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Cytokine mediated tissue fibrosis☆

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Abstract

Acute inflammation is a recognised part of normal wound healing. However, when inflammation fails to resolve and a chronic inflammatory response is established this process can become dysregulated resulting in pathological wound repair, accumulation of permanent fibrotic scar tissue at the site of injury and the failure to return the tissue to normal function. Fibrosis can affect any organ including the lung, skin, heart, kidney and liver and it is estimated that 45% of deaths in the western world can now be attributed to diseases where fibrosis plays a major aetiological role. In this review we examine the evidence that cytokines play a vital role in the acute and chronic inflammatory responses that drive fibrosis in injured tissues. This article is part of a Special Issue entitled: Fibrosis: Translation of basic research to human disease.

Keywords

Fibrosis; Cytokine; Macrophage; Fibroblast; Myofibroblast; Inflammation

1. Introduction

Physiological wound repair is a complex, highly orchestrated process that allows for the replacement of dead or damaged cells and is critically important in restoring homeostasis to a tissue after injury. Wound repair can be loosely defined by three overlapping stages; an initial response, a recovery of integrity, followed finally by resolution of the wound back to a functional epithelium. It requires a tightly regulated spatial and temporal response from key structural cells in the organ such as epithelial cells, endothelial cells and fibroblasts but also from immune and progenitor cells drawn from the circulatory system [1]. Acute inflammation is a recognised part of normal wound healing by serving as an innate immune response to the disrupted epithelial surface until it is reinstated. However, when inflammation fails to resolve and a chronic inflammatory response is established this process can become dysregulated resulting in pathological wound repair and the accumulation of permanent fibrotic scar tissue at the site of injury (Fig. 1). This fibrosis is characterised by the excessive accumulation of extra cellular matrix (ECM) components including collagens, fibronectin and hyaluronic acid at the site of tissue injury, leading to a decrease in organ

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function and, in some cases, organ failure and death [2]. It is estimated that 45% of deaths in the western world can now be attributed to diseases where fibrosis plays a major aetiological role [3]. Fibrosis can affect any organ including the lung, skin, heart, kidney and liver and may represent an aberrant response to a single major injury but more commonly is a response to a persistent or repetitive injury. In this review we examine the evidence for cytokines released as part of an acute or chronic inflammatory response in driving fibrosis in injured tissues.

2. Pathogen and damage associated molecular patterns in fibrosis

A functional epithelium provides an efficient barrier against microorganisms and other potentially harmful molecules *via* a wide range of mechanisms including mucociliary clearance, maintenance of epithelial adherence and tight junctions, homeostasis of ion and water transport and secretion of antibacterial, antimicrobial and antiprotease molecules [4]. However the epithelium is often located on vulnerable surfaces that receive significant challenges to their integrity such as the gut, skin and lungs. These tissues are routinely exposed to the external environment and a range of harmful molecules including bacteria and viruses, tobacco smoke, asbestos, silica and diesel exhaust that can lead to epithelial activation and, in cases of chronic exposure, epithelial damage, shedding and denudation.

Numerous fibrotic diseases are believed to have an infectious aetiology with bacteria (*Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*), viruses (HCV, Respiratory syncytial virus), fungi (*Aspergillus fumigatus*, *Cryptococcus neoformans*) and multi-cellular parasites (*Schistosoma mansoni*, *Toxoplasma gondii*) driving wounding, chronic inflammation and subsequent fibrosis in multiple organs [5–13]. Pathogen by-products including bacterial DNA and double stranded RNA, peptidoglycan, lipopolysaccharide and flagellin, collectively referred to as pathogen-associated molecular patterns (PAMPs), are recognised by pattern recognition receptors (PRR) on a wide range of cell types including immune cells (macrophages, neutrophils, T-cells, B-cells, dendritic cell, eosinophils) and structural cells (epithelial cells, fibroblasts, adipocytes) [14,15]. The interaction between PAMPs and PRR provides an evolutionarily conserved mechanism that provides the first line of defence against invading pathogens and activates numerous proinflammatory cytokine and chemokine pathways, leading to the eradication of the pathogen. The failure to clear the pathogen or its PAMPs provides a persistent source of tissue injury, chronic inflammation and creates an environment that might favour fibrosis. For example persistent colonisation of the allograft with *Pseudomonas aeruginosa* following lung transplantation is strongly associated with the subsequent development of bronchiolitis obliterans syndrome (BOS) [16] and prolonged infection with hepatitis C virus (HCV) or hepatitis B virus (HBV) leads to loss of liver architecture and function and ultimately cirrhosis [17,18].

It is also increasingly apparent that PRRs provide mechanisms for mounting inflammatory and wound-healing responses to sterile tissue trauma [19]. When epithelial cells are damaged or dying their membranes lose integrity and intracellular proteins leak into the external environment. These damage associated molecular pattern molecules (DAMPs) or alarmins include high-mobility box group 1 (HMGB-1), heat-shock proteins (HSP60, HSP70), interleukin (IL)-33 and IL-1α among others [20]. DAMPs can trigger innate immune responses in a wide variety of cell types *via* engagement of PRR and provides an important homeostatic mechanism by which the immune system can sense and mount wound-repair responses in damaged tissues [21]. However, there is also evidence that DAMPs can contribute to the pathogenesis of many inflammatory and fibrotic diseases. For example IL-33 is strongly associated with fibrosis in chronic liver injury [22] and is increased in systemic sclerosis patients, correlating with the extent of skin sclerosis and the severity of pulmonary fibrosis [23]. In addition HMGB-1 levels are elevated in the

bronchoalveolar lavage (BAL) of patients with idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis [24].

Fibroblasts express a number of PRR including toll-like receptors (TLR) and IL-1R therefore PAMPs and DAMPs can directly activate them and drive their differentiation to myofibroblasts [7,25,26]. It has also recently been suggested that there are differences in TLR expression and activation between normal fibroblasts and those isolated from patients with severe idiopathic pulmonary pneumonia [7,27]. Taken together these data suggest that myofibroblasts in a fibrotic environment may be maintained in a heightened state of readiness, primed to respond to small quantities of DAMPs and PAMPs. Therefore inhibiting TLR signalling may represent a novel approach to limit activation of the innate immune response, decrease inflammation and limit or reverse fibrosis.

3. Origin of myofibroblasts in fibrotic tissues

The origin of the myofibroblasts during fibrosis, and the relative contribution of myofibroblasts from each source to fibrosis, is a matter of on-going debate (Fig. 2). It was originally thought that the activation or proliferation of local resident stromal cells and their differentiation into myofibroblasts was the only source of myofibroblasts during fibrosis [28–33]. In healthy tissue fibroblasts are quiescent and are primarily involved in routine maintenance of the ECM during homeostasis. During both physiological and pathological wound repair the fibroblast is activated and differentiates into a myofibroblast [31,34,35]. Transforming growth factor-β1 (TGF-β1) continues to be regarded as the key growth factor involved in driving fibrosis [36] and can drive fibroblast to myofibroblast differentiation both *in vitro* and *in vivo* [37,38]. In addition to TGF-β1, a range of cytokines and growth factors have been shown to drive myofibroblast differentiation including IL-4, IL-13, and platelet derived growth factor (PDGF) among others [39–47].

It is also now widely believed that myofibroblasts could be derived from at least four other sources [48–50]. Fibrocytes were originally described as fibroblast like, peripheral cells that migrate into regions of tissue injury [51]. They express fibroblast specific proteins as well as the hematopoietic stem cell marker (CD34) and the leukocyte common antigen (CD45). Fibrocytes migrate to wound sites in response to different chemokine signals including secondary lymphoid chemokine (CCL21) and stromal cell-derived factor (CXCL12) [52–54].

Over time the expression of CD34 and CD45 is reduced and the cells differentiate into myofibroblasts [55,56]. Fibrocyte differentiation is regulated by multiple soluble mediators such as IL-4, IL-13 and PDGF [52,55]. There is significant literature indicating that fibrocytes are associated with organ fibrosis in pulmonary fibrosis, bronchial asthma, skin wounds, intimal hyperplasia and kidney fibrosis [49,52,54,57–60].

Epithelial to mesenchymal transition (EMT) describes the transdifferentiation of an epithelial cell to a cell with myofibroblast-like features. During EMT epithelial cells downregulate epithelial marker expression, upregulate mesenchymal markers expression and gain functional characteristics of mesenchymal cells [50,61,62]. TGF-β1 continues to be regarded as the masterswitch regulating fibrosis [63–65] and EMT driven by TGF-β1 has been suggested to play a role in fibrosis in multiple organs [66–68]. The ability of other cytokines to drive EMT directly remains more controvertial. For example there is conflicting data reqarding the ability of TNFα to drive EMT in the absence of TGF-β1 [62,69–73]. However, there is compelling evidence that cytokines including TNFα and Il-1β are able to accentuate TGF-β1 driven EMT in a range of cell types [71,73–78]. However, recently the contribution of myofibroblasts derived *via* EMT to fibrosis has been questioned due to contradicting reports using lineage tracing models in mice [68,79–91] (for a more

comprehensive assessment of the role of EMT in fibrosis please see Epithelial injury and lung repair – Harold Chapman, Renal epithelial injury – Wilhelm Kriz, Epithelium ER stress as a fibrotic stimulus – Timothy Blackwell also in this special issue).

More recently endothelial to mesenchymal transition (EnMT) has been suggested as a potential source of myofibroblasts during wound healing and fibrosis. EnMT was originally thought to be a phenomenon confined to embryonic development but in 2007, Zeisberg et al. published evidence suggesting that up to 35% of fibroblasts present in the fibrotic myocardium of mice with aortic banding originated from endothelial cells [92]. Consequently several other studies have suggested an important role for EnMT in cardiac, renal and pulmonary fibrosis [93–98]. Like EMT, EnMT can be driven by TGF-β1 and TGF-β2 [92] and accentuated by cytokines such as TNFα and IL-1β [99–101].

Pericytes are cells of mesenchymal origin that are intimately involved in the development, maturation, stabilisation and remodelling of the vasculature during homeostasis and angiogenesis [102]. Recently Lin and colleagues identified that pericytes are closely associated with the vasculature in normal kidney cortex and medulla. However in response to injury these pericytes detach from the vasculature, rapidly up regulate collagen production and α-smooth muscle actin (α-SMA) expression, migrate into the interstitial space and increase myofibroblast numbers [103]. A subsequent study adopting a genetic fate mapping approach to label pericytes (as well as mesangial cells and smooth muscle cells) identified that in a range of kidney injury models the number of labelled cells increased approximately 15 fold in 2–3 weeks strongly suggesting an important role for pericytes in fibrosis in the kidney [87]. Consequently a role for pericytes as a precursor for myofibroblasts has been proposed in other tissues including the spinal cord, lung, skin and skeletal muscle [91,104–107].

The challenge remains to effectively identify the quantity of myofibroblasts derived from each of the above sources in a fibrotic organ and determining the relative contribution of each to disease pathology to improve our understanding of the mechanisms driving fibrosis and allow the development of novel therapeutic targets.

4. TGF-β dependent and independent fibrosis

TGF-β is the most extensively studied molecule in fibrosis. There are three TGF-β isoforms (TGF-β1–3); all have similar biological activity, although each isoform is expressed in a distinct pattern under control of a unique promoter [108,109]. Although a wide variety of cell types produce and respond to TGF-β it is TGF-β1 that has been primarily linked to tissue fibrosis [110]. TGF-β1 is released from cells in a latent complex formed by binding to latency-associated protein (LAP), which holds TGF-β1 in an inactive state. To achieve an active state, TGF-β1 must dissociate from LAP, a process that can be catalysed by a range of agents including cathepsins, plasmin, calpain, thrombospondin, matrix metalloproteinases (MMPs) and integrins [109,111–114] (for a more comprehensive assessment of the role of integrin biology please see Integrin biology and ECM interactions – Dean Sheppard also in this special issue). Once activated TGF-β1 has been shown to signal primarily *via* heteromeric complexes of type II and type I serine/threonine kinase receptors which activate the SMAD signalling pathway, a homolog of the mothers against decapentaplegic drosophila proteins, and modulates the transcription of important target genes including pro-collagen I (COL1A1) and pro-collagen III (COL3A1) [115–118].

Numerous animal models have demonstrated an important role for TGF-β in the pathogenesis of fibrotic conditions [38,119–122]. For example TGF-β inhibition attenuated hepatic, renal and cardiac fibrosis in various animal models [123–125]. Extensive evidence suggests that the canonical TGF-β type I receptor (ALK5)/Smad3 pathway is critically

involved in the pathogenesis of fibrosis driven by TGF-β in many tissues. For example, the oral administration of an inhibitor of the kinase activity of ALK5 inhibited fibrosis in a rat model of TGF-β1-induced pulmonary fibrosis [126] and Smad3 null mice show attenuated fibrosis in bleomycin induced pulmonary fibrosis, renal interstitial fibrosis and cardiac fibrosis [127–130].

However, not all fibrosis is dependent on TGF-β1 and several Smad3/TGF-β1 independent mechanisms of fibrosis have been described in the lung and other tissues [131–133] suggesting that other mediators can act separately from the Smad3/TGF-β1 pathway.

5. Th2 cytokines in fibrosis

The T-helper 2 (Th2) cytokines IL-4 and IL-13 share many biological functions as both exploit the same IL-4Rα/Stat6 signalling pathway [134]; for example IL-4 and IL-13 can drive the differentiation of resident fibroblast and recruited fibrocytes to myofibroblast in a range of tissues [39,41,43–45]. However the development of IL-13 transgenic mice and knockout mice, as well as IL-13 specific antagonists, has revealed unique and non-redundant roles for IL-4 and IL-13 *in vivo* [135–138].

Although the contribution of IL-4 to fibrosis varies in different diseases it is considered a potent fibrotic mediator with one study suggesting that IL-4 is nearly twice as effective as TGF-β in inducing collagen synthesis from human skin derived fibroblasts [139]. One of the first *in vivo* reports to investigate the contribution of IL-4 in fibrosis was a study of Schistosomiasis in mice, in which neutralising antibodies to IL-4 were shown to significantly reduce the development of hepatic fibrosis [12]. Subsequently inhibitors of IL-4 were also found to reduce dermal fibrosis in a chronic skin graft rejection model and in a mouse model of scleroderma [140,141]. In addition IL-4 is found at increased levels in BAL fluids of patients with IPF, in the pulmonary interstitium of individuals with cryptogenic fibrosing alveolitis and in peripheral blood mononuclear cells (PBMCs) of patients suffering from periportal fibrosis of the liver [142–144].

When IL-4 and IL-13 were inhibited independently, IL-13 was identified as the dominant effector cytokine of fibrosis in several experimental models [135,145–149]. For example the overexpression of IL-13 in the lung triggered significant subepithelial airway fibrosis in mice in the absence of any other inflammatory stimulus [137]. In contrast, despite developing an intense inflammatory phenotype, the overexpression of IL-4 in the lung was not associated with evidence of subepithelial fibrosis [150]. Anti-IL-13 treatment has been shown to markedly reduce collagen deposition in the lungs of animals challenged with *Aspergillus fumigatus* conidia [146]. In schistosomiasis, collagen deposition was decreased by more than 85% following IL-13 blockade although the egg-induced inflammatory response was maintained, including no attenuation of IL-4 production [135,151,152]. IL-13 binds to two primary receptor chains, IL-13Rα1, which also binds IL-4 and thus accounts for the functional overlap of the cytokines, and IL13Rα2 [138,153]. IL-13Rα2 is generally considered to be a decoy receptor for IL-13 since it has a short cytoplasmic tail which is devoid of signalling activity [154]. IL-13Rα2 binds IL-13 with four orders of magnitude higher affinity and specificity than IL-13Rα1 [155] and is believed to exert an inhibitory function by blocking the formation of functional IL-13-IL13Rα1 complexes [138]. In agreement with this, mice lacking the IL-13Rα2 decoy receptor have enhanced IL-13 activity [156]. When infected with *Schistosoma mansoni* the IL-13Rα2-deficient mice had significantly increased liver fibrosis despite no change in the inflammatory response [157] suggesting that IL-13Rα2 directly inhibits the ECM-remodelling activity of IL-13. Soluble IL-13Rα2-Fc is a highly effective inhibitor of IL-13 that has been shown to ameliorate the

progression of established fibrotic disease [135,138,151,158] suggesting that modulation of the IL-13 signalling pathway may be a viable therapeutic target in fibrosis.

Macrophages demonstrate remarkable plasticity and change their physiology in response to the microenvironment [159]. Interestingly, selective depletion of macrophages in a model of liver fibrosis revealed distinct populations of macrophages associated with both injury and recovery phases of inflammatory scarring [160]. IL-4 and IL-13 promote the transition of resident macrophages into M2 or alternatively activated macrophages (AAMφ). *In vitro* and *in vivo* studies in mice have shown that this phenotype is characterised by elevated expression of the mannose receptor (CD206), Ym1, Relm-α, (also known as FIZZ-1), major histocompatibility complex class II antigens and arginase-1 [161]. Expression of arginase-1 by AAMφ is of particular interest because this enzyme controls L-proline production, which is required for collagen synthesis by activated myofibroblasts [162]. AAMφ have also been implicated in the development of Th2 effector responses, production of fibrogenic cytokines and recruitment of fibrocytes [163,164] leading to suggestions that AAMφ are important inducers of wound healing and fibrosis. However a recently published study employing $LysM^{Cre}IL\text{-}4R\alpha^{-/flox}$ mice, in which Cre-mediated recombination results in deletion of the IL-4Rα chain in the myeloid cell lineage and therefore macrophages cannot recognise IL-4 or IL-13, demonstrated that egg-induced granulomas and liver fibrosis developed normally in the absence of AAMφ following infection with *Schistosoma mansoni* [165]. Surprisingly depleting arginase-1 activity specifically in AAMφ exacerbated the development of liver fibrosis and increased the Th2 immune response suggested that AAMφ are required for the suppression and resolution of fibrosis [166]. The data suggest that AAMφ may compete with myofibroblasts for L-arginine which is required for collagen synthesis and could be exploited to ameliorate fibrotic disease. However first it will be important to investigate if AAMφ have similar inhibitory roles in other models of fibrosis.

IL-5 may also play an important role in fibrosis through the recruitment, differentiation and activation of eosinophils. IL-5 and eosinophils have been observed in a variety of diseases including skin allograft rejection and pulmonary fibrosis and eosinophils are an important source of pro-fibrotic cytokines and growth factors such as TGF-β1 and IL-13 [141,167,168]. Anti-IL-5 and IL-5 gene deletion have been shown to suppress eosinophilia and remodelling in murine models of allergic asthma [169–171] and decreased granuloma size in chronic Schistosoma mansoni infection [172]. However several other studies have failed to show a reduction in fibrosis in liver, skin and lung raising doubts about the importance of IL-5 and eosinophils in disease [173–175]. A recent study by Huaux et al. paradoxically demonstrated that IL-5−/− mice are not protected from bleomycin induced pulmonary fibrosis but that excessive amounts of IL-5 can exacerbate bleomycin induced pulmonary fibrosis [175] suggesting that IL-5 may be accentuating rather than driving fibrosis.

IL-10 is a multifunctional cytokine with diverse effects on most hemopoietic cell types that was first identified for its ability to inhibit the activation and effector function of T cells, monocytes, and macrophages. The primary function of IL-10 appears to be to limit and ultimately terminate inflammatory responses [176]. In agreement with a role as a suppressive cytokine, IL-10 deficient animals show significantly more severe hepatic and pancreatic fibrosis in response to challenge with carbon tetrachloride (CCL4) and cerulein respectively and mice treated with endogenous IL-10 develop significantly less liver, lung and pancreatic fibrosis [177–180]. This provides supportive evidence for the important role that pro-inflammatory inflammation can play in fibrogenesis. One potential mechanism of action for IL-10 may be *via* the direct inhibition of collagen synthesis and secretion from fibroblasts. For example culturing human scar derived fibroblasts with IL-10 induced a decrease in type1 pro-collagen protein and mRNA and the addition of anti-IL-10 to cultured

hepatic stellate cells caused enhanced collagen production under basal or stimulated condition [181,182]. Consequently some success in clinical studies have been reported including a reduction in serum alanine aminotransferase (ALT), hepatic inflammation and a reduction in fibrosis score in chronic hepatitis C patients treated with IL-10 [183].

6. IL-17A and its role in fibrosis

Th17 cells are a subset of CD4+ T-helper cells that differ from Th1 and Th2 cells in development and function and are characterised by the production of their signature cytokine IL-17. Differentiation of Th17 cells requires the combined actions of TGF-β, IL-6, and IL-21 in mice, whereas IL-6 and IL-21 can be replaced by IL-23 or IL-1β in humans [184–186]. These cytokines induce the expression of the orphan nuclear receptor RORγt that is the key transcription factor that orchestrates the differentiation of this effector cell lineage [187]. Once established the expression of IL-23 is required for stabilisation and expansion of these cells *in vivo* [188,189]. The development of Th17 cells can be suppressed by IFNγ, IL-2, IL-27 and IL-4 [190–194].

Il-17 is recognised as an inflammatory cytokine that exerts its function mainly on myeloid cells, epithelial cells and mesenchymal cells to induce the expression of a range of cytokines and chemokines, which in turn increase granulopoiesis and recruitment of leukocytes, mainly neutrophils, to the site of inflammation [195,196]. IL-17A antibody neutralisation reduced neutrophil influx during the early lung response to silica particles and endotoxin exposure [197,198] and intratracheal instillation of human recombinant IL-17 selectively recruited neutrophils into rat airways [199,200]. IL-17A expression is associated with the persistent neutrophilia observed in a variety of diseases including bacterial pneumonia and cystic fibrosis in the lung, acute lesions in atopic dermatitis and in renal allografts during acute rejection where the number of IL-17 positive cells are independent predictors of worse graft outcome [201–205]. IL-17 has also been shown to be elevated in the BAL of patients with IPF, with the recruitment of neutrophils to the BAL an important predictor of early mortality in IPF patients [206,207].

Several studies have suggested a possible contribution for IL-17A in the development of chronic fibroproliferative diseases [187]. For example Th17 cytokines are increased during the development of bleomycin induced skin fibrosis and IL-17A promotes the development of dilated cardiomyopathy, with blockade of IL-17A attenuating myocarditis-induced cardiac fibrosis and ameliorating ventricular function [208,209]. Several studies have also revealed that the development of hepatic granulomas in mice infected with Schistosoma mansoni is in part dependent on Th17 responses and that treatment with IL-17 neutralising antibodies significantly reduces granuloma formation in some strains of mice [210–212]. IL-17A has also been shown to be important for the development of pulmonary fibrosis after exposure to bleomycin. Detailed mechanistic studies in mice with bleomycin-induced fibrosis suggested that bleomycin-induced IL-17A production is also highly dependent on TGF-β1 signalling, and recombinant IL-17A-driven fibrosis is dependent on the downstream profibrotic activity of TGF-β1, suggesting co-dependent roles for IL-17A and TGF-β1 in the development of pulmonary fibrosis [207].

The aforementioned data suggest that targeting components of the IL-17A signaling pathway is a potential strategy for the development of novel therapeutic agents against fibroproliferative diseases.

7. Th1 cytokines in fibrosis

Numerous experimental models of fibrosis have documented potent anti-fibrotic functions of the archetypical Th-1 cytokine IFNγ. For example IFNγ inhibits the activation and

proliferation of hepatic stellate cells (HSC) and subsequent ECM deposition in a rat model of liver fibrosis induced by dimethylnitrosamine [213]. In addition IFNγ treatment ameliorates bleomycin induced lung fibrosis and reduced glomerulosclerosis and tubulointerstitial fibrosis in the rat subtotal nephrectomy model [213,214]. Mechanistically, IFNγ is believed to inhibit fibrosis by antagonising the pro-fibrotic activity of TGF-β1. TGF-β1 induced phosphorylation of Smad3 and its subsequent translocation to the nucleus is inhibited by IFNγ resulting in the decreased activation of TGF-β1 responsive genes. In addition, acting through Janus-associated kinase (Jak1) and Stat1, IFNγ induces the expression of Smad7, an antagonistic SMAD, which prevents the interaction of Smad3 with the TGF-β receptor, further attenuating TGF-β-induced signalling [215]. IFNγ can also directly inhibit fibroblast proliferation, TGF-β1 induced expression of the genes encoding procollagen I and procollagen III, and collagen synthesis in activated myofibroblasts as well as inhibiting the Th2 cytokine induced differentiation of fibrocytes into myofibroblasts [216,217]. Similar antifibrotic activity has been reported for IL-12, primarily *via* its ability to stimulate IFNγ production in Th1 cells. In schistosomiasis, treatment with recombinant IL-12 significantly reduced collagen deposition associated with chronic granuloma formation, while having no effect on the establishment of infection [218] and IL-12 treatment caused a significant reduction in the hydroxyproline content of the lung in the bleomycin mouse model of lung fibrosis [219].

However despite compelling evidence supporting an antifibrotic role for IFNγ both *in vitro* and *in vivo*, clinical studies employing the use of IFNγ have generated conflicting results. Positively, IFNγ treatment reduces liver fibrosis progression in people chronically infected with HCV [220]. In contrast, large randomised placebo controlled clinical trials in patients with IPF have revealed that treatment with IFNγ did not significantly improve survival, lung function, gas exchange, or the quality of life. In addition more patients in the IFNγ group had constitutional signs and symptoms (influenza-like illness, fatigue, fever, and chills) than those on placebo [221,222].

The M1 or classically activated macrophages (CAMφ) are produced during cell mediated responses and are a vital component of host defence. Such macrophage activation depends on IFNγ, a cytokine network involving IL-12 and exposure to microbial products. These macrophages secrete high levels of pro-inflammatory cytokines including TNFα and IL-1β and their activation must be tightly controlled because the cytokines and mediators they produce can lead to host-tissue damage [223]. TNFα and IL-1β have been identified as important targets in a variety of fibrotic conditions including IPF and asbestosis [224,225] and the overexpression of either TNFα or IL-1β in the lungs of mice leads to spontaneous pulmonary fibrosis [226,227]. Studies have subsequently identified that TNFα is essential for the development of bleomycin and silica induced pulmonary fibrosis, CCL_4 induced hepatic fibrosis and non-alcoholic steatohepatitis in mice [228–231]. Recently clinical trials employing inhibitors of the TNFα pathway such as etanercept and infliximab have been initiated to evaluate the potential clinical benefit for the treatment of IPF and other fibrotic diseases. One study in IPF reported that etanercept was well tolerated and showed a non-significant reduction in disease progression in several physiologic, functional, and quality-of-life endpoints [232].

Additionally, several studies have documented profibrotic activity for IL-1β in pulmonary fibrosis induced by bleomycin and silica, liver fibrosis in hypercholesterolemic mice, renal interstitial fibrosis resulting from unilateral ureteric obstruction and cardiovascular fibrosis after myocardial infarction [233–235]. IL-1β was found to be increased in the BAL of patients with IPF and acute respiratory distress syndrome (ARDS), with persistent elevation predicting poor outcome [207,236]. Recent studies have shown IL-1β driven pulmonary fibrosis to be dependent on IL-17A [207,237]. IL-1β has also been shown to drive EMT and

myofibroblast differentiation *via* a TGF-β1 dependent mechanism, confirming that it functions as a potent upstream driver of fibrosis [238]. In addition, IL-1β and TNFα have been demonstrated to accentuate TGF-β1 driven EMT and EnMT [71,73,74,76,99–101] highlighting another potential mechanism by which IL-1β and TNFα drive fibrosis.

8. Fibrosis – lessons from lung transplantation

The ability to investigate the role of the immune system in early fibrotic disease is limited as patients often present with established fibrosis and significant loss of organ function already. However there is a condition where it is possible to study the fibrotic remodelling process very early in disease and even before it has begun. Obliterative bronchiolitis (OB) affects 50% of lung transplant recipients limiting survival to a median of approximately 5 years and provides a valid human model of chronic inflammatory airway disease leading to dramatic fibrotic remodelling and loss of lung function over a short time course. The rate of disease progression, as measured by reduction in forced expiratory volume in 1 second (FEV1), can be ten times that seen in other chronic progressive inflammatory airways diseases such as chronic obstructive pulmonary disease [239]. Two decades of research of this condition have identified important mechanisms linking inflammation, the immune response and the development of fibrosis post lung transplant.

Several studies highlight an important role for innate immunity, PAMPs and PRR in the fibrotic remodeling seen in OB. For example, the acquisition of *Pseudomonas aeruginosa* in the transplanted airway is associated with an increased risk of developing in OB [16,240,241]. In addition, lung transplant patients with loss of function polymorphisms in TLR4 demonstrate significantly less acute rejection and a trend towards reduced severity of OB [242]. Similarly, patients with gain of function polymorphisms in CD14, which binds LPS and promotes signaling through TLR4, develop OB earlier after transplant and demonstrate increased OB related deaths. Interestingly, patients with a gain of function polymorphism in CD14 also have significantly greater TNFα and IFNγ in the peripheral blood implying a heightened state of innate immune activation drives the development of increased post-transplant rejection [243].

There is growing interest in the role of the macrophage as an effector cell in allograft injury and fibrosis. In the murine heterotopic tracheal transplant model depletion of recipient macrophages significantly abrogates obliteration of the transplanted airway [244]. Furthermore airway macrophages isolated from post-transplant patients secrete increased levels of pro-inflammatory cytokines compared to control patients [245,246] leading to an elevated expression of a variety of acute inflammatory cytokines in the BAL of patients with OB including TNFα, IL-1β and IL-8 [247,248]. It has been demonstrated that there is an early elevation in Th1-cytokines in lung transplant patients who developed OB compared to stable recipients and normal control subjects [249]. Furthermore, CD4+ T cells in patients who developed OB are of a Th1-phenotype suggesting that the microenvironment within the lung allograft may skew the immune response towards a Th1 phenotype that predisposes to OB [250].

Several studies have shown that the neo-macrolide azithromycin given at sub-m*inimum* inhibitory concentration for respiratory pathogens can reverse the decline in lung function in some patients with OB [251,252]. Furthermore a randomised placebo controlled study demonstrating that patients receiving azithromycin after lung transplantation had a lower incidence of OB compared with those receiving placebo in their first two years post transplantation [253]. Neo-macrolides are a group of antibiotics that are bacteriostatic and only bactericidal at high concentrations. Independently of their antimicrobial activity, macrolides possess immunomodulatory properties that may contribute to clinical benefits

observed in patients with OB. The mechanism of action was initially believed to be through a reduction in airway neutrophilia and IL-8 in the lung [254]. However, azithromycin has recently been shown to modulate inflammation by shifting macrophage polarisation towards an AAMφ phenotype identifying another possible mechanism of action [255].

The development of OB in a mouse model is associated with a significant elevation in TNFα levels at the onset of fibrosis [256] and neutralising antibodies to TNFα prevents the development of OB in this model [257]. Similar effects have also been reported in OB in rat tracheal allografts [258] and in a heterotopic porcine bronchial transplantation model [259]. However, to date there have been no reported trials of using biological agents targeting TNFα in OB post lung transplantation, although there is a single case report indicating improvement in wellbeing and FEV1 after Infliximab therapy in a child who developed OB following bone marrow transplantation [260]. Lung transplant recipients who go on to develop OB also showed an elevation in IL-17 compared to stable lung transplant recipients [247] and neutralising IL-17 prevented OB in the heterotopic tracheal transplant model in mice [261]. To date no studies have reported investigated the efficacy of anti-IL-17 therapies in OB.

TGF-β1 is present in elevated levels in the BAL of patients with OB [262] and blocking TGF-β1 or its downstream signalling inhibits OB in the heterotopic tracheal transplant model [263,264]. Mechanistically, bronchial epithelial cells isolated from stable lung transplant recipients undergo EMT when exposed to TGF-β1 [62] and this can be accentuated by the addition of TNFα or IL-1β or by co-culturing the cells with a *Pseudomonas aeruginosa* activated macrophage cell line [78,265]. In addition co-localisation of both epithelial (E-cadherin) and mesenchymal (α-SMA) markers in epithelial cells of post-transplant patients has been reported [62] highlighting EMT as one potential source of myofibroblasts in the development of OB. As well as an elevated level of TGF-β1, it has also been reported that IL-13 is elevated and biologically active in BAL during the development of BOS. Furthermore translational studies using a mouse model of OB showed that neutralisation of IL-13 reduced airway allograft matrix deposition and OB [148]. Both TGF-β1 and IL-13 can drive the differentiation of resident fibroblasts to myofibroblasts identifying a likely second source of myofibroblasts in OB. Finally, a higher proportion of circulating fibrocytes was measured in patients with OB Grade ≥ 1 than in those with OB Grade 0 (p) [266] highlighting a possible role for fibrocytes in allograft rejection. In agreement with this, inhibiting CXCL12 blocks fibrocyte migration and differentiation and attenuates OB in the murine heterotopic tracheal transplant model [267].

The aforementioned data highlight the complex nature of fibrosis in the transplant lung and the large number of potential therapeutic targets to limit disease progression. However, due to the ability to accurately identify patients that are likely to develop the disease before they present with clinical symptoms and the rapid decline in FEV1 as an experimental indication of disease progression, the development of OB after lung transplant could also be a valuable and cost effective tool for testing novel therapeutic strategies in the development of fibrosis.

9. Conclusion

A review of the literature pertaining to the role of cytokines in fibrosis highlights the wide range of functions a single cytokine can perform on numerous cell types and provides the possibility that targeting a single cytokine may provide a way of blocking/reversing at least some of the fibrotic process in disease. However the literature also tells us that a wide number of cytokines can perform several very similar functions and therefore compensate for the loss of another. How do we choose which cytokine/cytokines to target as potential therapeutics? And are these targets going to work universally or will there be organ specific

and disease specific roles identified? Given the diverse importance for Th1, Th17 and Th2 cytokines described in the literature in driving fibrosis in different organs, and indeed different diseases in the same organ, it seem unlikely that the treatment of fibrosis will be universal or organ specific and instead will likely be disease specific. Depending on the stage of disease when a patient is diagnosed, the treatment approach may also be tailored. For example if a patient presents early, i.e. with progressive fibrosis, the inhibition of ECM production is an obvious target to limit the development of fibrosis. However if a patient presents late in disease, i.e. with established fibrosis, the resolution of already deposited ECM is critical. Given the vast number of potential therapeutic targets and strategies it is important that a well-defined and considered approach to translating the wealth of experimental knowledge into clinically beneficial therapies is applied. The slow progression of many fibrotic diseases makes clinical trials expensive and prohibitive. Therefore quantitative clinical endpoints such as serum bio-markers and imaging techniques to accurately measure the rate of disease progression are desperately needed. The burden of diseases where inflammation and fibrosis plays an important role continues to grow and therefore the need for safe and effective anti-fibrotic therapies is great and is also likely to increase.

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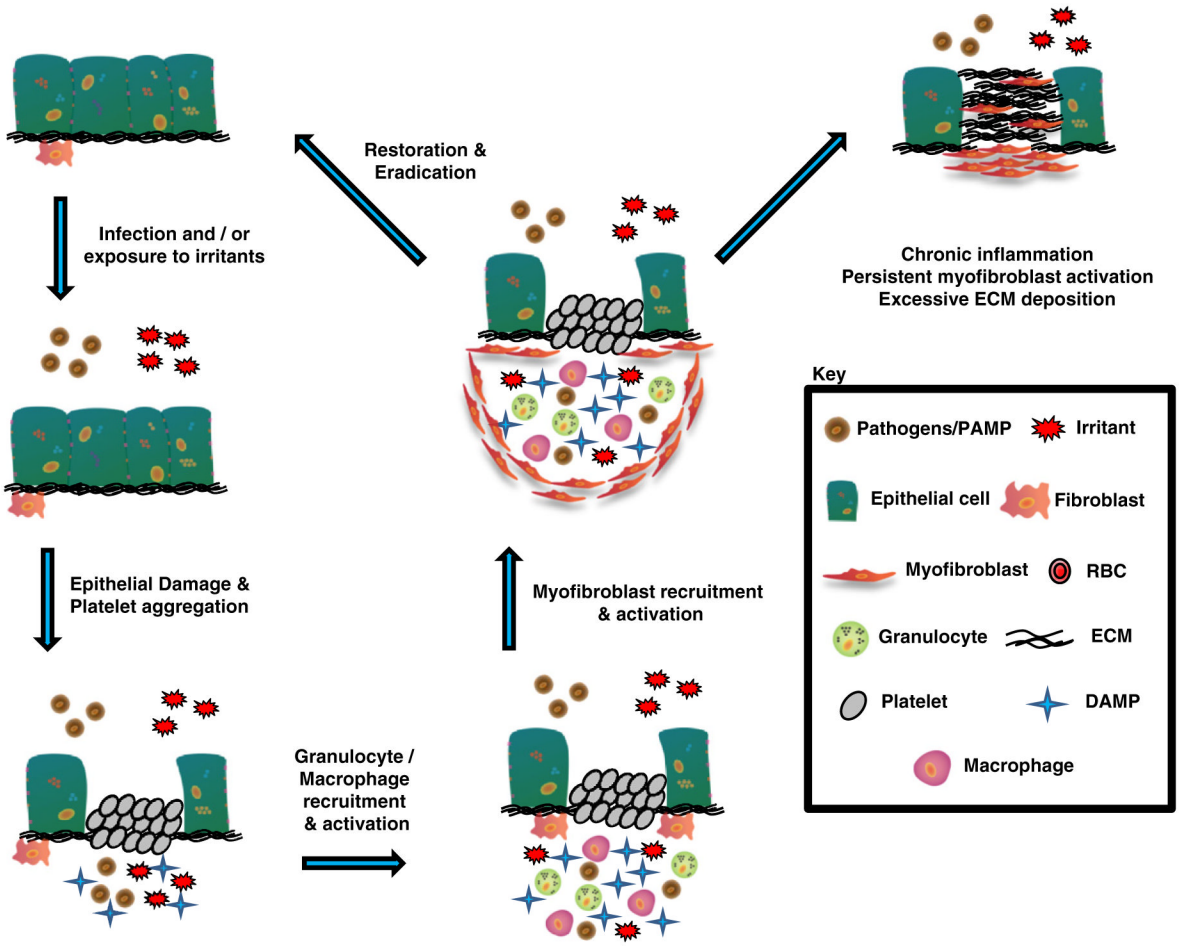

Fig. 1.

Pathological vs. physiological wound healing. Infection or exposure to harmful molecules can lead to epithelial damage and loss of epithelial integrity. Following injury fibroblasts, endothelial cells, and neighbouring epithelial cells release a range of soluble factors that trigger clotting and initiate the development of a provisional ECM. The aggregation and subsequent degranulation of platelets triggers increased blood flow, vasculature dilation and vasculature permeability allowing the effective recruitment of inflammatory cells to the site of tissue injury. The first responders are the neutrophils, eosinophils and basophils that are responsible for neutralising any invading pathogens *via* an oxidative burst response and eliminating cell debris/dying cells by phagocytosis. The granulocyte number in the site of epithelial injury peaks rapidly, within minutes, but is followed by a rapid decline. Once in the wound micro-environment the recruited monocytes mature to increase the number of macrophages in the wound and perform similar functions to those described for granulocytes. In addition they produce cytokines and chemokines that amplify the wound response by promoting the formation and stabilisation of a provisional ECM and promoting angiogenesis. Myofibroblast numbers are increased at the wound site from several sources (see Fig. 2). Once recruited to the wound area the myofibroblasts become activated and traverse the provisional ECM until they reach the edge of the wound and initiate contraction of the wound. Finally epithelial cells at the edge of the wound loosen adherence junctions and migrate over the ECM to restore a continuous epithelium and tissue homeostasis. At this point the myofibroblasts in the wound area undergo apoptosis and the macrophage numbers are significantly reduced *via* egress into the lymphatic system. Fibrosis occurs when the

initial wound is severe, the wound repair process becomes dysregulated or the source of epithelial damage persists resulting in repeated injury and chronic inflammation.

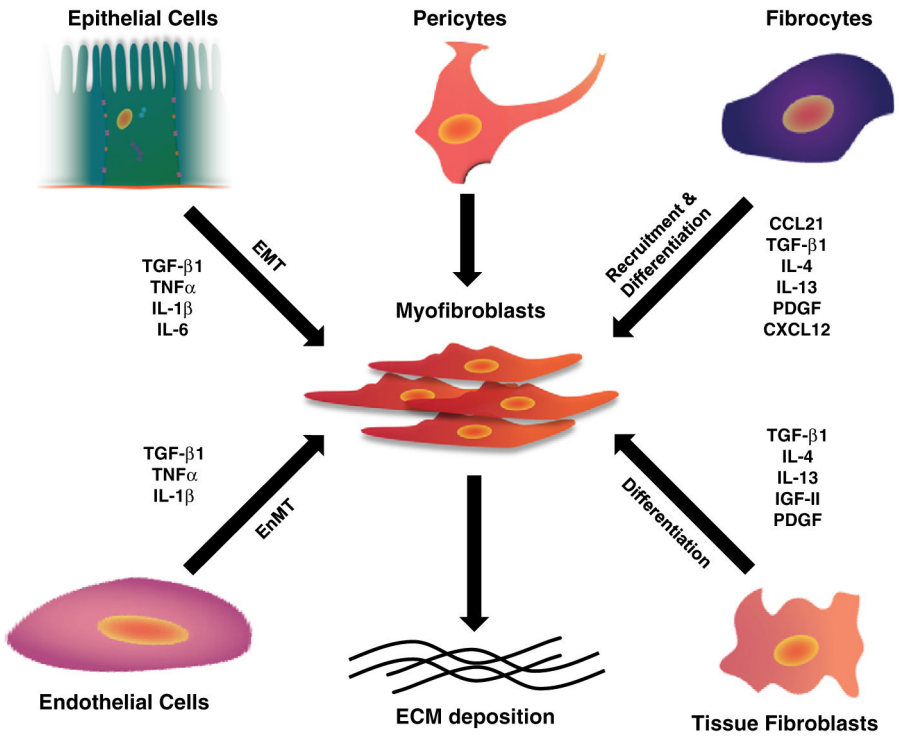

Fig. 2.

Proposed origins of myofibroblasts in fibrosis. It was originally thought that the activation or proliferation of local resident stromal cells and their differentiation in to myofibroblasts was the only source of myofibroblasts during fibrosis. However it is also now widely believed that myofibroblasts are derived from at least four other sources; through the recruitment and differentiation of fibrocytes, through the activation and proliferation of pericytes, *via* epithelial to mesenchymal transition (EMT) or *via* endothelial to mesenchymal transition (EnMT).