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Immune interactions in hepatic fibrosis. or “Leucocyte-stromal interactions in hepatic fibrosis”

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Synopsis

Liver cirrhosis is caused by iterative cycles of tissue injury, inflammation and repair. Although most causes of acute hepatitis resolve without scarring, chronic hepatitis is associated with persistent inflammation and matrix remodelling which leads to fibrosis and eventually cirrhosis. The mechanisms that govern wound healing are complex and involve interactions between both the innate and adaptive immune systems with stromal cells within a microenvironment comprised of cytokines, growth factors and modified matricellular proteins. As our understanding of the processes that govern inflammation and fibrosis improves it has become clear that the immune system plays a central role in the regulation of fibrosis, tissue repair and recovery that are vital for the maintenance of tissue homeostasis. Chronic inflammation and fibrosis are inextricably linked and the cellular interactions between immune effector cells, local fibroblasts and tissue macrophages at sites of scar formation determine the outcome of liver injury and the development of scarring.

Keywords

Liver; Inflammation; Stroma; Lymphocytes; Fibrosis; Hepatic Stellate Cell

Introduction

Far from being a static process chronic inflammation is a dynamic aggregate of lymphocytes, macrophages and stromal cells held together by autocrine and paracrine interactions. Consequently the role of leukocytes in wound healing must be considered within the context of these organ-specific stromal microenvironments. This is particularly the case in the liver where interactions between stromal cells and infiltrating leukocytes are critical in determining the outcome of liver injury as demonstrated by the observations that animals deficient in macrophages, T-cells or B-cells all show reduced fibrotic responses. The ability of leukocyte-derived cytokines to modulate the behaviour of liver fibroblasts is widely accepted but it has

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become clear that fibroblasts also contribute to the processes of leukocyte recruitment, positioning and survival at sites of chronic inflammation¹. Neutrophil recruitment in the immediate/early response to liver injury is preceded by rapid upregulation of adhesion molecules on stellate cells within the sub-endothelial space whilst in animals recovering from liver fibrosis resolution of liver inflammation is prefaced by apoptosis of activated liver myofibroblasts^{2,3}. Thus the induction, maintenance and resolution of liver inflammation and fibrosis is determined by interactions between infiltrating leucocytes and activated fibroblasts within the liver stroma^{4,5}. This review will discuss the immunological mechanisms that drive liver fibrosis and in particular the complex interactions that exist between cellular components of the stroma (macrophages and fibroblasts particularly) and the innate and adaptive immune systems.

Stromal microenvironments in chronic inflammatory disease

A characteristic feature of chronic liver disease is the presence of a persistent inflammatory infiltrate associated with regions of fibrosis and matrix remodelling. Whilst macrophages play an important role in the initiation and resolution of fibrosis fibroblasts are the most numerous cells within the liver stroma and are primarily responsible for the synthesis of the extracellular matrix⁶. Fibroblasts provide an architectural framework for tissues and regulate important homeostatic and developmental functions⁷. Until recently resident stromal cells were not thought to make significant contributions to immune responses within the liver, but it has become clear that fibroblasts and macrophages within the liver stroma play critical roles in regulating the inflammatory as well as the fibrotic reaction at sites of tissue injury^{1,8,9}. Disruption of these processes results in abnormal wound healing, scarring and perpetuation of inflammation¹⁰. This is particularly the case in human liver disease where reiterative cycles of tissue injury perpetuate stromal cell activation and provoke a switch from acute reversible inflammation to chronic inflammatory disease.

Monocytes demonstrate considerable plasticity and can differentiate into macrophages, dendritic cells (DC) and stromal cell lineages in response to microenvironmental activation signals^{11,12}. In humans CD16⁺ monocytes expressing high levels of the fractalkine receptor CX₃CR1 are recruited to tissues where they undergo further site specific differentiation into resident populations of cells such as Kupffer cells and interstitial DCs¹³ whereas CD16^{low} cells are recruited during inflammation (Figure 1). Most 'inflammatory' monocytes differentiate into macrophages in tissue and mediate clearance of pathogens and the resolution of inflammation, but some CCR7⁺ cells can emigrate from tissues via lymphatics to the draining lymph nodes where they acquire DC function. In the absence of inflammation, CD16⁺CX₃CR1^{high} monocytes enter the liver to replenish kupffer cells and DC populations¹². In addition a population of adherent CD14⁺ monocytes can be induced to differentiate along fibroblast lineages when cultured with T cells. The resulting cells known as fibrocytes, express both haematopoietic and mesenchymal markers (collagen-1+, CD11a/b, CD45+, CD34+). When recruited to inflamed tissues and exposed to cytokines such as TGFβ1^{11,14} they can differentiate into αSMA⁺ collagen-secreting contractile cells with characteristics of myofibroblasts. Fibrocytes are implicated in wound healing and inflammation in a variety of diseases including chronic liver disease^{15,16} and exemplify the cellular plasticity and divergence exhibited within the stroma.

Transcriptional profiling of human fibroblasts from different anatomical sites has demonstrated patterns of gene expression as divergent as those seen amongst the different leukocyte lineages^{17,18} and this biological diversity is reflected in differential patterns of growth factor and matrix expression¹⁹. The liver contains several phenotypically distinct fibroblasts^{20,21} which may be derived from distinct progenitor cells²². The intrinsic variation in phenotype and function of macrophages and fibroblasts within tissues may be an important factor that

predisposes tissues to scarring, and could explain why relapses in chronic inflammatory disease are often tissue and site-specific.

Macrophages regulate the induction and resolution of liver fibrosis

Many of the cells that play an important role in the immediate innate immune response, including neutrophils and mast cells, make little contribution to tissue fibrosis and NK-cells actually suppress fibrosis by killing activated myofibroblasts possibly by TRAIL dependent mechanisms^{23–25}. This may explain why many acute inflammatory reactions in the liver resolve without scarring^{26,27}. On the other hand macrophages are critical for fibrosis as demonstrated by depletion studies in animals which implicate hepatic macrophages in both the progression and resolution of fibrosis. Activation of fibroblasts by macrophage-derived TGF β 1 or insulin-like growth factor is an early feature of fibrogenesis which promotes a switch in fibroblast gene expression to initiate matrix remodelling²⁸ and macrophage-derived cytokines including IL-6 and TGF β maintain the proliferation and differentiation of liver fibroblasts^{29,30}.

The divergent roles played by macrophages within the liver are a consequence of distinct pathways of activation and differentiation determined by local cytokines and interactions with other liver cells³¹. Pathogen-associated molecular patterns (PAMP-s) are conserved sequences in microbes that stimulate TLRs and other pattern recognition receptors on macrophages and thereby activate innate immune responses. Classical activation resulting in so-called M1 macrophages is driven by IFN- γ and characterised by a signature of proinflammatory genes associated with Th1 lymphocyte responses and cell-mediated immunity. An alternative activation profile results in M2 macrophages associated with a Th2-type polarised response characterised by STAT-6 signalling, IL-4 and IL-13 secretion and upregulation of endocytic lectin receptors including the mannose receptor. Alternative activation is characteristic of parasitic infections, allergy, humoral immunity and fibrosis. The deactivation of macrophages is also important to promote resolution and this can be triggered by exposure to apoptotic cells, IL-10 and other regulatory mediators and glucocorticoids. Although there is a clear distinction in vitro between pro-inflammatory matri-lytic M1 and profibrotic M2 macrophages³² the situation in-vivo is more complex with the presence of both classical and alternatively differentiated macrophages in chronically inflamed liver tissue³³.

The importance of macrophages in liver fibrosis has been demonstrated by animal studies where macrophages were selectively depleted at different stages of fibrosis. Macrophage depletion at the induction of fibrosis, either by inhibiting CCL2-dependent recruitment or selective depletion of tissue macrophage populations with gadolinium chloride or diphtheria toxin, reduced scarring and resulted in fewer activated (α SMA⁺) HSC^{4,34,35}. By contrast depleting macrophages during the immediate/early phase of tissue recovery delayed matrix degradation and prolonged fibrosis. These divergent roles suggest that either different populations of macrophages are involved at each stage or more likely that macrophage function switches during fibrogenesis in response to cytokines in the microenvironment.

Whether macrophages revert to a quiescent phenotype following tissue recovery or undergo unidirectional activation followed by apoptotic death is unknown. The IkappaB kinase (IKK) complex which controls the activation of NF-kappaB transcription factors plays a pivotal role in switching off macrophage activation and the resolution of inflammation^{36,37} and in vitro macrophage deactivation can be induced by a variety of signals including IL-10 and TGF β , or ligation of the inhibitory receptors CD172 and CD200 which lead to anti-inflammatory cytokine production and a reduction in MHC class II expression¹².

Cross-talk between macrophages and liver fibroblasts leads to changes in function and in the patterns of cytokines secreted by both cell types^{38,39}. Survival of activated HSC in the fibrotic

liver is dependent on expression of TIMPs (which prevent degradation of matrix proteins) and survival signals from accumulated fibrillar Type 1 and 3 collagens. The failure to degrade collagen-1 critically impairs HSC apoptosis thereby perpetuating fibrosis⁴⁰. Recovery from liver fibrosis is associated with the secretion of macrophage-derived proteases that degrade cross-linked fibrillar collagens allowing other more promiscuous MMPs to accelerate matrilysis and thereby to remove the survival signals on which activated HSC depend^{4,41}. In addition macrophages may actively promote apoptosis of HSC by expressing TRAIL and other apoptotic stimuli that accelerate clearance of myofibroblasts at sites of scar formation⁴².

Thus the resident populations of macrophages within the liver direct different stromal responses to injury at different times but whether this reflects functionally distinct populations of cells derived from distinct blood monocyte precursors or is the result of a local intrahepatic switch in function is unknown.

Stromal cells modulate the differentiation and function of antigen presenting cells

We have previously reported that non-inflamed liver contains a subset of myeloid DCs which preferentially secrete IL-10 and induce IL-10 secretion in responding T cells^{43,44}. These DCs may play an important role in suppressing damaging immune responses in non-inflammatory states. In normal human liver most DCs are CD16⁺ and express high levels of CX3CL1. CD16⁺ circulating monocytes can differentiate into DCs during the process of transendothelial migration⁴⁵ and this process may be enhanced by interactions with fibroblasts⁴⁶. The interaction between stroma and leucocytes in human tissues has received relatively little attention although there is accumulating evidence to suggest that HSC and extracellular matrix provide differentiation signals to monocytes during recruitment into the liver. Human spleen-derived myofibroblasts express IL-15 that drives CD34⁺ blood cells to differentiate into activated natural killer (NK) cells and myeloid DCs after 3–4 weeks in culture demonstrating how stromal cells in tissues can shape the innate immune system⁴⁷. Such interactions will also determine the nature of the DCs generated. Murine splenic DCs can be generated from haematopoietic precursors by culture on splenic stromal cells⁴⁶ and these DCs have regulatory properties similar to those seen in human liver^{44,48}. Maturation was dependent on fibronectin-mediated adhesion and CXCL12 secreted by the fibroblasts. The DCs generated on the stromal cells mediated suppression of T cell proliferation in response to antigen presentation by mature DCs. These two studies show how different fibroblast-derived cytokines, IL-15 in the former study and CXCL12 in the latter may drive different differentiation pathways of interacting DCs and thereby shape the subsequent immune response.

Similar processes have been described in the skin where interactions between blood monocytes and human dermal fibroblasts mediated by β 2 integrins, ICAM-1 and Thy-1 (CD90) induce the maturation of DC with potent T cell activating capabilities⁴⁹. The importance of the local microenvironment is emphasised by our finding that culturing human monocyte-derived DCs in liver-conditioned media induced a regulatory phenotype similar to that seen in DCs isolated directly from the liver whereas culturing in skin-conditioned media had no such effect⁴⁴. IL-10 is the critical cytokine mediating this effect as defective hepatic DC function could be restored by inhibiting IL-10 but the precise role played by HSC in modulating these responses has not been determined⁵⁰. Thus the outcome of interactions between DC precursors and fibroblasts are likely to be tissue-specific responses modulated by local inflammatory signals that regulate DC differentiation and function.

Recent studies have demonstrated that HSC can themselves act as professional antigen-presenting cells (APC) by efficiently presenting antigens to MHC-I and MHC-II-restricted T cells and lipid antigens to CD1-restricted natural killer (NK) T cells. HSC share many

phenotypic similarities with follicular dendritic cells, a specialised antigen presenting cell located in secondary lymphoid tissue⁵¹. Unlike other resident liver APCs, notably sinusoidal endothelium and hepatocytes, the outcome of presentation by HSC appears to be full effector responses rather than tolerance. Thus HSC not only modulate the differentiation of other APC but can also directly stimulate immune responses. As immune responses develop and HSC differentiate into activated myofibroblasts their role may shift from direct antigen presentation to indirect effects on monocyte differentiation resulting in complex effects on intrahepatic immune responses⁵². Gene array studies have shown that as HSC mature they switch gene transcription from a fibrotic to a pro-inflammatory phenotype providing evidence that senescent HSC continue to modulate local inflammatory responses long after they have stopped remodelling the ECM in response to liver injury⁵³.

HSC activation in the liver promotes chronic inflammation

Liver myofibroblasts contribute to leukocyte recruitment to the injured liver by secreting cytokines, chemokines and other chemoattractants such as platelet activating factor and complement proteins^{54–56}. In most circumstances inflammation resolves once the initiating stimulus has been removed but in some conditions inflammation persists resulting in chronic hepatitis and fibrogenesis⁵⁷. The switch from acute resolving to chronic persistent hepatitis is associated with the development of a specialised microenvironmental niche that maintains leukocytes, particularly lymphocytes in close proximity with scar-associated fibroblasts at sites of tissue injury⁵. Chronic inflammation in other tissues including the kidney, lung and eye is also associated with the presence of closely associated lymphocytes and activated fibroblasts indicating that these links are a generic feature of fibrotic diseases^{1,58–60}.

To date the role of lymphocyte-fibroblast interactions in driving chronic inflammation has been most closely studied in rheumatoid arthritis but it is likely that general mechanisms defined in the joint will apply to chronic inflammation in the liver. Fibroblasts in rheumatoid joints shape the inflammatory infiltrate in several ways⁶¹. Fibroblast-derived chemokines can be presented on the endothelium of vessels in the joint thereby contributing to inflammatory cell recruitment from blood. In addition the local secretion of CXCL12 activates integrin-mediated adhesion of lymphocytes to fibroblast VCAM-1 thereby retaining lymphocytes in the joint^{62,63}. Other cytokines secreted by fibroblasts, particularly type-1 interferons provide survival signals for activated T cells and prevent them dying by apoptosis⁶⁴. Thus fibroblasts contribute to the persistence of chronic joint inflammation by promoting lymphocyte recruitment from blood, retaining infiltrating lymphocytes and by providing them with survival signals that prevent death by apoptosis, a critical component of immune resolution¹.

The interactions between fibroblasts and lymphocytes are bidirectional and T cell-derived cytokines are potent activators of fibroblasts in many chronic inflammatory diseases^{65–67}. The outcome of fibroblast activation will be determined by the activation of both disease-specific and tissue-specific signalling pathways and in humans polymorphisms in regulatory genes can result in major differences in response to particular stimuli⁶⁸. Thus LPS-mediated NFκB activation and CXCL8 secretion by liver myofibroblasts vary between subjects with liver disease^{69,70}. Failure to regulate activation of NFκB leads to sustained inflammation and a tendency towards a Th1 polarised immune response^{36,37} which may in part be driven by uncontrolled fibroblast activation. Thus mice with a selective deletion of RelB (a transcriptional regulator that stabilises the NFκB inhibitor IκB) in fibroblasts develop an overwhelming systemic inflammatory response to endotoxin which is characterised by persistent chemokine induction and leukocyte recruitment. Transfecting the wild type gene back into these animals attenuates inflammation by restoring Rel-B mediated stromal regulation⁷¹. More recently it has been shown that mice lacking NFκB1 (p50) treated with repeated injections of carbon tetrachloride develop a more severe inflammatory and fibrotic

reaction than wild type controls. This is associated with increased stellate cell expression of TNF- α in areas of inflammation, suggesting that the p50 subunit of NF- κ B may protect the injured liver by limiting TNF- α dependent inflammatory cell recruitment⁷².

Thus iterative cycles of fibroblast activation at sites of chronic injury result in the development of a stromal phenotype that perpetuates inflammation and leukocyte recruitment resulting in dysregulation of the normal processes of tissue repair¹⁰.

HSC activation is associated with leukocyte recruitment

Quiescent HSC are specialised pericytes found immediately adjacent to the sinusoids in the space of Disse. They maintain endothelial integrity by secreting angiogenic peptides secreted in response to bi-directional interactions with the overlying endothelium⁷³. The expression of markers associated with stem cells such as CD133, Thy-1 (CD90), nanog and OCT-4 and their ability to trans-differentiate into hepatocytes and endothelial-like cells suggest that HSC should also be regarded as progenitor cells^{73,74}. Quiescent HSC undergo a phenotypic switch in response to liver injury, becoming spindle-shaped and secreting pro-inflammatory cytokines and upregulating programmes of genes that result in excess deposition of fibrillar collagens^{6,75}. Whilst there is increasing evidence that human liver fibrosis is the product of both intra and extra-hepatic populations of fibroblasts, activated hepatic HSC are still regarded as the principle collagen producing cells in the fibrotic liver⁷⁶.

In liver fibrosis HSC-like cells are seen immediately adjacent to fibro-vascular membranes at the margins of fibrotic septa associated with the inflammatory infiltrate⁷⁷⁻⁷⁹. Thus while paracrine interactions with quiescent HSC maintain the overlying endothelium HSC activation results in fibrogenesis associated with an inflammatory infiltrate. HSC activation is precipitated in a variety of ways; reactive oxygen intermediates released from injured cells activate quiescent HSC whilst activation signals are generated within the stroma as latent TGF β 1 is activated via plasmin released by injured endothelial cells^{6,80}. Perpetuation of HSC activation is associated with increased sensitivity to local cytokine signals (particularly PDGF) and the effects of remodelling the existing ECM⁸¹. These enhanced cytokine responses typify activated HSC and are principally mediated by upregulation of receptor tyrosine kinases (RTKs)^{80,82}.

Migration and positioning of T-cells is determined by patterns of cytokine and chemokine secretion within the stroma

The liver responds to injury by recruiting leukocytes from the circulation, expanding populations of scar-forming fibroblasts and positioning the inflammatory infiltrate within the modified neo-matrix. An effective hepatic immune response requires that leukocytes are recruited to the liver and then appropriately positioned at sites of injury⁵⁷. Leukocyte adhesion to endothelial cells is a prerequisite for recruitment into tissue and is regulated by a sequence of interactions in which the leukocyte is captured from the flowing blood by carbohydrate-dependent mechanisms; activated by chemokines presented on the endothelial glycocalyx resulting in integrin-activation and arrest on the vessel wall allowing the cell to then migrate through endothelium into tissue by as yet poorly understood mechanisms. To trigger leukocyte recruitment chemokines must be presented at the endothelium where they can be delivered by transcytosis from underlying cells and then presented bound to proteoglycans in the endothelial glycocalyx⁸³. Thus the chemokines present on sinusoidal endothelium during liver inflammation may be derived from paracrine secretion by kupffer cells, other infiltrating leukocytes, hepatocytes and activated HSC. The situation of the latter immediately beneath the endothelium in close association with endothelium places them in an ideal site to “post” chemokines onto the overlying endothelium.

As a result of the complex microvascular anatomy of the liver, leukocytes can enter the liver via endothelial cells at three anatomical sites, in the portal tract, the sinusoids or the terminal hepatic veins⁸⁴. Although some branches of the hepatic artery and portal veins terminate in post-capillary venules, most blood is diverted to the liver parenchyma via the sinusoids before returning to the systemic circulation via hepatic veins and the sinusoidal bed provides the major route of entry into the parenchyma. Recent studies show that recirculating lymphocytes enter the liver via sinusoids and, if not retained, then migrate via the space of Disse to portal tracts before moving on to draining lymph nodes. Similarly dendritic cells, which enter via the sinusoids as monocyte precursors, take the same route via portal tracts to draining lymph nodes after activation⁸⁵. The lumen of the sinusoids is narrow and blood flow is regulated in part by stellate cells acting as pericytes which facilitates the attachment of flowing leukocytes^{86–88}. Leukocytes interact with sinusoids in a modified version of the adhesion cascade in which the capture phase is attenuated and does not involve selectins which capture leukocytes in other tissues^{89,90}. A different adhesion molecule, vascular adhesion protein 1 (VAP-1) which is constitutively expressed on hepatic endothelium mediates capture and transmigration of leukocytes on hepatic endothelium^{91,92}. Once captured and firmly adherent a lymphocyte must breach the endothelial barrier to enter tissue and under physiological shear stress VAP-1 and ICAM-1 promote lymphocyte transendothelial migration through sinusoidal endothelium⁹². Selection at the endothelial level is only one aspect of the processes that govern the development of an inflammatory infiltrate cells and retention of leukocytes within tissue is equally important although much less studied. Based on studies in other organs, particularly the thymus and bone marrow it is very likely that stromal cells will be integral in this process^{10,93}.

HSC accumulate at sites of tissue injury in response to PDGF and CCL2⁹⁴ and once activated secrete proinflammatory cytokines and chemokines that upregulate adhesion molecules such as ICAM-1, VCAM-1 and CX₃CR1 which facilitate interactions with local leukocytes^{3,5, 49,95}. HSC can modulate leukocyte recruitment in at least three ways 1) by paracrine interactions which prime overlying sinusoidal endothelial cells to recruit leukocytes 2) by secreting matrix which provides a substrate through which infiltrating leukocytes can migrate or be retained 3) by direct interactions with leukocytes to modulate their migratory behaviour or retain them at sites of injury.

Leukocytes entering the sub-endothelial space interact with stromal cells and extracellular matrix via cell surface integrins which not only provide anchorage but also mediate ‘outside-in’ signals that direct cellular activation and differentiation¹⁰. Injury to the liver stimulates vigorous infiltration of leukocytes as a result of cytokines and chemokines displayed on the endothelium and within the subendothelial stroma⁹⁶. Once in the sub-endothelial space lymphocytes undergo directed migration within the stroma by responding to signals displayed within the ECM. The distribution and spatial organisation of the hepatic infiltrate varies according to the inflammatory stimulus and reflects the expression of chemokines and adhesion molecules within the tissue⁵⁷. Thus in inflamed liver adhesion molecules and chemokines are increased and in the sinusoids both endothelial cells and activated HSC secrete the potent lymphocyte recruiting chemokines CXCL9 and CXCL10 in response to TNF α and IFN γ ⁹⁷.

Simply recruiting inflammatory cells is not enough to maintain an effective immune response because chronic inflammation requires that effector cells are retained in tissues. Adhesion molecules and chemokines including CXCL16 and Fractalkine (CX₃CR1) are expressed by target epithelial cells and stromal cells at sites of injury where they localise infiltrating T cells^{98–101}. Thus stromal expression of chemokines determines the position and shape of the resulting inflammatory infiltrate, and serves to localise leukocytes to areas of fibrosis.

Activated HSC modulate recruitment and positioning of leukocytes within the liver

By being situated between the endothelium and parenchyma HSC can participate in the recruitment of leukocytes into the liver. The first evidence that activated liver myofibroblasts contributed to liver inflammation came from the observation that CCL2 in HSC conditioned media promoted vigorous monocyte chemotaxis⁵⁴. CCL2 also recruits memory T cells, basophils and DCs suggesting it may be an important recruitment signal involved in chronic inflammation^{102,103}. Studies in rat models of liver fibrosis revealed that nearly all of the CCL2 generated came from activated HSC¹⁰⁴. Moreover activated HSC isolated from fibrotic human livers continue to secrete of very high concentrations of CCL2 in-vitro even in the absence of pro-inflammatory cytokines. Activated myofibroblasts secrete a wide variety of other chemokines, cytokines and matricellular proteins that regulate recruitment and subsequent differentiation of neutrophils, macrophages and lymphocytes (Figure 2). Our own unpublished data show that human hepatic myofibroblasts secrete not only CXCL8 and CCL2 but also CCL5, CCL11 and CCL3 and in response to $\text{INF}\gamma$ and $\text{TNF}\alpha$ large amounts of CXCL9 and CXCL10 (A. Holt et al submitted). These factors in cell supernatants from liver fibroblasts exert a powerful chemotactic effect on human lymphocytes in real time chemotaxis studies (Figure 3) increasing both the rate and magnitude of lymphocyte migration and demonstrating the potential of liver fibroblasts to direct lymphocyte migration within the stroma.

The importance of lymphocytes in fibrosis is illustrated by the fact that animals with targeted disruptions to chemokines that promote lymphocyte recruitment show reduced fibrotic responses to experimentally induced liver injury, whereas inhibition of neutrophil chemotaxis has no effect on fibrosis^{27,35}. Cytokine therapy with IL-10 antagonises pro-inflammatory chemokines and cytokines secreted by activated liver fibroblasts and reduces fibrosis in humans with chronic viral hepatitis and animals with experimentally induced hepatitis^{105,106}. Fibroblasts secrete other cytokines that can promote lymphocyte migration in addition to chemokines. IL-6, HGF and $\text{TGF}\beta$, all of which are secreted by αSMA + liver myofibroblasts¹⁰⁷ have lymphocyte chemotactic activity and could contribute to the recruitment and positioning of lymphocytes within the stroma^{108–111}.

Chronic inflammation is associated with the accumulation of T cell, B cells, and DCs in ectopic or tertiary lymphoid structures that resemble lymph nodes¹¹². Their development is a consequence of aberrant and sustained expression of cytokines that drive lymph node development including lymphotoxin and homeostatic chemokines. CCL21 is an important chemokine in lymph node development and function because it is critical for the recruitment of naïve T cells and dendritic cells to lymph nodes¹¹³. CCL21 is secreted by liver fibroblasts including those found associated with tertiary lymphoid structures in chronic inflammatory diseases including PBC, PSC and chronic hepatitis C^{99,114,115}. These findings suggest that activated fibroblasts may be responsible for the development and persistence of lymphoid follicles providing another mechanism by which they can modulate the recruitment of lymphocytes and determine the nature of chronic hepatic inflammation.

Thus activated HSC and liver fibroblasts at sites of tissue injury contribute to leukocyte recruitment by secreting chemokines and non-chemokine chemotactic factors that drive Gi-associated and Gi-independent mechanisms respectively. These operate throughout the inflammatory response, playing a role in early leukocyte recruitment and then in the remodelling of the stromal microenvironment to promote the recruitment and survival of T cells, B cells and dendritic cells as part of the chronic inflammation that accompanies fibrogenesis. Because fibroblasts are not all the same, site specific differences between stromal cells may play an important role in determining distinct patterns of inflammation seen in particular diseases or tissues^{17,19}.

Direct interactions with activated liver fibroblasts retain lymphocytes in tissue and prevent resolution of inflammation

The close temporal and spatial association between infiltrating lymphocytes and fibrosis has been recognised for many years, but the cellular interactions are poorly understood. In other tissues T-cells and B-cells rely on cellular elements within the stroma for structural anchorage and directed migration. Thus within secondary lymph nodes fibroblastic reticular cells provide the adhesive scaffold that facilitates lymphocyte migration within the lymph node and which maintains the zonal separation of T cells and B-cells¹¹⁶. These stromal cells bring together T-cells and dendritic cells by secreting chemokines that attract both cell types within the T zone in the medulla^{117,118}. Similarly stromal cells play a critical role in recruiting and positioning thymocytes within the thymus during lymphocyte development. It is very likely that liver fibroblasts play a similar role in directing lymphocyte recruitment to the inflamed liver. In human liver disease infiltrating lymphocytes are closely associated with myofibroblasts at the margins of fibrotic septa and with α SMA⁺ myofibroblasts within portal tracts. In mice with experimentally induced liver fibrosis lymphocytes are only found in the proximity of activated HSC/aLMF suggesting that there is a direct interaction between liver myofibroblasts and infiltrating lymphocytes⁵. Similarly, NK cells are found attached to α SMA⁺ HSC following fibrosis induction along the interface zone of fibrotic septa¹¹⁹ suggesting that paracrine interactions between fibroblasts and lymphocytes at sites of tissue damage facilitate the matricellular changes associated with fibrosis and chronic inflammation.

Studies in animals indicate that the release of CCL2 and expression of ICAM-1 by stellate cells immediately precedes leukocyte infiltration in the early phase of liver injury, indicating that HSC activation promotes the recruitment of leukocytes into the parenchyma¹²⁰. Part of this response is mediated by the secretion of cytokines and chemokines that promote recruitment through the overlying endothelium but recent evidence suggests that fibroblasts also modulate lymphocyte migration by direct contacts with the infiltrating cells. We have shown that if lymphocytes are placed in contact with myofibroblasts isolated from diseased human liver they show increased motility and migrate rapidly over and through the fibroblast monolayer in a process termed pseudoemperopolesis. Treatment of the fibroblasts with pro-inflammatory cytokines increases the kinetics of stromal transmigration which is dependent on increased expression of ICAM-1 and VCAM-1⁹⁵. Murine studies have demonstrated that HSC support lymphocyte adhesion in an ICAM-1 dependent manner and human HSC also express high levels of CD90 which supports adhesive interactions with dendritic cells^{49,95,121}. Thus in response to tissue injury liver myofibroblasts provide an adhesive template that facilitates stromal migration and approximates lymphocytes and fibrotic HSC-like cells in regions of matrix remodelling thus perpetuating the inflammatory reaction by promoting leukocyte recruitment at sites of tissue damage.

The development of a stable chronic inflammatory infiltrate depends upon the recruitment of leukocytes from blood and their subsequent survival at the site of inflammation outweighing the mechanisms of resolution which include local death of inflammatory cells by apoptosis and emigration out of the inflammatory site by lymphatics¹. Stromal cells modulate all of these processes including the survival of the inflammatory infiltrate as interactions with synovial fibroblasts induce expression of anti-apoptotic genes in T cells that prevent apoptosis. Fibroblasts can also keep neutrophils alive particularly by secreting cytokines including GM-CSF as well as type-1 interferons. These survival signals are amplified by adhesive interactions between neutrophil α 9 β 1 integrin and VCAM-1 on stromal cells^{63,122–124}. The cycles of tissue injury and repair associated with chronic liver disease leads to local secretion of cytokines including IL-6, IL-12 and TNF α by activated HSC, all of which can promote antigen-independent bystander T cell activation. Thus the cytokine milieu in the liver of patients with chronic hepatitis is shaped by activated HSC and favours not only lymphocyte survival but

also bystander activation of T cells - promoting effector T-cell function even in the absence of the original stimulating antigen^{125,126}. This amplifies antigen-specific responses within the tissue, but also increases the resistance of responder cells to apoptotic stimuli¹²⁷. As a result infiltrating T cells become increasingly resistant to apoptosis whilst bystander activation increases their effector function thereby exacerbating tissue damage and fibrosis¹²⁸.

Stromal interactions with infiltrating lymphocytes shape fibrogenesis in chronic hepatitis

Whilst interactions between macrophages and fibroblasts are critical for fibrosis induction and resolution¹²⁹ the role of the adaptive immune system is less well defined (Figure 4). Nevertheless there is good evidence that T-cells and B-cells are implicated in sustaining fibrotic responses in chronic liver disease¹³⁰. In animal models and diseased human liver lymphocytes are seen in close association with fibroblasts in inflamed portal tracts and fibrous septa suggesting the involvement of the adaptive immune response in fibrogenesis^{5,96}. Functional support for this hypothesis comes from animal studies in which depletion of T-cells and B-cells protects animals from liver fibrosis^{105,131}. Moreover mice deficient in both B-cells and T-cells (RAG2^{-/-}) are resistant to fibrosis in acute and chronic models of liver injury¹³¹. The specific role of particular lymphocyte subsets during fibrogenesis is less clear.

The contribution of intra-hepatic T-cell to fibrogenesis is accepted and adoptive transfer studies in a variety of murine models confirm the involvement of both CD4+ and CD8+ T-cells. CD4+ T-cells were originally identified as the source of a secreted 'fibrotic factor' in a T_H2-polarised mouse model of liver fibrosis which was subsequently shown to be IL-13^{132,133}. The polarity of the inflammatory response is critical for fibrogenesis, and different programmes of gene expression are induced when chronic inflammatory responses are dominated by TH1 or TH2 cytokines. Although TH1 cytokines characteristically generate an intense cellular response they cause little fibrosis¹³⁴, whereas TH2 cytokines increase transcription of several genes involved in fibrogenesis including pro-collagen I and III, MMP2, MMP9 and TIMPs³⁰, all of which are expressed in CCl₄ liver injury suggesting that mechanisms of fibrosis may share a common cytokine profile. The importance of T_H2 polarised T-cell subsets is confirmed by studies demonstrating that IL-13R α 2 blocking antibodies reduce liver fibrosis¹³² and inhibition of TH2 responses using targeted mutations of IL-4, STAT6, IL-4R α attenuate fibrosis in a mouse model of scleroderma³². Other studies have demonstrated that transfer of CD8+ (but not CD4+) T-cells from mice with CCl₄ induced liver fibrosis provokes significant liver injury in SCID-mice recipients¹⁰⁵. Conversely fibrosis is attenuated in transgenic mice that over-express IL-10 in hepatocytes and in animals depleted of T-lymphocytes. Although CD4+ T-cell and CD8+ make important contributions to fibrogenesis, their precise role is unclear. CD4 T-cells can modulate the local cytokine response to favour fibrogenesis via direct effects on stromal macrophages and fibroblasts and CD8 T-cells may promote fibrosis by amplifying tissue injury as part of a bystander response driven by the local cytokine milieu.

The role of B-lymphocytes in hepatic fibrosis is poorly understood although their ability to activate T-cells at low antigen loads and to secrete cytokines suggests they could be involved in shaping and maintaining the inflammatory response. Regulatory B-cells can direct the polarity of the local inflammatory response away from a Th1-like phenotype in autoimmunity and may contribute to local production of TGF β ₁^{135,136}. A recent study demonstrated an antibody-independent role for B-cells in liver fibrosis induced by two different agents CCl₄ or α -Naphthylisothiocyanate (ANIT). Surprisingly this study was unable to show any protective effect in CD4, CD8 or $\gamma\delta$ T-cell depleted mice but found that mice lacking both T cells and B-cells were resistant to experimental fibrosis despite mounting a similar inflammatory response to wild type animals¹³¹. A role for antibody was excluded because mice that were unable to

secrete immunoglobulin still showed reduced fibrosis when B cells were depleted¹³¹. The fact that T cell deficient mice showed normal fibrotic responses in this model argues against a role for B cells in antigen presentation. Therefore, it is most likely that B cells promote fibrosis by secreting cytokines or by contact-dependent interactions with other cells that favour a profibrotic microenvironment. Differentiation of naïve B-cells into Th1-like or Th2-like patterns of cytokine secretion is determined by similar cytokine microenvironments to those that regulate corresponding T-cell differentiation, and B-cells secrete the pro-fibrotic cytokines IL-4, IL-6, and IL-13 at levels similar to CD4+ T-cells¹³⁷. Whilst BCR ligation is required for optimal cytokine secretion, repetitive BCR-independent stimulation is sufficient to stimulate naïve B-cells to secrete cytokines in a polarised fashion^{138,139}. Thus the repeated cycles of tissue injury seen in human liver disease could maintain B-cell activation and allow the process to become self-perpetuating. Interactions between NKT cells, macrophages and B-cells within this fibrotic environment will also reinforce local cellular responses and secretion of TGF β ¹⁴⁰. Co-stimulatory signals provided by B cells to T-cells, NKTs and Kupffer cells may also be important.

CD40 is a strong candidate for a central role in these paracrine interactions. The ligand for CD40, CD154, is strongly expressed on lymphocytes and macrophages at sites of liver injury in several diseases^{31,141} and could activate B-cell CD40 resulting in cell survival, IL-6 secretion and paracrine activation of HSC proliferation and collagen synthesis. Dysregulated bystander activation of B-cells is usually prevented by activation of Fas-dependent apoptosis but survival signals such as BAFF/BLyS that are increased in the fibrotic microenvironment may inhibit Fas-mediated apoptosis allowing inappropriate survival of effector B-cells and amplification of fibrosis¹. However other animal studies have failed to show a major role for B cells in liver fibrosis. B cell depletion in a T_H2 dominated model of parasitic liver fibrosis did not provide any protection against fibrosis. This might be because the strongly polarised Th2 response in this model was unaffected by B cells. The example illustrates that different cellular mechanisms may be involved depending on the nature of liver injury and the pattern of inflammation¹⁴².

Recent interest has focussed the role of NK T cells in fibrogenesis. Invariant NKT cells are a subset of lymphocytes that recognize endogenous lipid ligands presented by CD1d. They regulate host responses to cell injury, tissue damage and viral infection and are potent secretors of cytokines. NKT cells are found in normal liver tissues but their numbers increase with necroinflammatory activity and in the context of chronic viral hepatitis they have been shown to secrete type 2 profibrotic cytokines including IL-4 and IL-13 which could drive progression of fibrosis. Part of this may be driven by increased expression of CD1d on APCs in chronically inflamed liver tissue¹⁴³. These studies are supported by animal experiments in which administration of the lipid β -glucosylceramide results in reduced intrahepatic NKT-cells associated with amelioration of fibrosis.¹⁴⁴ However there is likely to be further complexity because in other models IFN γ secreting NKT cells have an anti-fibrotic role¹⁴⁵. A recent study in the double negative TGF β RII mouse model of primary biliary cirrhosis shows a marked increase of NKT cells within the liver associated with tissue injury. When the same mice were crossed onto a CD1 $^{-/-}$ background, in which there are no CD1d-restricted NKT cells, liver inflammation and injury was markedly decreased suggesting NKT cells are involved in liver injury in this model. Furthermore they showed age-dependent differences in interferon- γ secretion by hepatic CD1d-restricted NKT cells suggesting that their precise role may change with ageing¹⁴⁶. Thus it is the combinations of leukocytes recruited and the outcome of complex cell-cell crosstalk and cytokine expression within the stroma which will determine the nature and progression of fibrosis regardless of the specific lymphocytes in the inflammatory infiltrate.

Summary

Advances in our understanding of liver fibrosis have highlighted the importance of interactions between liver fibroblasts and the innate and adaptive arms of the immune system in determining the outcome following tissue injury. Whilst many acute injuries to the liver result in self-limiting non-fibrotic inflammatory reactions, chronic hepatitis is associated with persistent inflammation and scarring. This is because the development of a stromal microenvironment comprising modified neomatrix, activated fibroblasts and macrophages recruits, retains and maintains lymphocytes at the site of tissue injury resulting in chronic persistent inflammation. The lymphocytes in turn secrete cytokines and interact directly with cellular components of the stroma to maintain the local pro-fibrotic environment. Strategies that aim to restore liver function, either by preventing fibrosis progression or replenishing hepatocytes from stem cell precursors, must take these complex bi-directional interactions into account. Future anti-fibrotic interventions in liver disease may utilise the complex interactions between fibroblasts and T-cells to ameliorate the production of scar tissue and prevent inflammation persisting. In this context the use of specific protein tyrosine-kinases inhibitors such as imatinib (Gleevec) to treat fibrotic liver diseases may offer particular promise both as a means of preventing proliferation of fibroblasts in response to chronic hepatitis, but also inhibiting stromal patterns of cytokine secretion.

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Glossary

HSC	hepatic stellate cell
aLMF	activated liver myofibroblasts
ECM	extra cellular matrix
NK cell	natural killer cell
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
IL	interleukin
CCl₄	Carbon tetrachloride
DC	Dendritic Cell

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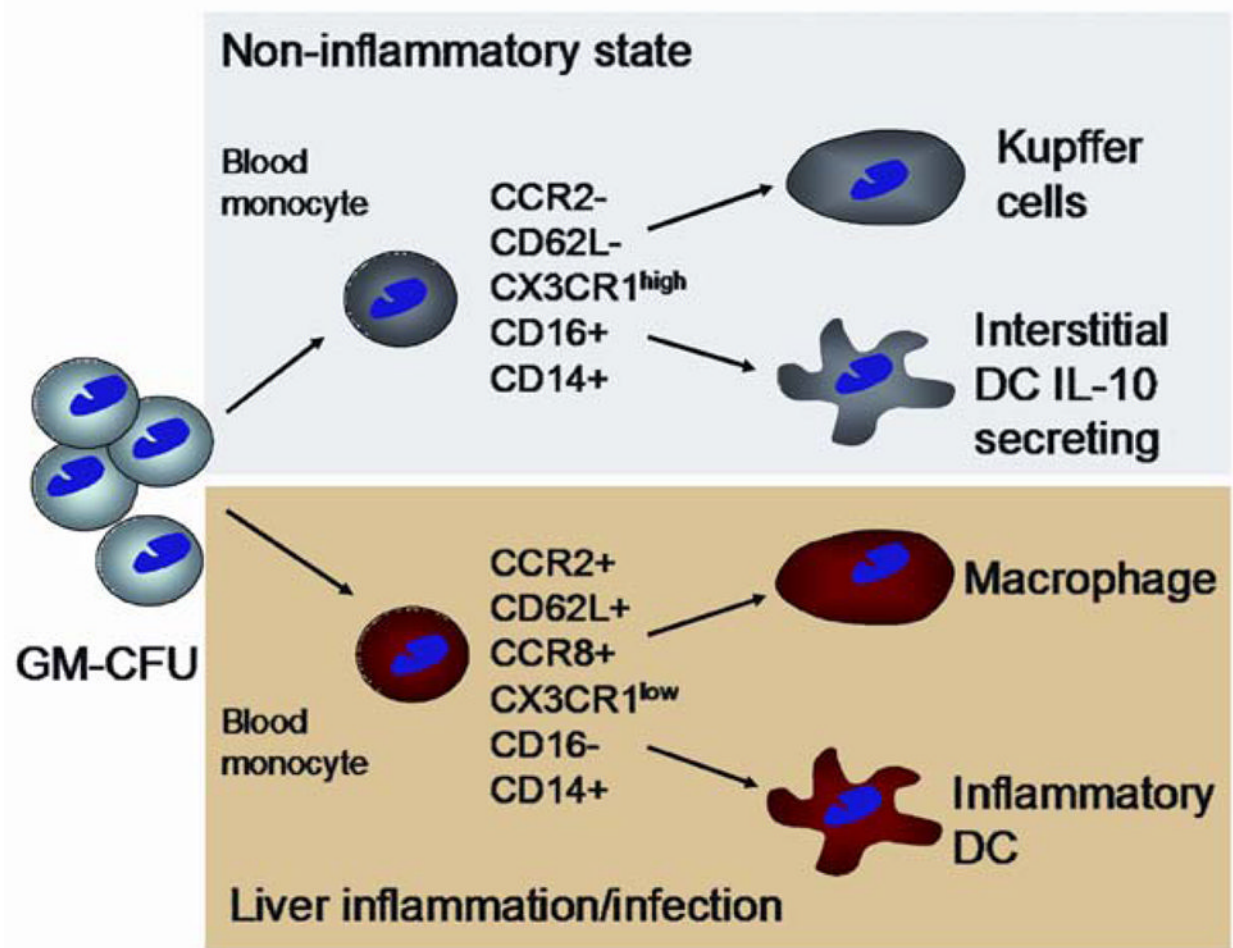


Figure 1.

Based on work in other tissues the figure shows the two main monocyte subsets in blood and how they can give rise to functionally diverse subsets of kupffer cells, macrophages and myeloid DCs after recruitment into the liver. Two distinct subpopulations of monocytes can be identified in the human circulation which show differences in expression of chemokine receptors and adhesion molecules involved in recruitment through sinusoidal endothelium. Adapted from ¹².

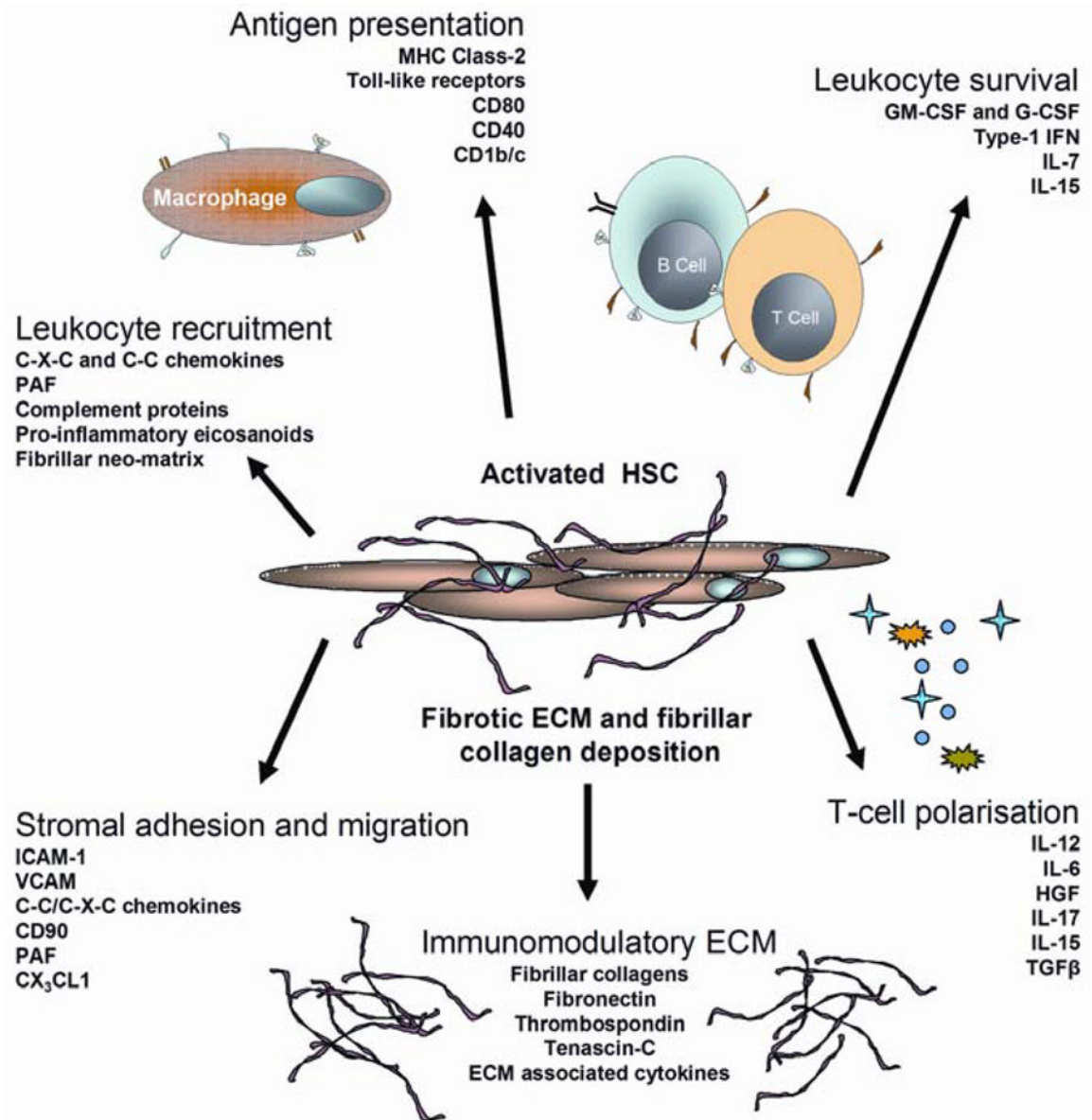


Figure 2. Activated hepatic stellate cells control many aspects of tissue inflammation

Following activation HSC undergo a phenotypic switch adopting a myofibroblast morphology and upregulating pro-inflammatory cytokines that regulate stromal immune responses as well as secreting fibrillar collagens and neo-matricellular proteins that generate tissue scarring. See text for further discussion.

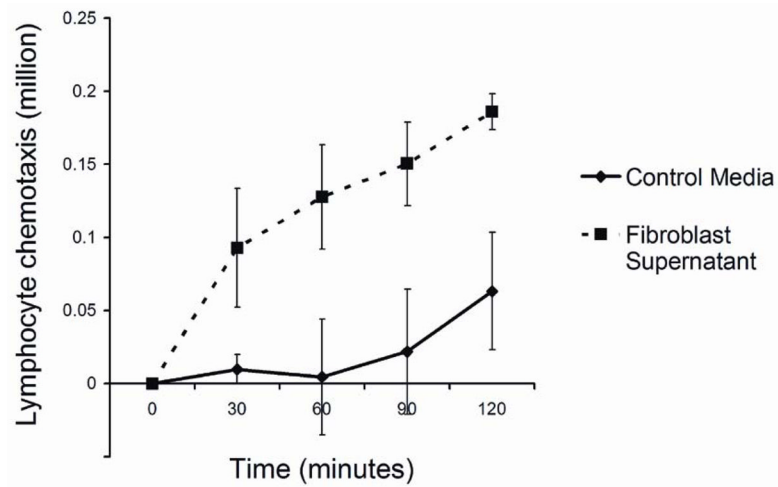


Figure 3. Real-time lymphocyte chemotaxis to liver fibroblast conditioned media

Directed real-time migration of fluorescently labelled human lymphocytes towards liver fibroblast conditioned media¹⁴⁷. Conditioned supernatant taken from human liver fibroblasts isolated from diseased livers contains soluble factors that promote rapid lymphocyte chemotaxis even in the absence of proinflammatory cytokine stimulation. Data represents mean \pm SEM 3 replicate experiments.

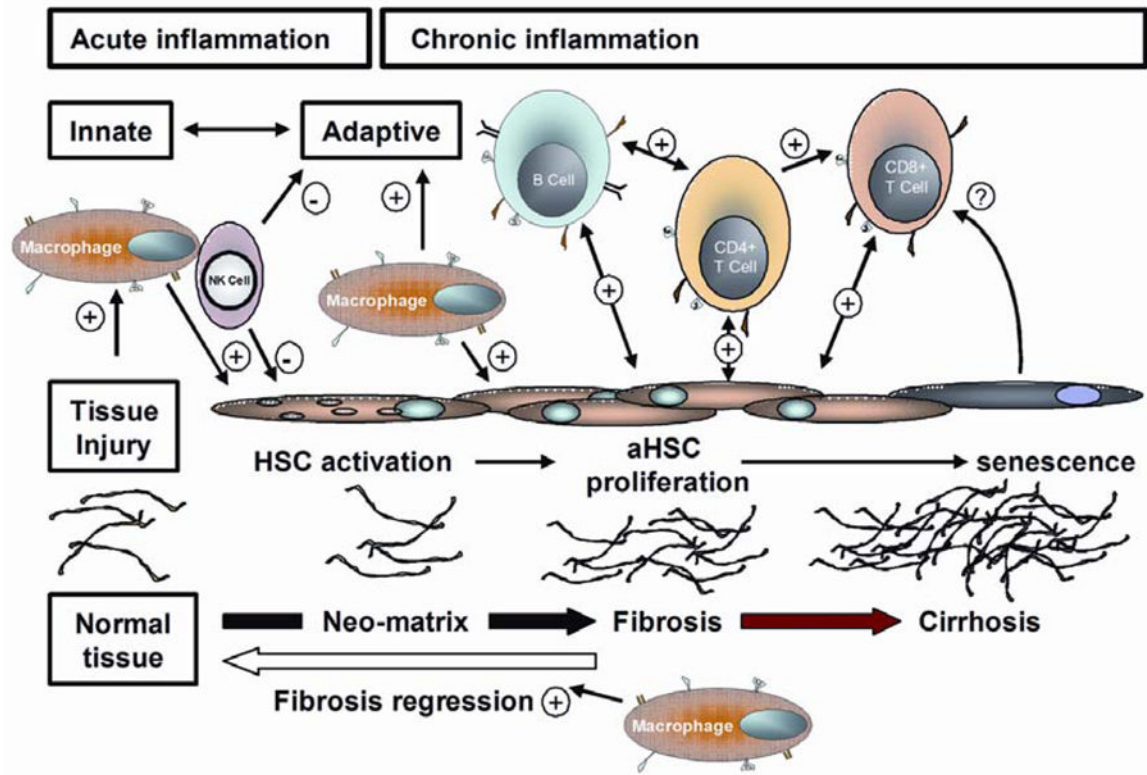


Figure 4. Liver fibrosis is the product of interactions between stromal cells and the innate and adaptive immune systems. For further discussion see text.