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INVITED REVIEW

Macrophage–stroma interactions in fibrosis: biochemical, biophysical, and cellular perspectives

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Abstract

Fibrosis results from aberrant wound healing and is characterized by an accumulation of extracellular matrix, impairing the function of an affected organ. Increased deposition of extracellular matrix proteins, disruption of matrix degradation, but also abnormal post-translational modifications alter the biochemical composition and biophysical properties of the tissue microenvironment – the stroma. Macrophages are known to play an important role in wound healing and tissue repair, but the direct influence of fibrotic stroma on macrophage behaviour is still an underinvestigated element in the pathogenesis of fibrosis. In this review, the current knowledge on interactions between macrophages and (fibrotic) stroma will be discussed from biochemical, biophysical, and cellular perspectives. Furthermore, we provide future perspectives with regard to how macrophage–stroma interactions can be examined further to ultimately facilitate more specific targeting of these interactions in the treatment of fibrosis.

Keywords: polarization; migration; phagocytosis; extracellular matrix; stiffness; stretch; shear stress; microstructure; profibrotic; antifibrotic

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Introduction

Fibrosis results from aberrant wound healing and is characterized by excessive deposition of extracellular matrix (ECM). Fibrosis can affect a wide range of organs and tissues, including but not limited to lungs, heart, liver, skin, and kidney. Increased deposition, disruption of degradation, and abnormal post-translational modifications of the ECM change the biochemical composition, availability of cell attachment domains, stiffness, and other biophysical properties of the tissue microenvironment – also known as the stroma (Figure 1). Stroma is composed of ECM and cells such as fibroblasts, pericytes, tissue-resident mesenchymal stromal cells (MSCs), adipocytes, mast cells, and macrophages [1]. Although the composition of ECM varies depending on the type of tissue, ECM is generally composed of collagens (mainly fibrillar collagens such as type I, II, III, V), elastin, fibronectin, and various glycosaminoglycan molecules. Mechanical strength is mainly provided by collagen fibres, while elastin fibres provide elasticity [2]. Traditionally, ECM is considered a network for structural support; however, recent findings indicate ECM to be far more bioactive and dynamic – orchestrating biochemical and biomechanical messages that regulate cell

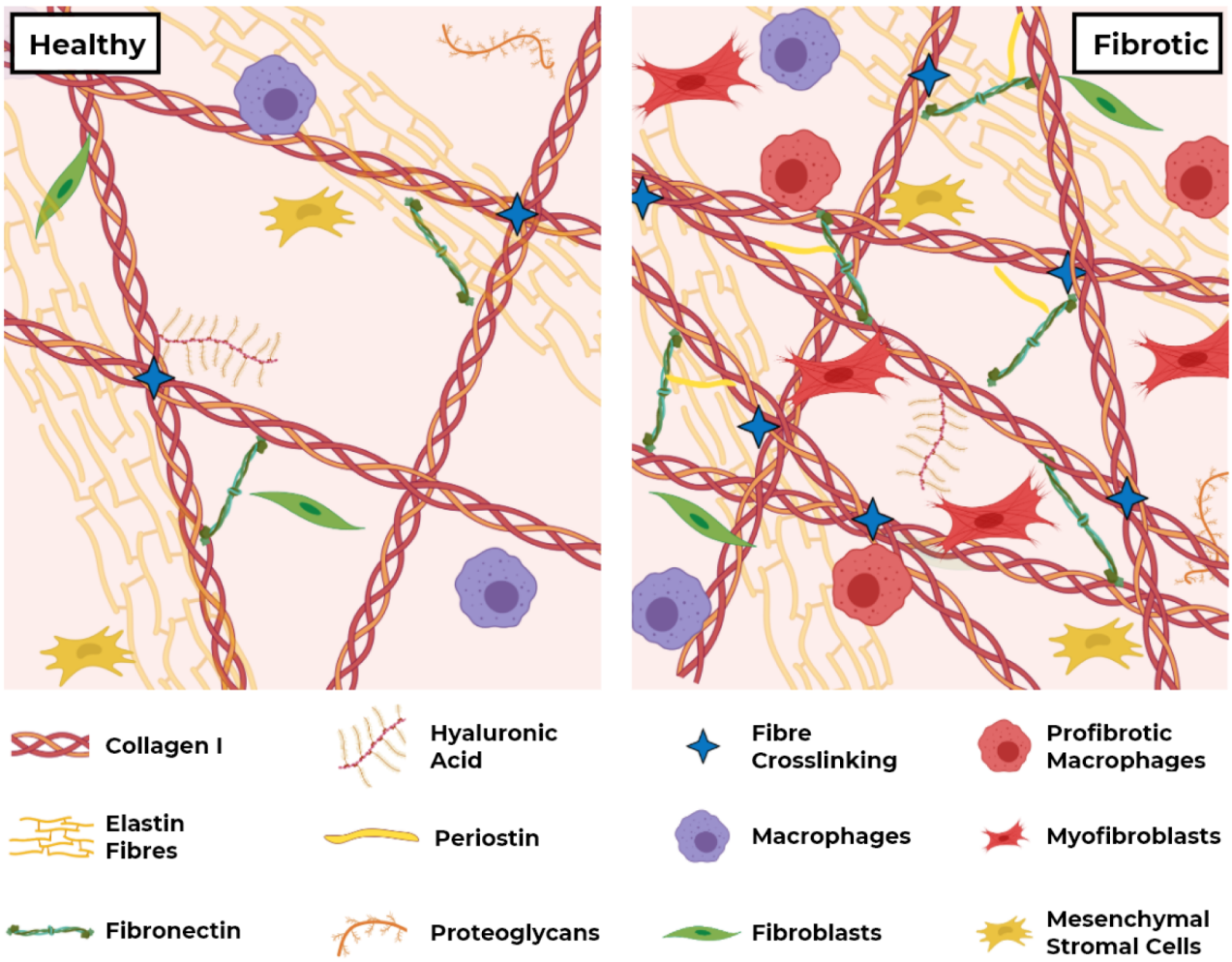

Figure 1. Schematic representation of stroma in healthy and fibrotic conditions. In fibrosis, aberrant ECM deposition and remodelling, as well as higher numbers and altered behaviour of fibroblasts and macrophages, change the biochemical and biophysical properties of stroma, and the consequential interactions with resident cells.

behaviour [1,3]. Dynamic remodelling of ECM is essential to preserve tissue homeostasis and dysregulation of ECM remodelling is an emerging research field, especially in cancer and in fibrosis [4–6].

Macrophages are crucial players in the maintenance of tissue homeostasis [7]. Their ability to polarize into a spectrum of phenotypes allows them to execute a wide variety of functions, all necessary to preserve tissue integrity (Figure 2) [8]. Initially, tissue injury will push macrophages towards a pro-inflammatory phenotype. Subsequent switching of macrophages to a wound healing phenotype that promotes ECM production by myofibroblasts and eventually polarizing to a pro-remodelling phenotype is required to ensure restoration of normal tissue architecture [9]. These phenotypes can be induced by cytokines such as IFN-γ and TNF-α (pro-inflammatory), IL-4 and IL-13 (wound healing), or IL-10 and TGF-β (pro-remodelling), amongst a plethora of other stimuli [10].

As macrophages contribute to all phases of wound healing, their potential role in the development and progression of fibrotic diseases is emerging [9]. Recent advances in single-cell RNA-sequencing have shone light on the heterogeneity of macrophages within and between healthy and fibrotic human tissues and allowed detailed identification of macrophage populations [11–13]. Macrophages that populate tissues can have two different origins, i.e. tissue-resident macrophages derived from embryonic progenitors and monocyte-derived macrophages (Figure 2). Whereas tissue-resident macrophages mostly arrive on site during organ development and are self-maintaining, monocytes migrate from blood to a tissue upon injury and can differentiate into macrophages throughout life [14]. In fibrosis, monocyte-derived macrophages appear to be an important contributor as large numbers of these macrophages have been described in fibrosis and depletion of these macrophages has been shown to attenuate fibrosis in several mouse models [15–18]. Although these monocyte-derived macrophages were long thought to be relatively short-lived, it recently became evident that these monocyte-derived macrophages can also adopt a phenotype similar to tissue-resident macrophages, supporting the emerging role of the tissue microenvironment in instructing macrophage behaviour [15,19]. Furthermore, we now know

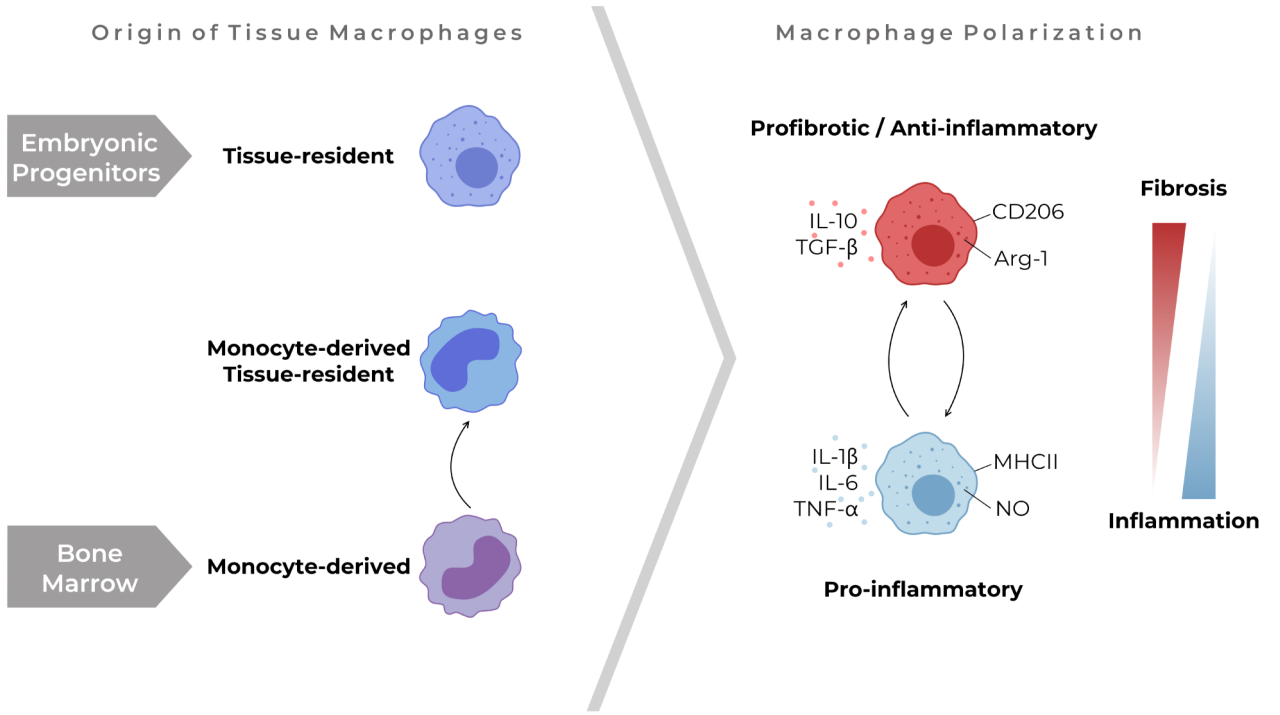

Figure 2. Macrophages in stroma. Tissue macrophages derive from different origins. Despite their differences, macrophages from all origins can change their polarization status to a more pro-inflammatory or profibrotic/anti-inflammatory phenotype upon stimuli from their microenvironment, as can be identified by metabolic changes (arginase-1 versus NO), the expression of surface markers (e.g. MHCII and CD206) or the expression and secretion of cytokines (e.g. TGF-β, IL-10, TNF-α, IL-1β, and IL-6). Arg-1, arginase-1.

that microenvironmental cues can have long-term effects on macrophage function: a concept called trained immunity [20]. These developments suggest that the direct effect of fibrotic stroma on macrophage behaviour may be an under-recognized element in the pathogenesis of fibrosis.

In this review, we summarize the current knowledge of macrophage–stroma interactions in biochemical, biophysical, and cellular perspectives. First, we focus on the recent advances related to influences by altered stromal composition on macrophages. Next, we outline the contribution of biophysical changes in the fibrotic stroma to macrophage behaviour and describe the influence of fibrotic stroma on macrophage–stromal cell interactions. Lastly, we provide future perspectives on how these interactions can be further examined to generate knowledge of their potential value as targets for the treatment of fibrosis.

Biochemical interactions between fibrotic stroma and macrophages

In fibrosis, aberrant deposition of ECM changes the biochemical composition of the stroma (Figure 1). Increased deposition and altered ratios of ECM proteins, such as fibronectin, collagen, periostin, and glycosaminoglycans, have been shown to influence the profibrotic behaviour of stromal cells [21–23]. Although studies have indicated effects of individual ECM proteins [24–27] and solubilized whole ECM on macrophages [28–30], the exact influence of fibrotic stroma on macrophages is still unknown. Emerging data from preclinical models indicate differential responses of macrophages in fibrosis: monocyte-derived macrophages were shown to be profibrotic in, for instance, models of peritoneal and hepatic fibrosis [17,18], while local microenvironmental cues were proposed as the driving force for the *in situ* transitioning of monocyte-derived macrophages towards an antifibrotic phenotype in a resolving model of CCl_4-induced liver fibrosis in mice [31]. In addition, $CD14^+$ monocytes and macrophages derived from $CD14^+$ monocytes have been shown to activate ECM-stored latent TGF-β, a well-known factor for inducing the progression of fibrosis, through integrin αvβ8-mediated pathways [32]. These observations suggest that the altered stroma present in fibrotic tissues may be a driving factor underlying changes in macrophage behaviour in fibrotic disease.

An important player in the altered fibrotic stroma is fibronectin. It regulates collagen organization, and higher deposition of fibronectin has been reported in pulmonary [33], hepatic [34], cardiac [35], and renal [36] fibrosis compared with non-fibrotic conditions. Although fibroblasts and other stromal cells are the predominant source of fibronectin [37], monocytes have also been shown to secrete fibronectin when pro-inflammatory cytokines are present in their microenvironment [38] and alternatively-activated macrophages were found to have high fibronectin expression at both gene and protein levels [39]. Fibronectin-adsorbed surfaces primed macrophages to a pro-inflammatory phenotype through activation of β1 integrin–PI3K/Akt

signalling [40]. In contrast, a fibronectin-rich environment has been shown to prime macrophages to adopt an anti-inflammatory profile in response to pathogen-associated molecular patterns through engagement of TLR2/4 coupled integrin β1-signalling *in vitro* [41]. These differential effects may possibly be caused by alternative effects of fibronectin-containing extra domain A (FN-EDA) and plasma fibronectin [42] on macrophages and require more investigation. Fibronectin can also promote the migration of macrophages, as demonstrated *in vitro* [43]. As infiltrated macrophages can also produce fibronectin in an inflammatory environment, a continuing cycle of inflammation, macrophage influx and priming, and fibronectin deposition may therefore contribute to fibrotic pathobiology.

In vitro systems show that instructions from altered and abnormal composition of fibrotic stroma direct macrophages towards more profibrotic responses, although these systems generally only allow a simplified version of the multitude of functions of macrophages in physiological conditions. Such models are also used to investigate the importance of the presence, number, and availability of cell binding motifs in stroma. Human monocytic THP-1 cells encapsulated in methacrylated gelatin (GelMA; collagen-based and therefore cell-binding) hydrogels had a more anti-inflammatory profile, with increased secretion of arginase-1 mediated through integrin α2β1 and possibly STAT6 signalling, compared with synthetic poly(ethylene glycol)dimethacrylate-based hydrogels, suggesting that the presence of cell binding motifs is linked to macrophage polarization [44]. Similarly, comparison of hyaluronic acid hydrogels to hyaluronic acid–gelatin hydrogels identified more attachment of peripheral blood-derived monocytes to the hyaluronic acid–gelatin hydrogels, due to the higher number of cell-binding motifs in the latter [45]. Moreover, coating surfaces with collagen type I induced polarization of alveolar macrophages towards a more anti-inflammatory $MHCII^{Lo}$-$CD206^{Hi}$ phenotype compared with macrophages cultured on uncoated surfaces [46]. Similarly, in an *in vitro* experiment with mouse bone marrow-derived macrophages, a collagen type I coating yielded more arginase-1-producing macrophages compared with fibronectin-coated surfaces, suggesting differential effects of distinct ECM proteins probably driven by their distinct cell-binding domains [26]. Post-translational modifications of collagen, such as fibre crosslinking, are important changes affecting the biochemical composition of stroma during fibrosis. These inevitably lead to changes in binding domain availability, fibre alignment, and other biophysical changes, although the exact influences on macrophages are unknown to date [47–51]. As with the positive feedback loop between fibrotic ECM and fibroblasts described by Parker *et al* [22], the above-mentioned findings indicate a possible profibrotic interchange between the presence, altered numbers, and types of cell-binding domains found in fibrotic stroma and macrophages.

Periostin, another important ECM matricellular protein/matrikine, is more abundantly expressed in pulmonary [52], hepatic [53], renal [54], and cardiac [55] fibrosis compared with healthy conditions. In an acute kidney injury model, periostin-overexpressing mice had more tissue-resident macrophages with a pro-regenerative phenotype than wild-type mice [56]. In the same study, periostin overexpression alone did not result in spontaneous kidney fibrosis, while periostin-KO mice had lower numbers of pro-regenerative macrophages. Conversely, in a bleomycin model of lung fibrosis, periostin-KO mice had less collagen in their lungs than did wild-type mice, in concordance with peripheral blood-derived monocytes having higher levels of periostin in patients with idiopathic pulmonary fibrosis (IPF) than non-disease control donors [52]. *In vitro* models of macrophage migration showed that periostin stimulated the migration of primary murine macrophages, which also had higher expression of integrin αV, one of the periostin receptors, in the lesion site compared with control. Moreover, periostin promoted the secretion of TNF-α from these macrophages, hypothetically through engagement of the focal adhesion kinase (FAK) pathway [57]. The exact influence of elevated periostin levels on both tissue-resident and monocyte-derived macrophages in fibrosis is yet to be understood, but the presented data suggest that periostin may disturb the balance between these two types of macrophages in favour of profibrotic monocyte-derived macrophages.

Glycosaminoglycans, non-fibrous components of the ECM, are also expressed at higher levels in fibrotic lungs [58], liver [59], kidney [60], and heart [61], compared with non-fibrotic conditions. Solubilized urinary bladder-derived ECM, which has high levels of hyaluronic acid, did not change TNF-α, NO, and arginase-1 levels in bone marrow-derived macrophages *in vitro* [29]. Once the hyaluronic acid in this ECM was degraded, however, more NO secretion was found while TNF-α secretion remained unchanged, suggesting that the hyaluronic acid content of the ECM plays a role in fine-regulating the pro-inflammatory responses of macrophages. In contrast, hyaluronic acid-containing collagen type I hydrogels promoted anti-inflammatory polarization of THP-1 macrophages through CD44 signalling and the STAT3 pathway compared with unmodified collagen type I hydrogels [62]. Monocyte-derived macrophages displayed lower expression of pro-inflammatory markers in hyaluronic acid-containing collagen type I hydrogels compared with collagen type I hydrogels without hyaluronic acid [63]. In addition, effects of post-translational modifications, such as additional sulphate groups on high-molecular-weight hyaluronan, on macrophage responses were investigated in the same study; however, no additional influence of the sulphate groups was observed. In contrast, another study comparing high sulphate with low sulphate content in hyaluronic acid-containing collagen type I hydrogels did find altered macrophage responses, as the production of pro-inflammatory cytokines was lower in high-sulphated hydrogels [64]. Although these studies had some opposing results, they do collectively suggest that the amount of, and modifications of, glycosaminoglycans could regulate macrophage responses.

In conclusion, differing macrophage responses may be driven by changes in the composition of the ECM and aberrant abundancies of ECM components during fibrosis. Fibrotic ECM–macrophage interactions could promote a profibrotic loop, stimulating further fibrotic responses leading to the recruitment of more profibrotic macrophages. In addition to elements determining the structure of ECM, bioactive factors (for example, growth factors) anchored in the ECM could also contribute to the biochemical interactions between macrophages and stroma. However, the challenge of distinguishing the effects of factors stored in ECM from paracrine effects of factors secreted by cells limits our understanding of the contribution of this reservoir function of (fibrotic) stroma to macrophage regulation. Even though there is increased momentum in the emergence of findings dissecting these elements, more studies are needed to address the following questions: (1) whether and what specific combinations of ECM proteins and their binding domains are responsible for regulating macrophage responses in fibrosis; and (2) which mechanisms underlie the priming of recruited macrophages towards a profibrotic profile by the fibrotic stroma?

Macrophage responses to biophysical changes

The altered biochemical composition of the stroma in fibrosis is also associated with changes in biophysical properties such as tissue stiffness, levels of shear stress, and microstructure [65]. However, knowledge on whether and how macrophages respond to these biophysical changes is still limited [66]. The knowledge available in the public domain is mainly based on biomaterials research, which has aimed at controlling macrophage behaviour to modulate the foreign body response by regulating biophysical properties of substrates or scaffolds [67].

Stiffness

Native tissue stiffness is highly dependent on the organ of interest, ranging from around 100 Pa in the brain to a few GPa in calcified bone [65,68]. Fibrosis is consistently associated with (local) increases in the stiffness of the tissue, independent of the organ of interest [65]. This increase is predominantly caused by an excess or a different ratio of ECM proteins. Moreover, post-translational remodelling such as crosslinking of ECM proteins and an increase in cellular stiffness have also been described to contribute to these stiffness alterations [69,70].

Several studies have indicated an effect of substrate stiffness on macrophage behaviour. Although the studied stiffnesses and compositions of the substrates varied greatly, stiffening of the substrate is generally associated with more macrophage adhesion and a bigger and more flattened phenotype as compared to a smaller and more rounded shape on soft substrates [71–82]. Nonetheless, these observed morphological changes on two-dimensional (2D) substrates may not be fully translatable to an *in vivo* situation. For instance, He *et al* reported that encapsulation of macrophages inside a stiff 3D hydrogel resulted in a more rounded shape compared with a softer hydrogel [83].

Related to these morphological changes, functional changes in the migratory behaviour and phagocytic activity of macrophages have also been observed. Infiltration and accumulation of monocyte-derived macrophages have been associated strongly with fibrosis, as inhibition of monocyte infiltration dampened the fibrotic response in experimental fibrosis models [18,84]. Higher substrate stiffness generally tends to stimulate macrophage migration and may therefore exacerbate fibrotic responses [74,75,82]. These investigations were all in 2D setups and the influence of substrate stiffness on movement in 3D is yet to be explored thoroughly. In addition to their migratory behaviour, the phagocytic capacity of macrophages is important in tissue homeostasis as well. For instance, impaired phagocytosis has been observed in airway macrophages from patients with IPF [85], whose lungs are stiffer than those of healthy individuals [58,86]. However, studies investigating whether substrate stiffness has a direct effect on the phagocytic capacity of macrophages reported variable results. Some studies indicated that higher substrate stiffness promoted phagocytosis compared with low stiffness [77,87], while others described a biphasic effect or no significant effect at all [74,78].

The effect of substrate stiffness on pro-inflammatory or profibrotic responses of macrophages has also not yet been shown conclusively. Most studies indicated more pro-inflammatory behaviour when substrate stiffness increased, based on the expression of markers such as IL-1β, IL-6, and TNF-α [75,78,80,81,83,88,89], although the opposite has also been described [77,82,87,90]. However, these studies all investigated short-term responses of macrophages to substrates of various stiffnesses. In the context of fibrosis, it would be of interest to study the long-term effects of stiffness on macrophage polarization.

Recently, several molecular mechanisms underlying macrophage mechanosensing and subsequent mechanotransduction have been elucidated. The mechanosensing abilities of macrophages have been attributed to transient receptor potential vanilloid type 4 (TRPV4), as deletion or inhibition of this mechanosensitive calcium channel abolished the observed effects of substrate stiffness on macrophage behaviour [87,91]. Interestingly, higher expression of TRPV4 has been shown in human fibrotic tissues and TRPV4-deficient mice were protected from fibrosis [92]. Furthermore, Previtera and Sengupta showed that stiffer substrates activated the TLR4-signalling pathway and thereby stimulated macrophage responses to pro-inflammatory stimuli [88]. Although the exact role of TLR4 in fibrosis still needs to be defined, several studies have indicated that TLR4 plays a role in the development of fibrosis in mice [93,94]. In addition to a possible role for these two receptors, substrate stiffness has also been associated with higher expression of integrins by macrophages, as well as a higher density of podosomes [75,78,81,82,95]. Podosomes are versatile, integrin-mediated adhesion structures that not only have mechanosensing and -transducing properties, but also have the ability to mediate local recruitment and the release of matrix-

degrading enzymes upon adhesion to a stiff substrate [95,96]. This direct matrix-degrading response of macrophages to substrate stiffness especially calls for further investigation into podosome function in fibrosis. Furthermore, in many non-myeloid cells, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) signalling is well known to be activated by stiff substrates [97]. Only recently, it was demonstrated that YAP signalling also plays a role in macrophage mechanotransduction, as macrophages cultured on stiffer hydrogels showed increased expression and nuclear translocation of YAP [89]. This nuclear translocation of YAP, which requires actin polymerization, has been associated with pro-inflammatory macrophage responses [89,98]. However, further elucidation of YAP/TAZ signalling in macrophage polarization is required, as a role in Wnt5a- and TGFβ1-mediated profibrotic macrophage polarization has also been described [99].

In summary, macrophages are able to detect changes in substrate stiffness, but exact responses of macrophages and the role of mechanotransduction in fibrosis remain unclear. Large differences in variables between the existing studies, such as substrate composition, viscoelasticity, porosity, ECM protein coating, macrophage origin, but also the experimental timeline, are likely to explain the inconsistent effects of stiffness on the macrophage responses observed thus far.

Shear stress and (cyclic) stretch

Pathologies like ventilator-induced lung injury and obstruction-induced fibrosis models, such as the bile duct ligation-induced liver fibrosis model, support the hypothesis that mechanical forces can play an important role in the development or progression of fibrosis [100–103]. However, the presence and magnitude of mechanical forces, such as (cyclic) stretch and shear stress, vary greatly depending on the organ of interest.

Shear stress is mainly caused by fluid flow, especially in the vasculature and ducts such as renal tubules [65]. In the lungs, airflow induces shear stress on the airway wall [104]. Tissue remodelling in fibrosis decreases the compliance of the vasculature, ducts, and airway walls, and can thereby increase shear stress [104]. Another shear stress-generating phenomenon, although very small, is interstitial flow [65]. Interstitial fluid pressure is highly dependent on factors such as blood pressure, cell density, and composition of the ECM, and has been described to increase during inflammation and wound healing [105,106]. It may also play a role in the progression of fibrotic disease as interstitial flow can stimulate myofibroblast differentiation, TGF-β production, and collagen alignment [106]. In macrophages, the application of interstitial flow induced higher expression of anti-inflammatory markers such as arginase-1, TGF-β, and CD206 through β1-integrin signalling compared with static conditions [107]. Furthermore, interstitial flow stimulated the migration speed of macrophages [107,108]. It is therefore not unlikely that the higher than normal interstitial fluid flow found in fibrosis contributes to perpetuation of the fibrotic response by stimulating macrophage infiltration and inducing a profibrotic phenotype.

Whereas shear stress is present in each organ, cyclic stretch or strain mainly plays a role in the heart (pulsatile haemodynamic loading) and lungs (respiration) [109,110]. Fibrosis in these organs results in lower compliance, thereby also reducing the cyclic stretch magnitudes [65]. Additionally, more subtle stretch forces can be generated by contractions of other cells such as myofibroblasts in wound healing and fibrosis [111]. *In vitro*, uniaxial cyclic stretch induced elongation of macrophages in the direction of the applied stretch [112,113]. Although macrophage elongation has previously been associated with an anti-inflammatory phenotype [114], it is not yet clear how cyclic stretch affects macrophage polarization and functional behaviour, as many contradicting results have been reported. Most studies described higher production of pro-inflammatory mediators upon cyclic stretch [112,115–120], while others found lower production [121], higher production of pro-remodelling factors [122] or a combination thereof [123]. Interestingly, TRPV4 was shown to regulate the secretion of pro-inflammatory cytokines by macrophages upon mechanical stretch [120]. Again, experimental variables such as macrophage origin, stretching regime, the way that macrophages were stimulated, and the experimental timeline likely contribute to the inconsistent results and complicate the translation of this information to *in vivo*.

Microstructural changes of the ECM

Abnormalities in the biochemical composition and post-translational modifications of the ECM in fibrosis result in a changed micro- and nano-structure of the tissue, which can be sensed by cells [70,124]. However, the number of studies investigating the interactions of these altered structures with macrophages is limited as the post-translational modifications observed in fibrosis are not well characterized and are difficult to mimic *in vitro*. From our own studies and studies in the field of biomaterials, we know that the surface or scaffold topography can modulate macrophage behaviour, but translation into a fibrotic context is challenging [46,67].

To better understand what topography cells can sense *in situ* in organs, second harmonic generation imaging can be used to analyse the organization and maturity of collagen fibres in tissue. For example, in IPF, thicker and more mature collagen fibres have been observed with this technique compared with control [48] and the effect of fibre diameter on macrophage polarization has been studied *in vitro*. Electrospun fibres with larger diameters (2–6 μm) were shown to induce a more anti-inflammatory macrophage phenotype in comparison to smaller diameters (<1 μm) [125,126]. Furthermore, high collagen density was recently described to induce a more immunosuppressive macrophage phenotype than low collagen density [127]. The high degree of collagen fibre alignment observed in fibrosis could also affect macrophage behaviour, as alignment of electrospun fibres resulted in lower expression levels of pro-inflammatory IL-1β by macrophages compared with randomly organized fibres

[119,128]. In addition to the changes in fibre diameter, density, and alignment, a study by Veres *et al* demonstrated that macrophages recognized mechanically damaged collagen fibres [129]. In fibrosis, higher levels of denatured, unfolded collagen chains have been detected compared with healthy tissues [130,131]. Binding of macrophages to uncoiled, denatured collagen fibres induced macrophage spreading and ruffling, thereby increasing the contact area with the damaged fibrils [129]. In addition, we have previously shown that alveolar macrophages behave differently on non-fibrous collagen type I layers compared with fibrous collagen type I layers [46]: macrophages on fibrous collagen layers transmigrated more, while showing an amoeboid-like phenotype, while non-fibrous collagen layers induced a more mesenchymal phenotype and higher expression of mannose receptor CD206, involved in collagen degradation and known to be upregulated on alveolar macrophages in patients with IPF.

Together, these studies indicate that macrophages are sensitive to remodelling-associated (micro)structural changes of collagen fibres in the ECM, although their subsequent responses and the consequences for fibrosis require further investigation.

Stromal regulation of macrophage–cell interactions in fibrosis

The biochemical and biophysical changes of fibrotic ECM alter the macrophage phenotype via stimulation of multiple mechanisms (summarized in Figure 3), but cells are also an important component of the stroma in addition to the ECM. In the fibrotic microenvironment, (activated) fibroblasts are the predominant cell type, although the activation status and the origins of these fibroblasts lead to different gene profiles and properties, increasing the heterogeneity of fibroblasts found in fibrotic tissues [132]. In addition to fibroblasts, endothelial cells, fibrocytes, and tissue-resident MSCs are found in both healthy and fibrotic stroma. Fibroblast–macrophage interactions in *in vitro* models of skin [133,134], hepatic fibrosis [135,136], and scaffold-free 3D spheroid models [137], as well as computational models [138], have shown (bidirectional) feedback mechanisms between these cell types. While direct cell–cell communications in fibrotic disease are outside the scope of this review, the influence of fibrotic stroma on these interactions now emerges as another key player in fibrosis.

Interplay between stromal cells and macrophages through the stroma could also affect the responses of these cells. In a recent study by Shook *et al*, both macrophages and the local microenvironment were shown to influence the heterogeneity of myofibroblast subpopulations during wound healing [139]. In a fibrocystin knockout mouse model of congenital hepatic fibrosis, depletion of macrophages using clodronate resulted in less accumulation of myofibroblasts and less deposition of collagen compared with non-treated mice [140]. Similarly, in cardiac fibrosis mouse models, macrophages produced secreted protein, acidic and rich in cysteine (SPARC, also

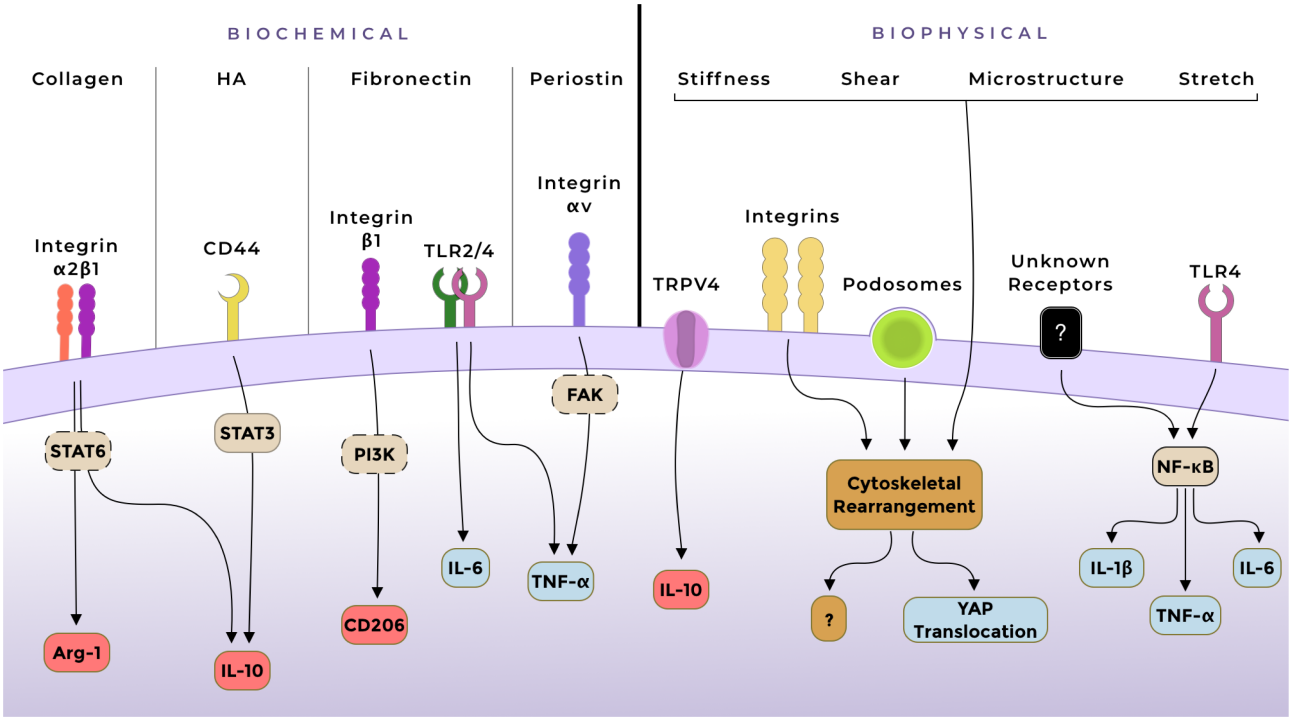

Figure 3. Schematic overview of the most described mechanisms involved in interactions between fibrotic stroma and macrophages. Hypothesised involvement of signalling pathways/molecules is illustrated with dashed frames. Upregulated pro-inflammatory markers are depicted in blue, and profibrotic/anti-inflammatory in red. Arg-1, arginase-1; FAK, focal adhesion kinase; TRPV4, transient receptor potential vanilloid type 4; YAP, Yes-associated protein.

known as osteonectin, an ECM glycoprotein) along with fibroblasts, which in turn promoted collagen production and maturation by fibroblasts [141]. Influences on macrophage behaviour through the modifications on the (alignment of) stroma have also been demonstrated: in a colitis mouse model, a subtype of mesenchymal cells regulated collagen and fibronectin fibre structures, guiding macrophages to profibrotic responses [142]. Also, in prostate cancer, cancer-associated fibroblasts were found to align fibronectin matrix into parallel fibres to guide the migration of cancer cells [143]. In addition, contraction of the ECM by myofibroblasts has been shown to prime latent TGF-β for later activation by proteases and integrin-mediated pathways [144,145]. For example, integrin αvβ8-mediated TGF-β activation by macrophages has been shown to result in perpetuation of the fibrotic response [32,146]. These findings taken together support the interplay between the cell-altered stroma and macrophages. Another demonstration of such interplay was in a study by Ford *et al*, in which mouse macrophages were stationary in dense collagen type I network hydrogels, while co-culturing them with fibroblasts in the same hydrogels increased their motility [147]. Direct and/or indirect communications between macrophages and fibroblasts in this 3D network resulted in improved directionality and increased speed of migration of macrophages, indicating that fibroblasts modified the microenvironment in favour of macrophages. In concordance, fibroblasts applied greater strains to stiffening collagen fibres [148], while a stiffer matrix promoted the profibrotic activation of fibroblasts [22]. Since macrophage behaviour is guided by both biochemical and biophysical cues in stroma, the above-mentioned interplay between cell-modified stroma and macrophages could therefore play an important role in regulating macrophage responses in fibrotic diseases.

The recruitment of monocyte-derived macrophages and their differentiation to profibrotic macrophages could be a key element in the progression of fibrosis. Alignment or modification of ECM proteins by fibroblasts was found to stimulate migration of profibrotic macrophages and therefore to sustain the fibrotic stroma. Mazur *et al* have shown that treating collagen surfaces with fibroblast-activating protein (FAP), a matrix-modifying enzyme, resulted in more attachment and spread of mouse peritoneal macrophages, compared with non-treated collagen surfaces [149]. *In vitro* experiments showed that FAP^{Hi} fibroblasts can have both ECM synthetic and proteolytic phenotypes. In line with this, increased FAP expression stimulated the migration of fibroblasts on collagen type I or fibronectin surfaces. Moreover, *in vivo* data suggest that FAP^{Hi} fibroblasts predominantly reside in fibronectin-rich regions [150,151]. Although the loss of FAP in different mouse models of lung fibrosis showed different results [152], elevated FAP levels were demonstrated in lung [153] and liver [154] fibrosis patients. These findings suggest that FAP-mediated regulation of stroma and therefore putative macrophage recruitment could influence the interactions between the fibrotic stroma and macrophages.

Recent studies indicate a contribution of (recruited) macrophages to the fibrotic response by differentiating into myofibroblasts [macrophage-to-myofibroblast transition (MMT)]. Differentiation of myeloid cells to fibroblast-like cells has been demonstrated in a skin wound healing model in mice [155]. In addition, MMT in a mouse renal fibrosis model was proposed as a mechanism for transitioning from an acute inflammatory phase to an active fibrotic phase. In concert, the majority of transitioned macrophages were profibrotic [156]. Similarly, MMT was also demonstrated in human renal allograft biopsy samples [157] as well as in a mouse cardiac fibrosis model, suggesting the involvement of MMT in many types of fibrosis [158]. Direct contribution of MMT was also indicated in a mouse model of myocardial infarction, in which the authors ruled out the contribution of fibrocytes to the new population of (myo)fibroblasts [158,159]. Furthermore, the lack of fibrocyte-cell surface marker CD34 on the majority of cells in the granulation tissue of wounds in mice also supports a macrophage origin [155]. Lastly, predominant macrophage markers on these new myofibroblasts found in the chronic renal allograft rejection samples support them being of macrophage origin in contrast to fibrocyte origin [157].

Overall, interactions between macrophages and cells in fibrotic stroma favour the recruitment of (profibrotic) macrophages and/or their profibrotic responses, while profibrotic macrophages in turn support proliferation and survival of activated fibroblasts. Although interactions between macrophages and tissue-resident MSCs have been shown in preclinical models [160–163], MSCs affecting macrophages in human fibrosis has yet to be proven. Furthermore, the involvement of MSCs in fibrosis needs deeper understanding. While there are still many unknown factors in the stroma–macrophage–cell interactions in fibrosis, the influence of cell-modified ECM on macrophage responses warrants more attention. Therefore, more studies are required on (1) the influence of stromal cells on the profibrotic response of newly recruited macrophages; (2) how alignment of ECM fibres in stroma could favour pro-regenerative macrophage recruitment; and (3) how the ECM composition affects MMT.

Concluding remarks

Based on *in vitro* studies, macrophages are highly responsive to changes in their microenvironment, regardless of whether these changes are biochemical, biophysical, or indirect via other stromal cells (Figure 4). It is almost inevitable that the altered stroma directly affects macrophage behaviour in fibrosis, but more sophisticated models are required to investigate how macrophages integrate a combination of signals, as biochemical or biophysical changes never come alone in the tissue environment.

The majority of studies that we have discussed were performed in the context of biomaterials and therefore did not

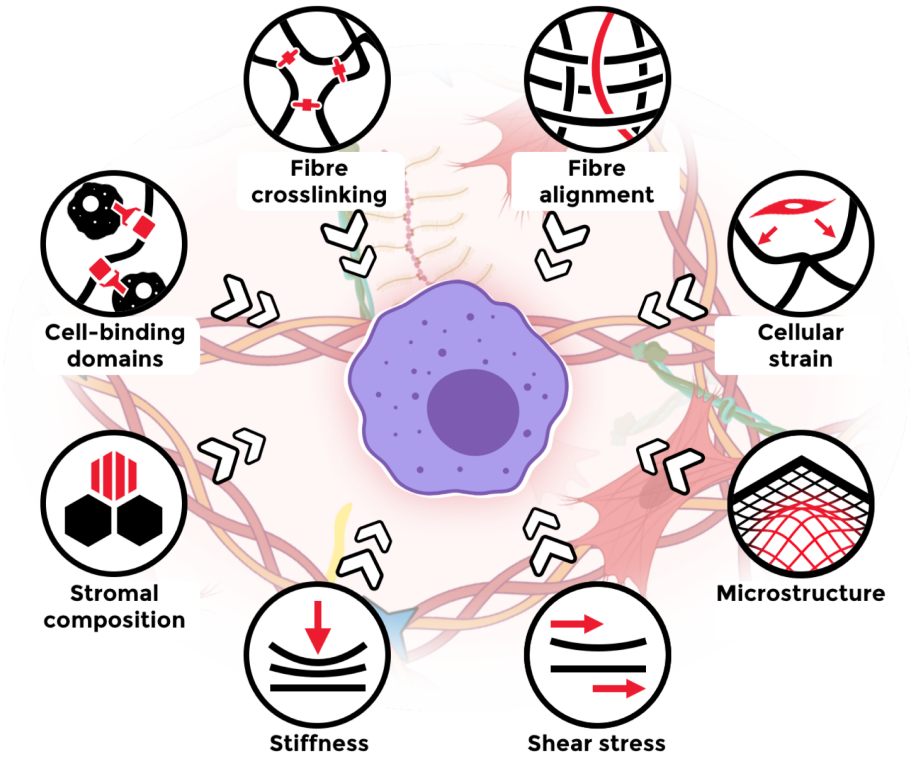

Figure 4. Graphical overview of fibrosis-related biochemical, biophysical, and cell-induced changes in stroma, concurrently influencing macrophage behaviour.

aim to mimic the stroma in a healthy or diseased state. Especially when studying the effect of biophysical properties, the chosen biochemical composition of the substrate may affect the response of macrophages in a synergistic or antagonistic manner. It is likely that the various contradictory results found between studies are a consequence of the impact of these kinds of interferences. It is also important to note that the majority of the discussed studies used 2D culture systems, complicating the translation to the 3D microenvironment *in vivo*. Furthermore, most studies investigated the first 24–48 h of macrophage responses, whereas studying later points in time may be more relevant for understanding the remodelling phase in fibrosis. Thus, although we can learn much from the biomaterials field, models that are more reflective of the organ environment are required to investigate how the fibrotic stroma affects macrophage behaviour.

The high variation in macrophage responses may also be due to different origins of the macrophages studied. Monocyte-derived macrophages and macrophage cell lines are both commonly used to study macrophage behaviour in response to microenvironmental changes, but their responses can vary, especially when compared with primary organ-specific macrophages. Given the evidence that myofibroblasts and macrophages isolated from fibrotic patients behave differently than macrophages from non-fibrotic patients, more studies are needed that investigate whether primary macrophages

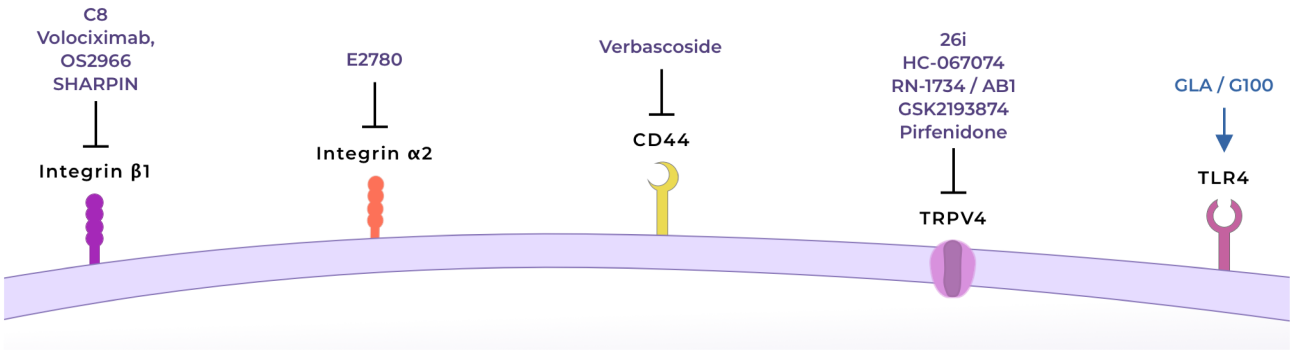

Figure 5. Putative targets and therapeutics to skew profibrotic macrophages to a more pro-inflammatory phenotype. Antagonists/inhibitors for integrin β1 [175–178], integrin α2 [179,180], CD44 [181] or TRPV4 [87,91,182–184]. Agonist for TLR4 [185–187]. TRPV4: transient receptor potential vanilloid type 4.

of fibrotic patients still respond to stimuli comparably to macrophages from non-fibrotic donors [164,165]. Such studies would also help towards understanding the contribution of macrophages to both the development and the progression of fibrosis.

Currently, the number of available antifibrotic therapeutics is limited. In 2014, nintedanib and pirfenidone were first approved by the FDA for the treatment of pulmonary fibrosis and their effectiveness in slowing down fibrosis in other organs is currently under investigation [166–169]. Interestingly, both pirfenidone and nintedanib have been shown to change macrophage behaviour and to interfere with collagen type I fibril formation [170–172]. Therapeutic strategies that specifically skew profibrotic macrophages towards a (slightly more) pro-inflammatory phenotype may present as an alternative or addition to existing treatment regimens, as recent studies have shown in mouse models of fibrosis [173,174]. Stimulating or inhibiting the pathways by which the fibrotic stroma affects macrophage behaviour (Figure 3), such as mechanosensing, may enable us to interfere with the fibrotic interplay between the stroma and macrophages (Figure 5). However, as the described pathways are not specific for macrophages, unexpected systemic effects need to be taken into account or circumvented by specifically targeting macrophages in the fibrotic area. Nonetheless, a better understanding of macrophage–stroma interactions and their role in the development and progression of fibrotic disease is required to specifically target macrophage function for the treatment of tissue fibrosis.

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Author contributions statement

GFV, MN, JKB and BNM conceived the concept and all the authors designed the manuscript. GFV and MN performed the literature search and drafted the manuscript and the figures. MN designed the figures. All the authors critically revised the manuscript and agreed with the final version.

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