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Patten, Daniel A; Shepherd, Emma L; Weston, Christopher J; Shetty, Shishir

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Novel targets in the immune microenvironment of the hepatic sinusoids for treating liver diseases

Corresponding Author

Dr Shishir Shetty MBChB PhD FRCP, Centre for Liver Research, 5TH Floor IBR, Institute of Immunology and Immunotherapy, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT

Email s.shetty@bham.ac.uk

Tel +441214158700

Fax +441214158701

Co-Authors

Dr Daniel A Patten PhD, Centre for Liver Research, 5TH Floor IBR, Institute of Immunology and Immunotherapy, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT

Dr Emma L Shepherd PhD, Centre for Liver Research, 5TH Floor IBR, Institute of Immunology and Immunotherapy, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT

Dr Christopher Weston PhD, Centre for Liver Research, 5TH Floor IBR, Institute of Immunology and Immunotherapy, Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT

Abstract

Immune dysregulation and accumulation of leukocytes is a hallmark of adult chronic liver diseases. Progressive hepatic inflammation can lead to fibrosis and cirrhosis with a high risk of liver failure or hepatocellular cancer (HCC). Recent advances have been made in the treatment of liver disease including the development of highly effective antiviral therapy for hepatitis C and the potential of immunotherapy for HCC. Despite this, the majority of other chronic liver diseases including alcoholic liver disease, fatty liver disease and cholestatic diseases do not respond to conventional anti-inflammatory therapies. Recent studies defining the organ-specific properties that contribute to resident immune activation and immune cell recruitment from the circulation in these conditions have identified novel hepatic inflammatory pathways which are now being targeted in clinical trials. Further understanding of how the immune microenvironment is regulated within the liver and how disease specific mechanisms alter this process will hopefully lead to combination therapies to prevent aberrant inflammation and also promote fibrosis resolution. In this review, we focus on the advances that have been made in identifying key components of the inflammatory pathway including the recognition of danger signals, the recruitment and retention of lymphocytes from the circulation and the pathways which promote resolution.

Main Concepts and Learning Points

1. The majority of adult chronic liver diseases are driven by inflammatory processes which are unresponsive to conventional anti-inflammatory therapies.

2. Recent work has highlighted the major role of macrophages, tissue resident Kupffer cells and recruited monocytes, in sensing hepatic damage which drives downstream immune responses.
3. Lymphocyte recruitment via the hepatic sinusoids contributes to hepatitis and is mediated by interactions with liver sinusoidal endothelial cells via typical and atypical adhesion molecules.
4. Clinical trials are targeting macrophage responses to epithelial damage and immune cell recruitment via adhesion molecules as novel anti-inflammatory approaches in chronic liver disease.
5. Further approaches to treat hepatic inflammation should take into account inflammatory pathways which mediate immune cell retention in liver tissue and promote resolution of fibrogenesis.

Adult inflammatory liver diseases lead to a major global burden on human health, and patients with progressive disease are at risk of developing fibrosis and cirrhosis which can culminate in end-stage liver failure or hepatocellular cancer (HCC), both of which are associated with extremely high mortalities[1]. Recent advances have been made in the treatment of liver disease, especially in the field of viral hepatitis. The development of direct-acting antivirals for the treatment of hepatitis C has demonstrated very high rates of viral eradication[2]. In the case of hepatitis B, current therapies are effective at suppressing viral replication and can reduce necroinflammation with reversal of fibrosis as well as reducing HCC risk[3,4]. In contrast, the inflammatory processes that drive other major liver diseases such as alcoholic liver disease, non-alcoholic steatohepatitis and cholangiopathies have

continued to be a major therapeutic challenge. For those patients who progress to advanced chronic liver disease there are limited options when they develop end-stage liver disease, with transplantation being the only choice in many cases[5]. New therapies are therefore urgently required to reduce the burden on transplantation and the associated high waiting list mortality.

Adult chronic liver diseases are driven by inflammation, which promotes epithelial damage and death leading to the activation of resident immune cells and the accumulation of circulating immune cells recruited from the circulation[6,7]. Each disease has a specific pattern of injury which is dependent on the site of initial damage. For example, NASH is triggered by hepatocyte damage characterised by sublethal injury associated with lipotoxicity, resulting in parenchymal inflammation associated with innate and adaptive immune responses[8]. In contrast, primary sclerosing cholangitis is driven by cholangiocyte injury leading to the localised release of chemokines and pro-inflammatory cytokines associated with portal inflammation and ductal proliferation and ductular loss[9]. These inflammatory processes are associated with the activation of hepatic stellate cells and if left unchecked lead to excessive deposition of extracellular matrix, fibrosis and persistent damage culminating in cirrhosis[10]. The site of injury determines the pattern of fibrosis with parenchymal diseases such as ALD/NASH presenting centrilobular and sinusoidal fibrosis and cholangiopathies associated with periportal fibrosis leading to irregular shaped nodules[11].

Targeting the inflammatory pathways that drive these conditions has the potential of inhibiting fibrogenesis, but the mechanisms involved are poorly understood. Autoimmune hepatitis for example is often responsive to steroid-based therapy and

immunomodulators, whereas other immune-mediated liver diseases such as primary sclerosing cholangitis and primary biliary cholangitis are currently unresponsive to these medications[9,12]. Furthermore, patients suffering from the major inflammatory liver diseases secondary to alcohol and non-alcoholic fatty liver disease do not derive benefit from current anti-inflammatory approaches. We therefore urgently require better understanding of the underlying core inflammatory pathways that drive these diseases to identify novel therapies which can prevent the progression to cirrhosis and end stage liver disease.

In this review, we focus on three major processes which are implicated in chronic inflammatory liver diseases, the immune response to danger signals released by persistent epithelial damage, the recruitment/retention of immune cells from the circulation and the factors which drive resolution and repair within the liver.

The immune response to danger signals released from epithelial damage.

Epithelial damage is a key factor in initiating inflammatory liver diseases. This involves cellular stress secondary to factors such as lipotoxicity in fatty liver disease, accumulation of breakdown products of alcohol and hepatotrophic viruses. These processes are associated with the release of danger signals or danger associated molecular patterns (DAMPs) into the microenvironment. How these danger signals are sensed and processed by the innate immune system is one of the key determinants of progression of these inflammatory conditions[13] (summarised in Figure 1).

Kupffer cell recognition of DAMPS

The major cellular population to sense and respond to these danger signals are the liver resident macrophages, Kupffer cells. Kupffer cells are the sentinels of the liver and are derived from yolk sac precursors which self renew[14]. They play a role in processing gut-derived products and mediating immune responses to microbes. Additionally, they sense sterile injury and associated DAMPS which are a characteristic of the major inflammatory liver diseases including alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH). DAMPs which have been associated with Kupffer cell activation include high mobility group protein B1 (HMGB1), ATP, uric acid, DNA fragments and cholesterol crystals[14]. Targeting the pathway of DAMP recognition is already underway in clinical trials. DAMPs are recognised by pattern recognition receptors including Toll-like receptors (TLRs) and scavenger receptors which are both highly expressed by macrophages. TLR4 has been studied extensively and a TLR4 antagonist, JKB-122, is currently undergoing assessment in the setting of NASH as an early phase II clinical trial NCT02442687. Galectin-3 expressed on Kupffer cells which is a member of the scavenger receptor family which recognise the terminal galactose residues on glycoproteins. Galectin-3 plays a key role in hepatic uptake of advanced lipoxidation and glycation end products[15]. An agent which binds galectin-3, GR-MD-02, is progressing through early stage clinical trials in the setting of NASH[16]. Other members of the scavenger receptor family which have been implicated in promoting hepatic inflammation include CD36 and Scavenger Receptor-A (SR-A). Targeted deletion of these receptors on myeloid cells, led to reduced levels of inflammation and fibrosis in models of fatty liver disease[17]. A recent study confirmed that the recognition of DAMPs, specifically products of lipid peroxidation such as malondialdehyde (MDA)-LDL, by CD36 and SR-A led to the release of pro-inflammatory cytokines[18]. Blocking

the action of these receptors may therefore be beneficial in the setting of NASH. Interestingly, the authors also targeted the DAMP directly, in this case the MDA epitope, by *in vivo* neutralization with antibodies. This approach was successful in reducing inflammation in their pre-clinical model of fatty liver disease.

The role of the inflammasome in chronic liver disease

Whilst the direct recognition of DAMPs is a viable pathway to target, there are also downstream pathways which play significant roles in the progression of chronic liver disease. The recognition of these danger associated ligands by pattern recognition receptors on Kupffer cells leads to the formation of the inflammasome. Inflammasomes are multi-protein complexes which are comprised of a nucleotide oligomerization domain (NOD)-like receptors and effector molecules including pro-caspase-1, and adaptor molecules e.g. apoptosis-associated speck-like CARD-domain containing protein (ASC)[19]. Following the formation of the inflammasome, Kupffer cells produce inflammatory mediators, such as interleukin 1beta and other pro-inflammatory cytokines and chemokines. Activation of inflammasome complexes have been confirmed in pre-clinical models of alcoholic liver injury and fatty liver disease[20]. This leads to the recruitment of other innate populations from the circulation such as neutrophils, monocytes and populations of T cells. Therefore targeting the pathway of inflammasome formation is also a rational approach to prevent progression of inflammatory liver diseases. Studies in pre-clinical models of alcoholic liver disease demonstrated that targeting the inflammasome pathway by pharmacological inhibition of IL-1R1 prevented the development and progression of alcoholic liver disease[21]. Additionally, one the most extensively studied inflammasomes in macrophages is the NOD-, LRR- and pyrin domain-containing 3

(NLRP3) inflammasome which has previously been shown to play a critical role in the progression of murine models of non-alcoholic fatty liver disease [22]. A recent study confirmed its role in driving liver inflammation and fibrogenesis by studying liver injury in mice with constitutive activation of NLRP3 in myeloid cells. Activation of the NLRP3 inflammasome led to excess production of TNF and IL-17 resulting in severe inflammation and fibrosis [23].

Recruitment of peripheral monocyte populations

Another major downstream consequence of Kupffer cell driven inflammation is the recruitment of other monocyte populations from the circulation via the CCL2-CCR2 axis[24,25]. The chemokine CCL2 promotes recruitment of $CCR2^+$ monocytes from the circulation, and this has been confirmed in experimental models of both alcoholic liver disease and fatty liver disease[26]. A recent study confirmed the increased accumulation of $CCR2^+$ macrophages within liver tissue parallels with fibrosis progression in fatty liver disease. These populations of cells were seen as aggregates of monocyte-derived macrophages around portal tracts[27]. Furthermore, gene analysis of these recruited (monocyte-derived macrophages) versus resident (Kupffer cells) confirmed that monocyte-derived macrophages were associated with multiple growth factors and cytokines leading to fibrosis progression, whereas Kupffer cells were characterised by factors associated with inflammation initiation. Therapeutic targeting of the recruitment of these $CCR2^+$ monocytes by administration of Cenicriviroc a CCR2/CCR5 dual chemokine receptor antagonist led to amelioration of hepatic inflammation and fibrosis in several models of NASH[27]. In keeping with these findings, there is encouraging clinical experience that Cenicriviroc could be a potential therapy for chronic liver disease. A phase 2b study of this agent

in patients with non-alcoholic steatohepatitis and established fibrosis demonstrated a significant improvement in fibrosis compared to placebo after 1 year of treatment[28]. Activated Kupffer cells also secrete several other chemokines including CCL25, CX3CL1, CXCL2 and CXCL8[14]; thus, targeting these chemokines may also influence the recruitment of other distinct immune populations from the circulation during inflammatory liver disease leading to other novel targets for treatment. An intriguing recent study has also identified the recruitment of immune populations from the peritoneal compartment. In a model of sterile liver injury a population of GATA6-positive macrophages were detected at a very early stage of tissue damage. These $GATA6^+$ macrophages migrated directly across the mesothelium and their recruitment was dependent on the adhesion molecule CD44 and adenosine triphosphate[29]. The role of these macrophages in the progression of chronic inflammatory liver diseases and their therapeutic potential is yet to be confirmed.

The activation of unconventional lymphocytes

In parallel to the initiation of inflammation by myeloid populations, there is gathering interest in the role of unconventional lymphocytes which are found highly enriched in epithelial tissues and have well established roles in anti-microbial immunity[30]. Their roles in early immune responses has led investigators to study if they could be pivotal in the triggering and regulation of progressive liver disease. $\gamma\delta$ T cells are predominantly generated in the thymus and characterised by a $\gamma\delta$ T cell receptor (TCR), they only account for 2-3% of all $CD3^+$ T cells in secondary lymphoid organs but have been found to be enriched in the liver[31]. $\gamma\delta$ T cells recognise conserved structures including non-peptide metabolites and heat shock proteins. They can rapidly release cytokines which are known to regulate adaptive immune populations

including conventional αβ T cells and therefore have been postulated as an additional link between innate and adaptive immune responses[32]. Experimental models of liver disease have demonstrated the accumulation of these cells during liver injury and their contribution to disease progression. In a murine model of autoimmune hepatitis, γδ T cells played a protective role associated with reduced liver damage and inflammatory cytokine levels. In this setting the protective mechanism was found to be regulated by IL-17 produced by γδ T cells downregulated the function of another family of unconventional T cells, natural killer T (NKT) cells[33]. Further support for the protective role of these cells in liver disease has been demonstrated in models of chronic liver injury. Murine models of fibrosis and steatohepatitis demonstrated that the $CCR6^+$ subset of γδ T cells prevented fibrosis by promoting the apoptosis of hepatic stellate cells[34].

As alluded to earlier, another subset of unconventional lymphocytes, NKT cells, appear to promote inflammatory liver disease. NKT cells are lymphocyte subsets which express cell surface markers associated with NK cells as well as the T cell receptor and they are characterised by their recognition of glycolipid antigens. They have been shown to localise to the hepatic sinusoids and demonstrate a crawling/patrolling phenotype[35]. NKT cells accumulated in models of liver injury and were shown to promote hepatic inflammation and contributed to progressive fibrosis[36]. Further studies focused on the potential contribution of NKT cells to fatty liver disease. Higher levels of NKT cells were detected in patients undergoing transplantation for NASH compared to order indications, this accumulation was also seen in murine models of NASH and mice deficient in NKT cells were protected from fibrosis in this model[37]. Subsequent studies implicated hepatic NKT cells in the

increased production pro-fibrogenic factors including osteopontin and hedgehog ligands[38].

Further understanding of the functional properties of another unique subset of innate-like T cells, mucosal-associated invariant T cells (MAIT) cells, has highlighted their potential as regulators of liver inflammation. MAIT cells are characterised by the expression of a semivariant TCR that recognises a MHC-like protein (MR-1)[39]. MR-1 presents vitamin B metabolites derived from commensal and pathogenic bacteria and thus MAIT cells can be activated by a variety of bacterial strains[40]. The high levels of these cells in human gut biopsies and accumulation in laminia propria led to them being named MAIT cells[41]. Subsequent studies have now shown that they are also enriched in the liver and have explored their antimicrobial properties in immune mediated liver disease and alcoholic liver disease[42-44]. This has led investigators to speculate that MAIT cells may make a significant immune contribution in the liver acting as a firewall between the host and gut derived bacteria[45]. However these reports have also shown that MAIT cells are highly activated in the liver and are the predominant IL-17 producers within the hepatic T cell compartment and could therefore be important drivers of aberrant hepatic inflammation. A recent study has studied the contribution of these cells in chronic liver injury. MAIT cells were found to be enriched in the periportal region and along the fibrotic septa in tissue from cirrhotic livers and in a carbon tetrachloride model of chronic liver injury these cells were found to be pro-fibrogenic by promoting the pro-inflammatory properties of both monocyte-derived macrophages and fibroblasts[46]. Unconventional lymphocytes are therefore a novel target to treat chronic inflammatory liver disease, but further work is clearly required to understand how to either manipulate their function or utilise them as cell therapy.

Lymphocyte recruitment via the liver sinusoids

The accumulation of adaptive immune cell populations within the liver is also a hallmark and driver of all adult chronic inflammatory liver diseases. A prerequisite for leukocyte recruitment from the circulation into organs is their interaction with endothelial cells lining blood vessels. In general, leukocyte migration from the blood into inflamed tissues occurs in post-capillary venules[47]; however, in the liver, this process occurs in the low shear flow microvasculature of the hepatic sinusoids which are lined by liver sinusoidal endothelial cells (LSEC)[7] (Figure 2). LSECs are a phenotypically and functionally unique population of endothelial cells. They are characterised by a minimal basement membrane and atypical cellular junctions as well as membranous pores organised in sieve plates called fenestrations[48]. Additionally, LSECs are also characterised by the expression of an array of scavenger receptors (SRs)[49]. These structural and phenotypic characteristics support the physiological functions of LSEC but they also influence the mechanisms of lymphocyte recruitment and thus are potential organ specific anti-inflammatory targets. The low shear stress environment of the hepatic sinusoids negates the requirement for the early rolling steps of the leukocyte adhesion cascade[7]. As a consequence, LSEC express negligible levels of selectins[50], a small family of transmembrane Ca^{2+}-dependent lectins which play an integral role in the initial stages of leukocyte recruitment in more conventional vascular beds[51]. A critical step in determining if lymphocytes accumulate at sites of inflammation is not only their adhesion to endothelium but also their subsequent transmigration across the

endothelial barrier. We now know that the process of transendothelial migration (TEM) in itself is a multi-step pathway involving a combination of receptor interactions which are potential therapeutic targets for inflammation[52]. The conventional route for TEM by leukocytes is via the paracellular route (in between cells, through cellular junctions), but it has also been shown that leukocytes can migrate via the transcellular route (directly through the endothelial body)[53]. Studies on human LSEC demonstrate that a significant proportion of lymphocytes migrate via the transcellular route[54]. Additional *in vitro* studies, demonstrated that the structure of these endothelial cells permits a novel migratory pattern, where lymphocytes were shown to migrate directly into LSEC and then migrate into adjacent endothelial cells[55]. This migration was dependent on interferon gamma and facilitated by the unique junctional complexes between LSEC. This work highlights that the sinusoidal vascular bed is not a simple barrier but plays an active role in regulating the immune microenvironment within the liver and the positioning of lymphocytes in liver tissue. Further work has elucidated some the molecular contributors to this process and their potential as novel anti-inflammatory targets.

Conventional adhesion molecules

Several studies have demonstrated that LSEC use a unique combination of both conventional endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and atypical adhesion molecules to mediate lymphocyte recruitment in chronic liver disease [56,57]. VCAM-1 binds the leukocyte-expressed $\alpha_4\beta_1$ integrin [58] and plays an important role in capturing lymphocytes from blood flow within the hepatic sinusoids and subsequently mediates stabilisation[59,60]. ICAM-1 supports firm adhesion of

lymphocytes, via binding to $\alpha_L\beta_2$ integrin (lymphocyte function-associated molecule-1 (LFA-1)) [61], and subsequently mediates their transmigration across LSEC[54,62]. Both VCAM-1 and ICAM-1 are significantly upregulated by proinflammatory factors, such as cytokines[63]; however, their adhesive function is largely dependent on the formation of endothelial adhesive platforms (EAPs)[64]. EAPs play an essential role in the spatial organisation of VCAM-1 and ICAM-1 within the cell membrane, resulting in concentrated areas of expression of the adhesion molecules in the contact area with adherent leukocytes[64]. The formation of EAPs has been proposed to be regulated by the tetraspanin family of receptors, which are able to laterally associate with adhesion molecules to form microdomains[64,65]. In support of this previous work, the tetraspanin CD151 associated with VCAM-1 within LSECs and was able to regulate lymphocyte adhesion under physiological flow conditions *in vitro*[66]. Due to their widespread constitutive expression in a number of cell types and tissues, VCAM-1 and ICAM-1 are unlikely to represent viable therapeutic targets; however, modulating their lateral interactions with tetraspanins, such as CD151, may present an attractive and organ-specific target for chronic inflammatory liver disease.

Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), which belongs to the immunoglobulin family along with VCAM-1 and ICAM-1, is known to bind to the $\alpha_4\beta_7$ integrin[67] and plays an important role in lymphocyte trafficking to the gut, via mucosal vessels[68]. Under normal physiological conditions, MAdCAM-1 is absent from the liver; however, previous studies have demonstrated that MAdCAM-1 can be upregulated through the enzymatic activity of an atypical adhesion molecule, vascular adhesion protein-1 (VAP-1), in LSEC in some chronic liver diseases[69]. This is particularly evident in primary sclerosing cholangitis (PSC), where it promotes the

recruitment of, gut-activated T cells which express high levels of $\alpha_4\beta_7$ integrin [70,71]. Its hepatic functionality is highly supportive of immunological crosstalk between the gut and the liver, and MAdCAM-1 might contribute to the pathophysiological link between inflammatory bowel disease (IBD) and PSC, a progressive autoimmune biliary disease which is associated with IBD in ~80% of cases. Currently, clinical trials are being considered to target MAdCAM-1/$\alpha_4\beta_7$ interactions in PSC using therapeutic antibodies originally developed for the treatment of IBD. Trials have included a selective humanised monoclonal antibody, Vedolizumab, to $\alpha_4\beta_7$. Prior clinical studies with Vedolizumab in the setting of IBD have confirmed that this drug can modulate lymphocyte recruitment to the gut in both ulcerative colitis and Crohn's disease leading to a reduction in inflammation and improved mucosal healing[72,73]. This has led to gathering interest in the use of Vedoluzimab in the setting of diseases where MAdCAM-1 has been shown to be upregulated, particularly PSC. Until recently, this had involved single centre case series with results suggesting safety and improvement of inflammatory parameters[74]. A multi-centre study has now been completed in patients with PSC and IBD which demonstrated clinical responses in the IBD pathology, and the drug was safely tolerated, but it did not lead to any detectable improvement in liver biochemistry[75]. Whether targeting the MAdCAM-1/$\alpha_4\beta_7$ interaction could improve long term outcomes in PSC, including prevention of progressive fibrosis, transplant-free survival and cancer incidence, still needs to be addressed.

Atypical adhesion molecules

Vascular adhesion protein-1 (VAP-1) is a membrane-bound amine oxidase that, under normal physiological conditions, is expressed in vascular endothelial cells,

smooth muscle cells, and adipocytes[76]. During homeostasis VAP-1 is localised to cytoplasmic vesicles in endothelial cells, but under inflammatory conditions the protein is trafficked to the cell surface[77]. Early studies of VAP-1 showed that it mediated leukocyte binding to high endothelial venules (HEVs), the specialised post-capillary venules found in lymph nodes[78]. Further studies confirmed that VAP-1 was expressed at high levels in chronically diseased liver tissues *ex vivo*[79] and directly mediated adhesion and transmigration across LSEC *in vitro*[80]. In addition, via its enzyme activity, VAP-1 can upregulate expression of other adhesion molecules (e.g. VCAM-1, ICAM-1 and MAdCAM-1) and chemokines (e.g. CXCL8) in LSECs, consequently enhancing leukocyte recruitment[69,81]. More recently, these results have been corroborated *in vivo*, confirming the multifaceted role of VAP-1 in leukocyte recruitment to the liver in murine models of liver injury, and described VAP-1 expression by hepatic stromal cell populations[82]. A number of preclinical studies targeting VAP-1 have confirmed that inhibition of its enzymatic activity and/or blockade of its adhesive function with therapeutic antibodies reduces leukocyte infiltration in a range of rodent models of inflammatory diseases[83].

Scavenger receptor that binds phosphatidylserine and oxidized lipids (SR-PSOX), which in its soluble form is also known as the chemokine, CXCL16, is expressed by LSEC[84] and is upregulated in both acutely[85,86] and chronically injured liver tissues[87]. CXCL16 is a specific ligand for the chemokine receptor CXCR6, thus enabling its membrane-bound form to interact with intrahepatic $CXCR6^+$ immune cells, such as effector T cells[87,88], natural killer (NK) cells[89,90] and NKT cells[84]. Genetic deficiency of SR-PSOX has recently been shown to reduce the extent of inflammation and necrosis in a murine model of acetaminophen (APAP)-induced acute liver injury[85].

Additionally, and perhaps more encouragingly, pharmacological intervention with neutralising antibodies against SR-PSOX has shown efficacy in reducing inflammation in preclinical murine models of sepsis-mediated[86,91] and carbon tetrachloride (CCl_4)-mediated[92] acute liver injury. Furthermore, Wehr and colleagues were also able to demonstrate the efficacy of SR-PSOX antibody therapy in a commonly used murine model of non-alcoholic steatohepatitis (NASH), showing a reduction in both macrophage infiltration and triglyceride levels. Therefore, targeting the SR-PSOX (CXCL16)/CXCR6 axis may hold promising potential for treatment of inflammation and subsequent fibrosis of the liver[92].

The class H scavenger receptor stabilin-1, also known as common lymphatic endothelial and vascular endothelial cell receptor (CLEVER-1), was originally shown to mediate lymphocyte transmigration across HEVs[93]. Given the phenotypic similarities between lymphatic endothelial cells and LSEC[50], stabilin-1 was found to be expressed in human liver and shown to be significantly upregulated in the hepatic sinusoids in chronic liver disease[54]. Following this, adhesion assays with lymphocyte subsets demonstrated that stabilin-1 specifically mediated transendothelial migration of T_{regs} and B-cells through LSECs *in vitro*, under conditions which mimic the physiological flow and proinflammatory microenvironment of the hepatic sinusoids during liver injury[54,62]. This was the first demonstration of a T_{reg}-specific adhesion molecule and transmigration of this lymphocyte subset was shown to be dependent on a combination of stabilin-1, VAP-1 and ICAM-1. T_{regs} play a vital role in promoting tolerance, they mediate immunosuppression through multiple mechanisms and prevent autoimmunity and counteract inflammatory reactions mediated by the effector arm of the immune system[94]. Therefore, in the context of inflammatory liver

diseases approaches to upregulate stabilin-1 or promote the function of stabilin-1 could promote T_{reg} accumulation as a strategy to prevent progressive hepatitis.

The expression of the stabilin-1 homologue, stabilin-2, has also been described in LSEC and was originally shown to act as a clearance receptor for hyaluronan from the blood[95,96]. Through a number of mutation experiments and antibody blockade studies *in vitro*, Jung *et al*. found that stabilin-2 was also able to mediate lymphocyte binding and identified the integrin $\alpha_M\beta_2$ as the lymphocyte-expressed ligand[97]. They also determined that stabilin-2 predominantly acts in the firm adhesion step of the leukocyte adhesion cascade as its silencing, via shRNA, did not affect lymphocyte rolling or transendothelial migration, but was still able to significantly reduce the number of adherent cells[97]. To date, the study by Jung *et al*. remains the sole investigation of the role of stabilin-2 in leukocyte recruitment to LSEC. Further work is required to understand how the stabilin receptor family expressed on LSEC contribute to lymphocyte recruitment in preclinical models of inflammatory liver disease.

Scavenger receptor class F, member 1 (SCARF1 or SR-F1), also known as scavenger receptor expressed by endothelial cells (SREC-I), has also been shown to be expressed in both murine and human LSEC[98,99]. Recently, it has been shown that SCARF1 plays a role in the selective recruitment of $CD4^+$ T cells to human LSEC, under physiological shear stress conditions *in vitro*[99]. In this study, SCARF1 contributed to the firm adhesion step of the leukocyte adhesion cascade, with endothelial surface expression of SCARF1 observed in adhesive cup structures formed on the surface of the LSEC[99]. However, SCARF1 is an understudied

scavenger receptor[100] and more research into the extent of the contribution of SCARF1 in immune cell recruitment is required before it can be considered as a therapeutic target. Nevertheless, SRs including SCARF1 have been shown to be upregulated in several human inflammatory liver diseases and appear to accumulate at the interface between inflammation/fibrosis and correlate with fibrosis progression.

Chemokines

Chemokines are an important component in the process of leucocyte recruitment and contribute to both firm of adhesion of leukocytes to endothelium and their subsequent migration across the endothelium. They are a family of small proteins which bind to G-protein coupled receptors on the leukocyte surface and induce conformational changes of intergrins which triggers firm adhesion[101]. They are also found within intraendothelial vesicles and promote transendothelial migration[102]. We have already highlighted their role in monocyte and NK/NKT populations but they also play a significant role on lymphocyte recruitment within the sinusoids. The most extensively investigated are the inflammatory chemokines CXCL9-11 which bind to the receptor CXCR3 and have been shown to be upregulated in a range of liver diseases[103-105] and functionally they contribute to the transendothelial migration of lymphocytes across primary human HSEC[103]. Previous studies have also shown that chemokines contribute to the compartmentalisation of lymphocytes in liver diseases with the CXCR3 ligands promoting recruitment into the parenchyma whereas CCR5 ligands (the chemokines CCL3-5) contribute to portal tract recruitment[103,106,107]. The contribution of chemokines to inflammation provides a clear rationale for targeting them as novel anti-inflammatories but a recent study highlights the difficulties of achieving sustained inhibition of chemokines. NI-0801 is

a human monoclonal antibody against the CXCR3 ligand, CXCL10, which was studied in the context of PBC[108]. Investigators completed a phase 2a study in patients with PBC with inadequate response to ursodeoxycholic acid with the aim of assessing the safety and efficacy of NI-0801. The study demonstrated that the drug was safely tolerated and led to pharmacological responses in the blood but there was no therapeutic benefit identified with repeated infusions.

An alternative approach would be to consider targeting lymphocyte subsets, focusing on pro-inflammatory subsets and allowing persistent recruitment of regulatory subsets in order to shift the balance in the hepatic microenvironment. Whilst CXCR3 ligands have been implicated in the recruitment of several subsets including both T_{regs} cells and subsets which secrete the pro-inflammatory cytokine IL-17 (Th17 cells)[109,110], other chemokines were implicated in the subsequent migration into hepatic tissue of these subsets. T_{reg} recruitment was regulated by the CCR4 ligands CCL17 and CCL22, whereas Th17 recruitment was regulated by CCL20, a CCR6 ligand[109,110]. In view of these findings, targeting the chemokine CCL20 rather than CXCR3 ligands may prove to be a more effective anti-inflammatory approach which will not alter T_{reg} recruitment. Recent studies highlight the importance of the Th17/Treg balance in determining progressive inflammatory liver disease[111-113].

Retention of immune cells in the stromal compartment

Following migration into the tissue, infiltrating immune cells are maintained in the local microenvironment. Complemetary to the role of the endothelial layer, the stromal compartment of the liver maintains a microenvironment which permits the recruitment and retention of inflammatory cells. The hepatic stellate cell (HSC)

population are a hepatic stromal cell type which resides in a quiescent state in the sub-endothelial layer between the endothelium and the parenchymal cells, namely the space of Disse. Release of stimulating factors from injured epithelial cells and infiltrating immune causes the HSCs to become activated, driving a programme of proliferation, migration and contractility of HSC controlled by a plethora of both paracrine and autocrine stimuli. The consequence of this activation is the synthesis of extracellular matrix (ECM) proteins and subsequent accumulation of scar tissue. In view of the key role played by HSC in fibrogenesis, there has therefore been a vast drive to investigate how these cells may be targeted as a therapeutic strategy in liver disease (reviewed in [114]).

In vitro activated primary human HSCs and *in vivo* activated liver myofibroblasts (aLMFs) secrete a range of cytokines, chemokines and growth factors which can recruit and position leukocytes by G-coupled receptor-dependent and –independent mechanisms[115]. When cultured in basal conditions, aLMFs and HSC secreted high levels of IL-6, HGF, VEGF, CCL2, and CXCL8 under control conditions and stimulation with pro-inflammatory cytokines TNFα and IFNγ enhanced all factors and induced secretion of additional chemokines including CCL5, CXCL9 and CXCL10. Moreover, aLMF- and HSC-conditioned supernatants promoted strong and rapid migration of lymphocytes towards these chemotactic factors under pro-inflammatory conditions and stimulated increased recruitment of lymphocytes across adjacent LSEC monolayers. These findings demonstrated that there are signals from HSCs which can recruit infiltrating immune cells which may be targeted to halt the progression of fibrogenesis. One such target which we have already discussed in the context of inflammation is VAP-1. VAP-1 is a dual functioning entity which, as

described, acts as an adhesion molecule as well as an enzyme which has a role in recruiting lymphocytes across endothelial cells[80]. More recent *in vivo* studies described a novel role of VAP-1 in hepatic inflammation and fibrogenesis through modulating HSC phenotype[116]. Soluble VAP-1 secreted by HSCs was enzymatically active and was able to recruit lymphocytes. VAP-1 modulation in the HSC cell line LX-2 increased transcription of profibrogenic genes such as collagen 1a1 as well as enhancing wound healing. These data were supported by murine models of liver injury in which VAP-1 knockout animals had less inflammation and fibrosis in response to injury[116]. The blockade of VAP-1 to treat primary sclerosing cholangitis (PSC) is currently being evaluated in the phase II clinical trial BUTEO (BUTEO NCT02239211).

Inflammatory pathways which promote fibrosis resolution and liver regeneration

We have covered some of the mechanisms which drive effector immune responses within the liver but it is also becoming clear that pathways which promote resolution of the inflammatory process play a key role in determining the severity of tissue injury. Targeting cellular populations that promote resolution could provide a novel anti-inflammatory approach. The resolution of inflammation and fibrosis is a highly co-ordinated, multifaceted process that is intended to eliminate remaining injurious agents responsible for the initial insult and shift the balance from a pro-inflammatory to an anti-inflammatory microenvironment (Figure 3). This is achieved through a sequence of events where selected immune cell populations are removed through apoptosis/necrosis/efferocytosis accompanied by recruitment and differentiation of pro-resolution immune subsets such as macrophages. Homeostasis is then restored

following repopulation of the injured area through regeneration of the hepatocyte pool, repopulation of the Kupffer cell niche and maintenance of hepatic tolerance, for example through T_{reg} recruitment and retention.

Immune cell intervention

Resolution of fibrosis is usually ascribed to the function of a specific macrophage population that secrete a range of pro-resolution mediators including matrix metalloproteinases, such as MMP-13[117], which promote the degradation of scar tissue. Duffield and co-workers used a transgenic CD11b-DTR mouse to selectively deplete $CD11b^{hi}$ macrophages in a reversible CCl_4-induced model of liver injury and described a biphasic injurious response; depletion of macrophages during ongoing injury reduced the extent of tissue damage, whereas depletion of the macrophage population following withdrawal of the toxin delayed recovery[118]. Building on these preliminary observations, hepatic macrophages have been shown to transition from pro-inflammatory $Ly6C^{hi}CCR2^{hi}CX_3CR1^{lo}$ expressing populations to pro-reparative $Ly6C^{lo}CCR2^{lo}CX_3CR1^{hi}$ subsets in mice, a process thought to be dependent on IL-4, IL-10 and phagocytosis[24,119]. Development of cellular therapy for liver cirrhosis through the provision of human phagocytic macrophage populations ($CD163^{hi}CD169^{hi}CD206^{hi}CCR2^{lo}$) is underway, with potential advantages over conventional monotherapeutic intervention strategies[120,121].

Adhesion receptors may also play a dual role in both the establishment and resolution of hepatic injury. Stabilin-1 has been discussed in the context of leukocyte recruitment, but this molecule is also expressed by a highly phagocytic macrophage population during resolution of chronic liver disease where it serves to limit further

inflammation and fibrosis by scavenging products of lipid peroxidation and suppressing secretion of CCL3[122]. Similar roles for other scavenger receptors are highly likely within the context of inflammatory liver disease[123].

Bile acids can signal through two major receptor pathways that regulate hepatic lipid and glucose metabolism, namely farnesoid X receptor (FXR) and TGR5 (a G protein-coupled bile acid receptor). Treatment of mice with the dual FXR/TGR5 agonist INT-767 induced a restorative intrahepatic macrophage phenotype ($Ly6C^{lo}CD206^{hi}$ and expression of *Retnla* and *Clec7a*)[124]. Provision of agonists for FXR and TGR5 have been suggested as potential therapeutics during liver regeneration where there is an excess bile acid pool[125] in NASH[126] or in cholestatic liver diseases[127] although some caution is required given the pleiotropic effects of these receptors, such as the role of TGR5 in the development of cholangiocarcinoma[128].

During acute liver failure (ALF), a marked increase in inflammatory macrophages is observed in areas of necrosis. However, patients with ALF exhibit an expanded population of macrophages with a resolution-like phenotype with suppressed innate and enhanced efferocytic/phagocytic responses that are present in both circulatory and tissue compartments. This functional switch was associated with the expression of the TAM family member Mer tyrosine kinase ($MerTK^{+}HLA\text{-}DR^{high}$) induced by secretory leukocyte protease inhibitor (SLPI) produced within the inflamed liver of both mice and humans following ALF. Such reprogramming of the myeloid population promotes neutrophil apoptosis and subsequent clearance through enhanced efferocytosis, and may be a target for future therapies[129]. Hepatocytes (and other liver resident cells) are also able to remove apoptotic and necrotic cells by

efferocytosis, although the relative contributions of this process to the resolution of chronic liver injury has not been determined fully[130].

Macrophages are not the sole mediators of hepatic resolution. NK cell cytotoxicity against early-activated or senescent-activated HSC via NK cell activating ligands (RAE-1 in mice; MICA in human), TRAIL receptors and production of IFN-γ, an inhibitor of HSC activation, promotes the resolution of liver injury[131]. Invariant NKT cells are thought to promote HSC killing, but can also be activated at the site of injury by self-antigens, leading to the production of IL-4 (but not IFN-γ), driving hepatocyte proliferation, a shift in the macrophage population from $Ly6C^{hi}$ to $Ly6C^{lo}$ expression and improved healing responses[132]. In mice, the regeneration of LSEC is dependent on the relative expression of the CXCL12 receptors CXCR4-7. During injury constitutive FGFR1 signalling increased the ratio of CXCR4:CXCR7 expression by LSEC, leading to an altered angiocrine response and proliferation of the stromal cell niche. Conversely, during resolution CXCR7 upregulation acts in concert with CXCR4 to induce the transcription factor Id1 with concomitant release of regenerative angiocrine factors and promotion of a pro-resolution environment[133].

Hepatic regeneration

Cellular repopulation of the hepatic niche following injury is essential to maintain not only the metabolic function of the organ, but also the ability to detoxify xenobiotics. Regeneration of the hepatocyte population is promoted by Kupffer cells through the production of IL-6 and TNF-α, driven by local recruitment of neutrophils in an ICAM-1 dependent process[134-136], production of complement proteins C3a and C5a[137] and local provision of growth factors such as HGF, VEGF and IL-1a[138]. Repopulation of

the hepatic niche usually occurs through self-replication of hepatocytes; however, in chronic liver disease hepatocyte proliferation is often impaired (for example through immune cell-derived IFN-γ [131,139,140]). Under these circumstances, the hepatocyte pool may be supplemented through a ductular reaction that regenerates functional hepatocytes from biliary cells, with important implications for therapeutic restoration of liver function[141].

Conclusion

We have highlighted several pathways and targets which could potentially contribute to new therapies for inflammatory liver disease. It is likely that combination therapies will be required to achieve significant clinical end points in terms of fibrosis regression and improvement in overall survival. An additional consideration is the dynamic and complex cycle of maldaptive wound repair which characterises advanced liver disease. It will be crucial that anti-inflammatory treatment for liver disease involves a personalised/precision medicine approach taking into account the stage of disease, inflammatory infiltrate and potential of driving fibrosis resolution. Whilst the benefits of inhibiting inflammation and driving resolution in chronic liver diseases are clear, the chronic nature of most liver diseases and the unique microenvironment of the liver promote the development of HCC. The future of developing novel anti-inflammatory agents in liver disease needs to take into account the potential of promoting HCC in the setting of subclinical malignancy or carcinoma-*in situ*. Previous studies have highlighted this potential risk in the setting of hepatitis C eradication with direct acting anti-viral therapy[142] and it is now becoming clear that HCC thrives in immunosuppressive microenvironments[143]. It is therefore important that we dedicate further research into understanding in which situations the

approach of suppressing inflammation in patients who have suffered liver disease for many years could potentially promote HCC. Nevertheless, we remain hopeful that the progress which has been made in understanding the regulators of inflammation in the liver microenvironment will lead to successful therapies to prevent the progression/reverse chronic liver disease.

Figure Legends

Figure 1 **Immune response to danger signals released from chronic epithelial injury**

Chronic epithelial damage in the liver leads to cellular stress and the release of danger signals. Pro-inflammatory pathways are triggered by Kupffer cell recognition of these danger signals by receptors including TLR-4, galectin 3 and CD36 as well as activation of the inflammasome. Subsequent recruitment of CCR2+ monocytes into liver tissue from the circulation leads to exacerbation of fibrogenesis. Unconventional T cells also play an important role in sensing cellular stress at epithelial surfaces. CCR6+ $\gamma\delta$ T cells prevent fibrosis by promoting hepatic stellate cell apoptosis whereas NKT cells and MAIT promote fibrogenesis with NKT cells releasing pro fibrogenic factors such as osteopontin and hedgehog ligands and MAIT cells activating proinflammatory and profibrogenic pathways in macrophages and hepatic stellate cells. DAMPS, danger associated molecular patterns; HMGB1, high mobility group protein B1; MDA-LDL, Malondialdehyde- low density lipoprotein; ATP, adenosine triphosphate; NLRP3, NOD-, LRR- and pyrin domain-containing 3; NKT cell, natural killer T cell; MAIT cell, mucosal associated invariant T cell; HSC, hepatic stellate cell; ECM; extracellular matrix.

Figure 2 **Lymphocyte recruitment and retention within the hepatic sinusoids during chronic liver injury**

All progressive chronic inflammatory liver diseases are associated with recruitment and retention of circulating lymphocytes into liver tissue. This recruitment occurs within the low shear stress environment of the hepatic sinusoids, where lymphocyte recruitment is triggered by selectin-independent capture and firm adhesion by VCAM-1 supported by CD151 on the endothelial surface. Other factors promote lymphocyte subset specific recruitment including aberrant adhesion of gut-homing lymphocytes (alpha4beta7+) to MAdCAM-1 and CD4 lymphocytes adhesion mediated by SCARF1. Presentation of chemokines including IP-10 to $CXCR3^+$ T cells and CXCL16 to $CXCR6^+$ T cells triggers activation and migration of T cells. The subsequent transendothelial migration step involves a combination of receptors including the atypical adhesion molecule VAP-1 with Treg specific recruitment occurring via transcellular pathway mediated by VAP-1 and stabilin-1. HSCs contribute to subendothelial retention of lymphocytes through the release of several chemotactic factors and contribution from VAP-1. T cell subset positioning in liver tissue is further regulated by chemokines including CCL20 for Th17 cells and CCL17 and CCL22 for Tregs. VCAM-1, vascular adhesion molecule-1; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; SCARF1, scavenger receptor class F, member 1; IP-10, interferon gamma-induced protein 10; VAP-1, vascular adhesion protein-1.

Figure 3 **Pathways which promote fibrosis resolution and liver regeneration**

The liver has the capacity to promote resolution of fibrosis and regenerative pathways. Kupffer cells have the capability to promote hepatocyte regeneration

through the release of several factors including IL-6 and TNFα. Liver sinusoidal endothelium can promote a pro regenerative pathway rather than pro-fibrotic through the upregulation of CXCR7 which induces the transcription factor Id1 leading to proregenerative angiocrine factors. NK cells can contribute to fibrosis resolution by directly killing senescence activated HSCs. Macrophages also play a pivotal role in fibrosis resolution through the release of several factors including MMP13 which degrades scar tissue. A key role is played by a subset of macrophages characterised by the pro-resolution phenoptype $Ly6C^{lo}CCR2^{lo}CX_3CR1^{hi}$. In chronic liver injury, uptake of products of lipid peroxidation such as oxLDLs by macrophages expressing stabilin-1 suppresses the release of pro-fibrotic factors. During acute liver injury the release of SLPI leads to the upregulation of MerTK on macrophages which promotes neutrophil apoptosis and subsequent clearance leading to resolution of inflammation. NK cell, natural killer cell; MMP-13, metalloproteinase-13; oxLDL, oxidised low density lipoprotein; SLPI, secretory leukocyte protease inhibitor; MerTK, Mer tyrosine kinase.

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