Chapter 1

General introduction

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Introduction

Macrophages are among the most abundant immune cells in the respiratory tract and these resident immune cells can be broadly divided into two populations depending on their localization: alveolar macrophages (AM) on the air-side that line the surface of alveoli and interstitial macrophages (IM) that reside in the lung tissue itself. The role of these macrophages in the lungs in steady state or diseased conditions has increasingly been the subject of various studies, but a lot remains unknown regarding their phenotype, function and contribution to lung diseases such as pulmonary fibrosis and chronic obstructive pulmonary disease (COPD). Therefore, in this thesis, we studied different macrophage phenotypes in COPD and tried to alter the (undesired) function of pulmonary macrophages (irrespective of their localization) in pulmonary fibrosis and COPD.

Resident macrophages

Recent studies have shown that alveolar macrophages are derived from embryonic progenitors (yolk sac macrophages and fetal monocytes) and self-maintain during steady-state conditions, i.e. they have the ability to divide and replenish the pool of resident AMs during life without contribution of adult monocytes derived from hematopoietic stem cells. AMs are situated in the inner lining of the lungs in order to respond quickly upon inhaled foreign particles or microbes that may potentially be pathogenic. They specialize in phagocytosing these foreign particles from the airway lumen and generate oxygen radicals and inflammatory cytokines as part of their host defense function.

Besides alveolar macrophages, interstitial macrophages populate the lungs within the lung tissue itself instead of the airway lumen. The origin of these IMs has not been defined yet. IMs have a higher turnover rate as compared to AMs, which may indicate that these cells are replenished from blood monocytes, but this is much debated. In addition, there is some evidence that IMs function as a pool for new AMs and may therefore also be derived from yolk sac macrophages and fetal monocytes.

Both AMs and IMs can have homeostatic properties. In steady state it was shown that both types of macrophages express MHC class II, even though the expression was higher on IMs than on AMs. Furthermore, they were found to produce high levels of IL-10 to suppress inflammation following an allergic event or activation by inflammatory cytokines. In addition, AMs are able to regulate the response to an inert antigen by controlling antigen presenting function of dendritic cells and suppress T cell activation. Even though AMs and IMs may have different origins they are both able of adopting different phenotypes depending on the signals they receive from their surroundings, which will be explained in more detail later on in this chapter.

Monocytes

In cases of tissue damage, steady state conditions change and tissue resident macrophages may be supplemented with macrophages derived from incoming monocytes. In mice, two populations of monocytes have been identified based on the expression of the surface mol-
Molecule Ly6C (lymphocyte antigen 6C). Monocytes with high expression of Ly6C are generally called classical or inflammatory monocytes and these patrol the extravascular tissues in homeostatic conditions. During this patrolling function they remain monocytic and do not commit to being macrophages. During inflammation, however, they respond by rapidly infiltrating into affected tissues and they can readily transform into macrophages with limited potential for migration. Monocytes with low expression of Ly6C are called nonclassical monocytes and patrol the blood vessels to monitor endothelial cell homeostasis. They develop from the Ly6C-hi subset and this can also take place in injured or inflamed tissue with subsequent conversion from inflammatory monocyte to wound-healing macrophages that can proliferate locally.

In humans, similar monocyte subsets are found based on the expression of CD14 and CD16. Classical monocytes express high levels of CD14 and no CD16, while nonclassical monocytes express low levels of CD14 and high levels of CD16. Both in humans and mice, an intermediate third subset is suggested to exist characterized in humans by high levels of CD14 and intermediate levels of CD16. The functions of this subset are not well understood, although they have been found to preferentially accumulate in inflamed human livers and have been postulated to play a role in fibrogenesis.

Macrophage activation states
In addition to their role in host defense mechanisms, macrophages in the lung have also been associated with many other processes, including tissue repair processes and destruction of tissue, fibrosis and downregulation of inflammation. In order to combine these diverse and sometimes contradictory activities, macrophages exert the ability to adopt the most effective phenotype based on signals from surrounding tissue. This results in a myriad of phenotypes among macrophages, irrespective of their origin, and the most investigated types are described below.

To distinguish the various macrophage phenotypes, a nomenclature was proposed similar to the Th1/Th2 dichotomy based on results from in vitro studies, with M1 macrophages being known as classically activated macrophages induced by interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-α) and M2 being known as alternatively activated macrophages induced by interleukin (IL)-4 and IL-13. Recent new insights led to changes within the macrophage field concerning the nomenclature of the macrophage phenotypes. It was already apparent that in vivo macrophage phenotypes appear as a continuum rather than discrete entities. In the new nomenclature, the macrophage phenotype is defined on the origin of the macrophage, the substance that induces the specific macrophage phenotype and/or on the markers it expresses. This in contrast to the previously used nomenclature that focused mainly on the expression of a single or a few markers or on the function of the macrophage, i.e. inflammatory macrophage (M1) versus the tissue repairing macrophage (M2). In this thesis, macrophage subsets will mostly be referred to according to the expression of specific markers or, if necessary, the old and new nomenclature will both be mentioned as some cited articles used the old nomenclature. The exact markers will be discussed in more detail later in this section.
IRF5+ M1 macrophages
This macrophage phenotype is activated by pro-inflammatory cytokines and microbial products like IFN\(\gamma\), TNF-\(\alpha\) and/or lipopolysaccharide (LPS) under the influence of the transcription factor interferon-regulatory factor 5 (IRF5) \(^{29}\). These macrophages are essential in host defense against intracellular pathogens by generating reactive oxygen species (ROS) and nitric oxide (NO) through upregulated expression of inducible nitric oxide synthase (iNOS) and amplifying Th1 immune responses by producing pro-inflammatory cytokines like IL-12, IL-1\(\beta\) and TNF-\(\alpha\) (see also figure 1) \(^{28}\). In addition, they show enhanced phagocytosis of micro-organisms, antigen presentation capabilities and enhanced production and secretion of matrix metalloproteinases (MMPs) such as MMP7 and MMP9 \(^{30-33}\). The secretion of MMPs enables macrophage migration during inflammatory responses, but excessive or unregulated production results in tissue damage \(^{26,33}\).

Figure 1. Schematic representation of three macrophage phenotypes and their characteristics. Abbreviations: IFN\(\gamma\): interferon gamma; TNF-\(\alpha\): tumor necrosis factor alfa; LPS: lipopolysaccharide; MHC class II: major histocompatibility complex class II; IL: interleukin; NO: nitric oxide; IRF5: interferon regulatory factor 5; Fe: iron; TGM2: transglutaminase 2; YM1: chitinase-3–like protein-3; FIZZ1/Relm\(\alpha\): resistin-like molecule-\(\alpha\); Arg-1: arginase-1; TGF\(\beta\): transforming growth factor beta; TLR: toll-like receptor; PGE2: prostaglandin E2; PPAR\(\gamma\): peroxisome proliferator-activated receptor gamma.
**M2 macrophages: CD206+ M2 or IL-10+ M2-like macrophages**

Alternatively activated or M2 macrophages were named to indicate their activation status was distinctly different from the classically activated macrophages. First discovered to be induced by IL-4 and IL-13 this phenotype was soon found to have more siblings, closely resembling each other but distinctly different in function. A variety of different names have been suggested according to the expression of various markers. They have suggested alternatively activated or M2 macrophages for the phenotype induced by IL-4/IL-13 and regulatory macrophages or M2-like cells for the phenotype characterized by high IL-10 production that are induced by a variety of stimuli. In this thesis, we will mainly distinguish between these two subsets and refer to them as CD206+ when mentioning the "M2-macrophages" and the "M2-like-macrophages" as IL-10+ macrophages.

1: CD206+ M2 macrophages

Macrophages induced by IL-4/IL-13 under the influence of the transcription factor IRF4, have a role in protection against helminthes and are considered wound-healing macrophages because of their association with physiological and pathological tissue remodeling. They are characterized by upregulated expression of mannose receptors (CD206) and transglutaminase 2 (TGM2) in man and mice and by upregulated expression of arginase-1, chitinase-3-like protein-3 (Chi3l3, also known as Ym1), and resistin-like molecule-α (Relmα, also known as FIZZ1) in mice only. They have poor antigen presenting capabilities and exhibit increased release of iron and increased clearance of apoptotic cells (efferocytosis) and extracellular matrix components.

2: IL-10+ M2-like macrophages

One of the CD206+ macrophage subtypes produces high levels of IL-10. These macrophages are induced by a number of stimuli that need to be combined with a Toll-like receptor (TLR) signal. The initial signals include glucocorticosteroids, prostaglandin E2 (PGE2), antibody immune complexes, transforming growth factor beta (TGFβ) and IL-10 itself. Transcriptional control of this phenotype is unclear but may involve peroxisome proliferator-activated receptor gamma (PPARγ) and the cAMP-responsive element-binding protein (CREB)-CCAAT/enhancer-binding protein-β (C/EBPβ)-axis. As a result of their high IL-10 production, these macrophages have strong anti-inflammatory activity. This can be beneficial during later stages of immune responses to limit inflammation, but may also permit tumor progression when associated with tumors. They may also be the macrophages that produce TGFβ in addition to IL-10, but this has not been rigorously shown due to the overlap in markers among the CD206+ macrophages. Only IL-10 production would be a reliable marker but is used seldom to identify these macrophages. Nevertheless, we will use IL-10 to identify this macrophage phenotype.

**Lung diseases**

The distinct phenotypes and functions of macrophages and the switching between them in lung tissue appear to have developed to maximize defense against external threats without impeding gas exchange. It therefore does not come as a surprise that changes in the number of macrophages and changes in macrophage phenotypes have been associated with many...
pulmonary diseases. Thus, depending on the function, macrophages could be beneficial or harmful. In this thesis we studied what macrophage functions/phenotypes are associated with disease processes and how we could change macrophage function/phenotype in order to have beneficial effects on lung diseases. Using the natural functions of macrophages may be an interesting novel opportunity to induce resolution of lung diseases.

The lung consists of conducting airways and an alveolar compartment. The latter is responsible for gas exchange between air and blood over a thin layer of ECM and epithelial cells (the interstitium). Increased deposition of ECM in the interstitium, as observed in patients with pulmonary fibrosis, or loss of interstitial tissue, as observed in patients with COPD, has an enormous effect on the gas exchange homeostasis and therefore lung function. The regulation of this interstitial ECM in the lung is poorly understood, but macrophages may play an important role in maintaining integrity of this thin layer. In addition, airway interstitial macrophages may also be involved in regulating processes leading to airway remodeling, another important characteristic of COPD. Below we describe what is known regarding the role of macrophages in the light of pulmonary fibrosis and COPD and which gaps in knowledge still remain.

**Pulmonary fibrosis**

Pulmonary fibrosis is a disease that encompasses a collection of restrictive pulmonary disorders characterized by progressive and irreversible destruction of lung architecture by excessive deposition of ECM in parenchymal areas of the lung. While ECM formation usually functions as an essential process of tissue repair after lung injury, continuous lung damage may result in abnormal wound healing and progress to fibrosis. Fibrosis of the interstitium ultimately leads to organ malfunction because of the disturbed architecture of the lung, leading to impaired gas exchange, and eventually death from respiratory failure. In some cases, fibrotic lesions remain localized to a limited area of the lung because the initial trigger is removed, for example after tuberculosis or a fungal infection, while in others such as in sarcoidosis and idiopathic pulmonary fibrosis (IPF) the fibrotic process continues to progress throughout the lungs in a diffuse manner.

Idiopathic pulmonary fibrosis (IPF) is the most common and most dangerous of the fibrotic interstitial lung diseases. The chronic and slowly progressing character of the disease together with an unknown etiology, makes it a difficult disease to diagnose and treat. The incidence of IPF appears to be increasing and is currently estimated at 7-16 cases per 100,000 persons. Patients diagnosed with IPF have a poor life expectancy with a median survival of 2-5 years. Currently there are no effective therapies available for these patients, as no therapy has yet been proven to cure or even halt the progression of fibrosis.

**Pathogenesis of pulmonary fibrosis**

To describe the pathogenesis of pulmonary fibrosis, tissue repair after injury can be divided into four different stages: the clotting phase for emergency wound healing, then the inflammatory phase to fight the inciting agent, followed by formation of scar tissue in the fibrotic phase for more permanent repair and eventually reduction of scar tissue and restoration.
of tissue homeostasis in the resolution phase. During fibrosis some or all of these stages of wound healing are dysregulated as will be discussed below and the same framework will be used to discuss the role of macrophages in fibrosis later on.

Pulmonary fibrosis is thought to be the result of repetitive injury to the epithelial cell layer lining the alveoli. This damage initiates a blood coagulation cascade to prevent severe blood loss and maintain some sort of homeostasis. This includes platelet accumulation and production of fibrin by epithelial cells, which is essential for fibrin-containing clot formation. To restore the function of damaged tissue, plasminogen activator (PA) eventually breaks down this fibrin matrix again. In pulmonary fibrosis, changes in both the coagulation cascade itself and the resolution of the wound-healing clot are known to affect the disease. Impaired fibrin degradation for instance has been shown to worsen epithelial cell survival, that can be caused by either the absence of PA or by increased production of PA inhibitors PAI-1 or PAI-2.

As a result of the cell damage, an inflammatory reaction is triggered. Unfortunately, it has been difficult to investigate the contribution of this phase to the fibrosis because patients usually present to the clinic with end-stage disease. Nevertheless, the inflammatory response has been extensively studied in LPS-induced inflammation in humans (reviewed by Rossol et al.). They concluded that epithelial cell damage induces the release of several cytokines and chemokines that trigger an influx of neutrophils, closely followed by monocytes to fight the inciting agent. Epithelial cells also release growth factors like TGFβ, TNF-α and epidermal growth factor alpha (EGFα) to stimulate tissue healing through the activation of fibroblasts, which are main producers of collagen and other ECM proteins.

Control of the inflammatory event, however, is crucial for a proper wound healing process. Apoptosis and subsequent removal of inflammatory macrophages, initiated by T cells, has proven to be essential in this step. Dysregulation of the inflammatory phase with a prominent role for M1 macrophages has long been thought to be important to the process of fibrosis. However, the fact that anti-inflammatory drugs such as corticosteroids have no therapeutic merit in patients with pulmonary fibrosis has made this assumption unlikely. Now the new prevailing hypothesis is that pulmonary fibrosis probably develops when the fibrotic phase itself and/or resolution phase become dysregulated.

To progress from the inflammatory phase to the next phase of tissue repair, inflammation needs to be dampened. The release of IL-10 and TGFβ by epithelial cells and macrophages dampen inflammation and promotes ECM production by myofibroblasts. Under the influence of TGFβ and PDGF, produced by damaged epithelial cells, platelets, and macrophages, fibroblasts differentiate into myofibroblasts, proliferate and produce ECM proteins. Furthermore, they start producing their own TGFβ to maintain the tissue healing process. In pulmonary fibrosis this phase is probably dysregulated as increased numbers of myofibroblasts and increased production of ECM are found in fibrotic lungs. Increased numbers of CD206+ macrophages are associated with this phase and these macrophages are therefore suggested to play an important role in the development of fibrosis.
Eventually repair of the epithelial cell barrier and removal of excess ECM are essential to recover normal lung function. To overcome the loss of alveolar epithelial type I cells (AEC I), alveolar epithelial type II cells (AEC II) become hyperplastic and provisionally restore the epithelial cell layer along ECM produced by myofibroblasts. Normally these type II cells would revert back to AEC I and homeostasis is restored. However, when injury is repetitive this does not seem to occur; ECM is produced continuously and AEC II continue to proliferate without differentiating to AEC I. In a proper wound healing response, the excess of ECM products is removed to gain full function of the lungs again. Macrophages play an important role in degrading and taking up ECM components. In order to do so, they produce cathepsins, MMPs and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs). A balance between the activities of proteolytic enzymes and their inhibitors determines the ECM-balance within the tissue. Although levels of both, MMPs, TIMPs and cathepsins are all elevated in patients and mouse models of pulmonary fibrosis, their balance is clearly disrupted as the net result is an excess of ECM in fibrotic lungs.

Macrophages and pulmonary fibrosis

Macrophages play an important role in the pathogenesis of lung fibrosis, but their role is complex. They are involved in many of the dysregulated tissue healing responses in fibrosis and adopt numerous phenotypes depending on the stage and condition of the tissue. This complicates studies into their role in fibrosis tremendously. In the next part we will discuss what is known about the contribution of each macrophage phenotype to what stage of fibrosis.

**IRF5+ M1 macrophages in pulmonary fibrosis**

To our best knowledge, we have found no studies reporting on the presence of IRF5+ macrophages in pulmonary fibrosis except for one study by Nagai et al. showing that folate receptor beta (FRβ)-positive macrophages were higher in patients with IPF as compared to controls. These macrophages have previously been shown to produce TNF-α and oxygen radicals and could therefore be IRF5+ macrophages.

Several lines of evidence suggest that IRF5+ macrophages may play a role in both the inflammatory phase as well as resolution phase of pulmonary fibrosis. In response to epithelial cell damage, monocytes are recruited to the site of inflammation and may differentiate into IRF5+ macrophages under the influence of pro-inflammatory cytokines. Once activated, IRF5+ macrophages themselves produce TNF-α, IL-1β, and oxygen radicals to kill and phagocytose microbes to fight an infection or remove an exogenous agent. Many studies indicate that these pro-inflammatory cytokines and oxygen radicals are associated with fibrosis development. In the study by Nagai et al. ablation of FRβ-expressing macrophages during the inflammatory phase of bleomycin-induced fibrosis, abrogated fibrosis development. However, the importance of the contribution of inflammation to established fibrosis has been challenged because anti-inflammatory drugs such as corticosteroids have no therapeutic effects in patients with pulmonary fibrosis. This view was confirmed by a study from Gibbons et al. They studied newly recruited inflammatory macrophages in a mouse model of bleomycin-induced lung fibrosis and showed that depletion of tissue-resident macrophages...
and/or circulating inflammatory monocytes during the inflammatory phase did not affect the onset or degree of fibrosis that developed after this inflammatory phase 87.

During the resolution phase, macrophages are involved in the process of ECM degradation and the uptake of matrix components 71,87,88. Depletion of macrophages during this recovery phase impaired the resolution of fibrosis by slowing down the degradation of ECM 87. It is unclear what type of macrophage is responsible for degradation of ECM, but a case can be made for IRF5+ macrophages as these have been shown to produce several cathepsins and MMPs including MMP7 and MMP9. Levels of MMP9 have been found increased in lungs of IPF patients and this may reflect a failing attempt of the lungs to remove excess ECM caused by a simultaneous increase of the inhibitor TIMP-1 89-91. In addition two other studies pointed out that IRF5+ macs may even be beneficial for fibrosis since they showed that the inflammatory cytokine TNF-α, mainly produced by the IRF5+ phenotype, promoted alveolar epithelial cell recovery and therefore also contributed to resolution 92. Furthermore, Redente et al. showed that TNF-α-knockout mice exhibited delayed resolution of bleomycin-induced pulmonary fibrosis, but they also showed that pulmonary delivery of TNF-α reduced the fibrotic burden in these mice 93. Furthermore, macrophages are also important in the subsequent removal of ECM components through endocytosis-mediated mechanisms. Again it is unclear if this is restricted to one particular phenotype but the receptors involved would suggest more of a CD206+ M2 phenotype and this will therefore be discussed in the next part.

In summary, IRF5+ (M1) macrophages are important in the inflammatory phase but their presence does not appear to affect the subsequent fibrotic phase. During resolution of scar tissue, macrophages are indispensable for degradation of ECM. This may be related to an IRF5+ phenotype and it may therefore be beneficial to stimulate recruitment of these macrophages to reverse fibrosis.

**CD206+ M2 macrophages in pulmonary fibrosis**

There is a great deal of evidence that Th2 responses are important in the development of fibrosis and it appears that IL-13 is the predominant cytokine of profibrotic responses 94-101. Levels of IL-13 are higher in patients with pulmonary fibrosis as compared to controls and macrophages isolated from these fibrotic lungs produce more IL-13 than macrophages from control lungs 102. It therefore comes as no surprise that CD206+ macrophages are associated with pulmonary fibrosis, although we could not find publications directly showing numbers of CD206+ macrophages are increased in lung tissue of patients with pulmonary fibrosis. We did find one study showing higher numbers of CD206+ macrophages and higher levels of other CD206 related markers in BALF of IPF patients as compared to controls and two studies showing higher numbers of insulin-like growth factor-I (IGF-I)-positive and PDGF+ interstitial macrophages in lung tissue of IPF patients as compared to controls 103-105. Both these markers are important profibrotic mediators and a recent study by Chen et al. showed that expression of IGF-I colocalized with arginase-1 and not with IL-10 expression in macrophages suggesting genuine CD206+ macrophages express IGF-I and not the IL-10+ M2-like subset 106. This was a study in mice. It therefore remains to be investigated whether this is also true in humans.
Other markers found on or produced by CD206+ macrophages have also been found increased in pulmonary fibrosis. Levels of galectin-3, a carbohydrate-binding lectin that is necessary for alternative activation, were higher in BALF of IPF patients as compared to control patients. Furthermore, macrophages from IPF patients produced more of the human marker CCL18, related to CD206+ M2 macrophages, than control macrophages and this correlated negatively with pulmonary function test parameters. IPF patients were also found to have higher serum and pulmonary levels of chitinase-like protein YKL-40 as compared to controls, although it is still unclear whether chitinases are true markers of alternative activation in human macrophages. This is also the case for arginase-1, which is a marker of the CD206+ M2 macrophages in mice, but its specificity is debated in humans. Nevertheless, lung tissue from IPF patients had higher expression of arginase-1 in macrophages than normal lung tissue. Lastly, circulating monocytes from systemic sclerosis patients with pulmonary fibrosis showed enhanced profibrotic phenotype by increased expression of CD163, a marker of alternative activation in humans.

Experimental models of pulmonary fibrosis have revealed more about the role of CD206+ macrophages in fibrosis in the lung. Depletion of macrophages during the fibrotic phase of lung fibrosis reduced the deposition of ECM in this organ. To confirm a role for CD206+ macrophages, levels of Ym1 and arginase-1 were measured before and after macrophage depletion. Both markers showed decreased expression in the lungs after removal of macrophages. The inflammatory macrophage marker iNOS did not show a reduction in expression, indicating that the Ym1+/Arg1+ macrophages are predominantly responsible for the development of fibrosis. Venosa et al. depleted macrophages that originate from the spleen by performing a splenectomy before an intratracheal nitrogen mustard exposure to induce fibrosis. The spleen functions as a reservoir of inflammatory monocytes that migrate into injured tissue and they found a decrease in the mature subset of M2 macrophages, accompanied by a larger M1 population and more tissue damage after splenectomy. Especially levels of IL-10, a key cytokine to dampen inflammatory reactions, were much lower in the splenectomy groups as compared with the sham group. Unfortunately, no further distinction was made between M2 and M2-like macrophages, but the reduction in IL-10 production suggests a reduction in the number of M2-like macrophages. Furthermore, the M2 marker MMP12 was shown to be essential in the development of fibrosis induced by excessive activation of Fas and in a model of IL-13 dependent fibrosis.

There is some evidence regarding the mechanisms behind CD206+ macrophages and their contribution to the development of fibrosis. The aforementioned production of IGF-I and PDGF contribute to proliferation of fibroblasts and stimulate their transformation towards ECM-producing myofibroblasts. Furthermore, FIZZ1 (also known as resistin-like molecule alpha (RELMα) expressed by alternative activated macrophages) was found to increase ECM production in fibroblasts. The same group found that FIZZ1 is induced in bleomycin-induced lung fibrosis and significantly less fibrosis was seen in FIZZ1 knock-out mice. Moreover, FIZZ1, expressed by skin macrophages, aids an effective wound healing process following injury when stimulated with IL-4. Here, FIZZ1 activation stimulated collagen fiber cross-linking that promotes tissue repair. However, a paper by Pesce et al. showed that FIZZ1 actually ameliorated fibrosis development by negatively regulating Th2-dependent
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responses\(^{120}\). These contradictory findings highlight other new findings that suggest that FIZZ1+ macrophages could be antifibrotic. A mechanistic study in a model of Schistosoma-induced liver fibrosis with specific deletion of IL-4R\(\alpha\) on myeloid cells, preventing alternative activation of macrophages, showed that IL-4-induced macrophages are not required for fibrosis development\(^{121}\). In addition, related studies with mice lacking arginase-1 in macrophages showed that the arginase-1-expressing macrophages were even required for suppression and resolution of fibrosis\(^ {120}\). This correlates well with findings that uptake of ECM components appears to be mediated by CD206+ macrophages in general. Uptake of these components is mediated by different mannose receptors and by glycoprotein milk fat globule epidermal growth factor 8 (Mfge8)\(^ {42,122}\). Mannose receptors are of course known alternative activation markers but for Mfge8 this is unclear, even though both the mannose receptor 2 and Mfge8 were shown to attenuate fibrosis in different models\(^ {122,123}\).

Besides the conventional cytokines that promote the development of the M2 macrophages, like IL-13 and IL-4, several studies have looked at additional regulatory mechanisms behind M2 polarization. The protein IL-1R-associated kinase-M (IRAK-M), upregulated by surfactant proprotein A (SP-A), was first identified as an inhibitor of TLR-mediated inflammation in human macrophages\(^ {124}\). Macrophages with upregulated IRAK-M expression produced less TNF-\(\alpha\) and IL-6 in response to LPS exposure. Then recently, Ballinger et al. showed that besides suppression of M1 cytokines, IRAK-M seems positively influence the differentiation of macrophages towards an M2 phenotype\(^ {125}\). IRAK-M expression in macrophages was higher following bleomycin exposure and was accompanied by an increased IL-13 production, while macrophages from IRAK-M\(-/-\) mice showed lower IL-13 production. In humans, IRAK-M expression was higher in IPF patients than in controls and correlated positively with arginase expression, suggesting a link between IRAK-M and fibrosis. In addition, inhibitory factors of M2 activation have also been revealed. Disrupted activity of tyrosine phosphatase ShP2 promoted alternative activation\(^ {126}\). ShP2 is able to suppress the JAK1/STAT signaling pathway that is activated after IL-4 stimulation, thereby suggesting that the phosphatase activity of Shp2 is able to inhibit alternative activation.

To summarize, CD206+ M2 macrophages are firmly associated with fibrosis development but new evidence suggests they may actually contribute to resolution of fibrosis. Their presence during fibrosis may be explained as a failing attempt to clear the excess ECM. The conflicting roles described in literature may be the result of difficulties separating the effects of CD206+ M2 and IL-10+ M2-like macrophages simply because these two subsets are difficult to distinguish. IL-10+ M2-like macrophages may be a more likely candidate for the promotion of fibrosis as will be discussed below.

**IL-10+ M2-like macrophages in pulmonary fibrosis**

The signature marker of these macrophages is IL-10, which is the canonical anti-inflammatory cytokine with profibrotic actions. Elevated levels of IL-10 and enhanced production of IL-10 by alveolar macrophages have been reported in several fibrotic diseases, including IPF and in systemic sclerosis patients with interstitial lung disease\(^ {112,127,130}\). Its anti-inflammatory actions in lung are illustrated by a study from Armstrong et al., showing that IL-10 inhibited TNF-\(\alpha\) production by alveolar macrophages after LPS stimulation\(^ {131}\). Interestingly, induction of the
IL-10 production by a low dose of LPS protected lung tissue against a subsequent lethal dose of LPS. In addition, several studies in mice using the model of bleomycin-induced fibrosis suggest that IL-10 attenuates bleomycin-induced inflammation and can thereby attenuate fibrosis development. However, overexpression of IL-10 in lungs of mice was found to be profibrotic. Sun et al. found that inducible IL-10 overexpression in Clara cells induced fibrosis by fibrocyte recruitment and activation of IL-13+IL10+ macrophages expressing Arg1. The increased levels of IL-10 found in lungs of IPF patients may therefore contribute to the fibrotic process.

MMP28 has also been shown to regulate M2 polarization and dampen the M1 polarization. MMP28−/− mice showed high levels of M1 markers at baseline and less M2 markers following bleomycin induced experimental fibrosis. Moreover, MMP28 influenced the IL-10 production; MMP28−/- mice showed lower basal levels of IL-10 compared to wild type. The macrophage receptor with collagenous structure (MARCO) also seems to play a role in the alternative polarization of macrophages in fibrosis. Following instillation of chrysotile asbestos, MARCO−/− mice expressed significantly lower levels of M2 markers: Ym1 and active TGFβ and had less fibrosis. In contrast, inflammatory mediators related to the M1 phenotype, TNF-α and IL-1β, were also increased in these knockout mice. Furthermore, patients with asbestosis, characterized by chronic inflammation and scarring of lung tissue, showed a predominant M2 phenotype with high levels of MARCO. The high levels of IL-10 and TGFβ that are associated with MARCO suggest that these macrophages are likely to be IL-10 M2-like macrophages. The high levels of the profibrotic, anti-inflammatory cytokine TGFβ that often coexist with high IL-10 levels would also fit with the role of dampening inflammation and promoting tissue repair by this macrophages subset. Whether TGFβ production is restricted to the IL-10-producing macrophage subtype remains to be investigated.

Figure 2. Schematic representation of the presence of IRF5+, CD206+, and IL-10+ macrophages in lung tissue during homeostatic conditions and after injury to the lung. Normally after lung injury a process of tissue repair is initiated with four distinct phases leading to homeostatic conditions again. In lung fibrosis this normal tissue repair response is suppressed or dysregulated leading to deposition of excess extracellular matrix and little resolution of scar tissue.
In summary, IL-10+ macrophages are likely candidates for the promotion of fibrosis. They may be recruited or induced by damage to the epithelium to dampen inflammation and start up tissue repair processes. In the event of on-going damage they are continually induced or recruited and may contribute to fibrosis by the overexpression of IL-10. Since corticosteroids are also capable of inducing IL-10+ macrophages, this would explain why these drugs are not effective against fibrosis and may even be disadvantageous. This was also illustrated by our finding that when corticosteroids are specifically delivered to liver macrophages in a model of liver fibrosis, fibrosis actually becomes worse.

Overall, current data on the role of macrophages in the development of pulmonary fibrosis show that macrophages are important cells in the pathogenesis of this disease (see also figure 2). IRF5+ M1 macrophages are important in the inflammatory phase and may also be important for resolution of the disease, although this hypothesis needs testing. CD206+ and CD206+IL-10+ macrophages are highly associated with fibrogenesis. However, new data suggest that the CD206+ macrophages may actually protect against development of fibrosis while the CD206+IL-10+ macrophages contribute to fibrosis. Therefore, key to understanding how these two phenotypes contribute to pulmonary fibrosis are studies differentiating between these two subpopulations. Eventually, macrophages may prove important in the resolution of fibrosis due to their expression of various proteolytic enzymes.

COPD
COPD is one of the most common respiratory diseases and affects around 320,000 people in the Netherlands (Annual Report 2011 Dutch Lung Fund). It is projected to be the fourth leading cause of death worldwide by 2030 and places a huge economic burden on society. COPD is caused by lung inflammation due to inhalation of noxious gasses and particles: in the Western World most commonly from cigarette smoking and in developing countries from indoor biomass cooking and heating. COPD is characterized by airflow limitation that is not fully reversible, which is caused by a combination of obstructive bronchiolitis (also known as chronic bronchitis) and destruction of alveoli resulting in airspace enlargement (also known as emphysema). The relative contributions of chronic bronchitis and emphysema to the COPD phenotype can vary from person to person.

Pathogenesis of COPD
Exposure to smoke and particles leads to an exaggerated chronic inflammation in lungs of people susceptible to the development of COPD. Excess mucus production and progressive narrowing of the respiratory bronchioles characterize chronic bronchitis. The mucosa, submucosa, and glandular tissue become infiltrated with inflammatory cells and the walls of the respiratory bronchioles become thickened because of edema and fibrosis. Chronic mucus hypersecretion is induced by goblet cell hyperplasia and hypertrophy of submucosal glands, which further contributes to occlusion of small airways. This progressive narrowing leads to obliteration or even complete disappearance of respiratory bronchioles. Not much is known about the role of macrophages in this part of the disease, but pigmented macrophages were found to cluster around small airways and these were associated with peribronchiolar fibrosis.
Within the peripheral compartment, alveolar destruction that characterizes emphysematous COPD is the result of the infiltration of inflammatory cells with a prominent role for macrophages. Both neutrophils and macrophages are being recruited to the lung because smoke/particle exposure injures epithelial cells that subsequently release cytokines and chemokines to recruit inflammatory cells. Neutrophils and macrophages have been postulated to be the main effector cells contributing to the excess tissue damage seen in emphysema because of their ability to produce proteolytic MMPs like neutrophil elastase and macrophage elastase (MMP12). Increased numbers of macrophages and neutrophils have been found in airways and lung parenchyma of patients with COPD. However, only the number of parenchymal alveolar macrophages was directly proportional to the severity of lung destruction in emphysematous lung tissue from COPD patients. Animal studies confirmed the dominant role for macrophages, because deletion of neutrophils in smoke-exposed rats did not prevent cigarette smoke-induced emphysema, whereas deletion of macrophages did. In addition, mice deficient in MMP12 (mainly produced by macrophages), were completely protected from cigarette-smoke-induced emphysema even though they could still produce neutrophil elastase. Similarly, inhibiting MMP12 reduced smoke-induced airway inflammation in mice.

Macrophages and COPD
The role of the different macrophage phenotypes in COPD is the topic of quite a few studies recently and the subject of much debate as the results have been somewhat counterintuitive. Based on studies in mice and results from patient studies, IRF5-directed polarization is expected to play an important role in the pathogenesis of COPD. However, the results of other studies have questioned this view and this is nicely illustrated by studies from Shaykhiev et al. and Hodge et al. The first ones recently studied the transcriptome of alveolar macrophages from healthy smokers and nonsmokers and compared them to alveolar macrophages from COPD smokers. Their results showed a mixed phenotype for alveolar macrophages after smoking with downregulation of genes associated with IRF5+ M1 macrophages and partial upregulation of CD206+ M2 genes, which was progressively worse in COPD. Hodge et al. showed a mixed phenotype in alveolar macrophages of smoking COPD patients with some M1 (MHC II expression) and M2 (efferocytosis) markers going down and some going up (pro-inflammatory cytokine production and DC-SIGN expression). In the next part we will touch upon this debate as we discuss the separate phenotypes in the pathogenesis of COPD.

IRF5+ M1 macrophages in COPD
Several lines of evidence support a role for IRF5+ macrophages, but also a role for dysregulated IRF5+ macrophages in the development of COPD, suggesting an undesired function or an actual genetic defect. First of all, exposure to compounds in smoke appears to induce inflammatory/M1 polarization of macrophages. Smoking is the most important risk factor for COPD and cigarette smoke contains many thousands of compounds, including LPS that can activate macrophages in the lung. Indeed, increased expression of iNOS in alveolar macrophages was found in COPD patients. Upregulation of iNOS increases ROS and NO production and can then cause oxidative stress, an important contributor to the pathogenesis
of COPD. Smoking itself can also cause oxidative stress and thereby add to the stress caused by the increase in iNOS activity in M1 macrophages.

Furthermore, many studies have shown that smoke exposure enhances the release of the M1 pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α. Inflammatory/M1-derived cytokines also play a role in the pathogenesis of COPD. IL-1β, IL-6, IL-8, and TNF-α have all been found elevated in COPD and in experimental settings have been found to contribute to the development of persistent airway inflammation, emphysema and mucus production. TNF-α was found to drive most of the emphysema development in the alveolar compartment in mice after smoking because mice lacking receptors for TNF-α only developed mild emphysema. This increase in TNF-α production was shown to correlate with the influx of inflammatory monocytes in mice in a mouse model for acute lung injury, together with high MMP12 levels in the lung. MMP12 has been indicated as an M2 marker, but the different phenotypes were not distinguished in this study. Though, both TNF-α and MMP12 are known to contribute to emphysema. Mice overexpressing TNF-α in lung tissue develop chronic inflammation and emphysema. However, in humans antibodies against TNF-α seem to be ineffective in COPD, questioning the relevance of this cytokine for human COPD. In addition to TNF-α, IL-1β also an inflammatory/IRF5+ macrophage cytokine, was found to play a role. Overexpression of IL-1β in lung caused lung inflammation, emphysema, mucus metaplasia, and airway fibrosis in mice. Taken together these data suggest cytokines produced by IRF5+ M1 macrophages at least play a role in the pathogenesis of COPD.

Another important inflammatory/M1-related cytokine with a role in COPD is IFNγ. It is produced by CD8+ T cells that infiltrate the lungs in COPD and can stimulate IRF5-directed polarization. Inducible overexpression of IFNγ in lungs of mice caused emphysema with alterations in the balance of MMPs and antiproteases. However, in human alveolar macrophages from smokers a reduction in IFNγ receptor expression and reduced IFNγ signaling were found, suggesting IRF5-directed polarization may be impaired after smoking. This of course is in line with the above-cited finding by Shaykhiev et al. that inflammatory genes are downregulated in alveolar macrophages of healthy smokers and smoking COPD patients as compared to nonsmokers.

IRF5+ M1 macrophages have also been found to produce MMP9, presumably to enable macrophage migration during inflammatory responses. In addition, MMP9 is associated with the breakdown of extracellular matrix in the parenchyma in COPD as macrophages from patients with COPD have a significantly higher production of MMP9 as compared to control macrophages. This was confirmed by Foronjy et al., showing that overexpression of human MMP9 in mouse macrophages induced emphysema and loss of alveolar elastin pointing at a role for these macrophages in COPD development.

Finally, an important property of IRF5+ macrophages that appears to be dysregulated is the phagocytosis of microorganisms. IRF5+ macrophages are geared towards killing and disposal of microbial threats and phagocytosis of micro-organisms is part of that function. COPD is often exacerbated by infections and there is accumulating evidence that reduced macrophage phagocytosis in COPD may be responsible for the persistence of micro-organisms in
the lungs. The cause of the impaired phagocytosis may be a result of reduced expression of essential proteins or a general genetic defect. Presence of micro-organisms even correlated positively with disease severity but seems to be pathogen specific. This dysfunction of phagocytosis is not restricted to micro-organisms but also appears to be present for CD206+ macrophage-related phagocytic functions such as efferocytosis and mannose receptor-mediated uptake. This overall inhibition of phagocytosis irrespective of macrophage phenotype was further confirmed by the later study of Hodge et al. that has already been mentioned before.

Taken together, the available data suggest that a dysregulated IRF5+ macrophage, either in function or a genetic defect, response plays a role in COPD rather than an increased number of these macrophages. Some aspects of the IRF5+ macrophage activation signature are upregulated in COPD (ROS generation, pro-inflammatory cytokines, production of MMP9), but some aspects and functions are downregulated (phagocytosis, IFNγ responsiveness).

CD206+ M2 macrophages in COPD
Overexpression of prototypical IRF5+ macrophage-inducer IFNγ may be able to induce emphysema, but so does overexpression of prototypical M2-macrophage inducer IL-13. Zheng et al. showed that overexpression of IL-13 in mouse lung tissue caused a pathology mirroring human COPD with macrophage- and lymphocyte-rich inflammation, emphysema, and mucus metaplasia. Unfortunately, macrophages were not further characterized in this study, so it is not known how IL-13 overexpression affected macrophage polarization. Further evidence for a role for CD206+ IL13-induced macrophages in emphysema came from a study by Kim et al. who showed that viral infections could induce an IL-13-producing phenotype through interactions with natural killer T cells leading to chronic airway inflammation. They also showed higher numbers of IL-13-positive macrophages in lung tissue of COPD patients.

In mice, CD206+ macrophages produce large amounts of chitinases like Ym1 and Ym2. Whether their human counterparts are also induced by alternative activation is unclear, but another member of this family, stabilin-1 interacting chitinase-like protein (SI-CLP), has been found upregulated following IL-4 and dexamethasone stimulation. Many members of the chitinase family associate with COPD. Chitotriosidase levels, for instance, were increased in bronchoalveolar lavage of smokers with COPD and they also had more chitotriosidase-positive cells in bronchial biopsies and an elevated proportion of alveolar macrophages expressing chitotriosidase as compared to smokers without COPD or never-smokers. Furthermore, macrophage chitinase-1 was selectively increased in a subset of patients with severe COPD and serum concentrations of YKL-40 were significantly higher in smokers with COPD as compared to nonsmokers or smokers without COPD and correlated negatively with lung function. Interestingly, YKL-40 also stimulated the production of pro-inflammatory cytokines and MMP9 by macrophages from COPD patients similar to the effects of TNF-α, suggesting YKL-40 itself actually induces a slightly different macrophage phenotype as compared to the CD206+ M2 phenotype.
Data from studies investigating MMPs indicate a possible role for CD206+ macrophages. As mentioned above MMP12 plays an important role in mouse emphysema and MMP12 was found specifically induced in IL-4-stimulated macrophages. Furthermore, Woodruff et al. showed increased CD206+ polarization of alveolar macrophages in smokers using MMP12 as a marker for alternative activation and many others showed that smoke induces MMP12 in macrophages. Interestingly, MMP12 production by macrophages was also found to be necessary to terminate both neutrophil and macrophage influx at the end of an inflammatory response and may therefore be an instrument of CD206+ macrophages to dampen inflammation to be able to start remodeling of damaged tissue. How that ties in with the potential pro-emphysematous role of M2 macrophages remains an open question.

Summarizing, there is some evidence for a role of CD206+ activation in COPD and this evidence points at a role contributing to the development of COPD. The data by Hodge et al. suggest that, similar to dysfunctional IRF5+ M1 activation, CD206+ M2 activation is also dysregulated with reduced efferocytosis but increased expression of M2 marker DC-SIGN.
**IL-10+ M2-like macrophages in COPD**

No attempts have been made to distinguish the roles of CD206+ and CD206+IL-10+ macrophages in COPD. Two studies reported on IL-10 in the context of COPD. A study by Hackett *et al.* showed diminished IL-10 production in lung tissue of COPD patients after LPS stimulation as compared to response in lung tissue of patients with normal lung function [223]. Takanashi *et al.* demonstrated that the level of IL-10 and the number of IL-10-positive macrophages in sputum from COPD patients and healthy smokers was decreased as compared to healthy non-smokers, although Li *et al.* found no difference in IL-10 levels in sputum from COPD patients as compared to controls [224,225]. This would suggest that IL-10+ macrophages are impaired in smoking and COPD and therefore cannot suppress the ongoing inflammation induced by smoke.

Combining the data available for IRF5+, CD206+ and IL10+ macrophages (see also figure 3), it appears that COPD is a disease of dysfunctional macrophages rather than a disease of one particular polarization state. Macrophages in COPD are promoting ongoing inflammation and tissue damage but are unable to effectively dampen inflammation because they have lost the ability to phagocytose microorganisms and apoptotic bodies and produce anti-inflammatory cytokines like IL-10. Currently, the most common treatment of COPD patients is by glucocorticosteroids, and this most common (chronic) treatment causes contradicting shifts in macrophage function [226]. Glucocorticosteroids do inhibit inflammation and may improve lung function, but also increase pneumonia susceptibility [227]. Thus, more insight into the role of macrophages in COPD could give direction to the search for more optimal treatment options.

**Scope of the thesis**

As discussed before, pulmonary fibrosis and COPD are diseases accompanied by changes in tissue structure, macrophage number and macrophage function in the lung. Macrophages are important in maintaining tissue ECM homeostasis in the lung and their dysfunction is related to the (over)production of ECM, contributing to pulmonary fibrosis and remodeling of airways in COPD, and with tissue destruction, contributing to emphysema. There is still debate regarding which macrophage phenotypes are involved in each of these processes. Our aim was therefore to identify the different macrophage subsets present in the diseased state and try to influence macrophage function in order to restore tissue homeostasis. Using an experimental model for pulmonary fibrosis, we aimed to stimulate the differentiation of proteolytic macrophages in order increase ECM degradation processes in the lung as described in chapter 2. In contrast, in chapter 3 we aimed to dampen the proteolytic activity of macrophages with the aid of a specific inhibitor of tartrate resistant acid phosphatase to improve our understanding of the role of macrophages in COPD. Furthermore, in chapter 4, we characterized blood monocytes in patients with COPD and compared them with healthy individuals to assess whether we can detect disease-related changes in these macrophage-precursors cells. In addition to the monocyte phenotypes, we also studied the presence of the three macrophage phenotypes described above in the central airways of COPD patients in chapter 5. Chapter 6 gives a general discussion on the findings in this thesis and we discuss future perspectives regarding the role of macrophages in lung diseases and their potential as targets for drug development.
References


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