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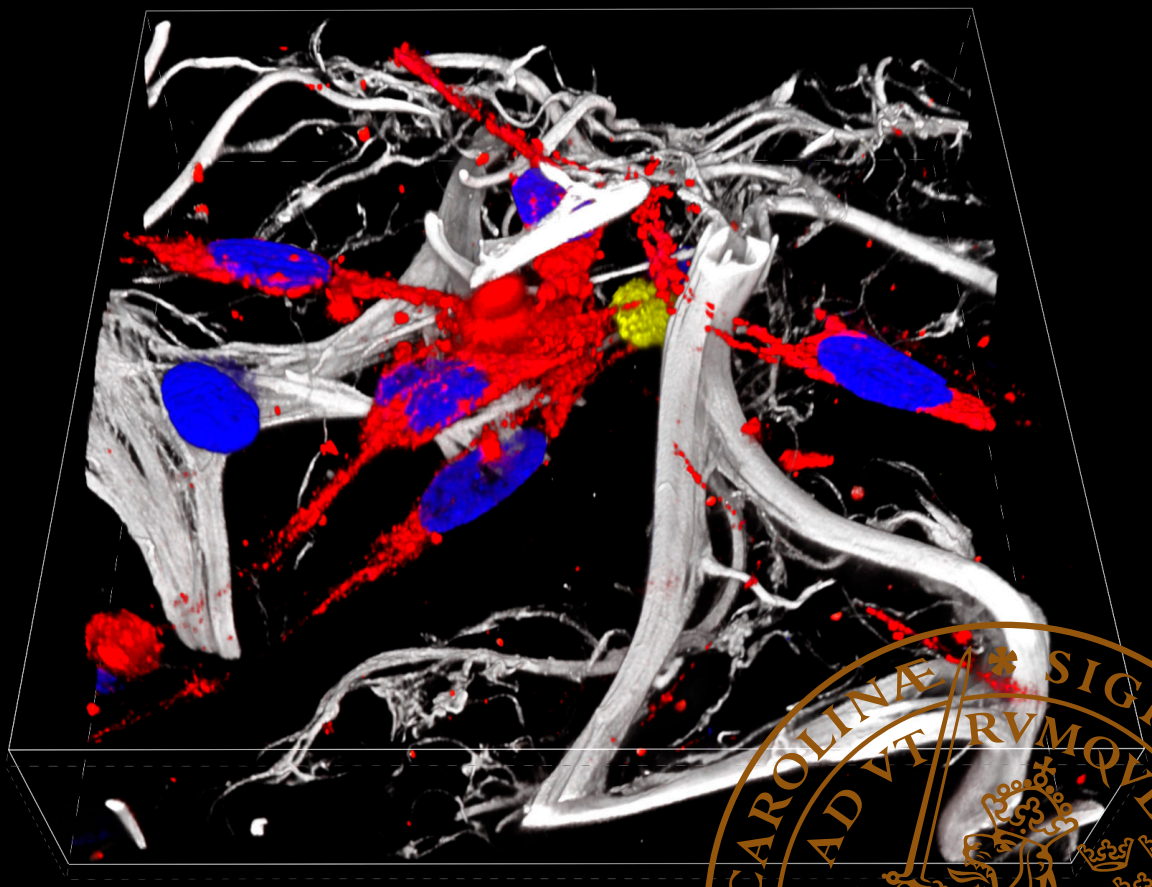

I believe in…

Optimism,
"Optimism is the faith that leads to achievement. Nothing can be done without hope and confidence."
Helen Keller

Creativity,
"Creativity is intelligence having fun."
Albert Einstein

Different thinking,
"Research is to see what everybody else has seen, and to think what nobody else has thought."
Albert Szent-Gyorgyi

…being the path to success.

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Mast cells and its crosstalk with mesenchymal cells in chronic lung diseases

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended In Belfrage lecture hall, BMC D15, Lund on the 14th of May 2019
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Abstract

The aim of this thesis is to demonstrate the complexity of cellular functions contributing to inflammation and remodelling in chronic lung diseases. Understanding the switch-over from physiological inflammation to chronic inflammatory processes with overproduction of mediators involved in remodelling is key to find novel therapeutic targets, regardless of the initial insult. Uncountable studies have reported mast cell involvement and high prevalence in allergy, asthma and cancer, whereas the role in chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary disease (IPF) is less described. Mast cells are great conductors, orchestrating immune and structural cell interactions during inflammation and remodelling. Fibroblasts are one of these cells interacting closely with mast cells and are known as major contributers to the remodelling processes due their ability to synthesise ECM. The goal is to delineate the crosstalk between mast cells and fibroblasts to understand the remodelling processes in chronic lung diseases.

The effects on migration, proliferation, morphology and mediator release in lung fibroblasts of mast cells and mast cell mediators such as proteases (tryptase and chymase), growth factors including vascular endothelial growth factor (VEGF) and transforming growth factor (TGFβ) was investigated in cells derived from healthy individuals and patients with IPF or COPD. Profibrotic TGFβ was used in order to induce a remodelling feature in our studies. The release of pro-inflammatory interleukin-6, angiogenetic feature of VEGF and anti-fibrotic hepatocyte growth factor (HGF) were investigated in our studies.

In this thesis we show that the protease activated receptor 2 (PAR-2), induced by tryptase, is a major regulator of fibroblast function, including migration, mediator release and cell morphology. Our findings indicate an important role of PAR2 in acute as well as chronic inflammatory diseases in which fibroblasts and mast cells are involved. Experiments performed in 3D lung scaffolds, confirmed the importance of extracellular matrix, as regulator of inflammatory mediators. The mast cell proteases, tryptase and chymase, showed different inflammatory mediator response on lung fibroblasts derived from healty individuals compared to patients with IPF, implicating that a turnover in mast cell subtypes from MC_T into MC_{TC} may occur during different inflammatory processes in chronic lung diseases.

These studies will help to better understand the complex mechanisms regulating the inflammation and remodelling processes in chronic lung disorders. Unwinding the crosstalk between mast cells and fibroblasts could improve therapeutic interventions for patients with chronic lung diseases.

Key words Mast cells, Fibroblasts, tryptase, chymase, extracellular matrix, Protease-activated receptor 2, Migration, remodelling,

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Image show: Confocal microscopy image of decellularized human lung scaffold (white) repopulated with fibroblasts (red) and mast cells (yellow).

Image By: Mariam Bagher (Experimental staining)
Sebastian Wasserström (Microscopy analyzation)

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“You are not a drop in the ocean.
You are the entire ocean in a drop”

by Rumi (Persian Poet)

Content

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Mast cells and mast cell tryptase enhance migration of human lung fibroblasts through protease-activated receptor 2. Bagher M, Larsson-Callerfelt AK, Rosmark O, Hallgren O, Bjermer L, Westergren-Thorsson G. *Cell Communication and Signalling*. 2018 Sep 15;16(1):59. doi: 10.1186/s12964-018-0269-3.

II. PAR-2 mediated interactions between mast cells and fibroblasts cause a switch in fibroblast morphology and cytokine profile. Bagher M, Rosmark O, Elowsson Rendin L, Nybom A, Wasserström S, Müller C, Hallgren O, Bjermer L, Larsson-Callerfelt AK, Westergren-Thorsson G. *Manuscript to be submitted in 2019.*

III. Mast cells and mast cell proteases alter cytokine and growth factor synthesis in healthy fibroblasts compared to IPF fibroblast. submitted 2019. Bagher M, Larsson-Callerfelt AK, Rosmark O, Nybom A, Hallgren O, Ahrman E, Malmström J, Bjermer L, Westergren-Thorsson G. *Manuscript.*

IV. VEGF synthesis is induced by prostacyclin and TGF-β in distal lung fibroblasts from COPD patients and control subjects: Implications for pulmonary vascular remodelling. Westergren-Thorsson G, Bagher M, Andersson-Sjöland A, Thiman L, Löfdahl CG, Hallgren O, Bjermer L, Larsson-Callerfelt AK. *Respirology*. 2018 Jan;23(1):68-75. doi: 10.1111/resp.13142.

Additional Peer-reviewed article, not included in the thesis

- Dermatan sulfate is involved in the tumorigenic properties of esophagus squamous cell carcinoma. Thelin MA, Svensson KJ, Shi X, Bagher M, Axelsson J, Isinger-Ekstrand A, van Kuppevelt TH, Johansson J, Nilbert M, Zaia J, Belting M, Maccarana M, Malmström A.
  Cancer Research. 2012 Apr 15;72(8):1943-52. doi: 10.1158/0008-5472.CAN-11-1351.

Selected abbreviations

HFL1	Human Feotal Lung Fibroblasts
LAD2	Laboratory of Allergic Diseases 2 (mast cell lines)
MC_T	Mucosal mast cells (Tryptase positive)
MC_{TC}	Connective tissue mast cells (Tryptase and chymase positive)
PBdMC	Peripheral blood derived mast cells
SCF	Stem cell factor
c-KIT	Tyrosine-protein kinase Kit Receptor
PAR-2	Protease-activated receptor 2
FCeRI	High affinity receptor for IgE
COPD	Chronic obstructive pulmonary diseases
IPF	Idiopathic pulmonary fibrosis
IgE	Immunoglobulin E
αSMA	Alpha-smooth muscle actin
VEGF	Vascular endothelial growth factor
HGF	Hepatocyte growth factor
IL-6	Interleukin 6
FGF2	Fibroblast growth factor 2
TGF-β	Transforming growth factor beta
ECM	Extracellular matrix

Preface

Mast cells, mostly known for their role in allergy as histamine releasing cells, have been studied in detail for decades. Histamine was first detected by the British scientist Henry H. Dale in 1910[1], while mast cells were already discovered in 1878 by the German scientist Paul Ehrlich[2]. Mast cells have evolutionary survived over 500 billion years, indicating their existence essential for most organisms. Mast cells are part of the innate immune system, and have different functions in protecting the host against venoms and parasites or regulating recruitment of other inflammatory cells. They are also involved in several processes, such as angiogenesis and wound healing, and important to adjust temporary homeostatic deviations. Upon activation, mast cells release different mediators including histamine, eicosanoids, proteases, cytokines and growth factors into the surrounding environment. During the past years mast cells and mast cell mediators have received attention regarding their involvement in physiological and pathological processes, mainly in asthma[3,4] but recently also in other chronic lung disorders such as chronic obstructive pulmonary disease (COPD) [5] and idiopathic pulmonary fibrosis (IPF)[6].

The general feature of chronic lung diseases are continuously persistent inflammation but also remodelling with an imbalance in turnover of extracellular matrix (ECM)[7,8,9,10,11]. Fibroblasts and phenotypes thereof, are the major producers of ECM proteins causing the maintained tissue remodelling. A continuous remodelling or defect wound healing process can cause emphysema or fibrosis with changed structure and elasticity of the tissue, which affects lung function. Importantly, parts of the lung tissue in these diseases have normal looking structure and composition of ECM whereas other parts have a clear pathological altered structure and ECM turnover[12]. These observations point at the importance of considering the microenvironment when studying cellular interactions.

Both mast cells and fibroblasts are considered to play an important role in the progression of chronic lung diseases. However, the crosstalk between these two cell types and how they influence each other, is poorly understood. The role of histamine is well-studied, but there are other mast cell mediators, such as tryptase and chymase, with less understood functions that are receiving more attention due to their altered expression in chronic lung diseases.

Therefore, the aim of this thesis was in particular to study in more detail the influence of mast cells on cellular functions and the crosstalk with lung fibroblasts derived from healthy individuals and patients with IPF or COPD. In particular, the role of the mast cell tryptase activating protease-activated receptor 2 expressed by fibroblasts was investigated. Different cellular functions including morphology, migration, synthesis of cytokines, growth factors and ECM proteins, were studied. Overall, our findings suggest a re-thinking about the role of mast cells and interactions with fibroblasts that can be of importance in understanding the mechanisms behind pathological conditions observed in chronic lung diseases. Findings, which in the future might be of importance for targeting new therapies for these serious diseases.

Introduction

Mast Cells

Mast cell origin and function

Mast cells were first discovered by the German scientist Paul Ehrlich in 1878, where he named them "Mastzellen" meaning well-fed cells, because their cytoplasm contained granular material[2]. Since then, knowledge and interest in mast cells has expanded during the last decades. Mast cells are inflammatory cells involved in the innate immune system, originating from the bone marrow. They circulate in the blood as $CD34^+$ hematopoietic progenitors and migrate to the targeted tissue where they differentiate and mature and become tissue specific mast cells[13, 14]. Mast cells are long-lived cells localized in all tissues in close connection to the external environment, such as lungs, skin, gastrointestinal tract, nasal mucosa and blood vessels. Several cytokines and growth factors are essential for mast cell survival, differentiation and maturation, however, the most important growth factor is stem cell factor (SCF)[15]. SCF binds to protein-tyrosine kinase receptor (c-KIT) expressed by mast cells, which has an important role in different intracellular communication and signalling[16]. Mast cells are multifunctional cells involved in a variety of different cellular functions and activities during vasodilation, angiogenesis, wound healing, angiogenesis, host defence against bacterial infections and parasites. Due to their capacity to release different multipotent mediators, they play an important role in regulating functions of different immune and structural cells such as dendritic cells, macrophages, T cells, B cells, fibroblasts, eosinophils, smooth muscle cells, endothelial cells and epithelial cells.

Mast cells contain many different mediators, such as cytokines (interleukin (IL)-3, IL-4, IL-13, IL-6, IL-17, SCF, FGF2, TNF-α)[17], serine proteases (tryptase, chymase, cathepsin G, Carboxypeptidase A3), Transforming growth factor (TGFβ), eicosanoids (prostaglandins and leukotrienes), proteoglycans (serglycin, heparin), histamine, serotonin and β-hexosaminidase[18, 19, 20]. There are two major types of mast cell degranulation, anaphylactic degranulation (AND) and piecemeal degranulation (PMD). AND is a rapid and explosive degranulation releasing the entire pre-stored content of the granules into the surrounding tissue. This is the common degranulation process during allergic reactions, which enables recruitment

of leukocytes and other inflammatory cells involved in the immune responses of body. PMD is a continuous and long-term degranulation process releasing specific mediators by small vesicles transporting the mediators to the plasma membrane and releasing them by fusion. Druing the last decade, more and more studies have suggested PMD to be involved in different diseases[21, 22, 23]. PMD has been suggested to have an important role in chronic processes such as wound healing, remodelling and other inflammatory diseases[24, 21]. Mast cells have been suggested to be involved in non-immunological mast cell degranulation, activated by cytokines and growth factors leading to increasing synthesis of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF)[25]. PMD has been identified during several different cellular events, including chronic psychosocial stress[26], CCL2[27], TLR stimulation[28]. However, the activation of PMD and its role remains to be fully understood. A third, even less studied mast cell degranulation process occurs via cell-cell interactions and is named trans-granulation.

Mast cell heterogeneity

There are two major subtypes of mast cells; mucosal mast cells (MC_T), mostly found in tissues of the lung and intestines and connective tissue mast cells (MC_{TC}) that are predominantly located in the skin, GI submucosa and blood vessels[29]. They are classified by their serine protease content, such that MC_T contain tryptase, and MC_{TC} contain both tryptase and chymase[30, 31]. However, a rare, third mast cell subtype has been reported as chymase positive, lacking tryptase, thus called MC_C[32]. Mast cell heterogeneity depends on the resident tissue where the maturation occurs and the microenvironmental conditions in the particular tissue.

Mast cell Proteases

One-third of all proteases is classified as serine proteases. As all proteases, they are enzymes that cleave proteins bounds connected to a serine side chain at the active site. Tryptase is a tetrameric neutral trypsin-like serine protease, consisting of four subunits, where each subunit has one active site. Although the name tryptase is similar to trypsin, there is a huge difference between them, such as physical and behavioural differences. There are two main subunits of tryptase, α-tryptase and β-tryptase, with 90% similar genetic sequence between them. Tryptase is pH and temperature sensitive, whereas it is stabilized by forming a complex with a glycosaminoglycan called heparin[29, 32]. Tryptase plays different roles in airway diseases, such as recruitment of other inflammatory cells, but may also act as mitogenic to fibroblasts[33]. They are also involved in airway homeostasis, vascular dilatation relaxation and contraction, airway smooth muscle activity and coagulation[34]. Previous studies have showed that mast cell tryptase may induces α-smooth muscle actin (αSMA) expression by fibroblasts[35].

Chymase, is chymotrypsin-type serine protease, but more destructive with an ability to cleave a range of different peptides. A lack of chymase induces an imbalance in the connective tissue, because of interaction with ECM such as fibronectin and non-helical collagens[36]. Chymase has an important role in tissue inflammation and remodelling, due to its proteolytic activity and degradation of ECM. Chymase binds to heparin and forms a molecular complex. Chymase may be important in activation and increasing the level of MMP-9, angiotensin II formation[37] and TGFβ[38, 39]. On the other hand, chymase may also be anti-inflammatory, being protective to venoms and parasites[40].

Mast Cells in Health and Chronic Diseases

Mast cells have an important role in the pathogenesis of both allergic and non-allergic chronic diseases. Mast cells and their mediators have been reported as key mediators in wound healing, fibrosis, cardiovascular disease and autoimmunity in addition to allergic inflammation[5,41]. Increasing numbers of mast cells have been observed in asthma[3, 4], idiopathic pulmonary fibrosis (IPF)[42], chronic obstructive pulmonary disorder (COPD)[5], especially those with smoking habits. Mast cells can be activated, not just by crosslinking of IgE-receptor, but also by different stimuli such as activation of other receptors as complement receptors (C3a and C5a), Toll-like receptors (TLR) and c-KIT receptor[43]. Mast cell activation results in a release of different mediators, such as cytokines, chemokines, and growth factors, proteases, into the surrounding tissue. Mast cells also contain bFGF and VEGF, which are involved in fibrosis and angiogenesis during tissue remodelling. By releasing histamine and cysteinyl leukotrienes, mast cells can recruit macrophages that can induce proinflammatory cytokines and lysosomal enzymes[5]. Mast cells recruit other inflammatory cells as eosinophils and lymphocytes[41]. Mast cells regulate many different processes in the immune system, whereas a persistant imbalance of these events may turn the tissue damage into chronic inflammation and remodelling. Mast cells contain the pro-fibrotic transforming growth factor beta (TGFβ), which promotes the migration, proliferation and differentiation of fibroblasts into myofibroblasts. Tryptase and chymase stimulates the proliferation of epithelial cells, smooth muscle cells, and fibroblasts. Kondo, S et al, indicated in their data, an important role for mast cell tryptase in ECM remodelling caused by renal fibroblasts, which can contribute to the development of renal interstitial fibrosis[42]. Mast cells are involved in inflammatory processes in fibrosis, however, a study suggested that both SCF and PAR-2 have an important roles in the recruitment of mast cells and maintenance of the pathology feature[44]. It has been suggested that a strong mast cell and fibroblast interaction is promoting the progression of IPF[44].

Fibroblasts

Fibroblast Origin and Phenotype

Fibroblasts are flat and spindle-shaped cells of mesenchymal origin, that are resident in all organ tissues[45, 46,47]. However, upon tissue injury, another source of fibroblasts originate from circulating myeloid-derived cells, fibrocytes, which can through paracrine activity induce fibroblast differentiation promoting remodelling[48]. Their morphology differ depending on which tissue they home to. Fibroblasts are major producers of ECM, building up the main structures of an organ-tissue. During wound repair and regeneration, fibroblasts can differentiate into myofibroblasts through an intermediate phenotype known as proto-myofibroblasts[49, 50]. All fibroblast subtypes can both produce and respond to a wide range of different cytokines, growth factors and matrix proteins. Myofibroblasts have some similarities to smooth muscle cells regarding their morphology. The characteristic feature of myofibroblasts is their high expression of αSMA stress fibres, which plays a crucial role in wound healing[51, 52, 53]. Uncontrolled proliferation or activation of these cells results in tissue fibrosis. However, beyond their ECM producing actions, fibroblasts also produce many different inflammatory mediators like cytokines, chemokines and growth factors, that in turn enhance the remodelling process in the tissue[12].

αSMA

As described earlier, myofibroblasts are the major producers of alpha smooth muscle actin (αSMA) fibres, which play an important role in cell division, motility and cell structure. They are a key feature in wound healing and tissue regeneration after tissue injury, regardless of the type of tissue[54 55] . During wound healing, a cascade of different cellular events starts. Fibroblasts migrate towards the site of injury, starting the production of new ECM, which creates a granulation tissue[56]. Fibroblasts differentiate into myofibroblasts that produce αSMA fibres, which are responsible for the tissue contractile activity. Because of their high ability to contract, the presence of myofibroblast/αSMA is a crucial step during wound healing, for wound closure [54 57 58]. During fibrosis, many different studies have reported high levels of αSMA as a result of enhanced myofibroblast presence. It has been suggested that ERK 1/2 and Rho associated kinase (ROCK) signalling pathways, are involved in cytoskeleton organization of actin filaments[59]. In lung diseases (particularly COPD), inflammation and remodelling is occurring, with high expression of αSMA compared to normal lung tissue[60, 61]. However, exactly how and by which mechanism the αSMA production is regulated remains to be elucidated.

Fibroblasts/Myofibroblasts In Health And Diseases

During normal wound healing, the differentiation of fibroblasts into myofibroblasts is crucial for tissue repair and regeneration. Once they have finished their "job", they undergo apoptosis in order to limit the ECM production and keep the balance of inflammatory and fibrotic mediators[62, 63]. However, the turnover from an apoptotic to a non-apoptotic myofibroblast remains still unclear.

Activated fibroblasts and myofibroblasts carry the main responsibility for tissue remodelling in chronic airway diseases, due to their high expression of matrix components[12]. The differentiation of lung fibroblasts into myofibroblast in chronic airway disease with ongoing remodelling, acts like a negative feedback loop. Fibroblasts are exposed to different profibrotic mediators that are released by already activated fibroblasts, which will further differentiate fibroblasts into ECM producers contributing to more remodelling and inflammatory mediator release[64]. The ECM produced by myofibroblasts is more unorganized, dense and in much higher levels compared to the ECM produced by fibroblast. Because of the large production of αSMA, myofibroblasts are more contractile, which in turn regulates the tissue architecture and stiffness[12, 65] .

Understanding the mechanisms behind the altered balance between myofibroblasts retained in the tissue for prolonged time, which allows them to continue their activities, and fate of apoptosis, may be the key to solve diseases involving tissue remodelling as a common feature[66.]

Extracellular Matrix

ECM is a complex three-dimensional scaffold, built of different proteins connecting to each other with strong molecular bindings. This scaffolds provides an environment and a protein-surface allowing to connect with by their receptors, which in turn induce a variety of different physiological cellular events[67]. By this, ECM and its interaction with the cells, is extremely important for controlling and regulating important cellular events in the body. ECM consists of many different proteins such as, proteoglycans, collagens, laminins, tenascin, fibronectin, elastin[68, 69, 70]. Extracellular matrix (ECM) is produced by several cells as airway smooth muscle cells, epithelial cells, fibroblasts and myofibroblasts, with the two latter responsible for the major production of ECM[12]. ECM provides structure and stability to the lung, and creates a well architected scaffold for the cells to grow on. This can be compared to a metaphor, as a house/lung scaffold for the people/cells to grow and establish themselves, where the ECM proteins act as building material for the house/lung[70].

As mentioned earlier, inflammation and remodelling are hallmarks of chronic lung diseases, where these go hand in hand over time during disease progression.

Airway remodelling involves alteration of the ECM composition, which due to its bioactivity[12], interacts with the surrounding cells and causes a homeostatic imbalance in the tissue. Dysregulation of ECM affects its shaping, which in turn affects different physiological (migration, proliferation, adhesion and differentiation) and pathological (cytokines, chemokine and growth factor release) cellular behaviours[12]. Different enzymatic and non-enzymatic biochemical reactions can regulate the assembly and dysregulation of ECM[12]. Many different ECM compartments have been reported to be involved in the pathogenesis of pulmonary diseases like asthma, COPD and IPF[70, 8, 9, 10, 11]. By recognizing, investigating and understanding the mechanisms behind these variations, we may get cues to lead us to new therapeutic targets.

Proteoglycans

During the past two decades, the importance of ECM proteoglycans (PG) have received lots of attention in chronic diseases. PGs act as organizers of biological tissue, where they provide perfect architected biological scaffolds for cell growth[71]. PGs consist of one heavy core protein with one or >100 covalently bound glycosaminoglycan (GAG) chains, which due to their negatively charged molecules, can create strong bindings to other proteins[72, 73]. These interactions are crucial not only for remaining the structure and function of ECM, but also for their role in regulating and organizing different biological activities required for a cell to act functionally correct[71, 74]. Recent years, the importance of GAGs, has been well documented and presented by different studies worldwide[75]. PGs exist both in ECM and on the cell surface, however until today, there is only one known PG that is present intracellularly, named as serglycin. Serglycin is located in the granules of inflammatory cells, mostly in mast cells and macrophages, and is the only PG that has heparin as sidechain. Serglycin, by its unique feature, acts as a linkage and connection to proteases located in the granules of inflammatory cells[76, 77]. This feature of serglycin allows it to regulate the activity of inflammatory cells, by controlling their mediator release, as well as cytokine and growth factor synthesis[78 49]. PGs have a variety of different activities in more or less complicated biological processes, like cell motility, proliferation, migration and differentiation. Some other important PGs in chronic inflammation and remodelling are biglycan, decorin and versican. During the past years, the role of GAGs and their influence in inflammatory immune response and modulators of cytokine and growth factor release, has received increasing attention. The most important GAGs in humans, are heparan sulfate/heparin (HS/Hep), chondroitin/dermatan sulfate (CS/DS) and hyaluronic acid (HA)[80, 74, 75]. Many different studies have reported increasing PG synthesis being involved in airway remodelling and hyperresponsiveness[81, 82, 83, 84, 85.]

Biglycan

Biglycan is a small PG with two GAG-chains consisting of CS/DS. Since the past 25 years, the importance of biglycan in inflammatory response during injury has been known. Already at 1993, Westergren-Thorsson et al., suggested that biglycan levels were enhanced during pulmonary inflammation[86]. Another study reported on biglycan playing an important role in cell morphology and αSMA distribution, thereby affecting fibroblast migration[87]. Biglycan also works as a pro-inflammatory factor promoting expression of IL-1β and IL-6[88, 89], demonstrating its important role as an inflammatory regulator[90, 91, 92].

Decorin

Decorin is a small, leucine-rich, extracellular CS/DS PG, involved in physiological and pathological processes in tissue. Interestingly, decorin has anti-fibrotic properties, due to its ability to bind to TGFβ[93,94]. Decorin also acts as a cross-linking molecule to keep collagen fibrils together. This means a decrease in decorin levels generates a looser collagen structure, while an increase of decorin renders thick and dense collagen structures as in fibrotic tissue[89]. Decorin has also been reported to interact with receptors important in inflammation and remodelling processes, such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (Met-receptor) and vascular endothelial growth factor 2 (VEGFR2). The latter one is involved in autophagy processes[95], suggesting that the interaction between decorin and VEGFR2 triggers autophagy and homeostasis in the tissue[95 96].

Versican

Versican is a well-studied and extracellularly located PG, with several CS/DS GAGs linked to its core protein. Versican remarkably increases during inflammation, and is known to interact both with inflammatory cells involved in immune responses of the body and also other ECM components[97,98]. Several studies have shown that versican stimulates inflammatory cells to release more cytokines and chemokines, which triggers cell migration, adhesion and differentiation[98]. Since tissue remodelling processes are dependent on the recruitment of inflammatory cells and their different mediators, versican acts as one of the important modulators and regulators of remodelling[98,84,99].

Perlecan

Perlecan is a large extracellular PG protein binding to a variety of different growth factors and basement membrane components, such as laminin and collagen IV. Perlecan are involved in different important cellular functions, due to their unique ability to bind to different heparin-binding molecules such as growth factors, proteases, ECM and basement membrane proteins[100, 101, 102].

Inflammatory Mediators Involved in Tissue Remodelling

TGFβ as a pro-fibrotic mediator

Transforming growth factor β (TGFβ), a member of a superfamily, is a growth factor essential for the cellular behaviours and activities such as differentiation, migration, adhesion, angiogenesis, wound healing, ECM production and apoptosis[103]. It is produced by a variety of different cells. TGFβ binds to one of its receptors, and activates different cellular activities and functions mostly through SMAD signalling pathway[103, 104]. Because of involvement in a wide range of important cellular activities, an alteration of TGFβ, may result in different diseases as cancer and fibrosis. However, because of this feature, TGFβ is mostly known as pro-fibrotic because of it accumulation of ECM, binding to biglycan, decorin and fibromodulin, contributing to tissue remodelling. TGFβ promotes fibroblast proliferation and differentiation into myofibroblasts, both as key players in fibrotic tissue. Thereby, TGFβ has an important role in the remodelling feature of chronic lung diseases, such as asthma[105], COPD[106,84] and IPF[107,108].

VEGF as an angiogenetic mediator

Angiogenesis is the biological process where new blood vessels are growing from already existing ones. Formation of new blood vessels, vascularisation, requires different growth factors and cytokines, whereas the most important is vascular endothelial growth factor (VEGF). VEGF is also involved in remodelling processes in chronic lung diseases[109,110,111,112]. Inflammatory cells, such as macrophages, lymphocytes and mast cells, and fibroblasts, produce important angiogeneic mediators that stimulate the processes of vascularisation[113]. IL-6 is one of these mediators that may trigger VEGF synthesis through different signalling pathways[114]. In an *in vitro* model of allergic airway disease, TGFβ synthesis was prohibited after VEGF inhibition, acting through PI3K/Akt signalling pathway[115]. Investigating the mechanism behind these events may provide knowledge about VEGF regulators, which can be used for preventing vascular remodelling in different chronic lung diseases.

IL-6 as a pro-inflammatory mediator

Interleukin-6 (IL-6), produced of a variety of cells, is a cytokine mostly known for its pro-inflammatory feature, but can also at some levels act as anti-inflammatory mediator in defence mechanisms[116,117]. During acute inflammation, IL-6 may act anti-inflammatory, contradictory to chronic inflammation, where it supports the pathogenesis of the disease[118]. In acute inflammation, IL-6 acts as a master cytokine through binding to its receptor, which is expressed by several inflammatory cells, thereby controlling and regulating other cytokines. The past years, a new term called myokine has been introduced as a cytokine produced and released by skeletal muscles. IL-6 has been identified both as a cytokine and a myokine, the latter suggesting a role in metabolism control[119]. IL-6 is involved in the pathogenesis of several chronic lung diseases such as asthma[120], IPF[121] and COPD[122]. Upon binding of IL-6 to IL-6R, different signalling pathways including JAK/STAT, Ras/Mitogen-Activated Protein Kinases (MAPK), Phosphoinositol-3 Kinase (PI3K)/Akt[123,124] may be activated. An anti-inflammatory drug, tocilizumab, blocking the IL-6 activity has showed efficacy for some inflammatory diseases like rheumatoid arthritis, systemic juvenile idiopathic arthritis, and Castleman's disease. Whether this anti-IL-6 drug is sufficient for pulmonary chronic lung disease, may not be easy to evaluate due to the dual contradictory effects of IL-6. However, more research in the role of IL-6 and other mediators in its signalling pathway is necessary to evaluate new treatment approaches[118, 124].

HGF as an anti-fibrotic mediator

Hepatocyte growth factor (HGF), also known as scatter factor, is a growth factor secreted by mesenchymal cells and acts mitogenic for a variety of cells such as fibroblasts, macrophages, smooth muscle cells, and epithelial cells in a variety of organs including heart, lung, kidney, liver, brain, and skin. HGF is involved in different cellular activities as proliferation, migration, differentiation, cell survival[125], and cytoskeletal organization[126]. HGF has been suggested to induce reorganization of the actin filament in epithelial cells, and act as a regulator for cytoskeletal construction and dynamics[127]. HGF binds to the cMET-receptor, which induces cellular response by activating Erk1/2 and Akt pathways. Erk1/2 in turn, has been reported to be involved in the SMAD signalling pathway, which regulates TGFβ activity[128]. Several studies have supported the hypothesis of HGF as an antifibrotic growth factor because of its feature as TGFβ regulator. Other studies have shown reduced TGFβ levels in animal models, preventing the progression of pulmonary[129] respectively myocardial fibrosis[130]. HGF has also been reported to reduce remodelling features caused by TGFβ in an asthma animal model[131,132]. Because of its wide distribution and expression in several cell types, and its

important feature as regulating TGFβ and remodelling, HGF pathway is an interesting therapeutic candidate for treating chronic lung diseases[128,129,133].

Protease-activated receptor 2 (PAR-2)

Protease activated receptor 2 (PAR-2), is a well characterized G protein-coupled receptor that is associated with inflammatory diseases, however it has an important role in tissue remodelling, Th2 cell activation, and other important roles in the progression of different inflammatory diseases. There are four different PARs, named PAR-1 to 4. PAR-1 is activated by thrombin, PAR-2 by trypsin and tryptase, PAR-3 by thrombin and PAR-4 by thrombin and trypsin. PAR-2 is expressed by several different cells such as fibroblasts, mast cells, endothelial cells, airway and vascular smooth muscle cells, macrophages and neutrophils[134]. Serine proteases such as tryptase, and trypsin[135], are the most common ligands for activation of PAR-2. PAR-2 is activated by proteolytic cleavage, by a ligand, which liberates its extracellular N-terminal domain and activates the loop II of the receptor, which in turn activates different signalling pathways such as MAP kinase ERK(1/2), JNK and p38 MAP kinase[136]. PAR-2 can also activate β-arrestin signalling pathway, independent of G-protein activation[137]. PAR-2 has been shown to be highly expressed in different chronic lung diseases as IPF [134, 138,139], compared to healthy lungs. A peptide, P2pal-18s, has been reported as a promising antagonist for the PAR-2 receptor expressed by human lung fibroblasts[140,141]. A previous study has demonstrated that PAR-2 inhibition by this peptide, reduces the pro-fibrotic and pro-inflammatory responses induced by PAR-2 activation[140]

Chronic Lung Disorders

Inflammatory Airway Diseases

One of the most advanced and architecturally complex organ in humans is the respiratory system, which is separated into upper and lower respiratory airways. The upper respiratory airways include the nose, nasal cavity, mouth, pharynx (throat) and larynx (voice box). The lower part of the respiratory airways includes the trachea and the lungs. The lungs are subdivided into central airways (bronchus), distal airways (bronchioles) and small airways (alveoli). The respiratory airways consist of a variety of different cells as fibroblasts, smooth muscle cells, epithelial cells, inflammatory cells (mast cells, macrophages, eosinophils, T-cells, B-cells, and dendritic cells). This multi-tasked organ is directly in contact with the inhaled air, thereby having an important host defence role.

Several different diseases affecting the airways are categorized as chronic lung diseases, and the most common ones are asthma and COPD, whereas IPF is a more rare lung disease.

These chronic lung diseases are associated with structural remodelling in the airways and parenchyma, affecting normal lung function[142]. However, the relationship between inflammation and remodelling, and which mediators and cells that are involved needs to be further clearified[143]. Investigating which type of cells respectively mediators involved in distribution and morphological changes that are responsible for these impaired lung functions, will help us to increase the understanding of underlying mechanisms of pathological and regenerative capacity of the lung. This will provide us with important knowledge for identifying and developing novel therapeutic targets for more effective treatment options[142].

Immune system

The human immune system of the body consists of innate immunity and adaptive immunity. Adaptive immunity, consisting of T cells and B cells, protects the body against specific pathogens and acts to kill pathogenic bacteria, fungi and virus. The innate immunity is evolutionary inherited and protects us from birth. The main function of the innate immunity is the recruitment of different inflammatory cells, after activation by stimuli from pathogens, allergens, irritations, and damaged cells.. The word inflammation comes from the latin word "*inflammare"* meaning "to set on fire". There are five classical criteria for inflammation, pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa). These are sign from the body to inform about the inflammation and start healing processes[144 145]. Different cells are involved in these cellular responses including macrophages, eosinophils, neutrophils, mast cells, and T cells (Th1 and Th2). The prevalence of T cells is differently distributed in asthma compared to COPD. In allergic asthma, the number of Th2 subtypes are predominant, while Th1 cells are the most common in COPD[146]. However, the balance of Th2/Th1 is not fully understood in IPF [147 48]. Both macrophages and neutrophils are sources of profibrotic cytokines and growth factors (TGFβ1, PDGF), chemokines and proteases (MMPs), which may affect remodelling processes[48].

Asthma

Disease characteristics

Asthma is a complex, heterogeneous disorder with chronic inflammation and remodelling of the airways[148]. Some characteristic features of asthma are airway hyperresponsiveness, high sensitivity against different stimuli and an increased

mucus secretion. The severity of asthma depends on the degree of airway remodelling and inflammation. This results in bronchoconstriction (contraction of smooth muscle cells), wheezing, coughing, and dyspnea (shortness of breath), and edema (microvascular leakage during acute exacerbations)[149].

The prevalence of asthma has enhanced drastically in developing countries, affecting 1 in 7 children and 1 in 12 adults[150]. Atopy associated asthma is more common in children than in adults with late onset of asthma[151]. Main reasons for the rise in asthma prevalence are suggested to be linked to increased allergen exposure, reduced childhood infections and poor bacterial exposure in the environment. Other suggestions may be changes in diet and increased antibiotic usage during childhood [151]. As a public health issue, asthma demands more efficient therapies, especially in more severe asthma. Therefore, it is important to clarify underlying cellular mechanisms behind disease phenotypes in order to be able to target the molecular pathways in asthma. The identification of new biomarkers is of utmost importance to be able to detect early signs of asthma, which hopefully could be suppressed by therapeutics and halt disease progression[151].

Pathophysiology

The pathology of asthma is heterogeneous and results in varying response to treatments. The different subtypes of asthma are characterized by specific clinical features that are caused by different predominant cell types. The eosinophilic Th2 type of asthma can appear both as allergic (atopy) and non-allergic, the latter one being more common in late-onset asthma while atopic asthma usually appears earlier in childhood, defined as early-onset asthma[152 153]. The different asthma types may appear similar, but are distinguished by the proportion of inflammatory cells involved[152 149] such as mast cells, eosinophils, neutrophils, and $CD4^+$ T-lymphocytes[152]. Interestingly, the increase of mast cell numbers in airway smooth muscles has been observed in asthma with bronchial hyperresponsiveness and obstructive airflow[151 154].

The neutrophilic asthma type is usually non-allergic and severe, characterized by high levels of neutrophilic inflammation. Regardless of the categorization of asthma into subtypes, some asthma features as airway inflammation, airway hyperresponsiveness and reversible obstructive airflow, are common for all of these subtypes. Asthma attacks can be triggered by different stimuli such as allergens or infections. Environmental factors such as air pollution and smoke cause injuries in airway epithelium that can worsen with virus infections.

A representative feature of asthma is the structural changes in the lung tissue, known as airway remodelling, which may be a response to the repair processes occurring due to the chronic inflammation. The airway remodelling is associated with airway wall thickening, preserved infiltration of inflammatory cells, release of growth factors, collagen deposition and hyperplasia. Some other characteristic feature of asthma due to remodelling is hyperplasia of nerves, microvessels,

angiogenesis, smooth muscle cells, and myofibroblasts. Increased numbers of smooth muscle cells and myofibroblasts have been observed to be directly proportional to asthma severity and chronicity in epithelial and mucosal regions. These cell types are mainly responsible for extracellular matrix deposition of proteins observed in the airways in lung explants from patients with asthma[152, 155, 156, 157].

COPD

Disease characteristics

COPD is the forth cause of death worldwide and is causing destruction and alteration of lung anatomy and structure. Patients with COPD have a 2-5 fold higher risk of developing lung cancer, compared with smokers without COPD[158 159]. COPD is classified by the Global initiative for chronic obstructive lung disease (GOLD) into five different stages, depending on the severity of the disease. The GOLD stages are defined by measuring lung capacity using spirometry, to define the volume of forcibly exhaled air in one second (FEV1) and forced vital capacity (FVC). The classification is based on a ratio between the measured FEV1 and FVC by spirometry. The lower this ratio is, the less severity of COPD[160 161].

Some characteristic features of COPD is a limitation in airflow and/or alveolar abnormalities, dyspnea, cough, wheezing, respiratory infections, mucus production, and mucociliary dysfunction. Exacerbations in COPD patients conduce to high morbidity and mortality[161]. The main cause of COPD is tobacco smoking, both active and passive. However, during the past years, more and more cases of COPD from non-smokers have been reported. Risk factors other than smoking are genetic factors, air pollution, biomass fuels, infection, accelerated aging, social and economic factors, bronchial hyperactivity (asthmatic smokers)[161 162]. Reduction in expiratory airflow and increased airflow resistance that is irreversible, is often the reason for remodelling and destruction of the airway tissue in small bronchioles (<2 mm diameter)[163 164 165].

Pathophysiology

One of the main features of COPD is pulmonary inflammation and remodelling with limiting airflow. Remodelling is associated with ECM accumulation and wall thickening (fibrosis) in the small airways, which is a result of uncontrolled tissue repair and excess of different inflammatory cytokines, proteases and proteinases. Contradictory to the bronchioles, the lung alveoli are undergoing emphysema, which is a condition with tissue destruction and enlarged airspaces in the distal airways extended beyond the terminal bronchioles[165]. Similar matrix alterations and αSMA positive cells have been observed both in the small and alveolar airways[165].

Remodelling and inflammation go hand in hand in COPD, where the abnormal balance of inflammatory cells have the potential to produce and proceed lung injury.

Different inflammatory cells both from the innate and adaptive immune system are involved during the inflammation in COPD[5 166]. Other inflammatory cells involved during inflammation are neutrophils and macrophages. The number of activated neutrophils are increased in COPD patients, however, their role is not totally defined. Neutrophils secrete serum proteinases, neutrophil elastase, cathepsin G, as well as matrix metalloproteinase 9 (MMP-9). Macrophages in the airways of COPD patients are much higher both in numbers and activity of inflammatory mediator release, compared to normal smokers. Some of these inflammatory mediators are tumour necrosis factor (TNF)-α, IL-8, CXC chemokines and MMP-9[5 166].

IPF

Disease characteristics

IPF is an interstitial lung disease with poor diagnosis with a median survival of 3 years. The prevalence is around 18-50 per 100,000 worldwide, and more common in men around the age of 65 year[167 168]. There are some evidence-based international guidelines developed by the respiratory societies around the world for the diagnostic criteria of IPF. According to those guidelines, the definition of IPF is a form of poor diagnosed chronic disease, limited to the lungs, with worsening lung function and dyspnea. The histological pattern of IPF is usual interstitial pneumonia[169]. A characteristic IPF lung feature is epithelial damage together with uncontrolled fibroblast proliferation and excess of cytokines released by inflammatory cells[64].

The cause of IPF is not known, however, environmental risk factors such as exposure to metal or wood dust, tobacco smoking, infections, genetic factors, and medications[64,170,171] have been suggested. There are no efficient therapies for IPF, and the therapeutic drug strategies for IPF have been widely debated during the last decade. Immunosuppressive and anti-inflammatory drugs have even worsened the prognosis of IPF. During the last decades, researchers around the world are in disagreement whether IPF is an inflammatory disease or not. There is evidence that IPF occurs long before diagnosed, however knowledge about the time frame between disease onset and time of diagnosis is still limited.

Pathophysiology

During normal wound healing, several mechanisms and cytokines are involved including the coagulation cascade, vascularization, fibroblast proliferation and migration, ECM and collagen synthesis. Different chemokines, growth factors and cytokines related to these mechanisms are released for recruitment of other inflammatory cells involved like mast cells, neutrophils, eosinophils and

monocytes. If the reparation and regeneration process becomes impaired and abnormal, the injury proceeds to inflammation, which in turn induces a release of inflammatory mediators (e.g. IL-1, IL-6, IL-8, TNF-α). The abundance of these cytokines and growth factors creates a severe loop, with chronic and persistent tissue remodelling[172]. Some genetic profiles of IPF fibroblasts remind of lung cancer fibroblasts. In line, several studies have reported a higher prevalence of lung cancer in IPF patients[64,173,174]. Following inflammation, the epithelium is left dysfunctionally with microinjuries which lead to regenerative actions involving epithelial and mesenchymal cells. In the IPF lung, normal repair mechanisms are disrupted with an imbalance between profibrotic and antifibrotic mediators[64]. This causes accumulation and overproduction of ECM resulting in remodelling and fibrosis[64 172]. Fibroblasts and myofibroblasts play a crucial role as major ECM producing cells in the pathogenesis of IPF. During normal wound healing and regeneration, the number of myofibroblasts is much lower compared to in the IPF lung, where they are excessively enhanced. Myofibroblasts contribute to the abnormal tissue structure in IPF lungs by overproduction of αSMA and characteristic contractile features[167,168,172]. This is contributing to the typical pattern of honeycombing observed in IPF-lungs[175]. One of the important inflammatory cells involved in the pathogenesis of IPF is the mast cell and its mediators. However their specific role in disease mechanisms is still elusive[176]. More research and knowledge about the interactions between inflammatory cells and mesenchymal cells, in the pathogenesis of IPF is warranted for better understanding of the disease progression and outcome[64].

Aims

The general aim of this thesis was to study the influence of mast cells on cellular functions of lung fibroblasts, and which role these interactions may have on inflammatory and remodelling processes in chronic lung diseases. Especially, my aim was to characterize the effect of tryptase and PAR-2 on migration, cytokine and growth factor profile along with fibroblast morphology in lung fibroblasts obtained from healthy individuals and patients with IPF or COPD.

The specific aims of the studies presented in this thesis were:

- To investigate the effects of mast cells and mast cell tryptase on the migratory capacity of fibroblasts, and the role of Protease-activated receptor-2 (PAR-2) (Paper I).
- To further study the role of PAR-2 antagonist and its effects on the cytokine profile and morphology of fibroblasts in presence and absence of mast cells (Paper II).
- To explore the effects of mast cells and the mast cell proteases, tryptase and chymase, on inflammatory mediators and ECM profile in lung fibroblasts derived from healthy subjects and IPF patients with (Paper III).
- To investigate the role of VEGF and TGFβ in ongoing vascular remodelling processes in lung fibroblasts derived from healthy subjects and patients with COPD patients (Paper IV).

Methodology

Different biological human materials and experimental techniques were used in this thesis in order to explore and investigate the inflammatory mediator profile and ECM interactions between fibroblasts and mast cells, together with pharmacological interventions (Fig 1).

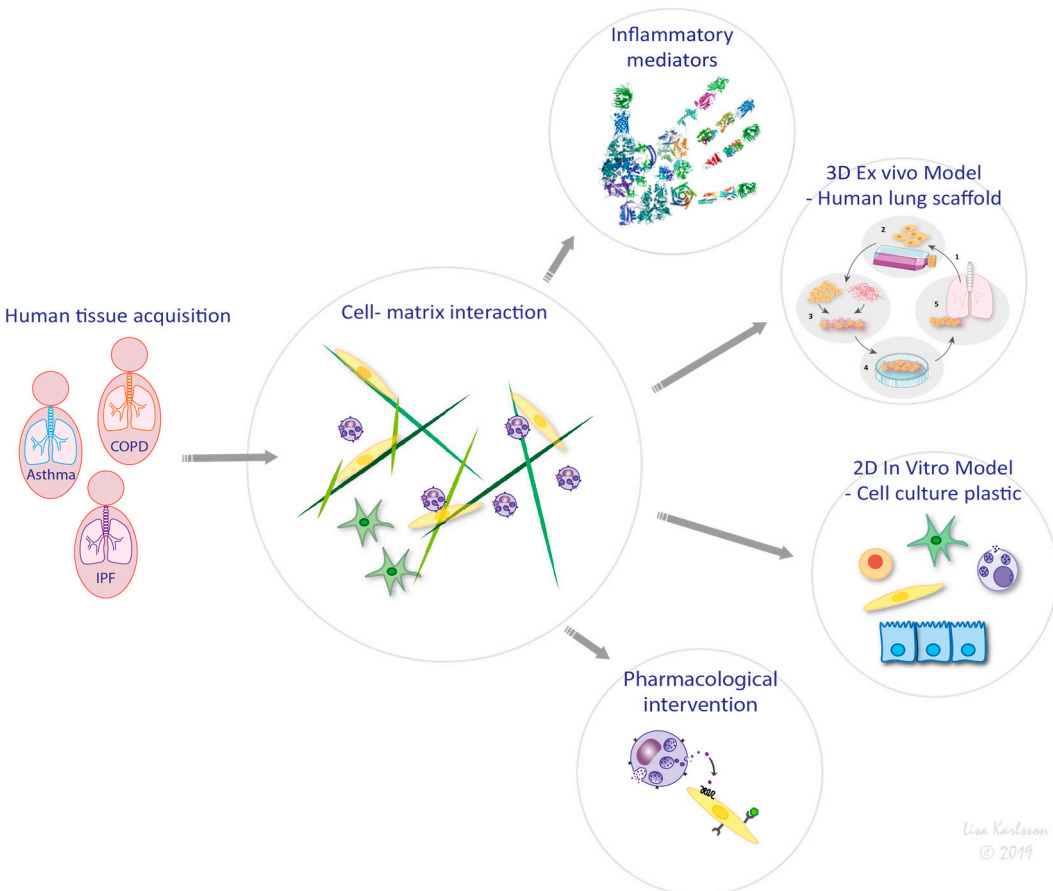

Figure 1. A variety of different biological matherials, techhnologies and methodologies were used in order to investigate the answers to the aims of this thesis. Fibroblasts obtained from human lung tissue were mono-cultured and co-cultured, both in vitro and ex vivo experimental setup. Inflammatory mediator relese and cellular functions were investigated, in presence and absence of pharmacological intervention with PAR2 antagonist (Illustration by: Lisa Karlsson).

Biological material

Mast cells and fibroblasts

Peripheral blood mononuclear cells were separated by Ficoll-plaque (21), followed by progenitor-separation using anti-CD34 magnetic beads with FcR blocking reagent. The progenitors were seeded in cell culture medium together with specific cytokines required for the mast cell differentiation as IL-3, IL-6 and (SCF). After 6 weeks in culture, the progenitors differentiated into mature mast cells (PBdMC), as a mix of both MC_{TC} and MC_T [177,178,179.] LAD2 mast cell lines were a gift from Dr. Arnold Kirshenbaum, US[180]. LAD2 mast cells express both tryptase and chymase, however, in a lower concentration than primary skin mast cells[181,182,183]. Human foetal lung fibroblasts (HFL-1; ATCC) were used between passages 16 and 21. Lung tissue explants from healthy organ donors, without any suitable recipient for transplantation, and from patients with IPF or COPD were used in this study. Primary distally-derived lung fibroblasts were isolated from the lung tissue explants and used in passages 4-7. The lung fibroblasts were cultured in DMEM supplemented with 10% fetal clone serum (FCIII), 1% antibiotics and 1% L-glutamine.

Patient and Ethical approval

The studies included in this thesis were approved by the Swedish Research Ethical Committee in Lund (FEK 213/2005, FEK 91/2006, FEK 413/2008, FEK675–12/2012 and KIT 2010-29). Written informed consent was obtained from the patient or from closest relatives. All experimental protocols were carried out in accordance with guidelines approved by the ethical committee.

Preparation, decellularization and repopulation of human lung scaffolds

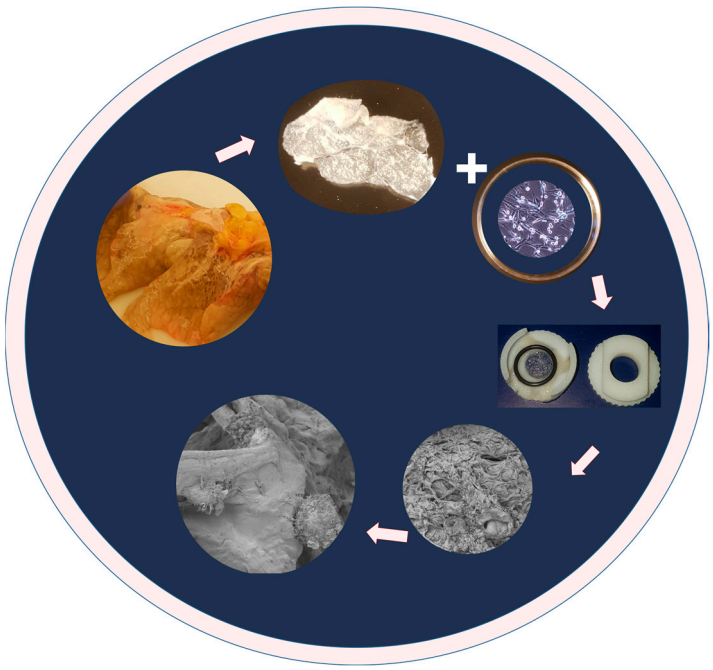

Figure 2. Preparation, decellularization and repopulation of human lung scaffolds. Lung tissue were dessected, cryosectioned, followed decellularixation treatments. The scaffolds were repopulated with fibroblasts and mast cells and mounted in scaffold-holders.

Briefly, the lung tissue was dissected (1 cm^3) from healthy lung, snap frozen and stored at -80°C. Lung slices of 350 µm were cryosectioned and treated with a detergent based decellularization solution followed by enzymatic DNA degradation. The decellularized lung scaffolds were washed in PBS and stored overnight at 4°C, until repopulation. The next day, lung scaffolds were incubated (37°C, 1 hour) in 12-well cell culture plastic (1 slice/well) in high-serum (10% FCIII) medium. Afterwards, fresh medium with HFL-1 was added to each scaffold slice, and placed on a shaker and preincubated (6 hours at 37 °C) before LAD2 cells were added and incubation continued overnight. The repopulated scaffolds were gently strapped to holders and incubated for 72 in fresh high-serum medium. After the incubation time, the cells were treated with PAR-2 antagonist (P2pal-18s) respectively TGFβ in fresh low-serum (0.4% FCIII) medium, followed by another 72 hours of incubation.

Afterwards the cell medium was collected and stored for further analyses. The scaffolds were fixed (4% paraformaldehyde) and stored in PBS at 4 °C until further analysis.

Cell migration Scratch assay

A scratch assay was used to investigate cell migration in vitro (Fig 3). A monolayer of fibroblasts was scratched using a tip to create a cross. The migration of fibroblasts towards this cell free area were observed by capturing images at different time points (0, 24 h, 48 h and 72 h). The migratory capacity of HFL-1 cells was measured as the percentage of cell-occupied space compared to the starting time.

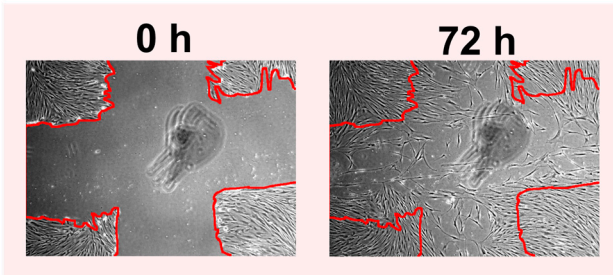

Figure 3. Cell migration experiment. The migratory capacity of HFL-1 cells was measured by scratch assay as the percentage of cell-occupied space compared to time (0 h, the starting point when the scratch was made). Image shows fibroblast migration at time point 0 respectively after 72 hours in culture.

Cell migration was performed in DMEM supplemented with low FCIII, in order to depress cell proliferation. In order to investigate the role of different mast cell specific proteases, fibroblasts were stimulated with tryptase respectively chymase. In order to mimic the effects of the mast cell subtype MC_{TC}, the fibroblasts were stimulated with a combination of tryptase and chymase. The fibroblasts were also stimulated with conditioned medium from mast cells. The interactions between mast cells and fibroblasts, were investigated in a co-culture and evaluated on the migration capacity of fibroblasts. Pharmacological interventions with the PAR-2 antagonist, P2pal-18s, were used in order to investigate the mechanism underlying the enhanced fibroblast migration caused by mast cells respectively mast cell tryptase. Fibroblasts treated with PAR-2 inhibitor, were stimulated with tryptase respectively mast cells and the migration capacity of fibroblasts were investigated by scratch assay[184, 185].

Cell Proliferation and Viability

Cell proliferation

In vitro cell proliferation assays were performed as previously described (31, 32). HFL-1 in mono-culture and in co-culture with mast cells were seeded in four 96-well plates, and incubated in medium supplemented with 10% respectively 0.4% serum, with and without different stimulations. Afterwards, the cells were fixed and stained with crystal violet dye, which binds to DNA in the cell nuclei. This allows for an indirect measurement of the amount of attached and viable cells. The absorbed dye from the cell nuclei was dissolved and measured, while the absorbance of dissolved crystal violet was directly proportional to the cell density[186,187].

WST

Cell viability was analysed using tetrazolium salt (WST-1). The supernatant was removed and WST-1 was added to the cell layer. Cells were incubated at 37 °C, 10% CO2 for a specific time interval. Absorbance was then measured at 450nm.

Immunohistochemistry

Chamber slides

Fibroblasts in mono-culture and in co-culture with mast cells were seeded on chamber slides and incubated for 72 hours, in high-serum (10%) medium. After the incubation time, the cells were treated with the PAR-2 antagonist respectively TGFβ, in fresh low-serum (0.4%) medium. Afterwards, the cells were formalin fixed and incubated in PBS in 4 °C until further immunocytochemistry (ICC) analysis. Afterwards primary antibodies against the molecule of interest were added to the cells and incubated, followed by washing steps and addition of secondary fluorescent antibodies. After further incubation and more washing steps, mounting medium containing Dapi were added to each slide. Different microscopy techniques were used for the visualisation of the molecules of interest. A VS120 slide scanner and confocal microscope were used for imaging the stainings. The images were analysed and processed using the imaging software VS-OlyVIA respectively NIS-elements.

Human healthy lung scaffolds

The same staining procedure as for cells seeded on chamber slides, was performed when staining the fixed lung scaffolds. The scaffolds still remaining in their holders were permeabilized with Triton-X100. Each scaffold was gently incubated with the primary antibody against the molecule of interest and incubated overnight at 4°C. The next day, each scaffold was washed in buffer several times and the secondary antibodies were added. The scaffolds were incubation for 48 hours at 4°C, followed by more washing steps. The scaffolds were gently removed from the holders and placed in special petri-dishes with a rounded cover glass on the bottom. The mounting medium containing cell nuclei staining, fluorescent Dapi, was added. Rounded cover glasses were placed above the scaffolds, before they were ready for microscopy visualization. Confocal microscope was used for imaging the scaffolds, while the images were analysed and processed using NIS-elements imaging software.

Mast cell degranulation by β-hexosaminidase

β-hexosaminidase is an enzyme localized in the granules of mast cells, which is released upon mast cell activation. This enzyme cleaves the terminal linked N-acetylhexosamine residues in N-acetyl-b-hexosaminides. Because of its feature, β-hexosaminidase is used as a mast cell marker. By immunological activation (IgE/Anti-IgE) of mast cells, the mast cells release their granule content consisting of different mediators including β-hexosaminidase, which in turn can hydrolyse 4-nitrophenyl-N-acetyl-β-D-glucosaminide. By this hydrolyzation, 4-nitrophenol is produced, which can be detected by a spectrophotometer at specific absorbance. The obtained enzyme activity provides an indirect measurement of the mast cell degranulation activity[188,189].

Gene expression, analysis for proteins and ELISA

Gene expression by qPCR

The basic principles of quantitative reverse transcription polymerase chain reaction (qPCR) is a method for expanding and making many copies of a single copy of DNA, coding for the gene of interest. This technique was used in our experiments in order to verify expression of specific genes for PAR-2 in fibroblasts respectively mast cells[190].

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method was used in our experiments in order to investigate the mediators released by the cells into the cell medium after different treatments. Three different cytokines and growth factors, IL-6, VEGF and HGF, were measured in the cell supernatants. All ELISA assays were performed according to the manufacturers' instructions, with all the required reagents included in the kit (R&D Systems).

Cytokine Multiplex Assay

Cytokine Multiplex Assay is a type of immunoassay, based on the same principles as ELISA. As the name Cytokine Multiplex Assay reveals, this method is used to detect several different cytokines and chemokines at the same time, in the same biological sample. However, this method uses magnetic beads for binding to different analyte-specific antibodies. These beads are coded with fluorescent color, which can be detected, measured and quantified.

This method was used in our experiments in order to detect several different proteins or mediators released by the cells after different treatments. The released mediators that were investigated in the cell supernatants by this method were; Hepatocyte growth factor (HGF), Vascular endothelial growth factor (VEGF-A), Vascular endothelial growth factor (VEGF-C), Fibroblast growth factor 2 (FGF2), Matrix metalloproteinase 9 (MMP-9), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 1α (IL-1α), SCF (Stem cell Factor) and cKIT. The multiplex cytokine assay was performed according to the manufacturers' instructions (Luminex Human Magnetic Assay 10-Plex, Biotechne).

Western blot analysis

Western blot is an analytical method for detecting and identifying proteins according to their molecular weight. Western blot analysis was used in our experiments in order to detect and quantify the protein levels of αSMA, in our cell culture models after treatment with PAR-2 antagonist respectively TGF-β. Briefly, the cells were seeded and collected after 72 hours with specific treatments. Lysis buffer was added to the cell lysate and collected using a cell scraper before storage at −20°C until further analysis. The proteins were visualized and quantified by specific image software (Odyssey FC and Image Studio). The protein intensities were normalized to total protein content for each sample.

Mass spectrometry analysis

Quantitative proteomic characterization of the lung ECM from healthy subjects and patients with IPF was performed. Resections from human distal lung tissue (10 mg wet weight/resection) were analysed. Protein extractions, MS sample preparation and LC-MS/MS analysis were all performed according to our previously published method[191].

Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorting (FACS) is a technique used for cell separation for cellular analysis based on cell morphology and expression of different types of surface proteins. These surface proteins can act as surface markers, also called clusters of differentiation (CD). FACS analyses were used in our studies in order to characterize our mast cells differentiated from CD34+ positive progenitor cells, isolated from peripheral blood (PBdMC). PBdMCs were harvested, washed and non-specific binding was blocked. Two different specific cell surface markers, identifying progenitor cells (CD34) respectively mast cell c-KIT receptor (CD117), were added to the cells. The samples were run on a FACSCalibur™ and analysed by software CellQuest.

Morphological characterization

SEM, TEM and Confocal microscopy

Different microscopy imaging techniques were used in order to investigate repopulated lung scaffolds and cells seeded on chamber slides. Confocal microscopy was used to detect the anti-body fluorescence stainings in our cell *in vitro* respectively *ex vivo* cell culturing models. Scanning electron microscopy (SEM) was used in order to better visualize the cell morphology in our cell *in vitro* respectively *ex vivo* cell culturing models. Samples were mounted and examined in a Jeol JSM-7800F FEG-SEM at Lund University Bioimaging Center (LBIC). Primary PBdMC were fixed with formaldehyde followed by further preparations and analysis of detailed cellular events, by using high resolution transmission electron microscopy (TEM). The images were analysed at Lund University Bioimaging Center (LBIC) using CM-10 TEM microscope (Philips, Eindhoven, Netherlands)[192].

Crystal Violet

Fibroblasts treated with different stimulations were stained with crystal violet and incubated 2 hours in room temperature or overnight in 4°C. Afterwards, cells were washed several times with H_2O, and allowed to dry. Images of each well were captured for analysis of cell shape. A ratio between length and width of each cell was calculated and presented[81].

Data analysis, calculations and statistical methods

Statistical analyses and graphs were generated using the GraphPad software (GraphPad Software Prism 7, La Jolla, USA). The non-parametric Mann–Whitney t-test was used to compare statistical differences between two patient groups. Two-ways repeated measurement analysis of variance (ANOVA) on ranks followed by the non-parametric Dunn's post hoc test were used to compare differences in pharmacological treatments between fibroblasts obtained from healthy subjects and patients with COPD. Data for HFL-1 are presented as mean +/- SEM and statistical analysis are performed with Student's t-test and one-way repeated measurement ANOVA followed by the Holm–Sidak post hoc test. To investigate migration over time and in response to stimulations and inhibitors, linear mixed models were used and performed in SPSS version 22 (SPSS, Inc., Chicago IL). P-values of $p < 0.05$ were considered as statistically significant.

Results

The crosstalk between fibroblasts and mast cells, and how they influence the effects of each other is poorly understood. There is increasing evidence from many studies, indicating the importance of fibroblast and mast cell interplay[42,44,193,194]. In this thesis, the interactions between mast cells and fibroblasts, in different *in vitro* and *ex vivo* co-culturing systems, were investigated.

Close cell-cell communication (Paper I, Paper III)

The close interaction between fibroblasts and mast cells has been evidenced by several imaging technologies in our co-culture systems. *In vitro* cultured mast cells are suspensions of cells floating in cell medium and requiring special medium with specific nutrients and cytokines (SCF, IL-6). However, in our experiments, mast cells and fibroblasts were seeded in the same medium as used for fibroblasts alone. An explanation to this can be fibroblasts providing mast cells with the cytokines and nutrients they need for survival. Images of co-cultures with fibroblasts and mast cells clearly indicate a close cell-cell interaction between these cells *in vitro* (Fig 4. A) and *ex vivo* (Fig 4. B). Immunofluorescence image of a co-localization of c-KIT receptor expressed by mast cells and SCF expressed by fibroblasts confirms further the close interaction between these two cell types (Fig 4. C).

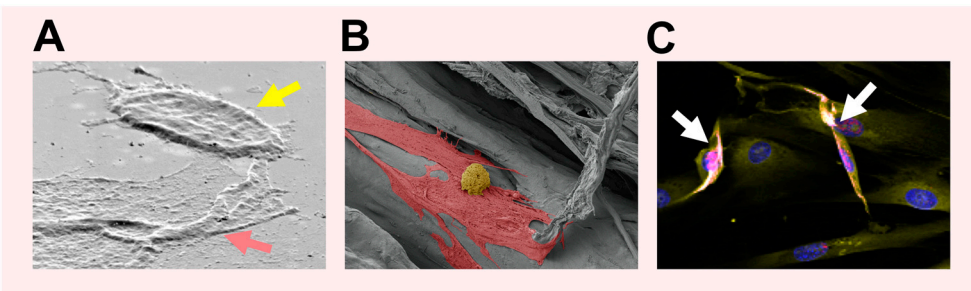

Figure 4. Close cell-cell interaction between mast cells and fibroblasts. SEM images of HFL-1 (pink arrow) and LAD2 (yellow arrow) cultured *in vitro* (**A**). Representative images of HFL-1 and LAD2 cultured *in vitro* shown with staining for SCF (yellow); c-KIT (fuchsia); mast cell specific tryptase (red); DAPI stained nuclei (blue) (**B**). SEM images of scaffold cultures, HFL-1 (pink), LAD2 (yellow) coloured for clarity (**C**).

Migratory and Proliferative effects of mast cells and mast cell tryptase (Paper I)

In order to further investigate the close interaction between mast cells and fibroblasts, migration respectively proliferation experiments were performed. The influence of mast cells and the mast cell protease, tryptase, on physiological functions of fibroblasts were investigated. Both PBdMC and LAD2 mast cells enhanced the migration of fibroblasts. Due to limitations with availability of PBdMC, LAD2 cells were used in our further experiments. LAD2 mast cells showed a concentration-dependent effect on the migration of fibroblasts (Fig. 5. A). Conditioned medium (CM) from mast cells also had a migratory effect on fibroblasts. The mast cell protease, tryptase, enhanced the migration of fibroblasts in a concentration-dependent manner (Fig. 5. B). Mast cells, CM and tryptase did not have any proliferative effects on fibroblasts when co-cultured in the same serum content (0.4 %) as migration experiments. This confirms that the enhanced migration was not due to proliferation. Interestingly, when the same proliferation experiments were performed in higher serum content (10%), tryptase enhanced the proliferation of fibroblasts, while mast cells contradictory to the effect of tryptase, decreased the proliferation of fibroblasts.

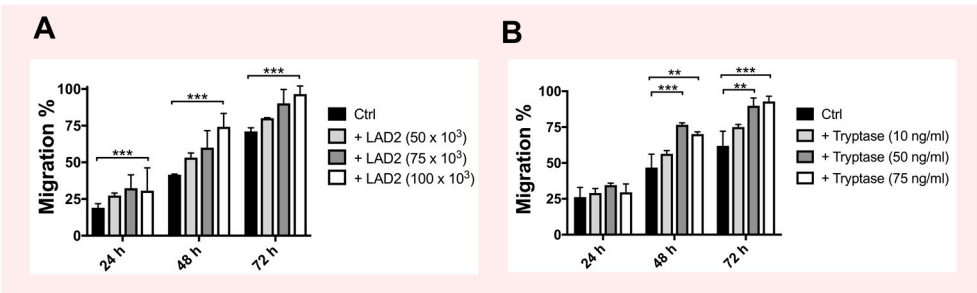

Figure 5. Effect of mast cells and tryptase on migratory capacity in human lung fibroblasts. LAD2 mast cells enhanced the migratory capacity of human lung fibroblasts, in a concentration-dependent manner, where the highest effect could be observed at $100x10^3$ cells (**A**). Mast cell tryptase, also enhanced the migration capacity of HFL-1 at 50 ng/ml and 75 ng/ml (**B**).

Pharmacological intervention with PAR-2 (Paper I)

The mechanism behind the migratory effects caused by mast cells, CM and tryptase was investigated by pharmacological intervention. PAR-2 is a receptor expressed by several cells, known for being activated by different serine proteases, like tryptase[195,196,197]. In order to investigate the mechanism behind the enhanced migration caused by LAD2 and tryptase, a PAR-2 antagonist, P2pal18s, was used in our experiments. The migratory capacity of PAR-2 antagonist-treated fibroblasts was investigated when co-cultured mast cells or stimulated with CM and tryptase.

Interestingly, the enhanced migratory effects from mast cells and tryptase, decreased after PAR-2 inhibition. Mast cells enhanced the migration of fibroblasts by 28%, while this effect decrease to 4% after PAR-2 inhibition. The same decrease could be observed after tryptase stimulation, where the enhanced migration decreased from 21% to 0.015% after PAR-2 inhibition (Fig 6. A). However, there was no difference in the migration capacity caused by CM, after PAR-2 inhibition. Expression of PAR-2 on fibroblasts was clarified both with gene expression analysis and by immunohistochemical analysis using antibody against PAR-2. Representative image of co-cultured fibroblasts with mast cells shows PAR-2 expression (Fig 6. B).

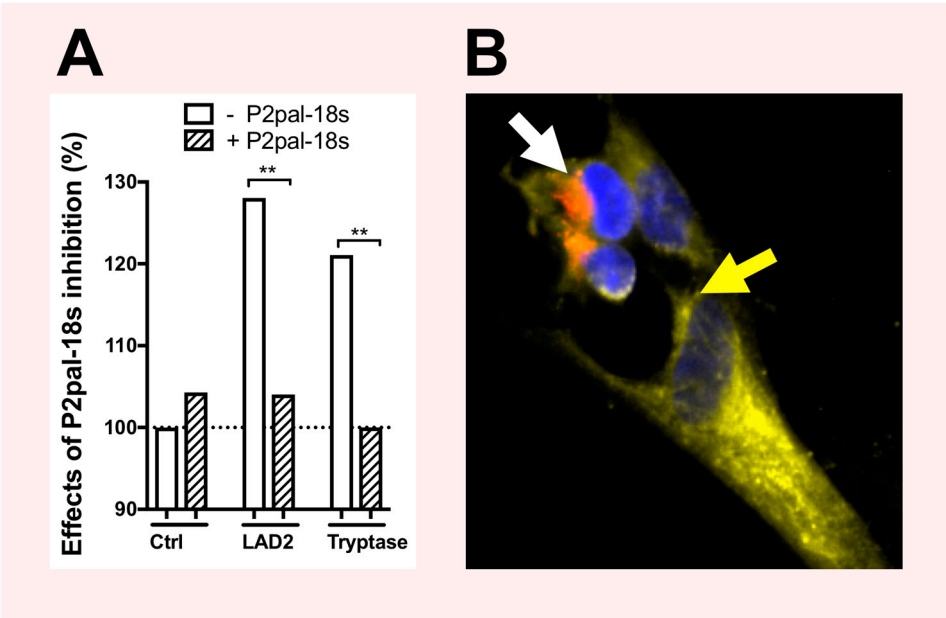

Figure 6. Effect of PAR-2 antagonist on migratory capacity of HFL-1. The PAR-2 antagonist P2pal-18S inhibited the pro-migratory effect of LAD2 mast cells respectively tryptase. The enhanced migration of HFL-1 decreased from 28 to 4% at 48 h for mast cells and from 21%, down to 0.015% for tryptase (**A**). Expression of PAR-2 examined in co-culture of HFL-1 and LAD2. Representative images are shown with PAR-2 staining in yellow, mast cell specific tryptase-staining in red and nuclei stained with DAPI in blue (**B**).

Phenotype alteration after pharmacological interventions (Paper II)

Together with the findings regarding the migratory capacity of fibroblasts by mast cells and mast cell tryptase via PAR-2, we observed another interesting feature of fibroblasts during these treatments. We could observe a morphological change in the fibroblast phenotype, when co-cultured with mast cells and treated with PAR-2 antagonist. We investigated these morphological changes of fibroblasts, by staining

the treated cells with crystal violet dye for better visualisation of the cell body. The longest and widest part of the fibroblasts were measured and a ratio between length/width of fibroblasts were calculated. Interestingly, cultured fibroblasts and mast cells treated with PAR-2 antagonist, showed a significant alteration in cell morphology of the fibroblasts (Fig 7.B) compared to the unstimulated (Fig 7.A). There was no difference in the ratio length/width after TGFβ stimulation (Fig 7.C), compared to the unstimulated (Fig 7.A).

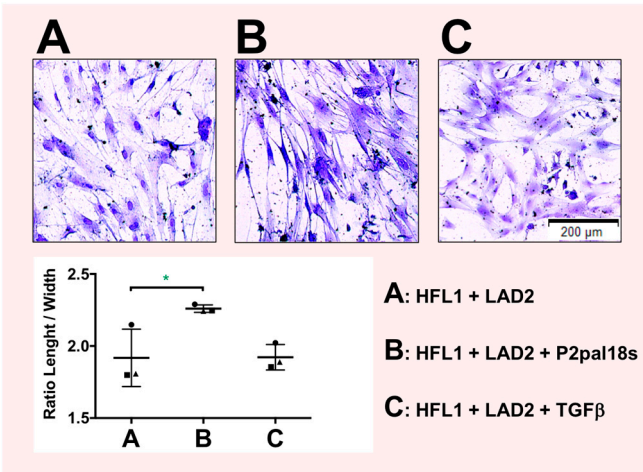

Figure 7. Cell morphology after the different treatments is shown by crystal violet staining. (A) Co-cultures of HFL-1 and LAD2 mast cells. Cultures included were treated with P2pal-18s antagonist (B), TGF-β stimulated (C) and untreated as controls. Change in HFL-1 morphology in response to treatments quantified by calculating length/width ratios of cells stained with crystal violet.

Morphological alterations are independent of αSMA (Paper II)

The morphological changes of fibroblasts induced by PAR-2 antagonist, reminded of a typical elongated myofibroblast phenotype, which is linked to the excess αSMA expression by these cells. We wanted to find out if these phenotype alterations observed in our findings were due to altered αSMA expression. Immunocytochemical staining by αSMA antibodies showed differently distributed and organized αSMA fibres, which were more elongated and thinner. The phenotype changes could be observed only in the co-culturing system, treated with PAR-2 antagonist (Fig 8.A) and not in mono-culture. These findings confirm the morphological changes we observed after crystal violet staining. However, in order to quantify the αSMA protein, western blot analysis was performed on cell extracts from the *in vitro* experimental settings. Interestingly, we could not see any significant difference in protein levels in the co-culturing system after treating with PAR-2 antagonist (Fig 8. B).

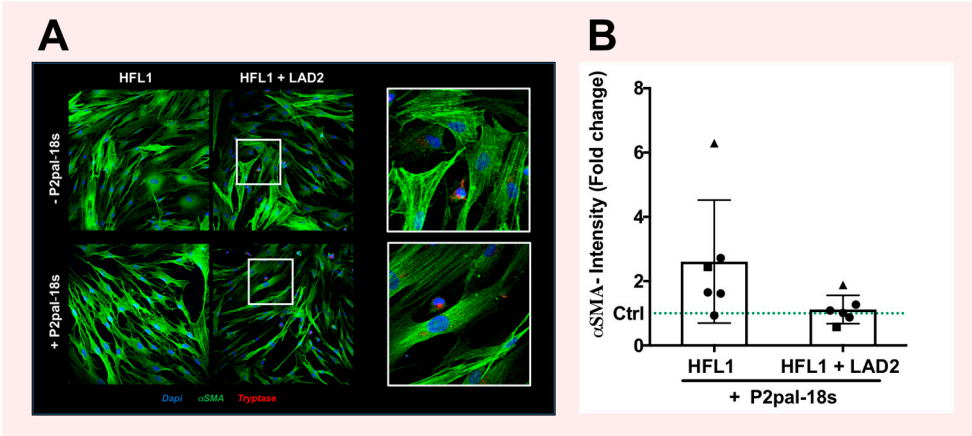

Figure 8. The cell morphology alterations are independent of αSMA. HFL-1 cells in mono-cultures and in co-cultures with LAD2 mast cells were treated with PAR-2 antagonist P2pal-18s. Representative images after each treatment are shown by confocal immunofluoresence microscopy, fixed cells were stained for nuclei (DAPI, blue); αSMA (green); tryptase (red) (**A**). There was no difference in the αSMA levels after P2pal18s treatment, compared to the untreated controls (**B**).

Inflammatory Mediator Profile in 2D and 3D (Paper II)

Our findings showed morphological alterations after PAR-2 antagonism that were independent of αSMA expression. To further try to explain our findings, we investigated the profile of different inflammatory mediators in our experimental cell culturing settings. Mono-cultured fibroblast and co-cultured with mast cells, were treated with PAR-2 antagonist (P2pal-18s). Stimulation with TGF-β was used as positive control in order to create a more profibrotic condition. The synthesis of proinflammatory (IL-6), angiogenetic (VEGF) and anti-fibrotic (HGF) mediators were investigated. The experiments were performed both in 2D *in vitro* with cells seeded on cell culture plastic, and also in 3D *ex vivo* with cells seeded on human healthy lung scaffolds. The *in vitro* studies showed a different cytokine synthesis in 3D compared to 2D, indicating an important role of ECM molecules in inflammatory response by the cells.

IL-6

The pro-inflammatory response was investigated by analysing IL-6 release after pharmacological intervention with PAR-2 antagonist. IL-6 synthesis increased both in mono-cultured fibroblasts and in co-culture with mast cells, when cultured on cell culture plastic (Fig 9. A). Interestingly, the IL-6 synthesis was much higher in fibroblasts/mast cells when co-cultured on human lung scaffold, compared to co-culturing on cell culture plastic. Contradictory to these results, the PAR-2 inhibited

mono-cultured fibroblasts, and did not show any significant difference in the IL-6 release compared to the untreated cells (Fig 9. B).

VEGF

The angiogenetic response of fibroblasts was investigated by analysing VEGF release. The PAR-2 antagonist did not have any significant effects on VEGF synthesis in either 2D or 3D monocultured fibroblasts. Interestingly the VEGF release increased in co-cultured fibroblast/mast cells, after PAR-2 inhibition in both 2D respectively 3D cell culturing system (Fig 9. C-D).

HGF

The anti-fibrotic effect of HGF on fibroblasts, induced by mast cells and mast cell mediators was investigated by analysing HGF synthesis in the different culturing systems. Mono-cultured fibroblasts and co-cultured fibroblast/mast cells, significantly release more HGF after treated with PAR-2 antagonist, in 2D cell culture tissue (Fig 9. E). Surprisingly, the HGF synthesis in PAR-2 antagonist treated mono-cultured fibroblasts, decreased when cultured in 3D lung scaffolds, compared to the untreated controls. There was no significant difference in the HGF release in the co-cultured PAR-2 antagonist treated fibroblast/mast cells when cultured in 3D lung scaffolds (Fig 9. F).

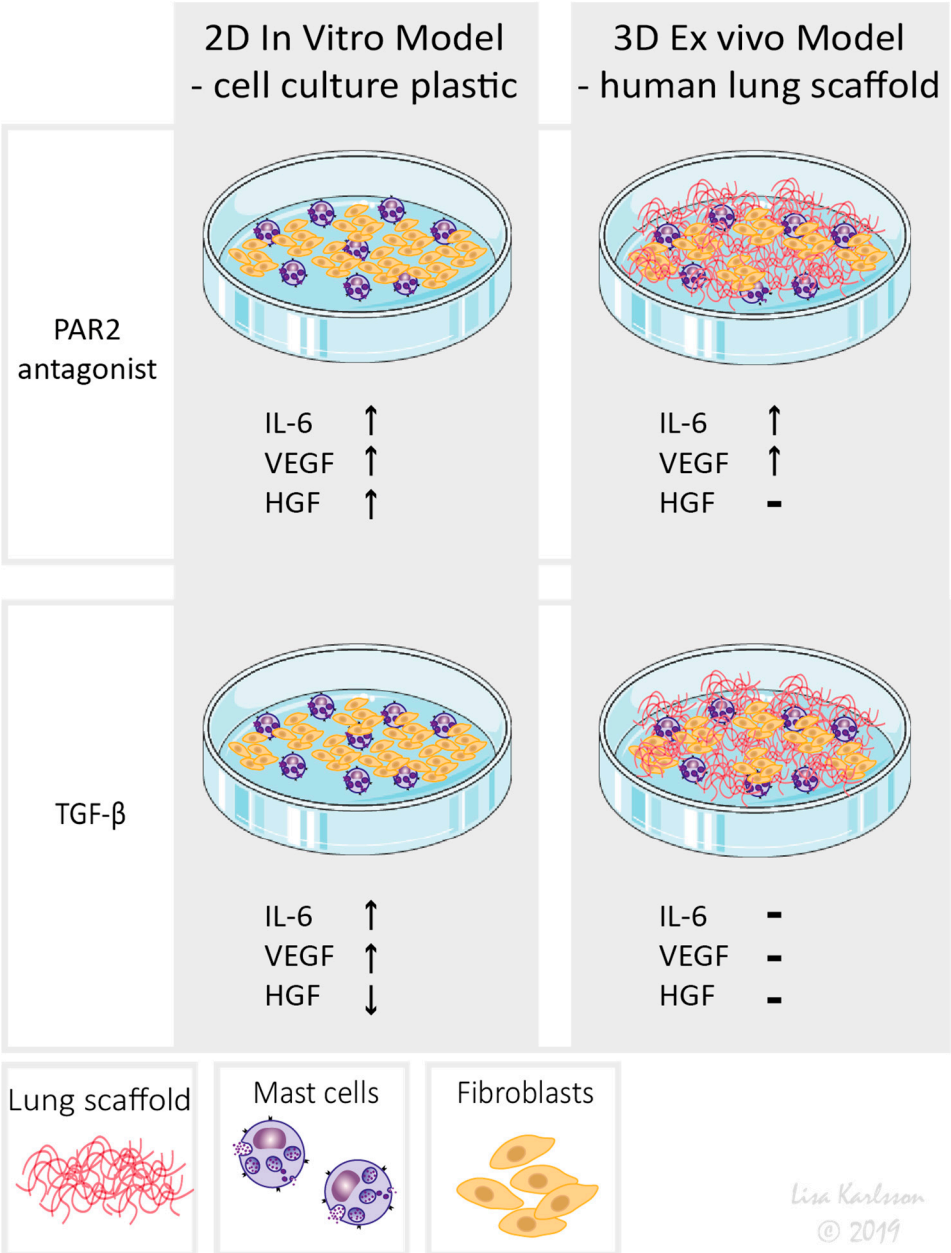

Figure 9. Mediator release in cells cultured in 2D cell culture plastic and 3D human lung scaffolds. The secretion of the different mediators IL-6, VEGF and HGF were analyzed after treatment with the PAR-2 antagonist P2pal-18s in 2D *in vitro* and 3D *ex vivo* experimental model *(Illustration by: Lisa Karlsson)*.

Tryptase and Chymase in healthy vs IPF lungs (Paper III)

Proteomic analysis by mass spectrometry performed on healthy respectively IPF lung tissue, showed differential distribution of the mast cell mediators, tryptase and chymase. The intensity levels of tryptase was much higher than chymase in both subject groups. In order to understand the distribution of MC_T and MC_{TC}, a ratio between the tryptase respectively chymase levels were calculated separately for each individual, in the healthy respectively IPF subject groups. Interestingly, a comparison between healthy and IPF lung tissue showed a decreasing tendency in tryptase/chymase ratio, that might be altered in IPF, although no significant differences were found in our material, due to limited subjects in the groups (Fig 10. A and B).

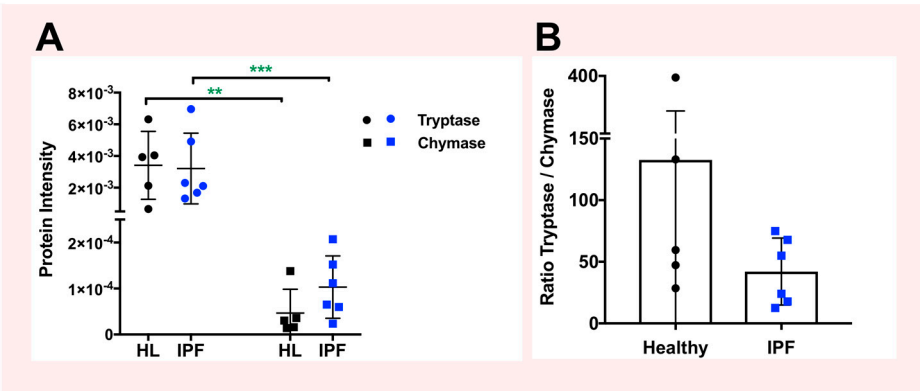

Figure 10. Tryptase and chymase levels compared between healthy and IPF lung tissue. Proteomic profiles of lung tissue from healthy individuals and IPF patients were investigated by mass spectrometry. Tryptase levels were much higer than chymase levels in human lung tissue, both in healthy and IPF lungs (**A**). A ratio of tryptase/chymase within the same donor was calculated (**B**).

Role of mast cell mediators in healthy vs IPF fibroblasts (Paper III)

These and previous findings showing alterations in the proinflammatory mediator profile of our culturing systems, conduced more investigations about the role of mast cells and mast cells mediators in the progression of chronic lung diseases as IPF. In order to investigated the role of tryptase and chymase in healthy respectively IPF, we stimulated fibroblasts from healthy respectively IPF lungs with these proteases and investigated the cytokine profile released by them. The VEGF release was upregulated after tryptase stimulation, both in healthy respectively IPF fibroblasts. Chymase did not have any significant effect on the VEGF synthesis, neither in healthy nor IPF fibroblasts. A mixture between tryptase and chymase also enhanced the VEGF release in both groups, indicating no effect from chymase (Fig 11. A). The HGF release increased after tryptase stimulation alone and a mixture of

tryptase and chymase in both healthy and IPF fibroblasts. Interestingly, chymase alone decreased the HGF release in IPF fibroblasts and showed an increased tendency in healthy fibroblasts (Fig 11. B).

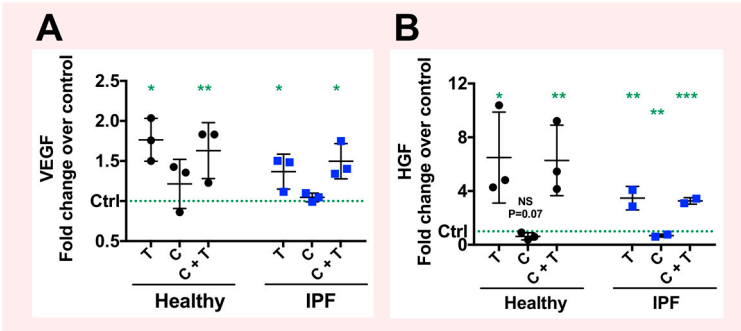

Figure 11. Mediator release from healthy and IPF fibroblasts. IPF fibroblasts were stimulated with tryptase (T), chymase (C) or a combination of tryptase and chymase (T+C), and VEGF (**A**) respectively HGF (**B**) were measured in the cell supernatants. Data is presented as fold changes compared to unstimulated controls.

VEGF synthesis in fibroblasts from healthy vs COPD lungs patients (Paper IV)

The role of VEGF in pulmonary vascular remodelling in primary distally-derived lung fibroblasts from healthy individuals and patients with severe COPD (GOLD IV) were investigated. TGFβ stimulated fibroblasts showed an increased synthesis of VEGF, but there were no significant differences between fibroblast from healthy *vs* diseased lungs.The migratory capacity and proliferation of HFL-1 fibroblasts and the effect on ECM synthesis were investigated after stimulation with different concentrations of $VEGF_{165}$. There was a significantly enhanced migration when stimulated with VEGF at a concentration of 100 ng/mL (Fig 12. A). Interestingly, the synthesis of the proteoglycans perlecan and biglycan was significantly increased (Fig 12. B-C).

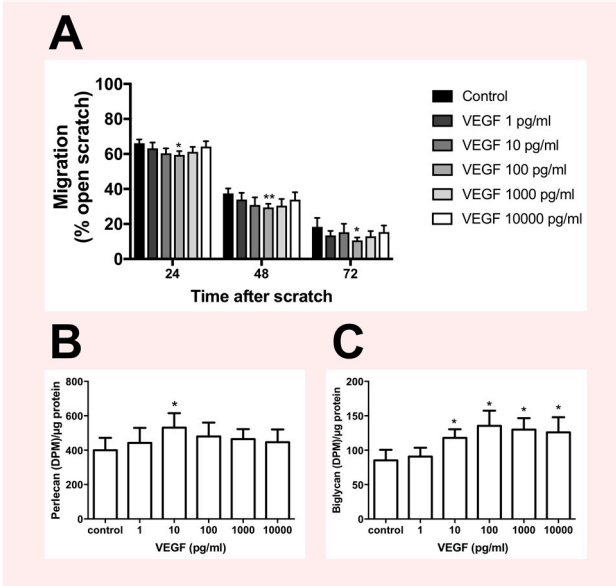

Figure 12. Effect of VEGF on fibroblast migration and synthesis of ECM proteins proteoglycans. VEGF 100 pg/mL increased migratory capacity of HFL-1, measured by scratch assay. VEGF (10–10 000 pg/mL) significantly increased synthesis of the individually measured proteoglycans perlecan (**B**) and biglycan (**C**).

Discussion

Whatever the source of injury, such as pathogens, allergens, irritations or damaged cells, numerous different cell types get involved in order to repair and regenerate the tissue. The innate immunity starts recruiting inflammatory cells, e.g. mast cells, macrophages and neutrophils. Fibroblasts are recruited to the inflamed tissue, resulting in myofibroblast differentiation, increased ECM production[12] and release of inflammatory mediators [62,63,64]. Usually, after these cells have finished the repair work, they undergo apoptosis, in order to create a controlled and balanced ECM production. If the cell apoptotic events are disrupted, the cells remain in the tissue and continues with an excess of ECM production and inflammatory mediators. The results of this positive feedback loop, turns the normal inflammation that clears automatically into chronic inflammation and remodelling of the tissue.

This thesis demonstrates the important role of mast cells and fibroblasts in different molecular event occurring during progression of chronic lung diseases. These studies have addressed the role of mast cell-fibroblast interactions in ongoing remodelling processes by investigating cellular functions such as migration, proliferation, morphological alterations and mediator profile. Our obtained results show a strong interplay between these two cells, which may be important for better understanding the outcome and progression of chronic lung diseases.

Mast cell function and cross-talk with fibroblasts

There are different secretory mechanisms of mast cell mediator release through degranulation. The most studied form of mast cell degranulation is anaphylactic degranulation by IgE stimulation[198], causing mast cells to rapidly release pre-stored mediators via fusion of granules with the plasma membrane[199]. By characterization of PBdMC using transmission electron microscopy, we could observe already degranulated mast cells without IgE-stimulation. However, when measuring tryptase levels in fibroblasts co-cultured with mast cells, we observed a time dependent increase in tryptase concentrations. Stimulation of fibroblasts with conditioned medium (CM) showed unchanged tryptase concentrations overtime.

Interestingly, Dvorak et al, suggested that mast cells may release mediators by piecemeal degranulation (PMD)[200], where instead vesicles containing selected mediators from granules fuse with the plasma membrane, leaving the morphology of the granules relatively unchanged [200] . A third, and even less investigated process,

is an even slower form of mast cell degranulation, which occurs through transgranulation between different cell types via cell-cell contacts. PMD and transgranulation of mast cell occurs via constitutive exocytosis of granules[201]. A previous study suggested that a chronic low-grade, partial activation of mast cells could be involved in pulmonary fibrosis [202]. SCF is an important cytokine important for mast cells proliferation and development. SCF, is expressed by fibroblasts and binds to c-KIT receptor on mast cells and is considered as a driving force for the recruitment of mast cells. In our studies, immunocytochemical stainings confirmed the expression and co-localization of SCF and cKIT in fibroblast and mast cells co-culture. Interestingly, several studies have reported that SCF activates mast cells, which induces degranulation[203,204,205]. Another study reported fibroblast/SCF and mast cell/c-KIT, carrying the responsibility for mast cell degranulation[203,206]. Based on these observations, we hypothesised that the interaction between these two cells in our cell systems may trigger the release of mediators through PMD.

An interesting finding from our protein analysis, triggered our curiosity regarding mast cell mediators. The levels of tryptase was much higher than chymase in human lungs, but interestingly, a ratio between tryptase and chymase within the same IPF patient, showed a tendency towards a decreasing ratio in IPF lung tissue (tryptase/chymase=40) compared to healthy control tissue (tryptase/chymase =130). This may indicate decreased tryptase levels followed by increased chymase levels. These data go in line with other studies suggesting a turnover of mast cell subtype from MC_T (tryptase) into MC_{TC} (tryptase and chymase) in IPF tissue [4]. A previous study suggested that mast cell tryptase induces alterations in fibroblast phenotype and differentiation into myofibroblasts, which are major producers of ECM contributing to tissue remodelling[207].

In the current study, we did demonstrated that mast cells and mast cell tryptase promote pro-migratory effects on human lung fibroblasts. In contrast, mast cell chymase did not show any significant effects on migration of fibroblasts. However, a combination of tryptase and chymase decreased the enhanced fibroblast migration caused by tryptase alone. Previous studies have reported that tryptase induces proliferative effects on fibroblasts [208, 193]. In order to find out whether these pro-migratory effects were due to soluble factors or cell-cell interaction between the cells, fibroblasts were stimulated with conditioned medium from mast cells. Our results showed a significantly enhanced fibroblast migration, however weaker, when stimulating with conditioned medium. An immunological (IgE/Anti-IgE) stimulation of mast cells and conditioned media in order to release and degranulate prestored granule contents, did not show any difference in pro-migratory effects on fibroblasts[198]. These findings, confirms our previously results and post the hypothesis that the mast cells are already degranulated by a non-immunologic activation.

In order to find out whether our findings were due to cell proliferation or migration, proliferation experiments were performed in medium with low serum

concentration, the same conditions as the migration experiments were performed in. As expected, neither mast cells or tryptase, had any proliferative effects on fibroblasts in medium with low serum concentration. Interestingly, when the same proliferation experiments were performed in medium with high serum concentration, tryptase enhanced proliferation of fibroblasts, while mast cells showed a decreasing effect on them. There can be several reasons behind this contradictory result. Conditioned media did not have any proliferative effects in either of the serum concentrations. These findings suggest a serum-dependent anti-proliferative property of mast cells, or other mediators released by these cells, being responsible for these contradictory proliferative effects on fibroblasts. Other reasons could be that different mast cell proteases interact with each other[209] or the presence of protein inhibitors or interfering growth factors. It can also be due to different anti-fibrotic growth factors synthesized by mast cells acting protective against remodelling[210]. However, the mechanisms underlying these activities remain unclear and further studies are warranted.

To further understand the cross talk between mast cells and fibroblasts, the role of PAR-2 was investigated. PAR-2 is a well characterized G protein-coupled receptor, involved in numerous different cellular events and plays an important role in tissue remodelling and progression of different inflammatory diseases. It activates the Th2 cell response together with several different components and signalling pathways[211,140]. PAR-2 has been shown to be expressed by both fibroblasts and mast cells and have several serine protease ligands including mast cell tryptase[212,213,214]. In our gene expression analysis, we showed that PAR-2 was expressed by fibroblasts, but not in LAD2 mast cells. By immunocytochemical staining, we confirmed the expression of PAR-2 on fibroblasts. One explanation to why we could not confirm PAR-2 expression on LAD2 mast cells may depend on different receptor expressions in different mast cell lines. Thereby, we hypothesized that the mechanism behind pro-migratory effects from tryptase and mast cells on fibroblast, could occur through tryptase/PAR-2 activation. To address this issue, we used the PAR-2 antagonist P2pal-18s, in order to inhibit PAR-2 activity[140,141]. Interestingly, the enhanced fibroblast migration caused by tryptase and mast cells that we previously observed, was reduced after PAR-2 inhibition with P2pal-18s. However, no changes in fibroblast migration stimulated with conditioned medium could be observed after PAR-2 inhibition. This could be explained by continuous degranulation (PMD) of mast cells, which is lacking in conditioned medium due to limited amounts of tryptase and no cell-cell contact. Previous studies have reported that PAR-2 activation induced pulmonary fibroblast migration, differentiation and ECM production, through ERK1/2 signalling pathway in fibroblasts [215]. Another study has reported that PAR-2 inhibition by P2pal-18s, reduced pro-fibrotic and pro-inflammatory responses in bleomycin-induced pulmonary fibrosis[140]. Previous studies have indicated that fibroblasts from IPF lungs exhibit increased PAR-2 expression [216].

PAR-2 inhibition induces phenotypic alterations in fibroblasts

We found that co-cultured fibroblasts with mast cells obtained a changed fibroblast morphology, resembling myofibroblasts, when treated with the PAR-2 antagonist, P2pal-18s. Myofibroblasts, differentiated form fibroblasts, are known for their major ECM production and important role in wound healing[217, 218]. In order to quantify these observations, the ratio between length and width of the cells was calculated and we found a significant difference in morphology between the co-cultured fibroblast after PAR-2 inhibition and untreated cells. Of note, TGFβ did not induce any difference in the ratio length/width in either mono-cultured fibroblasts or fibroblasts in co-culture with mast cells. An explanation to this could be that the ratio length/width may be unaffected because of increasing αSMA fibres in both directions (length and width) after TGFβ stimulation.

A characteristic feature of myofibroblasts is high abundance of αSMA synthesis and stress fibres causing contractility during wound healing and inflammation[24, 125]. Previous studies have suggested that mast cells and tryptase induce αSMA expression[219], while other mast cell features induce dermal fibroblast chemotaxis[213, 220, 221]. Interestingly, analysis by western blot showed unaffected αSMA levels in PAR-2 inhibited fibroblasts co-cultured with mast cells compared to mono-cultured fibroblasts, indicating that mast cells or PAR-2 antagonist did not have any significant effect on αSMA and that the morphological alterations in co-cultured fibroblast with mast cells are independent of αSMA expression. As expected, one mechanism of this morphological alterations could be activation of β-arrestin. PAR-2 activation triggers several different signalling pathways, where one is through β-arrestin, which in turn activates extracellular signal–regulated kinases 1 and 2 (ERK1/2). An interesting study has reported that activation of β-arrestin induces reorganization of the actin cytoskeleton[222,223,224]. The morphological alterations, independent of αSMA synthesis may thereby be explained by a β-arrestin activated pathway. Of note, TGFβ increased αSMA synthesis both in mono-cultured fibroblasts and in co-culture with mast cells. In line with our findings, several studies have reported high numbers of myofibroblasts in lung explants from patients with IPF and asthma[215,218] and increased PAR-2 activation resulted in increased αSMA expression[139].

Alteration in cytokine profile

To address further effects of mast cells, we investigated the release of inflammatory mediators in the cell culture systems. The pro-inflammatory cytokine IL-6 and the angiogenetic growth factor VEGF and HGF[225,226] were therefore measured in mono-cultured fibroblasts and fibroblasts co-cultured with mast cells in the presence and absence of PAR-2 inhibition, respectively TGFβ stimulation.

Another object to investigate was the role of mast cells and mast cell mediators as tryptase and chymase, played in the inflammatory mediator release from fibroblasts isolated from lung tissue from healthy subjects and patients with IPF. The cells were stimulated with the pro-fibrotic growth factor TGFβ, in order to act as a positive control for tissue remodelling.

The pro-inflammatory mediator IL-6 was significantly increased after PAR-2 inhibition, both in mono-cultured fibroblasts and in a co-culture with mast cells. These findings were in contrast to previous studies that instead reported increased IL-6 synthesis after PAR-2 activation and decreased synthesis upon inhibition of PAR-2 signalling pathway [227,228,229,230]. When stimulating fibroblasts isolated from healthy and IPF lungs with tryptase, chymase or a combination of tryptase/chymase, we could not see any significant difference in IL-6 release from either of these cells, suggesting a selection of specific fibroblasts or an effect of the changed ECM. Interestingly, TGFβ stimulation enhanced IL-6 production, however higher in mono-culture, suggesting that the presence of mast cells is an important regulatory factor for IL-6 secretion. Altogether, these findings indicate that IL-6 synthesis is dependent on cell-cell contact between fibroblasts and mast cells, and that it may act through another signalling pathway dependent on PAR-2 but independent of tryptase cleavage of PAR-2.

Another mediator of importance is the growth factor VEGF, an angiogenetic factor involved in pulmonary vascular remodelling processes[231] in chronic lung diseases[232]. Fibroblasts are one important source of VEGF synthesis and VEGF may also bind to specific matrix components.

VEGF secretion was higher in lung fibroblasts derived from patients with IPF compared to healthy subjects. However, we could not detect any differences in VEGF synthesis between fibroblasts derived from patients with very severe COPD compared to healthy subjects. TGFβ significantly increased VEGF synthesis in fibroblasts obtained from healthy subjects, respectively patients with COPD and IPF. Interestingly, in a recent study, increased serum VEGF levels correlated positively with more preserved lung function in patients with IPF, suggesting VEGF could have an anti-fibrotic role in IPF[233].

Previous studies have reported PAR-2 to be involved in VEGF synthesis in an *in vitro* model with human adipose stem cells[234] and glioblasma cancer cells[235]. In our studies, we observed an increased VEGF synthesis after PAR-2 inhibition in co-cultures of fibroblasts and mast cells. In contrast to our observed findings, other studies showed that VEGF was upregulated after PAR-2 activation, involving MAPK signalling pathway[236], in breast cancer cells[236] and glioblastoma[232]. Another study reported that PAR-2 activation induced VEGF secretion via PI3-kinase/Akt signalling pathway, suggesting a PAR-2 activation to be tissue or cell specific [234]. In the present study, tryptase, but not chymase, increased VEGF synthesis in fibroblasts isolated from healthy and IPF lungs.

Several studies have reported HGF as an anti-fibrotic growth factor[128, 225,226]. Our findings showed a significantly increased synthesis of HGF after treatment with PAR-2 antagonist, both in mono-cultured fibroblasts and in co-culture with mast cells. HGF has been shown to have anti-fibrotic properties due to interception with the SMAD signalling pathway and regulation of TGFβ activity. Previous studies have reported HGF to induce apoptosis of myofibroblasts and prevent fibroblast differentiation to myofibroblasts, which may have crucial roles in the progression of fibrosis[237, 238, 226, 225]. Interestingly, TGFβ stimulation in our *in vitro* cell cultures, decreased HGF secretion both in fibroblast mono-cultures and co-cultures with mast cells. These results are in line with other studies showing that TGFβ regulates the release of HGF, which in turn may suppress remodulatory effects of TGFβ[239, 240]. Healthy and IPF fibroblasts isolated from human lungs, showed an increased HGF synthesis after tryptase stimulation. Co-culture of fibroblasts from healthy respectively IPF lungs, with mast cells, showed no increase in HGF release compared to mono-cultured fibroblasts, however experiments with more subjects in the different groups are needed for better understanding. The protein analysis showed decreased levels of HGF receptor expressed in IPF lung tissue compared to healthy lung. Interestingly, in the present study chymase decreased HGF synthesis in fibroblasts from IPF patients. These contradictory data could partly be explained by the use of different model systems.

Interactions between inflammatory mediators and ECM

To further understand the crosstalk between mast cells and fibroblasts, the cells were investigated in a more physiological milieu using a 3D *ex vivo* lung scaffold model that was recently developed in our laboratory [241]. Decellularized lung tissue scaffolds obtained from healthy lungs were repopulated with fibroblasts and in co-culture with mast cells. The same experimental set up was performed in the scaffolds as in the *in vitro* culture system on plastic in the presence and absence of PAR-2 inhibitor respectively TGFβ. The same panel of inflammatory mediators as in our 2D *in vitro* cell cultures were analysed, in order to investigate the importance of ECM. Interestingly, IL-6 secretion was much higher in PAR-2 inhibited co-culture conditions in the lung scaffolds compared to 2D cell culture plastic. However, this effect was neutralized when stimulated with TGFβ. VEGF synthesis was upregulated in PAR-2 inhibited co-cultures, which followed the same pattern as in our 2D in vitro system. Interestingly, TGFβ-stimulation did not show the same pattern as in the 2D in vitro culture system. This can be a result of retention of added TGFβ or synthesised VEGF stored in the ECM of the decellularized lung scaffold, where proteoglycans are known to bind VEGF[242 243]. In our present study we showed that VEGF induced pro-migratory and proliferative effects on human lung

fibroblasts, and also increased proteoglycan synthesis such as perlecan and biglycan.

PAR-2 inhibited mono-cultured fibroblasts showed contradictory results on HGF release, with increased levels in 2D model, compared to a decreased pattern observed in the scaffold model. Also TGFβ stimulated co-cultures showed a different pattern. These data clearly indicate a strong influence of ECM components in inflammatory mediator release. PAR-2 signalling pathway appears to be modulated by the ECM, which go in line with what other researchers have reported. Interestingly TGFβ-stimulation also differed when comparing 2D culture on plastic with 3D culture in lung scaffolds. This may be due to the excess of matrix components that already exist in the lung scaffold and act as storage for cytokines and growth factors, which may increase more after stimulation with TGFβ. These findings suggest the presence of mast cells being important in the 3D ex vivo scaffold model, where ECM may have an important role in regulating proinflammatory responses of fibroblasts.

Concluding remarks

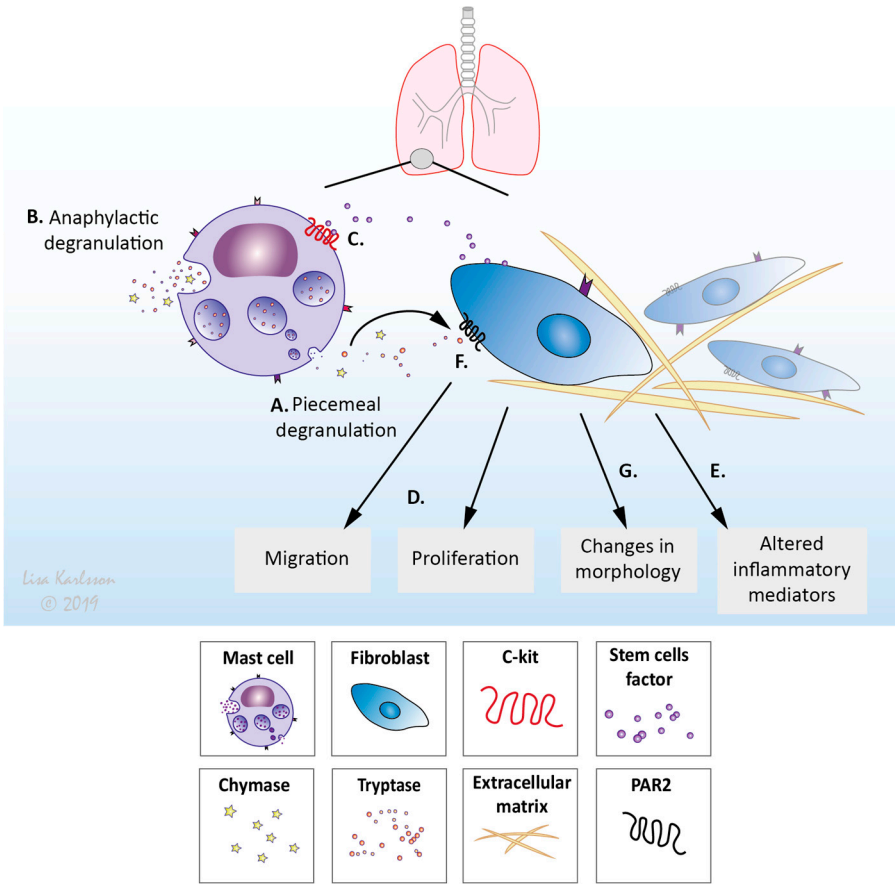

Figure 13. Summary of findings. We suggest that interactions between mast cells and fibroblasts are induces by piecemeal degranulation, which releases tryptase that stimulate PAR2-activation. This in turn, induce morphological alterations, cell migration and proliferation respectively inflammatory mediator release *(Illustration by: Lisa Karlsson).*

In conclusion, as proof of concept, this thesis demonstrates the important role of mast cell and fibroblast crosstalk in different molecular events in inflammatory and remodelling processes during the progression of chronic lung diseases. We propose an important role for cell-cell communication and signalling between mast cells and

fibroblasts, and that this interplay may trigger mast cell activation through a non-immunological pathway involving continuous degranulation defined as **piecemeal degranulation** (Fig 13. A), independent of anaphylactic degranulation (Fig 13. B). This activation may be due to an interaction between stem cell factor expressed/released by fibroblasts and c-KIT expressed by mast cells (Fig 13. B). We have shown that in human lung tissue, **tryptase levels are nearly ten times higher than chymase** levels, both in healthy donors and IPF patients. There was a trend that the ratio between tryptase and chymase in IPF lung tissue was lower compared to healthy controls, indicating more chymase positive mast cells in IPF lungs.

We have shown that **mast cells and mast cell tryptase have a pro-migratory** effect on fibroblast migration (Fig 13. C), while a combination of chymase and tryptase decrease this effect, suggesting chymase to have neutralizing effects on tryptase. Mast cell **tryptase increased VEGF and HGF** synthesis, while **chymase decreased HGF** and had no effect on VEGF synthesis in lung fibroblasts from both healthy donors and IPF patients (Fig 13. D). **TGF-β stimulation increased VEGF** synthesis in lung fibroblast from healthy donors respectively COPD and IPF patients. **VEGF** acts in an autocrine fashion on human lung fibroblasts, by **increasing their migration and proliferation** and ECM synthesis, components including **perlecan and biglycan**.

The mechanism behind enhanced migration induced by mast cells and mast cell tryptase, is suggested to go through **PAR-2 activation of signalling pathways** (Fig 13. E). We have shown that a PAR-2 antagonist (P2pal-18s) decreased the migration of fibroblasts induced by mast cells and tryptase. When fibroblasts and mast cells were in a co-culture, the **PAR-2 antagonist also induced morphological alterations** (Fig 13. F) towards a myofibroblast-like contractile and elongated phenotype. However, these changes were shown to be **independent of αSMA expression** in lung fibroblasts.

Lung tissue scaffolds, an *ex vivo* 3D model closer to physiological conditions, showed different inflammatory mediator responses compared to *in vitro* 2D cell culture models. **PAR-2 inhibition increased IL6, VEGF and HGF** in **both 2D and 3D** co-culture. However, IL-6 was even higher in the 3D model. TGF-β stimulation showed **increased secretion of IL-6, VEGF and a decrease of HGF in the 2D model**. However, this effect of TGF-β could not be observed in the 3D model, indicating the importance of an ECM environment.

Altogether, these findings support our hypothesis and provide a strong indication of mast cell/tryptase/PAR-2/fibroblast connection influencing the cellular functions by regulating migration, proliferation, differentiation, morphology and inflammatory mediator secretion of fibroblasts. Further studies are warranted to clarify the mechanisms behind the actions of the PAR-2 receptor and other mechanisms for mast cell interactions with fibroblasts, which may provide useful strategies for preventing or reducing uncontrolled tissue remodelling in the lung.

Future Perspective

In this thesis we have presented several new findings trying to understand the crosstalk between mast cells and fibroblasts in chronic lung diseases. Uncountable studies have reported that mast cells are involved in events during worsening or progression of disease. However, the phenomena that mast cells have evolutionarily remained in the innate immunity of the human body, indicates the prevalence of mast cells essential for regulating homeostasis in the body. Re-thinking about the role of mast cells in remodelling processes will definitely be a beginning to better understand the role of these cells in several diseases.

Future challenges will be to more in detail investigate the importance of mast cell-fibroblast interplay, such as better understand the role of mast cells and fibroblast crosstalk trough SCF/c-KIT interactions. Which role does SCF have in the activation of mast cells and if this interaction is triggering piecemeal degranulation in mast cells? How does mast cell-fibroblast cell-cell interaction affect the release of inflammatory mediators from mast cells respectively fibroblasts?

To find out the specific role of tryptase and chymase on a molecular level, and how these two mast cell mediators interact with each other and with fibroblasts in health and disease is an important step, and to investigate what the mechanisms behind this regulatory feature is. The protective role of chymase in cellular functions and ECM productions, has also to be investigated in more details. By which mechanism is chymase activating fibroblasts, and which is the cellular response in fibroblasts?

Another interesting future study will be to co-culture healthy respectively IPF lung fibroblasts together with mast cells in healthy lung scaffolds, and investigate the inflammatory mediator release from these cells. By using this 3D culture model, we can investigate the role of mast cells in a more physiological 3D environment. Also the ECM role and how the ECM interacts with mast cells and fibroblasts may be interesting to find out.

PAR2 is an interesting receptor that may be involved in many different diseases. More investigations are needed about intracellular signalling pathways independent of typical protein-coupling-activation of PAR2, such as activation of β-arrestin signalling pathway to identify the outcome, the initiator and driving force of chronic lung diseases, at cellular and molecular level.

Mast cells and their ability to orchestrate interactions between different cells and mediators at specific locations in the tissue, makes them unique and interesting. In particular, the crosstalk with fibroblasts may be of major importance during tissue remodelling in chronic lung diseases. There is still a significant need of research regarding the different mediators and mechanisms that mast cells are able to induce and activate. More united research is warranted to find therapeutic strategies for preventing or reducing uncontrolled tissue remodelling in chronic lung disorders.

Populärvetenskaplig sammanfattning

De flesta känner till att histamin ger upphov till allergiska reaktioner när kroppen visar överkänslighet mot till exempel pollen eller kvalster. Men vad många inte vet är att histamin produceras av en celltyp som kallas för mastcell. Dessa celler har även andra viktiga funktioner i kroppen. Mastcellen ingår i kroppens medfödda immunförsvar och finns framförallt i kontaktytan med yttre miljön, som till exempel i lungor, huden och tarmarna. Mastcellen innehåller små blåsor, så kallade granula som innehåller olika ämnen, bland annat histamin, heparin, enzymer samt en mängd olika tillväxtfaktorer, viktiga för att signalera till andra celler. När mastcellen träffar på ett främmande ämne i kroppen aktiveras den snabbt och släpper explosionsartat ut innehållet ur sina granula. Detta är det vanligaste händelseförloppet vid allergiska reaktioner, då kroppen reagerar på det främmande ämnet som den tror är en fara. Forskning kring mastcellernas roll vid allergiska reaktioner och astma är välstuderad, medan kunskap saknas kring deras betydelse vid icke-allergiska lungsjukdomar.

Numera har forskning visat att mastceller agerar som immunförsvarets dirigenter. De styr viktiga processer i kroppen vid skada eller inflammation. På senare år har man kunnat konstatera en ökad mängd mastceller även i kroniska lungsjukdomar som kroniskt obstruktiv lungsjukdom (KOL) och idiopatisk pulmonell fibros (IPF). Det som är gemensamt för dessa två sjukdomar är en kronisk pågående inflammation och förändrad produktion av bindvävsproteiner, så kallad remodellering. Inflammationen är kroppens sätt att försöka reparera den skadade lungvävnaden. Detta gör den med hjälp av ett samspel mellan olika celler, framförallt aktiveras så kallade fibroblaster. Fibroblaster är långlivade bindvävsceller som är kroppens största producenter av kollagen och andra bindvävsproteiner. Bindväven bygger upp strukturen i ett organ och utgör en viktig byggnadsställning för cellerna i lungan. Fibroblaster kommunicerar både med varandra och även med andra celler i lungan, såsom inflammatoriska celler som mastceller. Vid en fortlöpande och ihållande inflammation, fortsätter fibroblasterna att okontrollerat producera bindväv. Detta kan med tiden skapa en negativ loop, ju mer bindväv desto mer inflammatoriska ämnen bildas, som i sin tur bidrar till mer bindväv. Detta leder till ett kroniskt, inflammatoriskt tillstånd med förändrad bindväv, vilket sker vid KOL och IPF.

I denna avhandling har framförallt samspelet mellan fibroblaster och mastceller studerats. Detta samspel är väldigt viktigt för regenerering och reparation av skadad vävnad. Vi har kunnat visa att en av mastcellens granula innehållande enzymer, tryptas, ökar migrationen av fibroblaster. Tryptas påverkade även utsöndringen av flera viktiga inflammatoriska ämnen från fibroblaster, som i sin tur påverkade bindvävsdistributionen. Framförallt kunde vi visa att mastcellens enzymer, tryptas och kymas, hade olika påverkan på fibroblasternas inflammatoriska svar. Vi har även kunnat visa via vilken signalleringsväg tryptas reglerar fibroblasternas funktioner. Genom att hitta ledtrådar hur dessa celler samspelar med varandra så kan vi förstå vilka faktorer som är betydelsefulla för övergången från en vanlig inflammation till en kronisk inflammation.

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