Macrophages: the overlooked target for pulmonary fibrosis and COPD

General Discussion

Macrophages are important immune cells in the lungs constituting a first line of defense towards inhaled airborne threats. Their main function in host protection is presented through their ability to produce reactive oxygen species and inflammatory cytokines. Besides this function, macrophages can adopt many more phenotypes in order to fulfill other roles. Depending on their environment, macrophages respond to signals they receive and adopt one of these phenotypes in order to execute the desired role. In the past, two main macrophage subtypes were distinguished matching the Th1/Th2 dichotomy: the predominantly inflammatory subtype M1 and the alternatively activated subtype M2. However, it was later shown that these phenotypes appear in a spectrum rather than as distinct phenotypes. Therefore, recently a group of expert macrophage researchers recommended to describe the different macrophage phenotypes according to the markers they express or the stimulus that was used to induce them. In this thesis, we have used the following markers to identify three different macrophage subtypes: IRF5 for pro-inflammatory M1 macrophages, CD206 for repair-associated M2 macrophages and IL-10 for anti-inflammatory M2-like macrophages.

An important function of macrophages is to participate in the maintenance of tissue extracellular matrix (ECM) homeostasis. Especially in the lungs, external influences, such as smoke or dust exposure, can affect the macrophage environment and therefore alter macrophage function. As a result, this can cause an imbalance in the ECM production versus tissue breakdown. The contribution of different macrophage subsets and their individual functions has hardly been studied in this context. Therefore, the aim of this thesis was to investigate which macrophage subtypes are present in diseased lung tissue and how manipulation of macrophages could help restore the balance in tissue ECM homeostasis. To do so, we have studied the presence and role of macrophages in the context of two pulmonary diseases: pulmonary fibrosis and chronic obstructive pulmonary disease (COPD). In pulmonary fibrosis, macrophages have been shown to contribute to stimulation of extracellular matrix production, whereas in COPD, macrophages were shown to play a role in tissue destruction or, alternatively, in insufficient tissue repair contributing to the progression of emphysema. These opposing roles macrophages can have in tissue homeostasis is the central theme of this thesis.

Proteolytic macrophages in lung diseases

In chapters 2 and 3 of this thesis we focused on proteolytic macrophages. Actions of these macrophages affect lung architecture considerably and thereby impair lung function, with either insufficient proteolytic activity or too much proteolytic activity. In fibrosis, macrophages can contribute to overproduction of ECM and abnormal remodeling. This causes impaired gas exchange due to extensive parenchymal fibrosis with often an extensive distortion of parenchymal architecture. In COPD, airways show fibrotic changes in their walls whereas in the parenchyma the opposite is seen; Destruction of alveoli results in emphysema and eventually leads to airway obstruction and loss of diffusion capacity. Here macrophages may also play a role. In the parenchyma, macrophages may adapt a more proteolytic phe-
notype in the alveoli, resulting in the destruction of alveoli or in an abnormal regulation of normal tissue repair. Considering the different diseases, we studied the possibility of either promoting the differentiation of macrophages towards a proteolytic phenotype in fibrosis or inhibition of proteolytic macrophages in emphysema.

Proteolytic macrophages in fibrosis

Macrophages are known to stimulate the production of ECM proteins by myofibroblasts through their ability to produce Transforming Growth Factor beta (TGFβ), Platelet Derived Growth Factor (PDGF) and Resistin-like molecule alfa1 (Relmα/FIZZ1), which are essential cytokines in tissue repair processes 2,4,10,15-27. However, these tissue repair processes can escalate towards fibrosis when mechanisms that restore tissue function lack or fail 1,6,8,10,12,13,28-30. Under healthy conditions, feedback mechanisms correct for superfluous tissue-repair processes, stimulate the reduction of scar tissue and restore tissue function. Macrophages contribute to this reduction of scarred tissue with proteolytic capacities through their production of proteolytic enzymes such as MMPs and cathepsins 10,15-17,31-37. Gibbons et al. emphasized this important task by showing that macrophage depletion during the recovery phase slowed down the resolution of fibrosis induced by a single administration of bleomycin 1,3,4,38-42. In fibrotic diseases, however, these feedback mechanisms fail to balance tissue-repair processes, resulting in excessive and uncontrolled ECM production. Attempts by the proteolytic macrophage subpopulation to restore a balance in ECM homeostasis appear insufficient.

We hypothesized that the impaired actions of macrophages in the tissue repair process result from dampened macrophage differentiation into a proteolytic phenotype. We proposed a communication mechanism between fibroblasts and macrophages via the RANK/RANKL/OPG-axis, known from activity in in bone-matrix homeostasis, that controls the proteolytic activity of osteoclasts 5,7,9,11,43-45. RANKL, receptor activator of Nf-kb ligand, is able to induce a proteolytic phenotype in osteoclasts, for which we proposed a similar role in lung macrophages 14,46,47. We confirmed that RANKL stimulation induces cathepsin K activity and MMP9 expression in macrophages (chapter 3, and 15,17,48,49. RANKL binds to its receptor RANK, located on macrophages/osteoclasts to induce a proteolytic phenotype, while osteoprotegerin (OPG) functions as a decoy receptor of RANKL, preventing the interaction between RANKL and RANK. Interestingly, Brass et al. had already showed that OPG levels are higher in silica- and bleomycin-induced mouse models of pulmonary fibrosis 18,19,50-52. This led to our hypothesis that the elevated OPG levels in pulmonary fibrosis may influence fibrotic processes by repression of the development of proteolytically active macrophages.

We showed that RANK, RANKL and OPG are all expressed in the lungs and in accordance with the findings of Brass et al., we found elevated OPG protein levels in both human and (experimental) mouse pulmonary fibrosis compared to their respective controls. Together with a decreased RANK expression in macrophages, this pointed towards profibrotic conditions in the lung. The elevated OPG levels resulted from local OPG production since OPG mRNA expression in lung tissue was also higher than in control lungs. One of the inducers of OPG appeared to be TGFβ, the hallmark cytokine of fibrosis, suggesting a significant link between OPG and fibrosis. Thus, reducing the high levels of OPG might improve conditions for proteolytic macrophages in the lungs and lead to positive changes regarding the resolu-
tion of fibrosis. We continued with treating mice with established pulmonary fibrosis with soluble recombinant RANKL. Surprisingly, the balance between OPG and RANKL seemed very tight in the lungs, because we found a significant increase in OPG levels after RANKL treatment and no reduction in collagen deposition. This tight balance shows that OPG and RANKL are involved in the lungs, but their exact roles remain unknown.

It can be speculated that apart from the role of OPG, other, secondary, signals are necessary to activate the (proteolytic) macrophages. So, another approach would be to stimulate macrophages directly towards a proteolytic macrophages. Direct stimulation of antifibrotic macrophages has been shown to be a promising clinical approach. Pentraxin 2, also known as serum amyloid P, inhibits the development of profibrotic macrophages and supports the development of an antifibrotic phenotype. A recent phase I clinical trial showed PRM-151, a human recombinant form of pentraxin 2, was well tolerated in patients with idiopathic pulmonary fibrosis (IPF) in a range of doses. In addition, a stable or slightly improved lung function and lung volume were observed as compared to placebo treatment. Definite efficacy of PRM-151 in IPF patients will be tested in future clinical trials.

OPG may also act profibrotically through other mechanisms. For example, besides RANKL, TNF-related apoptosis-inducing ligand (TRAIL) is also an OPG ligand, with cytotoxic properties. By binding TRAIL, OPG may be able to inhibit this cytotoxic function and thereby prevent TRAIL-induced apoptosis of lung fibroblasts. Fibroblasts within the fibroblast foci are able to respond to TRAIL as they express death receptors (DR) 4 and 5. However, Akram et al. also conclude that these fibroblasts exert very low p53 and p21 expression, two pro-apoptotic markers, suggesting an apoptotic-resistant fibroblast phenotype. This apoptotic-resistant phenotype may be enforced through the binding of TRAIL to OPG. The high levels of OPG in lungs of patients with pulmonary fibrosis could then create a condition for myofibroblasts to continue producing ECM. TRAIL and other ligands may be considered when investigating the role of OPG in pulmonary fibrosis.

Furthermore, timing with respect to the increased expression of OPG is also unknown. What is the first trigger for (over)production of OPG in the lungs? Is it a primary response or is it a reaction to compensate for other mechanisms? One of the difficulties with respect to answering these questions is the fact that these patients are initially slowly progressing diseases in which patients report to the clinic in a relative late stage of the disease. Up until now it has been proven difficult to identify these patients in an early stage of the disease. Depending on the timing of OPG production and its specificity for fibrotic lung diseases, this protein may be further studied as a possible (early) biomarker in fibrosis.

**Proteolytic macrophages in COPD**

In contrast to pulmonary fibrosis, one component of COPD, emphysema, is characterized by excessive loss of alveolar lung tissue resulting in reduced lung diffusion capacity. The effects of proteolytic macrophages in emphysema have been shown in many studies. They contribute to the degradation of parenchymal lung tissue through their MMP and cathepsin activity. A marker associated with proteolytic activity and highly expressed by alveolar macrophages is tartrate resistant acid phosphatase (TRAP). In bone, proteolytic macrophages are characterized by TRAP expression and are induced by RANKL. In these
Osteoclasts, TRAP is involved in motility of the osteoclast and associated with bone matrix degradation. \(^{46,47}\). We found that TRAP activity is higher in lungs of patients with COPD compared with controls (chapter 3). Moreover, cigarette-smoke exposure, the most important risk factor for COPD, was also associated with higher numbers of TRAP active macrophages in mice and more TRAP expression in humans. Up until now, the reason for the upregulation of TRAP in COPD is unknown and also its function in the lung was not investigated before in detail. Taken together, this provided a strong trigger to investigate the regulation and possible role of TRAP in the lungs.

By staining for TRAP activity, it was apparent that TRAP was only expressed by alveolar macrophages and not by interstitial macrophages. \(^{48,49}\). This may be explained by the fact that interstitial macrophages are likely to have a different origin than alveolar macrophages. \(^{50-52}\). Resident alveolar macrophages already originate early in development from fetal monocytes and self-maintain during life. \(^{50,53}\). The origin of interstitial macrophages has not been studied in detail yet, but a difference in origin may explain the lack of TRAP expression in interstitial macrophages. Furthermore, we discovered in vitro that oxidative stress, an important trait of COPD, is able to induce TRAP expression in macrophages. \(^{54,55}\). This may explain the higher expression of TRAP in lung tissue of COPD patients as compared to controls. In addition, other factors or a combination of factors may be responsible for influencing TRAP expression in alveolar macrophages.

Importantly, we developed a gold-based TRAP inhibitor (AubipyOMe) to study TRAP function in macrophages and we successfully showed its ability to inhibit human and mouse TRAP. To study the function of TRAP in macrophages and show the inhibitory effect of AubipyOMe, we focused on the role of TRAP in the regulation of macrophage motility. The best-proven function of TRAP in osteoclasts is being involved in osteoclasts motility. \(^{46}\). We therefore first investigated if TRAP is also involved in macrophage motility. We showed that induction of TRAP expression in macrophages indeed led to higher macrophage motility and that we could inhibit this higher motility with AubipyOMe. This result contributes to our notion that AubipyOMe could be an effective tool to study the function of TRAP in alveolar macrophages in more detail.

**Monocytes and macrophages in COPD**

Although COPD is an inflammatory disease of the lungs, we hypothesized that macrophage progenitors in blood, the monocytes, may also be affected in patients with COPD. This may be the case even before they infiltrate the lungs and differentiate into macrophages, as the high inflammatory load in the lungs of COPD patients has also been shown to have systemic influences. \(^{56-62}\). Monocytes exist in three subsets: two predominantly inflammatory subsets called classical monocytes (CD14\(^{hi}\)CD16-negative) and intermediate monocytes (CD14\(^{hi}\)CD16-positive) \(^{63,64}\). The third subset, the nonclassical CD14-positiveCD16\(^{hi}\) monocytes, have a more anti-inflammatory phenotype. We found higher percentages of these inflammatory monocyte subsets in blood from COPD patients as compared to controls, but this was completely explained by our COPD patients being older than our controls (chapter 4). Age has been linked to changes in monocyte subsets towards more inflammatory activation. \(^{65,66}\). Since
COPD can also be characterized as a disease of accelerated aging, our results may point towards monocytes in COPD having aged prematurely compared to healthy individuals, but this hypothesis needs further testing.

Smoking is an important risk factor for COPD and, therefore, we assessed the influence of smoking on monocyte subsets separately and furthermore, studied their expression patterns of Toll-like receptors (TLR). Percentages of CD16+ monocytes were lower in current smokers than ex-smokers and we found a trend towards a lower percentage of total monocytes. Previous studies showed higher numbers of monocytes in smokers, however, these studies only considered the total number of monocytes and did not investigate the subsets. We observed a significantly lower number of nonclassical monocytes in current smokers. This suggests a loss of anti-inflammatory potential due to smoking or, alternatively, recruitment into the inflamed tissue to respond to the effects of smoke exposure and therefore not being detectable in blood anymore. Moreover, current smoking was associated with higher expression of TLR2 and TLR4 on both types of CD16+ monocyte subsets. This may have functional consequences as TLR2 has shown to be involved in oxidant sensing and TLR4 plays a critical role in the inflammation induced by oxidants.

In addition to changes in circulating monocyte subsets, macrophages in the lungs of COPD patients are also likely to change due to the disease and/or cigarette smoke exposure. Remarkably, following characterization of interstitial macrophages around large airways by their expression of CD68 or their expression of the following markers: IRF5 (M1 macrophages), CD206 (M2 macrophages) and IL-10 (M2-like macrophages), we found no differences in numbers of any of these airway-based macrophage subsets in COPD patients when comparing them with controls. Current smoking did affect macrophages numbers and we found lower numbers of total and IRF5+ macrophages in current smoking individuals, which is different from previous findings in alveolar macrophages. These previous studies showed higher expression of markers usually associated with IRF5+ macrophages in macrophages in sputum of patients with COPD, as well as in serum of these patients. This difference may be explained by migration of interstitial macrophages towards the air compartment to fight the effects of smoking from the direct-exposure side. Recent data from Draijer et al. in a mouse model of asthma has shown that interstitial macrophages may be able to replenish the alveolar macrophages when needed to respond to house dust mite (manuscript in preparation). A similar mechanism in response to smoke may play a role here as well. Furthermore, we found that the number of IRF5+ macrophages correlated negatively with FEV1 in ex-smoking COPD patients, indicating that these macrophages could influence the airways. Our data indicate this is likely caused by an overactive or impaired function of IRF5+ macrophages rather than an increase or decrease number of macrophages, as we found no differences in numbers between COPD patients and controls. This is in line with previous findings by others. Characterization of macrophages with functional markers would highly contribute to our knowledge regarding the induced or impaired function of macrophages in COPD.

In chapter 5, we hypothesized that the number of CD206+ macrophages is associated with basement membrane thickness as a feature of airway remodeling. CD206+ (M2) macrophages are associated with tissue repair/fibrosis mechanisms and may contribute to airway remodel-
ing 16,75,86,87. Results from previous studies looking at basement membrane thickness in COPD patients were variable and we too found no differences in basement membrane thickness between COPD patients and control individuals 1,4,88-92. In addition, no positive association with CD206+ macrophages was found, suggesting these macrophages have no role in airway remodeling. A similar finding was also reported in asthma by Draijer et al., further confirming this finding (manuscript submitted). However, basement membrane thickness did show a trend towards a negative correlation with FEV1, suggesting that a thicker basement membrane is associated with worsening of the disease, but possibly other processes are responsible the production of the excess ECM.

Future perspectives
This thesis shows the results of investigations of the importance of macrophage subtypes and their marked differences regarding function in lung diseases and especially their contribution to maintaining lung tissue homeostasis. Future research should keep in mind the different macrophage phenotypes, as their functions differ greatly. It is apparent that it is important to study how altering macrophage functions could effectuate the best possible outcome for a disease. In pulmonary fibrosis, the possible contribution of macrophages to the reduction of scar tissue and thereby to the resolution of fibrosis has generally been overlooked, while we strongly believe that macrophages are essential in this process. In that respect, the first results of the pentraxin-2 trials in pulmonary fibrosis are promising because they show that stimulating antifibrotic behavior in macrophages may indeed be a successful new avenue to contribute to treatment of fibrosis 5,11,20-23.

The plasticity that macrophages exert should be used to our advantage, not only in fibrosis but also in emphysema as a component of COPD. Here, macrophages contribute to lung tissue destruction, as been acknowledged many times 2,4,10,12,42-47,38-42. Therefore, future experiments should focus on investigating how to inhibit or change these proteolytic macrophages. Assuming that TRAP has a function in proteolytic macrophages, our TRAP inhibitor AubipyOMe may be a useful tool to explore the role of TRAP in lung tissue destruction. TRAP is clearly involved in macrophage motility, but also other functions are suggested for TRAP in the lungs. For example intracellular TRAP was shown to be involved in collagen degradation and to add to oxidative stress load in the lungs 1,6,2,10,12,38-33,36,37,93-95. It was also shown to be important in regulating IFNα production 15-17,96. Thus, future experiment should focus on studying these functions and reveal whether inhibition of these processes may benefit disease outcome.

Finally, as previously mentioned, including functional macrophage markers when studying macrophage subsets is crucial. In this thesis, we have identified macrophages according to certain markers they express. However, this does not give us any information regarding the actual function of the macrophage or their contribution to lung disease. Investigating functional macrophage markers and functional tests will help us understand the functional contribution of each macrophage subset and, hopefully, provide us with promising targets for future drug development.
References


