Chapter 2
Cellular and Molecular Mechanisms of Chronic Inflammation-Associated Organ Fibrosis

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Abstract Fibrotic or scar tissue represents a condition where normal tissue architecture has become distorted and been replaced by extracellular matrix (ECM). ECM deposition in injured tissues is a natural part of the wound healing process that facilitates efficient restoration of tissue integrity. However, if the injury persists, the excessive accumulation of ECM leads to the loss of organ function and eventual organ failure. Persistent injuries arise due to various causes, depending on the organ. Some fibrotic diseases are associated with the chronic inflammation that accompanies infection or autoimmune conditions. Other fibrotic diseases are triggered by chemical or pathophysiological insults to epithelial cells, or by unknown causes, as is the case for idiopathic pulmonary fibrosis (IPF). Although the etiology of fibrosis varies between specific diseases, the fibrotic process that takes place in each organ shares a number of common characteristics. In particular, it is widely accepted that excessive amounts of ECM components are produced by activated fibroblasts that accumulate in injured tissue. In the first half of this chapter, we discuss the controversial origin of activated fibroblasts as well as the mechanisms of their activation. In the second half of this chapter, we describe the cellular and molecular mediators that regulate fibrotic responses in the specific example of pulmonary fibrosis.

Keywords Bleomycin • Fibroblast • Fibrosis • Intratracheal transfer • IPF • Macrophage • Monocyte • Myofibroblast • Silica • Silicosis

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2.1 The Origin of Collagen-Producing Fibroblasts and Myofibroblasts

Tissue injury often occurs at the epithelium. The precursors of activated fibroblasts become activated following exposure to inflammatory mediators produced upon injury, then proliferate and migrate into the injured area. Under certain conditions, such as in skin injury, these activated fibroblasts differentiate into myofibroblasts, which are contractile cells that often express α-smooth muscle actin (α-SMA; Tomasek et al. 2002). Myofibroblasts proliferate and produce ECM components such as collagen I (Col1) in a range of fibrotic diseases and organs (Hinz et al. 2012). Because therapeutic options for fibrotic diseases are very limited, the origin of myofibroblasts has remained an active area of research. Classically mesenchymal cells such as tissue resident fibroblasts were proposed to be the precursors of myofibroblasts, but over the years various cell types have been reported to differentiate into myofibroblasts, making the origin of these cells a controversial question (Fig. 2.1).

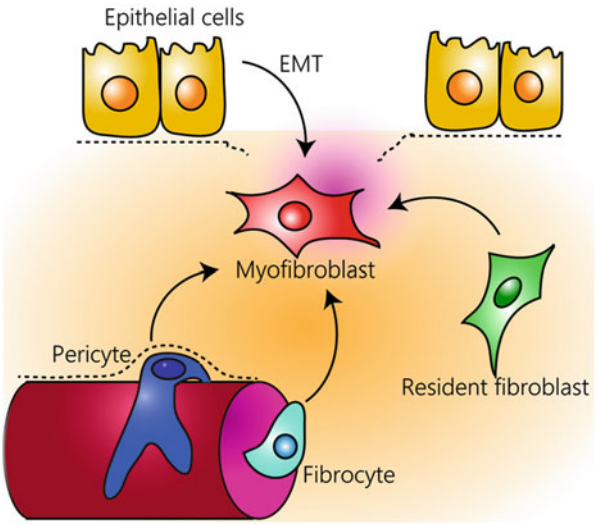

Fig. 2.1 The controversial origins of myofibroblasts. Multiple cell types have been reported to act as a source of myofibroblasts in organ fibrosis. Resident fibroblasts are classically considered to be the primary source of myofibroblasts. Epithelial cells may differentiate into myofibroblasts through EMT, although recent studies have failed to detect evidence of EMT. Bone-marrow–derived fibrocytes are supplied from the circulation and differentiate into myofibroblasts at sites of tissue injury tissue, but there is little evidence of collagen synthesis by these cells. Recent reports have demonstrated a pericyte origin of myofibroblasts in multiple organs

2.2 Myofibroblast Precursors from the Bone Marrow

Bone marrow-derived circulating cells have been reported to gain expression of Col1 in fibrotic organs. These cells are known as 'fibrocytes', and are defined as $CD45^+$ $Col1^+$ cells (Bucala et al. 1994). Recent studies using bone marrow chimeras or parabiosis have shown that only a small proportion of Col1-producing cells are supplied from the circulation in experimental models of fibrosis (Higashiyama et al. 2009; Kisseleva et al. 2006; Tsukui et al. 2013). Some myeloid cells internalise and degrade ECM components. These cells are detected as $Col1^+$ even though they may not synthesise Col1 themselves. A recent study revealed that haematopoietic cell-specific Col1 gene deletion does not reduce collagen deposition in bleomycin-induced pulmonary fibrosis (Kleaveland et al. 2014), suggesting that fibrocytes are not a significant source of Col1. Thus, previous studies reporting on $Col1^+$ $CD45^+$ fibrocytes may have been detecting myeloid cells that had internalised Col1 from their surroundings, rather than Col1-producing myeloid cells. However, it has also been reported that 35 % of myofibroblasts in the kidney come from the bone marrow, based on experiments using an α-SMA-RFP reporter in a unilateral ureteral obstruction model (LeBleu et al. 2013). Thus, further fibrocyte lineage tracing studies that address the synthesis and deposition of Col1 are necessary, and the extent to which bone-marrow–derived cells contribute to the activated fibroblast population remains unclear.

2.3 Myofibroblast Precursors from the Epithelium

It is widely accepted that epithelial cells can differentiate into mesenchymal cells via a process known as epithelial-to-mesenchymal transition (EMT) during development or cancer. It has also been reported that myofibroblasts differentiate from epithelial cells by EMT in experimental models of fibrosis. Most earlier studies suggesting a role for EMT in fibrosis were based on in vitro observations, whereas in vivo evidence implicating EMT in fibrosis consisted mainly of immunohistochemical staining showing coexpression of mesenchymal cell and epithelial cell markers. However, there were a number of technical limitations associated with these earlier studies (Kriz et al. 2011). Recent lineage-tracing studies have failed to observe EMT in the lung (Rock et al. 2011), liver (Taura et al. 2010), or kidney (Humphreys et al. 2010), and thus raise doubt as to whether epithelial cells are in fact precursors of activated fibroblasts. However, upon tissue injury epithelial cells upregulate transcription factors associated with EMT in a manner similar to that which occurs during development, and these phenotypic changes may promote mesenchymal crosstalk (Rowe et al. 2011). Epithelial cells also play an important role in the initiation of fibrosis. Thus, epithelial cells remain an important target of research that aims to elucidate the underlying mechanisms of fibrosis.

2.4 A Mesenchymal Origin of Myofibroblasts

In recent years, pericytes and perivascular cells have attracted considerable attention as likely precursors for myofibroblasts. Pericytes are embedded in the endothelial basement membrane and are characterised by their direct attachment to endothelial cells. Perivascular cells typically represent interstitial cells that exist in close proximity to blood vessels but outside the endothelial basement membrane. The perivascular cell population may be heterogeneous, and differs in each organ. In the liver, hepatic stellate cells localise to perivascular regions known as the space of Disse, and have characteristics of both pericytes and resident fibroblasts. Hepatic stellate cells represent a major source of myofibroblasts in carbon tetrachloride (CCl_4)-induced liver fibrosis (Mederacke et al. 2013). In the quiescent state, hepatic stellate cells store lipid droplets and do not express Col1. Upon activation, these cells lose their lipid droplets and begin to express Col1 and α-SMA. However, in cholestatic fibrosis, portal fibroblasts have also been suggested to act as myofibroblast precursors, and it remains to be determined which cell type represents the major myofibroblast precursor (Iwaisako et al. 2014).

In the kidney, pericytes are considered to be the most likely precursor for myofibroblasts. Humphreys et al. showed that Foxd1-CreER-labeled pericytes are the major progenitor of myofibroblasts in the unilateral ureter obstruction model (Humphreys et al. 2010). More recently, the Gli1-CreER transgene was shown to label a specific subset of perivascular PDGFRβ$^+$ cells in the kidney, and ablation of Gli1-CreER-labeled myofibroblasts ameliorated kidney fibrosis (Kramann et al. 2015). Gli1-CreER-labeled cells also expressed PDGFRα, CD146, and Nestin, but not NG2. In addition, like mesenchymal stem cells, this population possessed trilineage differentiation capacity. Although PDGFRβ is a widely used marker for pericytes, this study suggests that pericyte and perivascular populations have a degree of heterogeneity, and that specific subsets may possess higher profibrotic potential. Accordingly, further characterisation of subpopulations of cells (such as Gli1$^+$ cells) and comparison with other kidney cell subsets is required.

In the spinal cord, Glast-expressing type A pericytes have been shown to differentiate into scar-forming myofibroblasts (Goritz et al. 2011). Although Goritz et al. showed that type A pericytes are normally surrounded by basal lamina, another group reported recently that type A pericytes in the spinal cord express Col1 even in the quiescent state, which is a characteristic of resident fibroblasts (Soderblom et al. 2013). Dulauroy et al. revealed that ADAM12-Cre-labelled cells are major myofibroblast precursors in models of dermis or muscle injury, and that these cells migrate into injured areas from the perivascular niche (Dulauroy et al. 2012). ADAM12-Cre-labelled cells localised in the perivascular region, surrounded by endothelial basement membrane. After injury, the labelled cells detached from the blood vessels and emerged from the basement membrane, although it was difficult to distinguish these cells from perivascular cells that acquired ADAM12 expression after the injury had occurred. ADAM12-Cre-

labelled cells were PDGFRα$^+$, which is a marker used to identify resident fibroblasts in other organs. However, at present, the characteristics of mesenchymal populations such as fibroblasts and pericytes are poorly understood, and it is difficult to generalise subsets and markers across organs. Many studies have shown that myofibroblasts express PDGFRβ, but myofibroblast progenitors are not necessarily PDGFRβ$^+$, because PDGFRβ expression is induced in mesenchymal cells upon injury (Henderson et al. 2013). Because there is no single marker that defines these mesenchymal cell subsets, elucidation of the basic molecular signatures of pericytes and resident fibroblasts will facilitate further characterisation of these cells in different organs. The same principle applies to the characterisation of activated fibroblasts, and a range of different activation markers for Col1-producing cells has been reported, such as α-SMA, Fn1, S100a4, and Spp1. It remains unclear whether any of these fibroblast activation markers define subpopulations with specific functions in fibrosis.

2.5 The Origin of Col1-Producing Cells in the Lungs

For the last several years, we have been studying the origin and dynamics of activated fibroblasts in pulmonary fibrosis. Following bleomycin-induced lung injury, Col1-producing activated fibroblasts form massive clusters and destruction of the alveolar structure is observed (Tsukui et al. 2013). The precursors of these activated fibroblasts appear likely to be resident fibroblasts or pericytes. Rock et al. reported that NG2-CreER-labelled pericytes proliferate after injury, but that these cells do not express high levels of α-SMA (Rock et al. 2011). Instead, they observed the proliferation of PDGFRα$^+$ cells, suggesting that resident fibroblasts rather than NG2$^+$ pericytes are the major progenitors of activated fibroblasts in the lungs. Hung et al. used Foxd1-Cre mice to examine pericytes in pulmonary fibrosis (Hung et al. 2013). Foxd1 is expressed only during development and labels PDGFRβ$^+$ cells in the alveolar regions more efficiently than NG2-CreER. Among Foxd1-Cre-labelled cells, around 60 % are NG2$^+$ whereas greater than 80 % express PDGFRβ. This study showed that 68 % of α-SMA$^+$ cells in fibrotic lesions were derived from Foxd1-Cre-labelled cells at day 14 after bleomycin treatment. The discrepancy between the results of the studies of Rock et al. and Hung et al. may be related to the markers used. Because not all PDGFRβ$^+$ cells express NG2, it is possible that NG2$^-$ PDGFRβ$^+$ pericytes have the potential to differentiate into myofibroblasts. Moreover, both studies lacked in vivo evidence of Col1 production by proliferating or α-SMA$^+$ mesenchymal cells. Histological analysis has only limited capacity for the examination of multiple markers and functions. For future studies, it may be useful to combine histological analysis with flow cytometric analysis and ex vivo assays in order to characterise activated fibroblast populations.

Many reports suggest that myofibroblasts are the key player in fibrosis. It remains unclear the extent to which other profibrotic cell populations contribute

to fibrosis, or even if other profibrotic cell populations exist. Our studies have demonstrated that only a small proportion of Col1-producing cells become α-SMA$^+$ after bleomycin-induced lung injury, whereas Col1-producing cells dramatically upregulate profibrotic genes and organise fibrotic lesions (Tsukui et al. 2013). The function of the α-SMA molecule is to mediate cell contraction. Although cell contraction and ECM production are closely associated elements of the wound healing process, α-SMA expression is not necessary for ECM production itself. Thus, direct evidence of excessive ECM deposition by myofibroblasts or other cell populations is necessary for understanding the contributions of profibrotic cell populations to pulmonary fibrosis.

The structure of the lungs is characterised by thin alveolar walls that enable efficient gas exchange. Because of these thin alveolar walls, interstitial cells including resident fibroblasts are very sparsely distributed throughout the alveolar walls in the quiescent state. The mechanism by which these interstitial cells generate clusters and fibrotic lesions following epithelial injury is important for understanding the origin of profibrotic cells. Our experiments using BrdU have confirmed that Col1-producing cells proliferate following bleomycin treatment (Tsukui et al. 2013). However, the proliferation of these cells was not robust enough to explain fully their formation of clusters, and histological analysis suggested that Col1-producing cell clusters might result from cell migration as well as proliferation. It has been reported previously that resident fibroblasts migrate into the alveolar airspaces following epithelial injury (Fukuda et al. 1985; Fig. 2.2a). The migration of fibroblasts after injury is well characterised in the skin (Tomasek et al. 2002), and the lungs may undergo a similar process of wound healing. Some reports suggest that fibroblastic foci in IPF patients are formed by the migration of resident fibroblasts into the alveolar airspaces (Noble 2005).

Our group conceived an experimental design to investigate whether activated fibroblasts that migrate into the alveolar airspaces can become interstitial fibroblasts that form fibroblastic foci. In this experiment, fibroblasts were delivered intratracheally into alveolar airspaces that were undergoing tissue remodelling following bleomycin-induced injury (Tsukui et al. 2015). We purified resident fibroblasts from Col1-GFP reporter mice, then intratracheally transferred the purified resident fibroblasts into wild-type mice that had been treated with bleomycin several days earlier. We found that the transferred fibroblasts dramatically upregulated profibrotic genes, displayed activated morphology, and formed fibroblastic foci (Fig. 2.2b), suggesting that activated fibroblasts in the alveolar airspaces eventually form fibrotic lesions. Interestingly, pericytes that were purified according to their expression of the NG2 reporter did not differentiate into Col1-producing activated fibroblasts following intratracheal transfer. Thus, as reported previously (Rock et al. 2011), NG2$^+$ pericytes in the lungs are unlikely to act as progenitors for myofibroblasts, or NG2$^+$ pericytes may be unable to respond to environmental changes in the alveolar airspaces in the same way as resident fibroblasts.

In our experiments, the intratracheal transfer of resident fibroblasts partly recapitulated the activation of Col1-producing cells, suggesting that exposure to

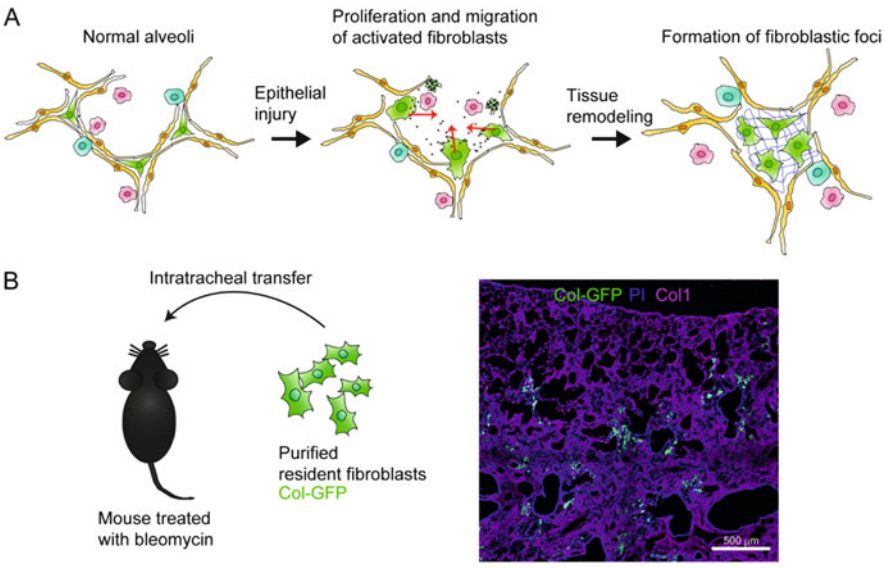

Fig. 2.2 Mechanisms of fibroblastic foci formation in pulmonary fibrosis. (**a**) Following epithelial injury, activated fibroblasts proliferate and migrate into injured alveolar airspaces. Activated fibroblasts form clusters and produce ECM after migration, generating fibroblastic foci at the site of injury. (**b**) Intratracheal cell transfer experiments have shown that fibroblasts in alveolar airspaces are able to become activated fibroblasts in fibroblastic foci. Purified resident fibroblasts from collagen I GFP (Col-GFP) reporter mice were transferred intratracheally into wild-type mice 10 days after intratracheal instillation of bleomycin. The lungs were analysed by immunohistochemistry 21 days after bleomycin instillation. Transferred fibroblasts (*green*) formed fibroblastic foci in the regions of collagen I (*magenta*) deposition. Nuclei were visualised by propidium iodide (PI, *blue*). Scale bar: 500 μm

alveolar airspaces may be pivotal to the process of fibroblastic foci formation by activated fibroblasts. Migratory fibroblasts may form clusters in injured alveolar airspaces, produce ECM components, and then contract, leading to coalescence of the alveolar walls and the formation of fibrotic lesions (Noble 2005). This hypothesis also explains the lack of robust proliferation of activated fibroblasts in bleomycin-induced pulmonary fibrosis, and the loss of lung volume that is observed in the later stages of IPF. However, many important questions remain about the mechanisms that underlie pulmonary fibrosis. If a subset of the pericyte population contributes to the formation of activated fibroblasts, what are the identifying features of that subset, and are those pericytes able to respond to changes in the alveolar airspace environment? The mechanisms of fibroblast migration and fibroblastic foci formation also remain poorly understood. For example, we found that osteopontin defines a subset of activated fibroblasts that form the leading edge of fibroblastic foci and encroach upon the alveolar airspaces (Tsukui et al. 2013), but the factors that elicit osteopontin^{+} activated fibroblasts remain unknown. Further delineation of the process of fibroblastic foci formation may lead to the identification of novel therapeutic targets for pulmonary fibrosis.

2.6 Cellular and Molecular Mediators of Pulmonary Fibrosis

Wound healing following lung injury is often skewed towards fibrotic responses rather than towards the normal regeneration of the lung architecture (King 2005). Various cellular and molecular mediators, including activated tissue cells (epithelial cells, endothelial cells, and fibroblasts), leukocytes, and soluble mediators participate in a sequential cascade of wound healing responses. In the course of normal tissue regeneration, damaged cells release soluble mediators such as CC chemokine ligand 2, IL-1β, and IL-33 (Cavarra et al. 2004; Mercer et al. 2009; Pichery et al. 2012), which recruit and activate various leukocytes in the damaged tissues. These activated leukocytes play important roles in the clearance of the pathogens, debris, and foreign particles responsible for the injury (Forbes and Rosenthal 2014). In addition, these damaged tissue cells and activated leukocytes also promote tissue repair by secreting cytokines and growth factors such as IL-13, platelet-derived growth factors, and TGF-β1 (Bonner et al. 1991; Huaux et al. 2003). These profibrotic factors activate fibroblasts, inducing the production of temporary ECM that forms a scaffold for lung regeneration. During the resolution phase of the inflammatory response, the remaining leukocytes, particularly macrophages, clear the temporary ECM scaffolds and extracellular debris (Gibbons et al. 2011; Liang et al. 2012), and possibly also clear activated fibroblasts (Redente et al. 2014). This process of clearance is an essential step preceding tissue regeneration (Duffield et al. 2013). Pulmonary fibrosis arises as a result of the dysregulation of wound repair processes (Duffield et al. 2013). In the remainder of the chapter, we discuss the involvement of tissue cells, leukocytes, and inflammatory mediators in the development of pulmonary fibrosis.

2.7 The Role of Granulocyte Responses in the Induction of Lung Injury and Fibrosis

In the early stages of lung injury, granulocytes such as neutrophils and eosinophils infiltrate the injured lung (Wynn 2011). Neutrophils play a major role in the priming of acute inflammatory responses through the production of proinflammatory cytokines, reactive oxygen species, and reactive nitrogen species (Dostert et al. 2008; Hasan et al. 2013; Kolaczkowska and Kubes 2013). Neutrophils also promote tissue remodelling and directly degrade elastic fibers and the basement membrane through the production of proteases such as neutrophil elastase (Kang et al. 2001). Eosinophils enhance the recruitment of other inflammatory cells, such as effector T cells, and exacerbate inflammatory responses, particularly in allergic lung inflammation (Humbles et al. 2004). In addition, eosinophils promote fibrotic responses by producing the profibrotic cytokines IL-13 and TGF-β1 (Huaux et al. 2003; Minshall

et al. 1997). Collectively, these granulocytes are likely to play important roles in the induction and progression of fibrotic responses following acute lung injury.

2.8 The Role of Macrophage Subsets in Pulmonary Fibrosis

Following the infiltration of granulocytes into the lung in acute lung injury, monocyte-derived macrophages (MMs) from the bone marrow infiltrate the lung (Forbes and Rosenthal 2014). In parallel with the accumulation of granulocytes and MMs, alveolar macrophages (AMs) become activated after phagocytosing the irritants and cellular debris that are present during lung injury (Hussell and Bell 2014). These macrophages have been shown to ameliorate or exacerbate pulmonary fibrosis in mice in a context-dependent manner (Gharaee-Kermani et al. 2003; Liang et al. 2012; Moore et al. 2001; Murray and Wynn 2011; Wynn 2011; Wynn and Barron 2010). In the bleomycin-induced acute model of pulmonary fibrosis, MMs and AMs promote lung inflammation and the activation of tissue cells in the early stages of pulmonary fibrosis through the secretion of proinflammatory mediators and growth factors such as TNF-α, IL-1β, IL-6, TGF-β, platelet derived growth factor, insulin-like growth factor-1, and vascular endothelial growth factor (Wynn et al. 2013). Because MMs and AMs are a major source of collagenolytic enzyme matrix metalloproteinases (MMPs), the infiltration of MMs and activation of AMs results in the disruption of lung architecture (Hussell and Bell 2014; Wynn and Barron 2010). On the other hand, MMs and AMs also suppress excessive damage and ECM deposition in the lungs through the secretion of anti-inflammatory mediators (Gibbons et al. 2011; Redente et al. 2014; Tighe et al. 2011), the clearance of fibrous connective tissue, and possibly through the clearance of proinflammatory extracellular debris, such as apoptotic cells (Liang et al. 2012). Indeed, depletion of AMs in the resolution phase of bleomycin-induced pulmonary fibrosis delays the clearance of fibrotic lesions (Gibbons et al. 2011). In a model of silica-induced pulmonary fibrosis, we recently demonstrated that MMs continuously infiltrate the silica-treated lungs through to the chronic phase of the disease. MMs selectively accumulated in the periphery of silica-induced fibrotic lesions, and notably, MMs limited the expansion of the fibrotic area and the induction of diffuse pulmonary fibrosis (Shichino et al. 2015). We also found that MMs suppress the expression of human IPF-related genes, particularly tissue remodelling-related genes, in the silica-treated lungs. In addition, the changes in the expression of IPF-related genes appear to be suppressed by MM-derived inflammatory mediators (unpublished observations). These observations suggest that MMs and AMs exert pathogenic effects at the onset of lung injury, but might exert protective effects during the development of chronic pulmonary fibrosis. These insights into the role of MMs in pulmonary fibrosis raise concerns about

the potential adverse effects of MM-targeted therapies for pulmonary fibrosis, including anti-CCL2 therapy.

At least two possible hypotheses could explain the protective role of macrophages during chronic pulmonary fibrosis. One hypothesis is that macrophages undergo phenotypic conversion from a proinflammatory/profibrotic pattern to an anti-inflammatory/antifibrotic pattern. The anti-inflammatory phenotype is characterised by the increased expression of IL-10, arginase-1, and RELMα (Murray and Wynn 2011). The anti-fibrotic features of this phenotype include increased expression of MMP9, MMP12, and MMP13, and/or the increased capacity to phagocytose extracellular debris (Murray and Wynn 2011). Indeed, in the CCl_4-induced liver fibrosis model, MMs and resident Kupffer cells promote the resolution of fibrosis (Duffield et al. 2005; Mitchell et al. 2009) and switch from a proinflammatory/profibrotic phenotype to an anti-inflammatory/antifibrotic phenotype (Mitchell et al. 2009; Ramachandran et al. 2012). Another hypothesis is that there are dual roles for inflammatory mediators in lung fibrosis and repair. For example, TNF-α, one of the major proinflammatory mediators produced by MMs, not only promotes inflammation but also suppresses collagen production by fibroblasts (Inagaki et al. 1995; Siwik et al. 2000). In addition, a reduction in MM numbers or TNF-α inhibition leads to disrupted organisation of the granuloma in *M. tuberculosis*-infected mouse lungs (Chakravarty et al. 2008; Peters et al. 2001), consistent with our observations in the silica-induced pulmonary fibrosis model (Shichino et al. 2015). Moreover, the resolution of bleomycin-induced pulmonary fibrosis is delayed in TNF knockout mice (Redente et al. 2014). These observations suggest that overall, MMs may exert a protective effect on the pathological course of pulmonary fibrosis, possibly due to MM-associated 'beneficial inflammation' (Fig. 2.3). The molecular mechanism(s) that underlie the protective properties of MMs remain poorly understood. Further studies on the protective aspects of MMs may support the development of novel therapeutic strategies for chronic pulmonary fibrosis.

2.9 Adaptive Immune Cell Responses in Pulmonary Fibrosis

After the acute inflammatory phase of pulmonary fibrosis, effector T cells and B cells infiltrate into the injured lung and modulate inflammatory and fibrotic responses. $CD4^+$ T_H17 cells and T_H2 cells produce IL-17A and IL-13, respectively (Lo Re et al. 2013). Because IL-17A has proinflammatory properties and induces TGF-β1 expression (Wilson et al. 2010), and because IL-13 has profibrotic effects (Chiaramonte et al. 1999), these T-cell subsets are thought to promote lung injury and fibrosis. Conversely, $CD4^+$ T_H1 cells play a protective role in pulmonary fibrosis because they produce the antifibrotic cytokine IFN-γ (Giri et al. 1986). On the other hand, there are mixed reports regarding the effects of $CD4^+$ regulatory

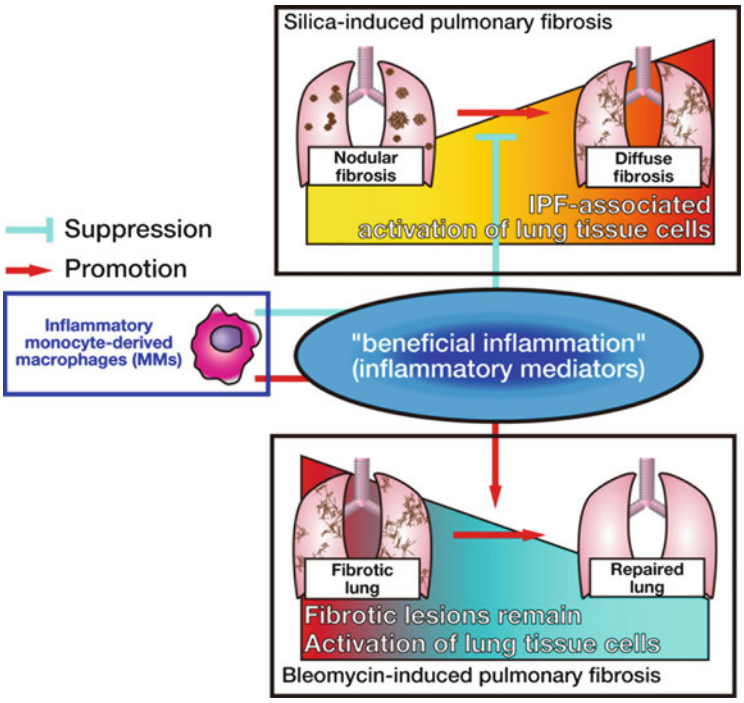

Fig. 2.3 Role of MMs in the progression/resolution of PF. MMs inhibit the development of diffuse PF and IPF-related transcriptomic signatures in silica-induced chronic PF. MMs also accelerate the resolution of bleomycin-induced self-limiting PF. Putative MM-mediated 'beneficial inflammation' in PF might mediate the protective effect of MMs by suppressing IPF-related tissue cell activation

T cells (T_{reg}) in pulmonary fibrosis. Because T_{reg} produce both the anti-fibrotic cytokine IL-10 (Kitani et al. 2003; Wilson et al. 2010) and the profibrotic cytokine TGF-β1 (Boveda-Ruiz et al. 2013), the mixed effects of T_{reg} might be explained by the predominance of these cytokines in different aspects of fibrotic responses. B cells also have mixed effects on the progression of pulmonary fibrosis. B cells accumulate in the lungs in bleomycin- or silica-induced pulmonary fibrosis, and fibrosis is alleviated in B-cell–deficient mice (Arras et al. 2006; Komura et al. 2008). On the other hand, the protective effect of IL-9 overexpression in silica-induced pulmonary fibrosis is negated in B-cell–deficient mice (Arras et al. 2006). These observations suggest a possible role for B cells in the progression of pulmonary fibrosis, the mechanisms of which remain largely unknown. Thus, overall, depending on their subset, T cells and B cells may influence the course of fibrotic responses. However, as discussed below, their involvement in the progression of irreversible pulmonary fibrosis is only minor in some experimental models.

2.10 Requirement for Leukocyte Subsets in Pulmonary Fibrosis: Lessons from Depletion Studies

Numerous studies using experimental models of pulmonary fibrosis have demonstrated that lung leukocyte subsets modulate lung injury, repair, and fibrosis (Wynn 2011). On the other hand, IPF, the most common type of idiopathic interstitial pneumonia, progresses even with less inflammatory cell infiltration than is observed in other types of interstitial pneumonia (Raghu et al. 2011). IPF is usually poorly responsive to various anti-inflammatory and immunosuppressive therapies including corticosteroids, anti-TNF biologics, and cyclosporin A (Raghu et al. 2011). The poor response of IPF to anti-inflammatory and immunosuppressive therapies raises doubts about the functional consequences of inflammatory leukocyte infiltration in the progression of chronic pulmonary fibrosis. Several types of immunodeficient mice, such as NOD/SCID and *Rag1*$^{-/-}$ mice lacking T and B cells and *Il5*$^{-/-}$ mice lacking eosinophils, still develop experimental pulmonary fibrosis (Hao et al. 2000; Helene et al. 1999; Hubbard 1989). In addition, the depletion of neutrophils or NK/NKT cells in the acute inflammatory phase of the disease does not hinder the development of experimental pulmonary fibrosis, and bleomycin-induced pulmonary fibrosis is not completely prevented by the depletion of AMs in the inflammatory or fibrotic phase (Beamer et al. 2010; Clark and Kuhn 1982; Gavett et al. 1992; Gibbons et al. 2011). Moreover, we recently found that the degree of infiltration into the lungs of CD4^{+} T cells, CD8^{+} T cells, eosinophils, and NK cells, and the number of AMs present in the lungs, were equivalent between a self-limiting bleomycin-induced pulmonary fibrosis model and a progressive silica-induced pulmonary fibrosis model, suggesting that these cells do not alter the course of the disease (Fig. 2.4, unpublished observations and Shichino et al. 2015). In contrast, MM infiltration increased to a greater extent in the silica model compared to the bleomycin model (Fig. 2.4; Shichino et al. 2015). However, *Ccr2*$^{-/-}$ mice, in which the infiltration of MMs into the lung is deficient, still displayed progression to silica- and bleomycin-induced pulmonary fibrosis (Shichino et al. 2015). The necessity for the development of pulmonary fibrosis of other leukocyte subsets such as CCR2-independent monocyte-derived macrophages, interstitial macrophages, and lung-resident dendritic cells remains unclear due to the lack of subset-specific depletion systems.

Recently, it was shown that fetal-liver–chimeric mice with CSF1R-deficient haematopoietic cells lack CCR2-independent monocyte-derived macrophages in their peripheral tissues (Alexander et al. 2014). In addition, long-term administration of an anti-CSF1R antibody also depletes tissue-resident macrophages (including macrophages located in the interstitium), but has minimal effect on dendritic cell subsets in most lymphoid tissues (MacDonald et al. 2010). These systems may present useful tools for investigating the role of CCR2-independent macrophages in the development of pulmonary fibrosis. Nevertheless, the experimental data available to date suggest that although adaptive immune cells, eosinophils, neutrophils, MMs, AMs, NK cells, and NKT cells in the lung may be involved in the

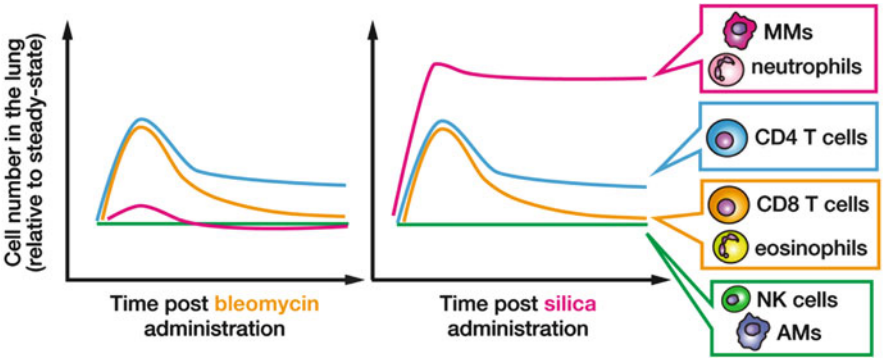

Fig. 2.4 Leukocyte subset kinetics differ between bleomycin-induced PF and silica-induced PF. The total number of $CD4^+$ T cells, $CD8^+$ T cells, eosinophils, NK cells, and AMs does not differ significantly between the two models. In contrast, MM and neutrophil numbers are dramatically elevated in the silica-treated lung, but not in the bleomycin-treated lung

pathogenesis of pulmonary fibrosis, these cells might not be essential for the progression of pulmonary fibrosis.

2.11 Conclusions and Future Perspectives

In recent years, the controversy surrounding the major source of myofibroblasts in fibrosis has been narrowed down to two candidates: tissue-resident fibroblasts and pericytes. However, the relative contributions of these potential precursors to the myofibroblast population remains elusive across a range of fibrosis models. In addition, there is a need for further characterisation of the functional heterogeneity within the activated fibroblast population, which is currently represented by α-SMA^+ myofibroblasts. Differences in inflamed tissue microenvironments might influence the differentiation or activation of fibroblasts and determine whether the wound healing process skews towards fibrosis or regeneration. Although interventions targeting specific subsets of inflammatory leukocytes have not been sufficient to ameliorate experimental pulmonary fibrosis, further elucidation of the spatiotemporal regulation of fibrotic responses by the various leukocyte subsets may reveal new points of therapeutic intervention for chronic inflammation-associated pulmonary fibrosis.

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