. *Nature* 2006, **440**(7081):233-236.
95. Özören N, Masumoto J, Franchi L, Kanneganti TD, Body-Malapel M, Erturk I, Jagirdar R, Zhu L, Inohara N, Bertin J *et al*: **Distinct roles of TLR2 and the adaptor ASC in IL-1 β /IL-18 secretion in response to *Listeria monocytogenes***. *J Immunol* 2006, **176**(7):4337-4342.
96. Mouse Genome Sequencing C, Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M *et al*: **Initial sequencing and comparative analysis of the mouse genome**. *Nature* 2002, **420**(6915):520-562.
97. Chandrasekar B, Mummidi S, Mahimainathan L, Patel DN, Bailey SR, Imam SZ, Greene WC, Valente AJ: **Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF- κ B- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin**. *J Biol Chem* 2006, **281**(22):15099-15109.
98. Kim HJ, Kim MY, Hwang JS, Kim HJ, Lee JH, Chang KC, Kim JH, Han CW, Kim JH, Seo HG: **PPAR δ inhibits IL-1 β -stimulated proliferation and migration of vascular smooth muscle cells via up-regulation of IL-1Ra**. *Cell Mol Life Sci* 2010, **67**(12):2119-2130.
99. Wang ZB, Kong LW, Kang J, Vaughn DM, Bush GD, Walding AL, Grigorian AA, Robinson JS, Nakayama DK: **Interleukin-1 β Induces Migration of Rat Arterial Smooth Muscle Cells Through a Mechanism Involving Increased Matrix Metalloproteinase-2 Activity**. *J Surg Res* 2011, **169**(2):328-336.
100. Fix C, Bingham K, Carver W: **Effects of interleukin-18 on cardiac fibroblast function and gene expression**. *Cytokine* 2011, **53**(1):19-28.
101. Hagood JS, Mangalwadi A, Guo B, MacEwen MW, Salazar L, Fuller GM: **Concordant and discordant interleukin-1-mediated signaling in lung fibroblast thy-1 subpopulations**. *Am J Respir Cell Mol Biol* 2002, **26**(6):702-708.
102. Postlethwaite AE, Raghov R, Stricklin GP, Poppleton H, Seyer JM, Kang AH: **Modulation of fibroblast functions by interleukin 1: increased steady-state accumulation of type I procollagen messenger RNAs and stimulation of other functions but not chemotaxis by human recombinant interleukin 1 α and β** . *J Cell Biol* 1988, **106**(2):311-318.
103. Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, Trembath RC, Jennings S, Barker L, Nicklin P *et al*: **Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension**. *Circulation* 2010, **122**(9):920-927.
104. Eda H, Burnette BL, Shimada H, Hope HR, Monahan JB: **Interleukin-1 β -induced interleukin-6 production in A549 cells is mediated by both phosphatidylinositol 3-kinase and interleukin-1 receptor-associated kinase-4**. *Cell Biol Int* 2011, **35**(4):355-358.
105. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F: **Principles of interleukin (IL)-6-type cytokine signalling and its regulation**. *Biochem J* 2003, **374**(Pt 1):1-20.
106. Paulin R, Meloche J, Bonnet S: **STAT3 signaling in pulmonary arterial hypertension**. *JAKSTAT* 2012, **1**(4):223-233.
107. Yoshio T, Masuyama JI, Kohda N, Hirata D, Sato H, Iwamoto M, Mimori A, Takeda A, Minota S, Kano S: **Association of interleukin 6 release from endothelial cells and pulmonary hypertension in SLE**. *J Rheumatol* 1997, **24**(3):489-495.
108. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB: **Interleukin-6 overexpression induces pulmonary hypertension**. *Circ Res* 2009, **104**(2):236-244, 228p following 244.
109. Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S, Eddahibi S: **Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice**. *Respiratory research* 2009, **10**:6.
110. Raffetto JD, Khalil RA: **Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease**. *Biochem Pharmacol* 2008, **75**(2):346-359.

111. Schermuly RT, Kreisselmeier KP, Ghofrani HA, Samidurai A, Pullamsetti S, Weissmann N, Schudt C, Ermert L, Seeger W, Grimminger F: **Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafertrine in chronic experimental pulmonary hypertension.** *Circ Res* 2004, **94**(8):1101-1108.
112. Lee FY, Lu HI, Zhen YY, Leu S, Chen YL, Tsai TH, Chung SY, Chua S, Sheu JJ, Hsu SY *et al*: **Benefit of combined therapy with nicorandil and colchicine in preventing monocrotaline-induced rat pulmonary arterial hypertension.** *Eur J Pharm Sci* 2013, **50**(3-4):372-384.
113. Boriushkin E, Wang JJ, Li J, Bhatta M, Zhang SX: **p58IPK suppresses NLRP3 inflammasome activation and IL-1 β production via inhibition of PKR in macrophages.** *Sci Rep* 2016, **6**:25013.
114. Jefferis BJ, Papacosta O, Owen CG, Wannamethee SG, Humphries SE, Woodward M, Lennon LT, Thomson A, Welsh P, Rumley A *et al*: **Interleukin 18 and coronary heart disease: prospective study and systematic review.** *Atherosclerosis* 2011, **217**(1):227-233.
115. Yamaoka-Tojo M, Tojo T, Inomata T, Machida Y, Osada K, Izumi T: **Circulating levels of interleukin 18 reflect etiologies of heart failure: Th1/Th2 cytokine imbalance exaggerates the pathophysiology of advanced heart failure.** *J Card Fail* 2002, **8**(1):21-27.
116. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, Seidler T, Hasenfuss G, Chvatchko Y, Shah AM *et al*: **Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure.** *FASEB J* 2004, **18**(14):1752-1754.
117. Bujak M, Frangogiannis NG: **The role of IL-1 in the pathogenesis of heart disease.** *Arch Immunol Ther Exp (Warsz)* 2009, **57**(3):165-176.
118. Van Tassell BW, Raleigh JM, Abbate A: **Targeting interleukin-1 in heart failure and inflammatory heart disease.** *Curr Heart Fail Rep* 2015, **12**(1):33-41.
119. Wang M, Markel TA, Meldrum DR: **Interleukin 18 in the heart.** *Shock* 2008, **30**(1):3-10.
120. MacLennan DH, Kranias EG: **Phospholamban: a crucial regulator of cardiac contractility.** *Nat Rev Mol Cell Biol* 2003, **4**(7):566-577.
121. Murray DR, Mummid S, Valente AJ, Yoshida T, Somanna NK, Delafontaine P, Dinarello CA, Chandrasekar B: **β 2 adrenergic activation induces the expression of IL-18 binding protein, a potent inhibitor of isoproterenol induced cardiomyocyte hypertrophy in vitro and myocardial hypertrophy in vivo.** *J Mol Cell Cardiol* 2012, **52**(1):206-218.
122. Galiè N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M *et al*: **2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT).** *Eur Heart J* 2016, **37**(1):67-119.
123. Mandel J, Poch D: **In the clinic. Pulmonary hypertension.** *Ann Intern Med* 2013, **158**(9):ITC5-1-16.
124. Kvale PA, Conway WA, Coates EO, Bower GC, Khaja FU, Adams KM, Lee JA, Kopacz M, Reeves J, Cugell DW *et al*: **Continuous or nocturnal oxygen therapy in hypoxemic chronic obstructive lung disease: a clinical trial. Nocturnal Oxygen Therapy Trial Group.** *Ann Intern Med* 1980, **93**(3):391-398.
125. Stuart-Harris C, J.M.Bishop, Clark TJH, Dornhorst AC, Cotes JE, Flenley DC, Howard P, Oldham PD: **Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party.** *Lancet* 1981, **1**(8222):681-686.
126. Cottin V: **Treatment of pulmonary hypertension in interstitial lung disease: do not throw out the baby with the bath water.** *Eur Respir J* 2013, **41**(4):781-783.
127. Rich S, Brundage BH: **High-dose calcium channel-blocking therapy for primary pulmonary hypertension: evidence for long-term reduction in pulmonary arterial pressure and regression of right ventricular hypertrophy.** *Circulation* 1987, **76**(1):135-141.

128. Hinderliter AL, Willis PWt, Barst RJ, Rich S, Rubin LJ, Badesch DB, Groves BM, McGoon MD, Tapson VF, Bourge RC *et al*: **Effects of long-term infusion of prostacyclin (epoprostenol) on echocardiographic measures of right ventricular structure and function in primary pulmonary hypertension. Primary Pulmonary Hypertension Study Group.** *Circulation* 1997, **95**(6):1479-1486.
129. Roeleveld RJ, Vonk-Noordegraaf A, Marcus JT, Bronzwaer JG, Marques KM, Postmus PE, Boonstra A: **Effects of epoprostenol on right ventricular hypertrophy and dilatation in pulmonary hypertension.** *Chest* 2004, **125**(2):572-579.
130. Lang M, Kojonazarov B, Tian X, Kalymbetov A, Weissmann N, Grimminger F, Kretschmer A, Stasch JP, Seeger W, Ghofrani HA *et al*: **The soluble guanylate cyclase stimulator riociguat ameliorates pulmonary hypertension induced by hypoxia and SU5416 in rats.** *PLoS One* 2012, **7**(8):e43433.
131. Kolb TM, Hassoun PM: **Right ventricular dysfunction in chronic lung disease.** *Cardiol Clin* 2012, **30**(2):243-256.
132. Aziz F, Tk LA, Enweluzo C, Dutta S, Zaeem M: **Diastolic heart failure: a concise review.** *J Clin Med Res* 2013, **5**(5):327-334.