

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

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DOI:

[10.1055/s-0039-1678727](https://doi.org/10.1055/s-0039-1678727)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Patten, DA, Shepherd, EL, Weston, CJ & Shetty, S 2019, 'Novel targets in the immune microenvironment of the hepatic sinusoids for treating liver diseases', *Seminars in Liver Disease*, vol. 39, no. 2, pp. 111-123.
<https://doi.org/10.1055/s-0039-1678727>

[Link to publication on Research at Birmingham portal](#)

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Checked for eligibility: 03/05/2019

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

3

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26

27 **Abstract**

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

46

47 **Main Concepts and Learning Points**

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

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

63

64

65 Adult inflammatory liver diseases lead to a major global burden on human health,
66 and patients with progressive disease are at risk of developing fibrosis and cirrhosis
67 which can culminate in end-stage liver failure or hepatocellular cancer (HCC), both of
68 which are associated with extremely high mortalities¹. Recent advances have been
69 made in the treatment of liver disease, especially in the field of viral hepatitis. The
70 development of direct-acting antivirals for the treatment of hepatitis C has
71 demonstrated very high rates of viral eradication². In the case of hepatitis B, current
72 therapies are effective at suppressing viral replication and can reduce
73 necroinflammation with reversal of fibrosis as well as reducing HCC risk^{3,4}. In
74 contrast, the inflammatory processes that drive other major liver diseases such as
75 alcoholic liver disease, non-alcoholic steatohepatitis and cholangiopathies have

76 continued to be a major therapeutic challenge. For those patients who progress to
77 advanced chronic liver disease there are limited options when they develop end-
78 stage liver disease, with transplantation being the only choice in many cases⁵. New
79 therapies are therefore urgently required to reduce the burden on transplantation and
80 the associated high waiting list mortality.

81

82 Adult chronic liver diseases are driven by inflammation, which promotes epithelial
83 damage and death leading to the activation of resident immune cells and the
84 accumulation of circulating immune cells recruited from the circulation^{6,7}. Each
85 disease has a specific pattern of injury which is dependent on the site of initial
86 damage. For example, NASH is triggered by hepatocyte damage characterised by
87 sublethal injury associated with lipotoxicity, resulting in parenchymal inflammation
88 associated with innate and adaptive immune responses⁸. In contrast, primary
89 sclerosing cholangitis is driven by cholangiocyte injury leading to the localised
90 release of chemokines and pro-inflammatory cytokines associated with portal
91 inflammation and ductal proliferation and ductular loss⁹. These inflammatory
92 processes are associated with the activation of hepatic stellate cells and if left
93 unchecked lead to excessive deposition of extracellular matrix, fibrosis and
94 persistent damage culminating in cirrhosis¹⁰. The site of injury determines the pattern
95 of fibrosis with parenchymal diseases such as ALD/NASH presenting centrilobular
96 and sinusoidal fibrosis and cholangiopathies associated with periportal fibrosis
97 leading to irregular shaped nodules¹¹.

98 Targeting the inflammatory pathways that drive these conditions has the potential of
99 inhibiting fibrogenesis, but the mechanisms involved are poorly understood.

100 Autoimmune hepatitis for example is often responsive to steroid-based therapy and

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

109

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

114

115 **The immune response to danger signals released from epithelial damage.**

116 Epithelial damage is a key factor in initiating inflammatory liver diseases. This
117 involves cellular stress secondary to factors such as lipotoxicity in fatty liver disease,
118 accumulation of breakdown products of alcohol and hepatotropic viruses. These
119 processes are associated with the release of danger signals or danger associated
120 molecular patterns (DAMPs) into the microenvironment. How these danger signals
121 are sensed and processed by the innate immune system is one of the key
122 determinants of progression of these inflammatory conditions¹³ (summarised in
123 Figure 1).

124

125 *Kupffer cell recognition of DAMPS*

126 The major cellular population to sense and respond to these danger signals are the
127 liver resident macrophages, Kupffer cells. Kupffer cells are the sentinels of the liver
128 and are derived from yolk sac precursors which self renew¹⁴. They play a role in
129 processing gut-derived products and mediating immune responses to microbes.
130 Additionally, they sense sterile injury and associated DAMPS which are a
131 characteristic of the major inflammatory liver diseases including alcoholic
132 steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH). DAMPs which
133 have been associated with Kupffer cell activation include high mobility group protein
134 B1 (HMGB1), ATP, uric acid, DNA fragments and cholesterol crystals¹⁴. Targeting
135 the pathway of DAMP recognition is already underway in clinical trials. DAMPs are
136 recognised by pattern recognition receptors including Toll-like receptors (TLRs) and
137 scavenger receptors which are both highly expressed by macrophages. TLR4 has
138 been studied extensively and a TLR4 antagonist, JKB-122, is currently undergoing
139 assessment in the setting of NASH as an early phase II clinical trial NCT02442687.
140 Galectin-3 expressed on Kupffer cells which is a member of the scavenger receptor
141 family which recognise the terminal galactose residues on glycoproteins. Galectin-3
142 plays a key role in hepatic uptake of advanced lipoxidation and glycation end
143 products¹⁵. An agent which binds galectin-3, GR-MD-02, is progressing through
144 early stage clinical trials in the setting of NASH¹⁶. Other members of the scavenger
145 receptor family which have been implicated in promoting hepatic inflammation
146 include CD36 and Scavenger Receptor-A (SR-A). Targeted deletion of these
147 receptors on myeloid cells, led to reduced levels of inflammation and fibrosis in
148 models of fatty liver disease¹⁷. A recent study confirmed that the recognition of
149 DAMPs, specifically products of lipid peroxidation such as malondialdehyde (MDA)-
150 LDL, by CD36 and SR-A led to the release of pro-inflammatory cytokines¹⁸. Blocking

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

155

156 *The role of the inflammasome in chronic liver disease*

157 Whilst the direct recognition of DAMPs is a viable pathway to target, there are also
158 downstream pathways which play significant roles in the progression of chronic liver
159 disease. The recognition of these danger associated ligands by pattern recognition
160 receptors on Kupffer cells leads to the formation of the inflammasome.
161 Inflammasomes are multi-protein complexes which are comprised of a nucleotide
162 oligomerization domain (NOD)-like receptors and effector molecules including pro-
163 caspase-1, and adaptor molecules e.g. apoptosis-associated speck-like CARD-
164 domain containing protein (ASC)¹⁹. Following the formation of the inflammasome,
165 Kupffer cells produce inflammatory mediators, such as interleukin 1beta and other
166 pro-inflammatory cytokines and chemokines. Activation of inflammasome complexes
167 have been confirmed in pre-clinical models of alcoholic liver injury and fatty liver
168 disease²⁰. This leads to the recruitment of other innate populations from the
169 circulation such as neutrophils, monocytes and populations of T cells. Therefore
170 targeting the pathway of inflammasome formation is also a rational approach to
171 prevent progression of inflammatory liver diseases. Studies in pre-clinical models of
172 alcoholic liver disease demonstrated that targeting the inflammasome pathway by
173 pharmacological inhibition of IL-1R1 prevented the development and progression of
174 alcoholic liver disease²¹. Additionally, one the most extensively studied
175 inflammasomes in macrophages is the NOD-, LRR- and pyrin domain-containing 3

176 (NLRP3) inflammasome which has previously been shown to play a critical role in
177 the progression of murine models of non-alcoholic fatty liver disease²². A recent
178 study confirmed its role in driving liver inflammation and fibrogenesis by studying
179 liver injury in mice with constitutive activation of NLRP3 in myeloid cells. Activation
180 of the NLRP3 inflammasome led to excess production of TNF and IL-17 resulting in
181 severe inflammation and fibrosis²³.

182

183 *Recruitment of peripheral monocyte populations*

184 Another major downstream consequence of Kupffer cell driven inflammation is the
185 recruitment of other monocyte populations from the circulation via the CCL2-CCR2
186 axis^{24,25}. The chemokine CCL2 promotes recruitment of CCR2⁺ monocytes from the
187 circulation, and this has been confirmed in experimental models of both alcoholic
188 liver disease and fatty liver disease²⁶. A recent study confirmed the increased
189 accumulation of CCR2⁺ macrophages within liver tissue parallels with fibrosis
190 progression in fatty liver disease. These populations of cells were seen as
191 aggregates of monocyte-derived macrophages around portal tracts²⁷. Furthermore,
192 gene analysis of these recruited (monocyte-derived macrophages) versus resident
193 (Kupffer cells) confirmed that monocyte-derived macrophages were associated with
194 multiple growth factors and cytokines leading to fibrosis progression, whereas
195 Kupffer cells were characterised by factors associated with inflammation initiation.
196 Therapeutic targeting of the recruitment of these CCR2⁺ monocytes by
197 administration of Cenicriviroc a CCR2/CCR5 dual chemokine receptor antagonist led
198 to amelioration of hepatic inflammation and fibrosis in several models of NASH²⁷. In
199 keeping with these findings, there is encouraging clinical experience that Cenicriviroc
200 could be a potential therapy for chronic liver disease. A phase 2b study of this agent

201 in patients with non-alcoholic steatohepatitis and established fibrosis demonstrated a
202 significant improvement in fibrosis compared to placebo after 1 year of treatment²⁸.
203 Activated Kupffer cells also secrete several other chemokines including CCL25,
204 CX3CL1, CXCL2 and CXCL8¹⁴; thus, targeting these chemokines may also influence
205 the recruitment of other distinct immune populations from the circulation during
206 inflammatory liver disease leading to other novel targets for treatment. An intriguing
207 recent study has also identified the recruitment of immune populations from the
208 peritoneal compartment. In a model of sterile liver injury a population of GATA6-
209 positive macrophages were detected at a very early stage of tissue damage. These
210 GATA6⁺ macrophages migrated directly across the mesothelium and their
211 recruitment was dependent on the adhesion molecule CD44 and adenosine
212 triphosphate²⁹. The role of these macrophages in the progression of chronic
213 inflammatory liver diseases and their therapeutic potential is yet to be confirmed.

214

215 *The activation of unconventional lymphocytes*

216 In parallel to the initiation of inflammation by myeloid populations, there is gathering
217 interest in the role of unconventional lymphocytes which are found highly enriched in
218 epithelial tissues and have well established roles in anti-microbial immunity³⁰. Their
219 roles in early immune responses has led investigators to study if they could be
220 pivotal in the triggering and regulation of progressive liver disease. $\gamma\delta$ T cells are
221 predominantly generated in the thymus and characterised by a $\gamma\delta$ T cell receptor
222 (TCR), they only account for 2-3% of all CD3⁺ T cells in secondary lymphoid organs
223 but have been found to be enriched in the liver³¹. $\gamma\delta$ T cells recognise conserved
224 structures including non-peptide metabolites and heat shock proteins. They can
225 rapidly release cytokines which are known to regulate adaptive immune populations

226 including conventional $\alpha\beta$ T cells and therefore have been postulated as an
227 additional link between innate and adaptive immune responses³². Experimental
228 models of liver disease have demonstrated the accumulation of these cells during
229 liver injury and their contribution to disease progression. In a murine model of
230 autoimmune hepatitis, $\gamma\delta$ T cells played a protective role associated with reduced
231 liver damage and inflammatory cytokine levels. In this setting the protective
232 mechanism was found to be regulated by IL-17 produced by $\gamma\delta$ T cells
233 downregulated the function of another family of unconventional T cells, natural killer
234 T (NKT) cells³³. Further support for the protective role of these cells in liver disease
235 has been demonstrated in models of chronic liver injury. Murine models of fibrosis
236 and steatohepatitis demonstrated that the CCR6⁺ subset of $\gamma\delta$ T cells prevented
237 fibrosis by promoting the apoptosis of hepatic stellate cells³⁴.

238 As alluded to earlier, another subset of unconventional lymphocytes, NKT cells,
239 appear to promote inflammatory liver disease. NKT cells are lymphocyte subsets
240 which express cell surface markers associated with NK cells as well as the T cell
241 receptor and they are characterised by their recognition of glycolipid antigens. They
242 have been shown to localise to the hepatic sinusoids and demonstrate a
243 crawling/patrolling phenotype³⁵. NKT cells accumulated in models of liver injury and
244 were shown to promote hepatic inflammation and contributed to progressive
245 fibrosis³⁶. Further studies focused on the potential contribution of NKT cells to fatty
246 liver disease. Higher levels of NKT cells were detected in patients undergoing
247 transplantation for NASH compared to other indications, this accumulation was also
248 seen in murine models of NASH and mice deficient in NKT cells were protected from
249 fibrosis in this model³⁷. Subsequent studies implicated hepatic NKT cells in the

250 increased production pro-fibrogenic factors including osteopontin and hedgehog
251 ligands³⁸.

252 Further understanding of the functional properties of another unique subset of
253 innate-like T cells, mucosal-associated invariant T cells (MAIT) cells, has highlighted
254 their potential as regulators of liver inflammation. MAIT cells are characterised by
255 the expression of a semivariant TCR that recognises a MHC-like protein (MR-1)³⁹.
256 MR-1 presents vitamin B metabolites derived from commensal and pathogenic
257 bacteria and thus MAIT cells can be activated by a variety of bacterial strains⁴⁰. The
258 high levels of these cells in human gut biopsies and accumulation in lamina propria
259 led to them being named MAIT cells⁴¹. Subsequent studies have now shown that
260 they are also enriched in the liver and have explored their antimicrobial properties in
261 immune mediated liver disease and alcoholic liver disease⁴²⁻⁴⁴. This has led
262 investigators to speculate that MAIT cells may make a significant immune
263 contribution in the liver acting as a firewall between the host and gut derived
264 bacteria⁴⁵. However these reports have also shown that MAIT cells are highly
265 activated in the liver and are the predominant IL-17 producers within the hepatic T
266 cell compartment and could therefore be important drivers of aberrant hepatic
267 inflammation. A recent study has studied the contribution of these cells in chronic
268 liver injury. MAIT cells were found to be enriched in the periportal region and along
269 the fibrotic septa in tissue from cirrhotic livers and in a carbon tetrachloride model of
270 chronic liver injury these cells were found to be pro-fibrogenic by promoting the pro-
271 inflammatory properties of both monocyte-derived macrophages and fibroblasts⁴⁶.

272 Unconventional lymphocytes are therefore a novel target to treat chronic
273 inflammatory liver disease, but further work is clearly required to understand how to
274 either manipulate their function or utilise them as cell therapy.

275

276

277

278 **Lymphocyte recruitment via the liver sinusoids**

279 The accumulation of adaptive immune cell populations within the liver is also a
280 hallmark and driver of all adult chronic inflammatory liver diseases. A prerequisite
281 for leukocyte recruitment from the circulation into organs is their interaction with
282 endothelial cells lining blood vessels. In general, leukocyte migration from the blood
283 into inflamed tissues occurs in post-capillary venules⁴⁷; however, in the liver, this
284 process occurs in the low shear flow microvasculature of the hepatic sinusoids which
285 are lined by liver sinusoidal endothelial cells (LSEC)⁷ (Figure 2). LSECs are a
286 phenotypically and functionally unique population of endothelial cells. They are
287 characterised by a minimal basement membrane and atypical cellular junctions as
288 well as membranous pores organised in sieve plates called fenestrations⁴⁸.
289 Additionally, LSECs are also characterised by the expression of an array of
290 scavenger receptors (SRs)⁴⁹. These structural and phenotypic characteristics
291 support the physiological functions of LSEC but they also influence the mechanisms
292 of lymphocyte recruitment and thus are potential organ specific anti-inflammatory
293 targets. The low shear stress environment of the hepatic sinusoids negates the
294 requirement for the early rolling steps of the leukocyte adhesion cascade⁷. As a
295 consequence, LSEC express negligible levels of selectins⁵⁰, a small family of
296 transmembrane Ca²⁺-dependent lectins which play an integral role in the initial
297 stages of leukocyte recruitment in more conventional vascular beds⁵¹. A critical step
298 in determining if lymphocytes accumulate at sites of inflammation is not only their
299 adhesion to endothelium but also their subsequent transmigration across the

300 endothelial barrier. We now know that the process of transendothelial migration
301 (TEM) in itself is a multi-step pathway involving a combination of receptor
302 interactions which are potential therapeutic targets for inflammation⁵². The
303 conventional route for TEM by leukocytes is via the paracellular route (in between
304 cells, through cellular junctions), but it has also been shown that leukocytes can
305 migrate via the transcellular route (directly through the endothelial body)⁵³. Studies
306 on human LSEC demonstrate that a significant proportion of lymphocytes migrate via
307 the transcellular route⁵⁴. Additional *in vitro* studies, demonstrated that the structure
308 of these endothelial cells permits a novel migratory pattern, where lymphocytes were
309 shown to migrate directly into LSEC and then migrate into adjacent endothelial
310 cells⁵⁵. This migration was dependent on interferon gamma and facilitated by the
311 unique junctional complexes between LSEC. This work highlights that the sinusoidal
312 vascular bed is not a simple barrier but plays an active role in regulating the immune
313 microenvironment within the liver and the positioning of lymphocytes in liver tissue.
314 Further work has elucidated some the molecular contributors to this process and
315 their potential as novel anti-inflammatory targets.

316

317 *Conventional adhesion molecules*

318 Several studies have demonstrated that LSEC use a unique combination of both
319 conventional endothelial adhesion molecules, such as vascular cell adhesion
320 molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and atypical
321 adhesion molecules to mediate lymphocyte recruitment in chronic liver disease^{56,57}.
322 VCAM-1 binds the leukocyte-expressed $\alpha_4\beta_1$ integrin⁵⁸ and plays an important role
323 in capturing lymphocytes from blood flow within the hepatic sinusoids and
324 subsequently mediates stabilisation^{59,60}. ICAM-1 supports firm adhesion of

325 lymphocytes, via binding to $\alpha_L\beta_2$ integrin (lymphocyte function-associated molecule-1
326 (LFA-1))⁶¹, and subsequently mediates their transmigration across LSEC^{54,62}. Both
327 VCAM-1 and ICAM-1 are significantly upregulated by proinflammatory factors, such
328 as cytokines⁶³; however, their adhesive function is largely dependent on the
329 formation of endothelial adhesive platforms (EAPs)⁶⁴. EAPs play an essential role in
330 the spatial organisation of VCAM-1 and ICAM-1 within the cell membrane, resulting
331 in concentrated areas of expression of the adhesion molecules in the contact area
332 with adherent leukocytes⁶⁴. The formation of EAPs has been proposed to be
333 regulated by the tetraspanin family of receptors, which are able to laterally associate
334 with adhesion molecules to form microdomains^{64,65}. In support of this previous work,
335 the tetraspanin CD151 associated with VCAM-1 within LSECs and was able to
336 regulate lymphocyte adhesion under physiological flow conditions *in vitro*⁶⁶. Due to
337 their widespread constitutive expression in a number of cell types and tissues,
338 VCAM-1 and ICAM-1 are unlikely to represent viable therapeutic targets; however,
339 modulating their lateral interactions with tetraspanins, such as CD151, may present
340 an attractive and organ-specific target for chronic inflammatory liver disease.

341

342 Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), which belongs
343 to the immunoglobulin family along with VCAM-1 and ICAM-1, is known to bind to
344 the $\alpha_4\beta_7$ integrin⁶⁷ and plays an important role in lymphocyte trafficking to the gut, via
345 mucosal vessels⁶⁸. Under normal physiological conditions, MAdCAM-1 is absent
346 from the liver; however, previous studies have demonstrated that MAdCAM-1 can
347 be upregulated through the enzymatic activity of an atypical adhesion molecule,
348 vascular adhesion protein-1 (VAP-1), in LSEC in some chronic liver diseases⁶⁹. This
349 is particularly evident in primary sclerosing cholangitis (PSC), where it promotes the

350 recruitment of, gut-activated T cells which express high levels of $\alpha_4\beta_7$ integrin^{70,71}. Its
351 hepatic functionality is highly supportive of immunological crosstalk between the gut
352 and the liver, and MAdCAM-1 might contribute to the pathophysiological link
353 between inflammatory bowel disease (IBD) and PSC, a progressive autoimmune
354 biliary disease which is associated with IBD in ~80% of cases. Currently, clinical
355 trials are being considered to target MAdCAM-1/ $\alpha_4\beta_7$ interactions in PSC using
356 therapeutic antibodies originally developed for the treatment of IBD. Trials have
357 included a selective humanised monoclonal antibody, Vedolizumab, to $\alpha_4\beta_7$. Prior
358 clinical studies with Vedolizumab in the setting of IBD have confirmed that this drug
359 can modulate lymphocyte recruitment to the gut in both ulcerative colitis and Crohn's
360 disease leading to a reduction in inflammation and improved mucosal healing^{72,73}.
361 This has led to gathering interest in the use of Vedoluzimab in the setting of diseases
362 where MAdCAM-1 has been shown to be upregulated, particularly PSC. Until
363 recently, this had involved single centre case series with results suggesting safety
364 and improvement of inflammatory parameters⁷⁴. A multi-centre study has now been
365 completed in patients with PSC and IBD which demonstrated clinical responses in
366 the IBD pathology, and the drug was safely tolerated, but it did not lead to any
367 detectable improvement in liver biochemistry⁷⁵. Whether targeting the MAdCAM-
368 1/ $\alpha_4\beta_7$ interaction could improve long term outcomes in PSC, including prevention of
369 progressive fibrosis, transplant-free survival and cancer incidence, still needs to be
370 addressed.

371

372 *Atypical adhesion molecules*

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

375 smooth muscle cells, and adipocytes⁷⁶. During homeostasis VAP-1 is localised to
376 cytoplasmic vesicles in endothelial cells, but under inflammatory conditions the
377 protein is trafficked to the cell surface⁷⁷. Early studies of VAP-1 showed that it
378 mediated leukocyte binding to high endothelial venules (HEVs), the specialised post-
379 capillary venules found in lymph nodes⁷⁸. Further studies confirmed that VAP-1 was
380 expressed at high levels in chronically diseased liver tissues *ex vivo*⁷⁹ and directly
381 mediated adhesion and transmigration across LSEC *in vitro*⁸⁰. In addition, via its
382 enzyme activity, VAP-1 can upregulate expression of other adhesion molecules (e.g.
383 VCAM-1, ICAM-1 and MAdCAM-1) and chemokines (e.g. CXCL8) in LSECs,
384 consequently enhancing leukocyte recruitment^{69,81}. More recently, these results have
385 been corroborated *in vivo*, confirming the multifaceted role of VAP-1 in leukocyte
386 recruitment to the liver in murine models of liver injury, and described VAP-1
387 expression by hepatic stromal cell populations⁸². A number of preclinical studies
388 targeting VAP-1 have confirmed that inhibition of its enzymatic activity and/or
389 blockade of its adhesive function with therapeutic antibodies reduces leukocyte
390 infiltration in a range of rodent models of inflammatory diseases⁸³.

391

392 Scavenger receptor that binds phosphatidylserine and oxidized lipids (SR-PSOX),
393 which in its soluble form is also known as the chemokine, CXCL16, is expressed by
394 LSEC⁸⁴ and is upregulated in both acutely^{85,86} and chronically injured liver tissues⁸⁷.
395 CXCL16 is a specific ligand for the chemokine receptor CXCR6, thus enabling its
396 membrane-bound form to interact with intrahepatic CXCR6⁺ immune cells, such as
397 effector T cells^{87,88}, natural killer (NK) cells^{89,90} and NKT cells⁸⁴. Genetic deficiency of
398 SR-PSOX has recently been shown to reduce the extent of inflammation and
399 necrosis in a murine model of acetaminophen (APAP)-induced acute liver injury⁸⁵.

400 Additionally, and perhaps more encouragingly, pharmacological intervention with
401 neutralising antibodies against SR-PSOX has shown efficacy in reducing
402 inflammation in preclinical murine models of sepsis-mediated^{86,91} and carbon
403 tetrachloride (CCl₄)-mediated⁹² acute liver injury. Furthermore, Wehr and colleagues
404 were also able to demonstrate the efficacy of SR-PSOX antibody therapy in a
405 commonly used murine model of non-alcoholic steatohepatitis (NASH), showing a
406 reduction in both macrophage infiltration and triglyceride levels. Therefore, targeting
407 the SR-PSOX (CXCL16)/CXCR6 axis may hold promising potential for treatment of
408 inflammation and subsequent fibrosis of the liver⁹².

409

410 The class H scavenger receptor stabilin-1, also known as common lymphatic
411 endothelial and vascular endothelial cell receptor (CLEVER-1), was originally shown
412 to mediate lymphocyte transmigration across HEVs⁹³. Given the phenotypic
413 similarities between lymphatic endothelial cells and LSEC⁵⁰, stabilin-1 was found to
414 be expressed in human liver and shown to be significantly upregulated in the hepatic
415 sinusoids in chronic liver disease⁵⁴. Following this, adhesion assays with lymphocyte
416 subsets demonstrated that stabilin-1 specifically mediated transendothelial migration
417 of T_{regs} and B-cells through LSECs *in vitro*, under conditions which mimic the
418 physiological flow and proinflammatory microenvironment of the hepatic sinusoids
419 during liver injury^{54,62}. This was the first demonstration of a T_{reg}-specific adhesion
420 molecule and transmigration of this lymphocyte subset was shown to be dependent
421 on a combination of stabilin-1, VAP-1 and ICAM-1. T_{regs} play a vital role in promoting
422 tolerance, they mediate immunosuppression through multiple mechanisms and
423 prevent autoimmunity and counteract inflammatory reactions mediated by the
424 effector arm of the immune system⁹⁴. Therefore, in the context of inflammatory liver

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

427

428 The expression of the stabilin-1 homologue, stabilin-2, has also been described in
429 LSEC and was originally shown to act as a clearance receptor for hyaluronan from
430 the blood^{95,96}. Through a number of mutation experiments and antibody blockade
431 studies *in vitro*, Jung *et al.* found that stabilin-2 was also able to mediate lymphocyte
432 binding and identified the integrin $\alpha_M\beta_2$ as the lymphocyte-expressed ligand⁹⁷. They
433 also determined that stabilin-2 predominantly acts in the firm adhesion step of the
434 leukocyte adhesion cascade as its silencing, via shRNA, did not affect lymphocyte
435 rolling or transendothelial migration, but was still able to significantly reduce the
436 number of adherent cells⁹⁷. To date, the study by Jung *et al.* remains the sole
437 investigation of the role of stabilin-2 in leukocyte recruitment to LSEC. Further work
438 is required to understand how the stabilin receptor family expressed on LSEC
439 contribute to lymphocyte recruitment in preclinical models of inflammatory liver
440 disease.

441

442 Scavenger receptor class F, member 1 (SCARF1 or SR-F1), also known as
443 scavenger receptor expressed by endothelial cells (SREC-I), has also been shown to
444 be expressed in both murine and human LSEC^{98,99}. Recently, it has been shown that
445 SCARF1 plays a role in the selective recruitment of CD4⁺ T cells to human LSEC,
446 under physiological shear stress conditions *in vitro*⁹⁹. In this study, SCARF1
447 contributed to the firm adhesion step of the leukocyte adhesion cascade, with
448 endothelial surface expression of SCARF1 observed in adhesive cup structures
449 formed on the surface of the LSEC⁹⁹. However, SCARF1 is an understudied

450 scavenger receptor¹⁰⁰ and more research into the extent of the contribution of
451 SCARF1 in immune cell recruitment is required before it can be considered as a
452 therapeutic target. Nevertheless, SRs including SCARF1 have been shown to be
453 upregulated in several human inflammatory liver diseases and appear to accumulate
454 at the interface between inflammation/fibrosis and correlate with fibrosis progression.

455

456 *Chemokines*

457 Chemokines are an important component in the process of leucocyte recruitment
458 and contribute to both firm of adhesion of leukocytes to endothelium and their
459 subsequent migration across the endothelium. They are a family of small proteins
460 which bind to G-protein coupled receptors on the leukocyte surface and induce
461 conformational changes of integrins which triggers firm adhesion¹⁰¹. They are also
462 found within intraendothelial vesicles and promote transendothelial migration¹⁰². We
463 have already highlighted their role in monocyte and NK/NKT populations but they
464 also play a significant role on lymphocyte recruitment within the sinusoids. The most
465 extensively investigated are the inflammatory chemokines CXCL9-11 which bind to
466 the receptor CXCR3 and have been shown to be upregulated in a range of liver
467 diseases¹⁰³⁻¹⁰⁵ and functionally they contribute to the transendothelial migration of
468 lymphocytes across primary human HSEC¹⁰³. Previous studies have also shown
469 that chemokines contribute to the compartmentalisation of lymphocytes in liver
470 diseases with the CXCR3 ligands promoting recruitment into the parenchyma
471 whereas CCR5 ligands (the chemokines CCL3-5) contribute to portal tract
472 recruitment^{103,106,107}. The contribution of chemokines to inflammation provides a
473 clear rationale for targeting them as novel anti-inflammatories but a recent study
474 highlights the difficulties of achieving sustained inhibition of chemokines. NI-0801 is

475 a human monoclonal antibody against the CXCR3 ligand, CXCL10, which was
476 studied in the context of PBC¹⁰⁸. Investigators completed a phase 2a study in
477 patients with PBC with inadequate response to ursodeoxycholic acid with the aim of
478 assessing the safety and efficacy of NI-0801. The study demonstrated that the drug
479 was safely tolerated and led to pharmacological responses in the blood but there
480 was no therapeutic benefit identified with repeated infusions.

481

482 An alternative approach would be to consider targeting lymphocyte subsets, focusing
483 on pro-inflammatory subsets and allowing persistent recruitment of regulatory
484 subsets in order to shift the balance in the hepatic microenvironment. Whilst CXCR3
485 ligands have been implicated in the recruitment of several subsets including both
486 T_{regs} cells and subsets which secrete the pro-inflammatory cytokine IL-17 (Th17
487 cells)^{109,110}, other chemokines were implicated in the subsequent migration into
488 hepatic tissue of these subsets. T_{reg} recruitment was regulated by the CCR4 ligands
489 CCL17 and CCL22, whereas Th17 recruitment was regulated by CCL20, a CCR6
490 ligand^{109,110}. In view of these findings, targeting the chemokine CCL20 rather than
491 CXCR3 ligands may prove to be a more effective anti-inflammatory approach which
492 will not alter T_{reg} recruitment. Recent studies highlight the importance of the
493 Th17/Treg balance in determining progressive inflammatory liver disease¹¹¹⁻¹¹³.

494

495 **Retention of immune cells in the stromal compartment**

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

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

510

511 *In vitro* activated primary human HSCs and *in vivo* activated liver myofibroblasts
512 (aLMFs) secrete a range of cytokines, chemokines and growth factors which can
513 recruit and position leukocytes by G-coupled receptor-dependent and –independent
514 mechanisms¹¹⁵. When cultured in basal conditions, aLMFs and HSC secreted high
515 levels of IL-6, HGF, VEGF, CCL2, and CXCL8 under control conditions and
516 stimulation with pro-inflammatory cytokines TNF α and IFN γ enhanced all factors and
517 induced secretion of additional chemokines including CCL5, CXCL9 and CXCL10.
518 Moreover, aLMF- and HSC-conditioned supernatants promoted strong and rapid
519 migration of lymphocytes towards these chemotactic factors under pro-inflammatory
520 conditions and stimulated increased recruitment of lymphocytes across adjacent
521 LSEC monolayers. These findings demonstrated that there are signals from HSCs
522 which can recruit infiltrating immune cells which may be targeted to halt the
523 progression of fibrogenesis. One such target which we have already discussed in the
524 context of inflammation is VAP-1. VAP-1 is a dual functioning entity which, as

525 described, acts as an adhesion molecule as well as an enzyme which has a role in
526 recruiting lymphocytes across endothelial cells⁸⁰. More recent *in vivo* studies
527 described a novel role of VAP-1 in hepatic inflammation and fibrogenesis through
528 modulating HSC phenotype¹¹⁶. Soluble VAP-1 secreted by HSCs was enzymatically
529 active and was able to recruit lymphocytes. VAP-1 modulation in the HSC cell line
530 LX-2 increased transcription of profibrogenic genes such as collagen 1a1 as well as
531 enhancing wound healing. These data were supported by murine models of liver
532 injury in which VAP-1 knockout animals had less inflammation and fibrosis in
533 response to injury¹¹⁶. The blockade of VAP-1 to treat primary sclerosing cholangitis
534 (PSC) is currently being evaluated in the phase II clinical trial BUTEO (BUTEO
535 NCT02239211).

536

537 **Inflammatory pathways which promote fibrosis resolution and liver** 538 **regeneration**

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

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

553

554 *Immune cell intervention*

555 Resolution of fibrosis is usually ascribed to the function of a specific macrophage
556 population that secrete a range of pro-resolution mediators including matrix
557 metalloproteinases, such as MMP-13¹¹⁷, which promote the degradation of scar
558 tissue. Duffield and co-workers used a transgenic CD11b-DTR mouse to selectively
559 deplete CD11b^{hi} macrophages in a reversible CCl₄-induced model of liver injury and
560 described a biphasic injurious response; depletion of macrophages during ongoing
561 injury reduced the extent of tissue damage, whereas depletion of the macrophage
562 population following withdrawal of the toxin delayed recovery¹¹⁸. Building on these
563 preliminary observations, hepatic macrophages have been shown to transition from
564 pro-inflammatory Ly6C^{hi}CCR2^{hi}CX₃CR1^{lo} expressing populations to pro-reparative
565 Ly6C^{lo}CCR2^{lo}CX₃CR1^{hi} subsets in mice, a process thought to be dependent on IL-4,
566 IL-10 and phagocytosis^{24,119}. Development of cellular therapy for liver cirrhosis
567 through the provision of human phagocytic macrophage populations
568 (CD163^{hi}CD169^{hi}CD206^{hi}CCR2^{lo}) is underway, with potential advantages over
569 conventional monotherapeutic intervention strategies^{120,121}.

570

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

575 inflammation and fibrosis by scavenging products of lipid peroxidation and
576 suppressing secretion of CCL3¹²². Similar roles for other scavenger receptors are
577 highly likely within the context of inflammatory liver disease¹²³.

578

579 Bile acids can signal through two major receptor pathways that regulate hepatic lipid
580 and glucose metabolism, namely farnesoid X receptor (FXR) and TGR5 (a G protein-
581 coupled bile acid receptor). Treatment of mice with the dual FXR/TGR5 agonist INT-
582 767 induced a restorative intrahepatic macrophage phenotype (Ly6C^{lo}CD206^{hi} and
583 expression of *Retnla* and *Clec7a*)¹²⁴. Provision of agonists for FXR and TGR5 have
584 been suggested as potential therapeutics during liver regeneration where there is an
585 excess bile acid pool¹²⁵ in NASH¹²⁶ or in cholestatic liver diseases¹²⁷ although some
586 caution is required given the pleiotropic effects of these receptors, such as the role of
587 TGR5 in the development of cholangiocarcinoma¹²⁸.

588

589 During acute liver failure (ALF), a marked increase in inflammatory macrophages is
590 observed in areas of necrosis. However, patients with ALF exhibit an expanded
591 population of macrophages with a resolution-like phenotype with suppressed innate
592 and enhanced efferocytic/phagocytic responses that are present in both circulatory
593 and tissue compartments. This functional switch was associated with the expression
594 of the TAM family member Mer tyrosine kinase (MerTK⁺HLA-DR^{high}) induced by
595 secretory leukocyte protease inhibitor (SLPI) produced within the inflamed liver of
596 both mice and humans following ALF. Such reprogramming of the myeloid
597 population promotes neutrophil apoptosis and subsequent clearance through
598 enhanced efferocytosis, and may be a target for future therapies¹²⁹. Hepatocytes
599 (and other liver resident cells) are also able to remove apoptotic and necrotic cells by

600 efferocytosis, although the relative contributions of this process to the resolution of
601 chronic liver injury has not been determined fully¹³⁰.

602

603 Macrophages are not the sole mediators of hepatic resolution. NK cell cytotoxicity
604 against early-activated or senescent-activated HSC via NK cell activating ligands
605 (RAE-1 in mice; MICA in human), TRAIL receptors and production of IFN- γ , an
606 inhibitor of HSC activation, promotes the resolution of liver injury¹³¹. Invariant NKT
607 cells are thought to promote HSC killing, but can also be activated at the site of injury
608 by self-antigens, leading to the production of IL-4 (but not IFN- γ), driving hepatocyte
609 proliferation, a shift in the macrophage population from Ly6C^{hi} to Ly6C^{lo} expression
610 and improved healing responses¹³². In mice, the regeneration of LSEC is dependent
611 on the relative expression of the CXCL12 receptors CXCR4-7. During injury
612 constitutive FGFR1 signalling increased the ratio of CXCR4: CXCR7 expression by
613 LSEC, leading to an altered angiocrine response and proliferation of the stromal cell
614 niche. Conversely, during resolution CXCR7 upregulation acts in concert with
615 CXCR4 to induce the transcription factor Id1 with concomitant release of
616 regenerative angiocrine factors and promotion of a pro-resolution environment¹³³.

617

618 *Hepatic regeneration*

619 Cellular repopulation of the hepatic niche following injury is essential to maintain not
620 only the metabolic function of the organ, but also the ability to detoxify xenobiotics.
621 Regeneration of the hepatocyte population is promoted by Kupffer cells through the
622 production of IL-6 and TNF- α , driven by local recruitment of neutrophils in an ICAM-1
623 dependent process¹³⁴⁻¹³⁶, production of complement proteins C3a and C5a¹³⁷ and
624 local provision of growth factors such as HGF, VEGF and IL-1a¹³⁸. Repopulation of

625 the hepatic niche usually occurs through self-replication of hepatocytes; however, in
626 chronic liver disease hepatocyte proliferation is often impaired (for example through
627 immune cell-derived IFN- γ ^{131,139,140}). Under these circumstances, the hepatocyte
628 pool may be supplemented through a ductular reaction that regenerates functional
629 hepatocytes from biliary cells, with important implications for therapeutic restoration
630 of liver function¹⁴¹.

631

632 Conclusion

633 We have highlighted several pathways and targets which could potentially contribute
634 to new therapies for inflammatory liver disease. It is likely that combination therapies
635 will be required to achieve significant clinical end points in terms of fibrosis
636 regression and improvement in overall survival. An additional consideration is the
637 dynamic and complex cycle of maldaptive wound repair which characterises
638 advanced liver disease. It will be crucial that anti-inflammatory treatment for liver
639 disease involves a personalised/precision medicine approach taking into account the
640 stage of disease, inflammatory infiltrate and potential of driving fibrosis resolution.
641 Whilst the benefits of inhibiting inflammation and driving resolution in chronic liver
642 diseases are clear, the chronic nature of most liver diseases and the unique
643 microenvironment of the liver promote the development of HCC. The future of
644 developing novel anti-inflammatory agents in liver disease needs to take into account
645 the potential of promoting HCC in the setting of subclinical malignancy or carcinoma-
646 *in situ*. Previous studies have highlighted this potential risk in the setting of hepatitis
647 C eradication with direct acting anti-viral therapy¹⁴² and it is now becoming clear that
648 HCC thrives in immunosuppressive microenvironments¹⁴³. It is therefore important
649 that we dedicate further research into understanding in which situations the

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

655

656 Figure Legends

657 Figure 1 **Immune response to danger signals released from chronic epithelial** 658 **injury**

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

674

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

677 All progressive chronic inflammatory liver diseases are associated with recruitment
678 and retention of circulating lymphocytes into liver tissue. This recruitment occurs
679 within the low shear stress environment of the hepatic sinusoids, where lymphocyte
680 recruitment is triggered by selectin-independent capture and firm adhesion by
681 VCAM-1 supported by CD151 on the endothelial surface. Other factors promote
682 lymphocyte subset specific recruitment including aberrant adhesion of gut-homing
683 lymphocytes ($\alpha 4\beta 7+$) to MAdCAM-1 and CD4 lymphocytes adhesion
684 mediated by SCARF1. Presentation of chemokines including IP-10 to CXCR3⁺ T
685 cells and CXCL16 to CXCR6⁺ T cells triggers activation and migration of T cells.
686 The subsequent transendothelial migration step involves a combination of receptors
687 including the atypical adhesion molecule VAP-1 with Treg specific recruitment
688 occurring via transcellular pathway mediated by VAP-1 and stabilin-1. HSCs
689 contribute to subendothelial retention of lymphocytes through the release of several
690 chemotactic factors and contribution from VAP-1. T cell subset positioning in liver
691 tissue is further regulated by chemokines including CCL20 for Th17 cells and CCL17
692 and CCL22 for Tregs. VCAM-1, vascular adhesion molecule-1; MAdCAM-1,
693 mucosal vascular addressin cell adhesion molecule-1; SCARF1, scavenger receptor
694 class F, member 1; IP-10, interferon gamma-induced protein 10; VAP-1, vascular
695 adhesion protein-1.

696

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

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

700 through the release of several factors including IL-6 and TNF α . Liver sinusoidal
701 endothelium can promote a pro regenerative pathway rather than pro-fibrotic through
702 the upregulation of CXCR7 which induces the transcription factor Id1 leading to
703 proregenerative angiocrine factors. NK cells can contribute to fibrosis resolution by
704 directly killing senescence activated HSCs. Macrophages also play a pivotal role in
705 fibrosis resolution through the release of several factors including MMP13 which
706 degrades scar tissue. A key role is played by a subset of macrophages
707 characterised by the pro-resolution phenotype Ly6C^{lo}CCR2^{lo}CX₃CR1^{hi}. In chronic
708 liver injury, uptake of products of lipid peroxidation such as oxLDLs by macrophages
709 expressing stabilin-1 suppresses the release of pro-fibrotic factors. During acute
710 liver injury the release of SLPI leads to the upregulation of MerTK on macrophages
711 which promotes neutrophil apoptosis and subsequent clearance leading to resolution
712 of inflammation. NK cell, natural killer cell; MMP-13, metalloproteinase-13; oxLDL,
713 oxidised low density lipoprotein; SLPI, secretory leukocyte protease inhibitor; MerTK,
714 Mer tyrosine kinase.

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