

# Efferocytosis in liver disease

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## Summary

The process of dead cell clearance by phagocytic cells, called efferocytosis, prevents inflammatory cell necrosis and promotes resolution and repair. Defective efferocytosis contributes to the progression of numerous diseases in which cell death is prominent, including liver disease. Many gaps remain in our understanding of how hepatic macrophages carry out efferocytosis and how this process goes awry in various types of liver diseases. Thus far, studies have suggested that, upon liver injury, liver-resident Kupffer cells and infiltrating monocyte-derived macrophages clear dead cells, limit inflammation, and, through macrophage reprogramming, repair liver damage. However, in unusual settings, efferocytosis can promote liver disease. In this review, we will focus on efferocytosis in various types of acute and chronic liver diseases, including metabolic dysfunction-associated steatohepatitis. Understanding the mechanisms and consequences of efferocytosis by hepatic macrophages has the potential to shed new light on liver disease pathophysiology and to guide new treatment strategies to prevent disease progression.

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## Introduction

The clearance of dead cells, termed efferocytosis, is an essential process to maintain normal tissue homeostasis and to restore homeostasis following tissue damage.<sup>1–4</sup> Impaired efferocytosis and subsequent accumulation of dead cells promotes tissue necrosis, inflammation, and defective resolution and repair, and has been shown to contribute to numerous inflammatory diseases in humans.<sup>1–4</sup> Cell death occurs at both the onset and during progression of acute liver injury (ALI) and chronic liver diseases.<sup>5–8</sup> Hepatic macrophages, including both resident Kupffer cells (KCs) and infiltrating monocyte-derived macrophages (MoMΦs), are primarily responsible for efferocytosis of dead cells in the liver, but other liver cells, such as hepatic stellate cells (HSCs) and hepatocytes, can also carry out efferocytosis, at least *in vitro*.<sup>9–12</sup> Understanding the mechanisms of efferocytosis and why defective efferocytosis occurs in the setting of chronic inflammatory diseases has emerged as a major area of research,<sup>1–3</sup> but many gaps remain in our knowledge of the role of efferocytosis in the pathogenesis of liver disease.<sup>9</sup> Here, we will review the discoveries that have been made to date on the roles of efferocytosis in normal liver physiology and various types of liver disease. We will highlight the knowledge gaps in this important area of research and suggest key topics for future investigation, including in therapeutic translation.

## The fundamentals of efferocytosis (Fig. 1)

Efferocytosis is the process by which phagocytic cells engulf and ingest dead or dying cells. This process is carried out mostly by professional phagocytes, like macrophages and dendritic cells, but non-professional phagocytes, such as epithelial and mesenchymal cells, can also engulf dead cells.<sup>1–4</sup> Efferocytosis is a multi-step process involving the recognition, binding, internalisation, and digestion of dead cells.<sup>13</sup> In the first stage, dead cells release so-called find-me molecules, e.g., sphingosine-1-phosphate and nucleotides, which promote the migration of macrophages to dead cells. Macrophages then recognise the dead cells through efferocytosis receptors, such as the TAM (Tyro-Axl-MerTK) family receptors; T cell/transmembrane, immunoglobulin, and mucin (TIM) family members, such as TIM4; triggering receptor expressed on myeloid cells 2 (TREM2); LDL receptor-related protein 1; and BAI1 (brain-specific angiogenesis inhibitor 1). These receptors interact with molecules on the surface of dead cells, e.g. phosphatidylserine and calreticulin, either directly or indirectly through bridging molecules, such as Gas6, protein S, and MFG-E8 (milk fat globule epidermal growth factor 8). The processes of internalisation and digestion are mediated by Rac1-actin activation followed by phagosomelysosome fusion. Interestingly, the debris resulting from the phagolysosomal degradation of dead cells,

Keywords: apoptotic cells; macrophages; Kupffer cells; hepatic stellate cells; liver inflammation; liver fibrosis; tissue resolution; acute liver injury; metabolic dysfunction-associated steatohepatitis (MASH)

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upon transport to the macrophage cytoplasm, can activate signalling pathways that reprogramme macrophages to clear multiple dead cells (continual efferocytosis) and to carry out resolution and repair functions.<sup>1–4</sup>

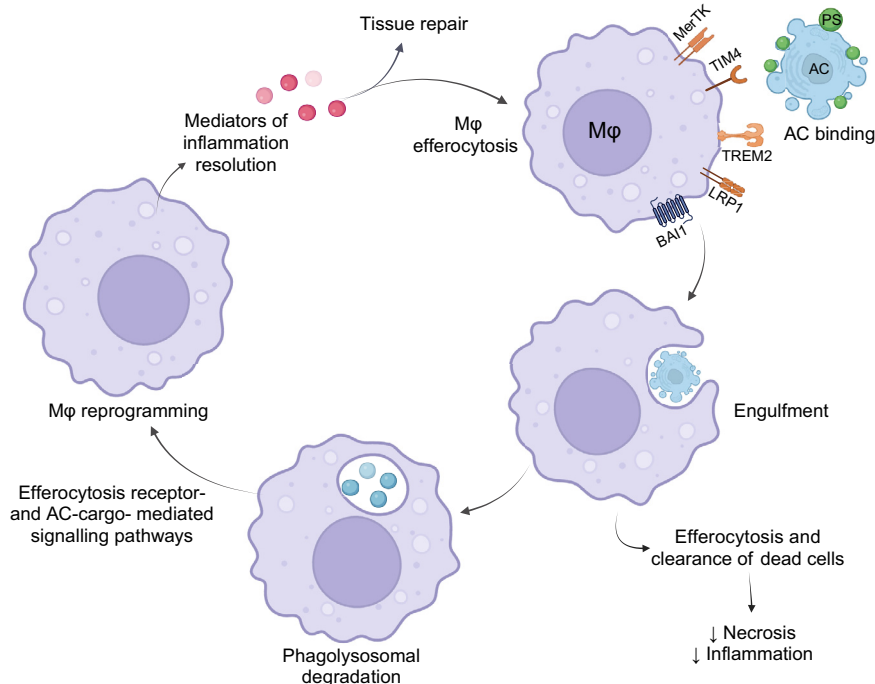
The main function of efferocytosis is to prevent the release of cellular contents (necrosis), protect against inflammation, and promote inflammation resolution through the release of resolution mediators such as transforming growth factor- $\beta$  (TGF $\beta$ ), interleukin-10 (IL-10), and lipid mediators of resolution.<sup>1–3,14,15</sup> The links between efferocytosis and resolution constitute a positive-feedback cycle, as resolution mediators are not only produced by efferocytosing macrophages but can also further promote efferocytosis.<sup>16,17</sup> Accumulating evidence suggests that impaired efferocytosis contributes to human chronic inflammatory diseases.<sup>1–3</sup> Mechanisms of impaired efferocytosis include defects in dead cell recognition by inappropriate expression of “don’t-eat-me” signals, such as CD47 and signal regulatory protein- $\alpha$  (SIRP $\alpha$ );<sup>18</sup> loss of efferocytosis receptors via proteolytic cleavage;<sup>19,20</sup> and defective dead cell internalisation.<sup>21</sup>

### Cell death in liver disease

Multiple forms of liver cell death, including necrosis, apoptosis, necroptosis, pyroptosis, and ferroptosis, have been reported in various types of liver disease,<sup>5–8,22,23</sup> including ALI and metabolic dysfunction-associated steatohepatitis (MASH; formerly NASH). Caspase-dependent apoptosis involves membrane condensation and then rapid macrophage-mediated clearance

### Key points

- Both resident Kupffer cells and monocyte-derived macrophages recruited to the liver can clear dead liver cells and their debris.
- In certain setting, hepatic stellate cells, hepatocytes, and liver sinusoidal endothelial cells may also be able to engulf dead liver cells, but less is known about this area.
- Most studies have suggested that liver efferocytosis, particularly macrophage-mediated clearance of dead neutrophils or hepatocytes, promotes the resolution of liver injury. Hence, if efferocytosis becomes impaired during disease progression, e.g. as is thought to occur in alcohol-related liver disease and metabolic dysfunction-associated steatohepatitis (MASH), liver injury and fibrosis does not resolve.
- The two major mechanisms thought to explain the beneficial effects of macrophage-mediated efferocytosis in the liver are the prevention of inflammation and injury from uncleared dead cell debris and the reprogramming of macrophages into a pro-resolving phenotype.
- In contrast to the situation with macrophage efferocytosis, efferocytosis by hepatic stellate cells, e.g. in viral hepatitis, may activate these cells and thereby promote liver fibrosis.
- Many studies in this area of research are based on *in vitro* efferocytosis data with or without correlation with liver injury endpoints rather than *in situ* quantification of hepatic efferocytosis *per se*.
- Future studies exploring possible human genetic links between efferocytosis-related gene variants and progression of various types of liver disease would help bridge the gap between experimental studies and human liver disease.
- Despite these limitations, this area of research has substantial translational potential, as new strategies are emerging to boost efferocytosis and resolution in liver disease as a way to help resolve liver injury.



**Fig. 1. Fundamentals of efferocytosis.** Phagocytes, particularly M $\Phi$ s, capture ACs through recognition of signals on the ACs, notably PS, by receptors, e.g., MerTK, TIM4, and TREM2. AC internalisation and phagolysosomal degradation clear tissues of dead cells, which prevents necrosis and inflammation. In addition, efferocytosis reprogrammes M $\Phi$ s into a pro-resolving phenotype driven by efferocytosis receptor signalling and pathways triggered by the degraded AC cargo, e.g. amino acids, lipids, and nucleic acids. The reprogrammed M $\Phi$ s secreted resolution mediators, e.g. interleukin-10, that both repair tissues and further stimulate efferocytosis. The figure was generated using [Biorender.com](https://www.biorender.com). ACs, apoptotic cells; M $\Phi$ s, macrophages; PS, phosphatidylserine; MerTK, MER proto-oncogene, tyrosine kinase; TIM4, T cell immunoglobulin and mucin domain containing 4; TREM2, Triggering receptor expressed on myeloid cells 2; LRP1, LDL receptor related protein 1; BAI1, Adhesion G protein-coupled receptor B1.

unless efferocytosis is defective.<sup>1,2,4</sup> Hepatocyte apoptosis is a feature of viral hepatitis, alcohol-associated liver disease (ALD), and MASH.<sup>24,25</sup> Apoptosis in liver disease can also occur in KCs,<sup>26–28</sup> HSCs,<sup>29,30</sup> liver sinusoidal epithelial cells,<sup>31,32</sup> and infiltrating neutrophils.<sup>33</sup> Necroptosis is an MLKL-RIP3-dependent, caspase-independent form of cell death in which the dead cells become leaky and promote inflammation.<sup>34</sup> Hepatocyte necroptosis has been shown to play a role in the pathogenesis of ALD,<sup>35,36</sup> MASH,<sup>37–39</sup> and liver cancer.<sup>40</sup> However, the role of necroptosis in the pathogenesis of ALI is controversial.<sup>41</sup> A recent study also showed that sublethal necroptosis in hepatocytes contributes to inflammation and liver cancer.<sup>42</sup> Pyroptosis refers to cell death mediated by caspase 1-mediated inflammasome signalling, leading to pore formation in cells by gasdermin D and NINJ1.<sup>43</sup> Pyroptosis has been implicated in ALD,<sup>44</sup> MASH,<sup>45–47</sup> and ALI.<sup>22,48,49</sup> Lastly, cell death regulated by iron-dependent lipid peroxidation, termed ferroptosis,<sup>50</sup> may contribute to the pathogenesis of MASH<sup>51,52</sup> and ALI.<sup>53–55</sup>

### Hepatic macrophage heterogeneity in liver disease

In healthy liver, resident KCs represent the major type of hepatic macrophage, and they can initiate an inflammatory response upon sensing liver injury.<sup>56,57</sup> In addition to resident KCs, circulating monocytes in the blood can infiltrate the liver and differentiate into MoMΦs to mediate a chronic inflammatory response in diseases such as ALD and MASH – this process is often associated with the loss of KCs.<sup>58–60</sup> As will be discussed below, the efferocytic capabilities of resident KCs and recruited MoMΦs can differ.<sup>61</sup> In the past decade, single-cell RNA sequencing has revealed hepatic macrophage heterogeneity in mouse models as well as humans.<sup>62</sup> For example, two functionally different subsets of murine KCs, a major CD206<sup>low</sup>ESAM<sup>-</sup> population (KC1) and a minor CD206<sup>hi</sup>ESAM<sup>+</sup> population (KC2), were identified in both the steady-state and in diet-induced obesity.<sup>63</sup> In addition, lipid-associated macrophages, which express osteopontin and desmin, were found in steatotic livers and were located in the regions of the liver with reduced numbers of KCs.<sup>59</sup> Five macrophage subclusters have been identified in murine MASH livers, including so-called scar-associated macrophages (SAMs), characterised by high expression of TREM2 and CD9.<sup>60,64</sup> SAMs were found to reside near areas of fibrosis, and they have been implicated in carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis via the expression of matrix metalloproteinases, which, despite the name, can be pro-fibrotic in the liver.<sup>65</sup> In addition, TREM2<sup>+</sup>CD9<sup>+</sup> SAMs were also identified in human cirrhotic livers, and conditioned media from cultured SAMs activated primary HSCs.<sup>66</sup> As will be described below, TREM2 can function as an efferocytosis receptor in MASH livers. For a detailed overview of hepatic macrophage subpopulations, the reader is referred to excellent reviews published elsewhere.<sup>56,57,67</sup>

### Efferocytosis in liver diseases (Fig. 2)

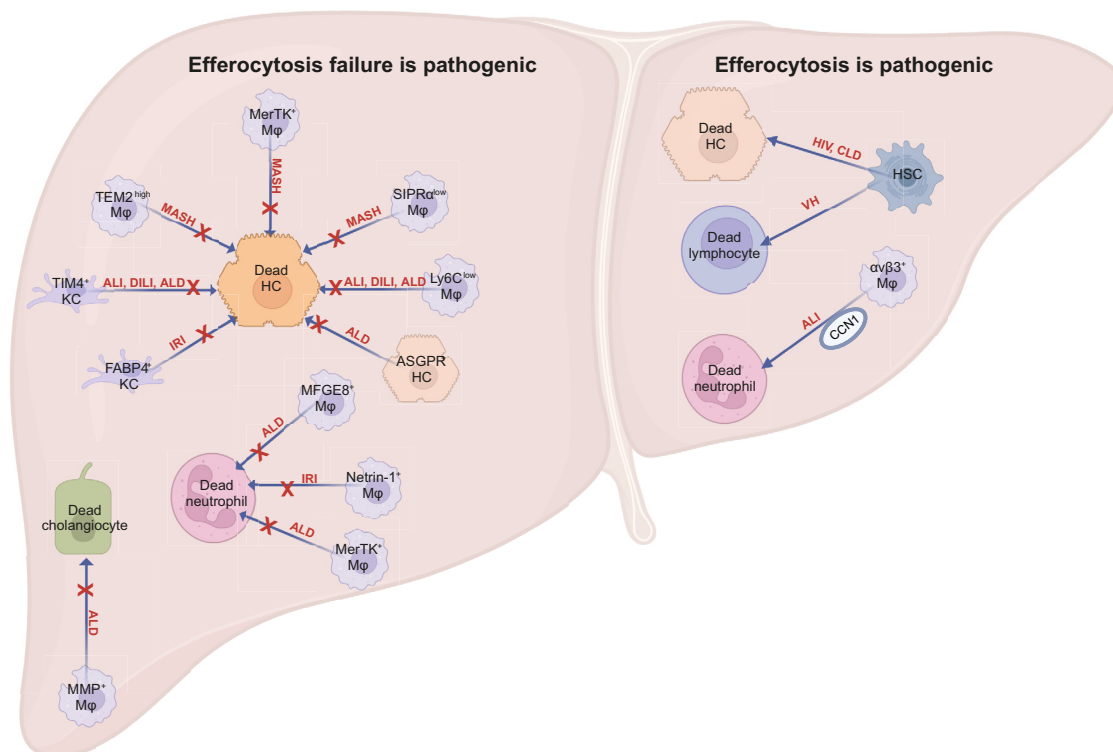
Clearance of dead cells or debris by KCs<sup>68–70</sup> and MoMΦs<sup>71</sup> in the liver, and possibly by hepatocytes<sup>72–74</sup> and liver endothelial cells,<sup>75,76</sup> is required to maintain normal hepatic function,<sup>9,77,78</sup> while dead cell engulfment by HSCs may lead to HSC activation.<sup>10,11,79–81</sup> Here, we discuss the mechanisms and consequences of dead cell clearance carried out by different liver cells in various types of liver disease (Table 1).

### Efferocytosis in toxicant-induced ALI

Exposure to certain chemicals can cause hepatocyte death and liver damage, inflammation, and occasionally fibrosis. CCl<sub>4</sub> is a hepatotoxic agent that is classically used to induce liver injury and fibrosis in mice. CCl<sub>4</sub>-induced hepatotoxicity results in the production of free radicals that cause oxidative stress and lipid peroxidation, leading to hepatocyte damage, inflammation, and the formation of fibrotic scar tissue in the liver.<sup>82</sup> Investigators used this model to investigate a protein called fatty acid binding protein 7 (FABP7), which is a fatty acid binding protein localised to KCs. In CCl<sub>4</sub>-treated mice, Fabp7 knockout caused elevated plasma alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, indicative of liver injury, as well as lower KC numbers in areas of liver necrosis and elevated liver fibrosis.<sup>83</sup> The authors showed that efferocytosis was impaired in KCs from Fabp7-knockout mice compared with wild-type KCs *ex vivo*, and this was associated with lower expression of the efferocytosis receptor CD36 in the knockout KCs. While these *ex vivo* findings could provide a possible mechanism for the *in vivo* findings, direct evidence linking defective efferocytosis *in vivo* to liver damage and fibrosis in the Fabp7-knockout mice remains to be demonstrated.

In a model of reversible hepatic fibrosis in which mice were treated with CCl<sub>4</sub> for 4 weeks and then followed for up to 10 days thereafter, two subsets of liver-infiltrating macrophages, CD11b<sup>high</sup>F4/80<sup>int</sup>Ly6C<sup>high</sup> and CD11b<sup>high</sup>F4/80<sup>int</sup>Ly6C<sup>low</sup>, were identified during liver fibrosis resolution.<sup>84</sup> The Ly6C<sup>low</sup> subset, which was derived from the Ly6C<sup>high</sup> subset, increased progressively during the fibrosis-resolution period and showed high expression of genes associated with extracellular matrix degradation, notably genes encoding certain types of metalloproteinases, and phagocytosis. These Ly6C<sup>low</sup> macrophages also showed evidence of having ingested dead cell debris in the livers of the fibrosis-resolving mice. Most importantly, experimental depletion of this so-called "restorative macrophage subset" blunted fibrosis resolution. Conversely, these macrophages could be expanded in the livers of CCl<sub>4</sub>-treated mice by the administration of phagocytosis-stimulating liposomes, and this treatment enhanced the resolution of fibrosis. Efferocytosis of dead hepatocyte debris by bone marrow-derived macrophages *ex vivo* recapitulated the restorative macrophage phenotype through an ERK signalling pathway. In another study, the authors focused on an efferocytosis-activated STAT3/IL-10/IL-6 autocrine-paracrine pathway in macrophages that promoted a restorative phenotype and further enhanced efferocytosis.<sup>85</sup> The authors presented evidence that this pathway promoted inflammation resolution and liver repair and regeneration in CCl<sub>4</sub>-treated mice.

While these studies suggest that efferocytosis by hepatic macrophages can improve fibrosis in ALI, efferocytosis-mediated tissue repair can also lead to a fibrogenic response<sup>86</sup> that, in the wrong setting, can be pathogenic. In this context, a recent study focused on the secretion of TGFβ1 by macrophages following ingestion of apoptotic neutrophils, as TGFβ1 is a potent activator of HSCs and inducer of liver fibrosis.<sup>33</sup> Efferocytosis in this scenario was dependent on the bridging molecule CCN1 (cellular communication network 1), which binds phosphatidylserine (PS) on apoptotic cells and integrin αvβ3 on macrophages. When the investigators caused an impairment in efferocytosis via a mutation in CCN1 in CCl<sub>4</sub>-treated mice, apoptotic neutrophils were increased in the liver, but there were decreases in TGFβ1, HSC



**Fig. 2. Proposed roles of efferocytosis in liver disease.** (Left) Examples of clearance of dead hepatocytes, neutrophils, and cholangiocytes, mostly by various types of hepatic M $\phi$ , which are proposed to become defective in liver disease and thereby promote disease progression. (Right) In certain types of liver disease, mostly *in vitro* studies have suggested that efferocytosis by HSCs promotes their activation, leading to pathologic liver fibrosis. Clearance of dead neutrophils by a subpopulation of hepatic M $\phi$ s may also be pathogenic. As explained in the text, many of these processes should be considered hypotheses awaiting further proof *in vivo*. The figure was generated using [Biorender.com](https://www.biorender.com). ALI, acute liver injury; ALD, alcohol-related liver disease; CLD, cholestatic liver disease; DILI, drug-induced liver injury; HC, hepatocyte; HIV, human immunodeficiency virus; HSC, hepatic stellate cell; IRI, ischaemia-reperfusion injury; KC, Kupffer cell; MASH, metabolic dysfunction-associated steatohepatitis; M $\phi$ s, macrophages; VH, viral hepatitis; MerTK, MER proto-oncogene, tyrosine kinase; SIRP $\alpha$ , Signal-regulatory protein alpha; Ly6C, Ly6-C antigen; ASGPR, Asialoglycoprotein receptor 1; FABP4, Fatty acid binding protein 4; TIM4, T cell immunoglobulin and mucin domain containing 4; TREM2, Triggering receptor expressed on myeloid cells 2; MMP, Matrix metalloproteinase; MFGE8, Milk fat globule EGF and factor V/VIII domain containing; CCN1, Cellular communication network factor 1.

activation, and liver fibrosis. Thus, the role of efferocytosis in experimental ALI is complex, suggesting that certain mechanisms of efferocytosis can promote fibrosis resolution, while other mechanisms can have the opposite effect. A key issue, yet to be resolved, is what happens to efferocytosis in the liver in specific types of ALI in humans, which, if biopsy material were available, could be assessed by using validated assays of *in situ* efferocytosis in liver sections.<sup>39</sup>

### Efferocytosis in drug-induced liver injury

Drug-induced liver injury (DILI) is an acute form of liver injury and, if not reversed, can result in liver failure.<sup>87</sup> DILI can be caused by conventional drugs, herbal medications, dietary supplements, and other xenobiotics.<sup>87</sup> The pathogenesis of DILI is predominantly characterised by death of hepatocytes, although cholangiocyte or endothelial cell death may also occur. The release of damage-associated molecular patterns (DAMPs) from dead cells induces a pro-inflammatory response, resulting in the recruitment of neutrophils and then, if the insult is removed, MoM $\phi$ s to promote the clearance of dead cell debris and liver regeneration.<sup>5</sup>

As mentioned previously, MerTK is an efferocytosis receptor whose activation and participation in apoptotic cell uptake can promote resolution signalling. Patients with acetaminophen

(APAP)-induced acute liver failure exhibited an increase in resolution-like MerTK<sup>+</sup>HLA-DR<sup>high</sup> monocytes and hepatic macrophages, which are characterised by a suppressed inflammatory response and enhanced efferocytic/phagocytic responses.<sup>88</sup> A similar phenotype of hepatic macrophages (MerTK<sup>+</sup>MHCII<sup>high</sup>) with increased efferocytic capabilities was found during the resolution phase of APAP-induced ALI in mice.<sup>88</sup> A protective role for MerTK was suggested by data showing persistent liver injury and inflammation, and accumulation of activated neutrophils following APAP overdose in MerTK-deficient mice. *In vitro*, MerTK<sup>+</sup>HLA-DR<sup>high</sup> monocytes were able to efficiently efferocytose apoptotic neutrophils. These data suggest that MerTK on resolving-type monocytes/macrophages participates in apoptotic cell clearance and resolution of inflammation in APAP-induced liver injury, but analysis of apoptotic neutrophil clearance by macrophages in liver tissue will be necessary to bolster this conclusion.

Similar to toxicant-induced liver injury, infiltrating Ly6C<sup>high</sup> monocytes differentiate into Ly6C<sup>low</sup> MoM $\phi$ s during the resolution phase of acute APAP-induced liver injury, with increased expression of a specific repertoire of bridging molecules and receptors that allow for engulfment of apoptotic neutrophils.<sup>89</sup> When circulating Ly6C<sup>high</sup> monocytes were ablated in APAP-overdose mice, the subsequent decrease in liver

**Table 1. Efferocytosis in various types of liver disease.**

Types of liver disease	Experimental model(s)	Efferocyte cell type/receptor	Type of dead cell	Consequences of efferocytosis	Ref.
Toxicant-induced ALI	CCl <sub>4</sub> -induced liver fibrosis progression	KCs/CD36	Apoptotic thymocyte	Impaired efferocytosis increases liver injury (elevated ALT, AST, and LDH), hepatic necrotic area, and liver fibrosis.	83
	CCl <sub>4</sub> -induced liver fibrosis resolution	MoMΦs CD11b <sup>high</sup> F4/80 <sup>int</sup> Ly6C <sup>low</sup> (restorative macrophage)	Apoptotic hepatocyte	Efferocytosis of hepatocyte debris induces increased restorative MΦ subset, which enriches in a gene expression profile favouring liver fibrosis resolution (matrix degradation, phagocytosis); STAT3-IL10-IL6 autocrine-paracrine pathway involves in efferocytosis-promoted MΦ phenotypic conversion.	84,85
	CCl <sub>4</sub> -induced liver fibrosis progression	KCs/integrin αβ3	Apoptotic neutrophil	Efferocytosis of apoptotic neutrophil increases MΦ Tgfb1 secretion, resulting in HSC activation and liver fibrosis.	33
DILI	APAP-induced liver injury resolution	Monocytes and MoMΦs: MerTK <sup>+</sup> HLA-DR <sup>high</sup> (human); MerTK <sup>+</sup> MHCII <sup>high</sup> (mouse)	Apoptotic neutrophil	Efferocytosis reduces neutrophil accumulation, pro-inflammatory response, and liver injury.	88
	APAP-induced liver injury resolution	Monocytes and MoMΦs: Ly6C <sup>low</sup>	Apoptotic neutrophil	Efferocytosis promotes a gene expression profile of apoptotic cell bridging molecules and receptors, favouring dead neutrophil clearance and Ly6C <sup>high</sup> /Ly6C <sup>low</sup> transition.	89
	APAP-induced liver injury resolution	KCs and MoMΦs	Necrotic hepatocyte	Efferocytosis promotes MΦ maturation and differentiation, and enhances inflammation resolution.	90,91
	APAP-induced liver injury resolution	Alternatively activated MΦs	Necrotic hepatocyte	Efferocytosis promotes necrotic hepatocyte clearance, inflammation resolution and reduces necrosis and liver injury.	92
Hepatic IRI	IRI	KCs	Apoptotic neutrophil	Efferocytosis promotes inflammation resolution and decreases liver injury.	94
	IRI	KCs and MoMΦs	Apoptotic neutrophil	Efferocytosis promotes apoptotic neutrophil clearance, specialised pro-resolving lipid mediators and growth factor production, resulting in inflammation resolution.	95,96
	IRI	KCs/TIM4	Apoptotic thymocyte	Efferocytosis promotes IL10 production and decreases inflammation, resulting in resolution of liver IRI.	99
	IRI	KCs/MerTK	Apoptotic hepatocyte	Efferocytosis promotes apoptotic hepatocyte engulfment, reduces DNA accumulation, causing decreased MΦ STING activation-induced inflammation post-IRI.	101
	IRI	MoMΦs/Trem2	Apoptotic cell	Trem2 mediated efferocytosis promotes the resolution of inflammation post-IRI through regulating Rac1 signal.	104
Viral hepatitis-induced liver injury	HBV/HCV	HSCs/ICAM-1	Disease-associated lymphocyte	Efferocytosis induces HSC activation.	79
	HIV plus Alcohol	HSCs/Axl	Apoptotic bodies from HCV-infected hepatocyte	Efferocytosis promotes HSC activation.	80
	HCV	HSCs	Apoptotic bodies from hepatocyte	Efferocytosis promotes oxidative radicals, resulting in HSC activation.	10
CLD	BDL	KCs	Apoptotic bodies from hepatocyte	Efferocytosis promotes Fas ligand production by KCs, inducing inflammation and HSC activation.	106
	BDL	HSCs	Apoptotic bodies from hepatocyte	Efferocytosis promotes HSC activation.	10
	BDL	HSCs/Galectin-3	Apoptotic bodies from hepatocyte	Galectin-3 modulates efferocytosis-induced HSC activation and fibrosis.	107
	Reversible BDL	CD68 <sup>+</sup> MΦs	Apoptotic cholangiocyte	Efferocytosis promotes extracellular matrix remodelling by induction of subsets of MMPs.	108

(continued on next page)

Table 1 (continued)

Types of liver disease	Experimental model(s)	Efferocyte cell type/receptor	Type of dead cell	Consequences of efferocytosis	Ref.
ALD	Ex vivo	Peritoneal MΦs	Apoptotic thymocyte	Alcohol exposure reduces MFG-E8 expression, causing impaired efferocytosis.	112
	ALD	MoMΦs	Apoptotic hepatocyte	Efferocytosis increases pro-resolving Ly6C <sup>low</sup> MΦs subset, promotes inflammation resolution and protects against liver injury.	113
	Ex vivo ALD	Hepatocytes/ASGPR KCs/MoMΦs	Apoptotic lymphocyte Neutrophil extracellular trap	Alcohol exposure inhibits efferocytosis. Increased "don't-eat me" CD47 signal prevents clearance of alcohol-induced neutrophil extracellular trap by MΦs, causing inflammation.	12 114–116
MASH	High-fat/high-cholesterol diet	MoMΦs/Trem2	Apoptotic hepatocyte	Trem2-mediated efferocytosis promotes apoptotic hepatocytes clearance; Trem2 deficiency impairs efferocytosis and accelerates liver fibrosis.	20,129
	MCD diet	KCs/MoMΦs	Apoptotic hepatocyte	Impaired clearance of mito-DAMPs from apoptotic hepatocytes causes mito-DAMPs accumulation that induces HSCs activation and liver fibrosis.	123
	FPC and HF-CDAA diet	KCs/MoMΦs	Necroptotic hepatocyte	Blockade of "don't-eat me" CD47-SIRPα axis improves necroptotic hepatocyte clearance and liver fibrosis.	39

ALD, alcohol-associated liver disease; ALI, acute liver injury; APAP, acetaminophen; AST, aspartate aminotransferase; BDL, bile duct ligation; CCl<sub>4</sub>, carbon tetrachloride; CLD, cholestatic liver disease; DAMPs, damage-associated molecular patterns; DILI, drug-induced liver injury; HSC, hepatic stellate cells; IRI, ischaemia-reperfusion injury; KCs, Kupffer cells; LDH, lactate dehydrogenase; MASH, metabolic dysfunction-associated steatohepatitis; MoMΦs, monocyte-derived macrophages.

Ly6C<sup>low</sup> MoMΦs was associated with an accumulation of apoptotic neutrophils.<sup>89</sup> Similar findings were reported in other studies using different methods to prevent entry of monocytes or restorative-type macrophages into the livers of APAP-overdose mice.<sup>90,91</sup> Conversely, when mice with APAP-induced liver injury were infused with alternatively activated macrophages, which *in vitro* can engulf necrotic hepatocytes, there was a decrease in hepatocellular necrosis.<sup>92</sup> Further, treating a rat KC line with erythropoietin promoted KC phagocytosis of beads or bacteria, which was postulated to mediate the protective effects of erythropoietin in acute APAP-induced liver injury in mice.<sup>93</sup> While these studies suggest that increasing resolution-type, pro-efferocytic macrophages in the liver may enhance recovery from APAP-induced liver injury, the actual beneficial role of efferocytosis *per se* is unproven, as quantification of *in situ* efferocytosis of dead cells by macrophages in the liver was not reported in any of these studies. Accordingly, future studies that include measurements of efferocytosis by specific subsets of hepatic macrophages *in situ* will be needed to further understand the roles, mechanisms, and therapeutic potential of efferocytosis in DILI.

### Efferocytosis in hepatic ischaemia-reperfusion injury

Hepatic ischaemia-reperfusion injury (IRI) is one of the primary causes of early liver dysfunction and failure following liver transplantation, and studies have suggested that impaired phagocytosis of dead cells in the liver by resident KCs plays a key role in the pathogenesis of hepatic IRI. Analysis of post-IR livers revealed macrophages with numerous long pseudopodia and distribution of the Golgi complex towards the damaged tissue, suggesting that the macrophages were phagocytosing dead cells.<sup>94</sup> The macrophage pseudopodia were dependent on urokinase-type plasminogen activator (u-PA) and plasminogen, and mice deficient in u-PA or plasminogen showed impaired macrophage phagocytosis and impaired liver tissue repair.<sup>94</sup> Another study showed that treatment of IRI-affected mice with the pro-resolving lipid mediator resolvin D1, acting through its receptor ALX/FPR2, caused a shift from inflammatory to resolving-type macrophages in the liver, improved efferocytosis of dead Gr-1<sup>+</sup> myeloid cells, and lessened liver injury.<sup>95</sup> Another link between efferocytosis and protection from IRI comes from a study indicating a pro-efferocytic function of netrin-1, as deletion of netrin-1 impaired efferocytosis of apoptotic polymorphonuclear neutrophils, while exogenous netrin-1 treatment enhanced efferocytosis both *in vitro* and in IR livers and promoted repair and regeneration of injured livers.<sup>96</sup>

TIM4 is an efferocytosis receptor expressed in several types of myeloid cells, including KCs.<sup>97</sup> The role of TIM4 in IRI is complex. One study found that TIM4 expression was elevated in IRI-affected mice and that deletion or antibody-mediated neutralisation of TIM4 blocked inflammatory macrophage entry into the liver and lessened liver tissue damage.<sup>98</sup> On the other hand, TIM4-deficient bone marrow-derived macrophages showed a defect in the engulfment of necrotic hepatocytes *in vitro*, but analysis of dead cell uptake by macrophages in post-IR livers was not reported. A second study showed that TIM4 was expressed exclusively by KCs in post-IR livers and that KC-TIM4 deficiency worsened IR liver injury and prevented tissue resolution.<sup>99</sup> The efferocytic function of KC-TIM4 was demonstrated *ex vivo*, but, as above, an *in situ* analysis of efferocytosis in post-IR livers was not reported. Thus, the role of TIM4 in efferocytosis and resolution in the setting of hepatic IRI

requires further analysis, including careful analysis of dead cell uptake by macrophages.

MerTK is another efferocytosis receptor that has been studied in hepatic IRI. One group reported that deletion or inhibition of macrophage Gsk3 $\beta$  (glycogen synthase kinase 3 $\beta$ ) promoted the reparative phenotype of hepatic macrophages in IR and lessened liver injury.<sup>100</sup> These protective effects were diminished in MerTK-deficient mice, but precise links among Gsk3 $\beta$ , MerTK regulation, and MerTK-mediated resolution and efferocytosis remain to be elucidated. Another study suggested a link between exacerbated IR liver damage in aged mice and A disintegrin and metalloprotease 17 (ADAM17)-mediated MerTK cleavage,<sup>101</sup> which is a mechanism of impaired efferocytosis and resolution in the setting of inflammation.<sup>19</sup> In aged IRI-affected mice, the accumulation of apoptotic hepatocytes was increased, accompanied by increases in DNA, activation of STING (stimulator of interferon genes), and liver injury. Treatment of the mice with ADAM17 small-interfering RNA mitigated these processes, including decreasing the number of apoptotic cells in the liver. Although *in situ* efferocytosis was not analysed, another study exploring a different disease featuring defective efferocytosis (atherosclerosis) showed that prevention of MerTK cleavage enhances *in situ* efferocytosis and tissue resolution.<sup>19</sup> Thus, ADAM17-mediated MerTK cleavage may contribute to the pathophysiology of IR-induced liver injury. These findings should not be confused with another study showing that a non-efferocytic signalling function of MerTK can promote liver fibrosis in MASH by increasing the production of TGF $\beta$ ,<sup>102</sup> which is consistent with human genetic data.<sup>103</sup> Interestingly, the efferocytosis receptor TREM2 may cooperate with MerTK in IR-induced liver injury, e.g. by enhancing Rac1-mediated apoptotic cell engulfment, to promote efferocytosis-induced reprogramming of hepatic macrophages in the resolution phase of IRI.<sup>104</sup>

### Efferocytosis in viral hepatitis-induced liver injury

Studies of livers from patients with viral hepatitis have suggested that dead liver cells can be engulfed by HSCs and that this process may promote HSC activation and liver fibrosis. In HBV- and HCV-infected livers, ICAM-1-mediated engulfment of disease-associated lymphocytes, but not control lymphocytes, by an HSC line was observed *in vitro* and led to HSC activation.<sup>79</sup> When internalisation of these lymphocytes was prevented by blocking ICAM-1 or the engulfment proteins Cdc42 or Rac1, HSC activation was prevented. In another study, apoptotic bodies shed by a hepatocyte line infected with HCV were also engulfed by HSCs *in vitro*, leading to HSC activation in a manner that was dependent on hepatocyte-derived growth factor in the apoptotic bodies.<sup>81</sup> These findings are consistent with the results of prior *in vitro* studies.<sup>10,11</sup> Thus, it is possible that the engulfment of diseased lymphocytes and/or hepatocyte-derived apoptotic bodies by HSCs contributes to viral hepatitis-induced liver fibrosis, but the difficulty in creating mouse models of viral hepatitis limits the ability to show causation *in vivo*. Finally, in people living with HIV, alcohol abuse can promote liver fibrosis. In this context, investigators found that HSCs engulfed apoptotic bodies derived from hepatocytes that were both infected with HIV and exposed to the alcohol metabolite acetaldehyde.<sup>80</sup> The efferocytosis receptor Axl, which is in the same family as MerTK, mediated the uptake of these apoptotic bodies, and HSC activation occurred via activation of ROS-JNK-ERK1/2 and IL6-JAK-STAT3 pathways. As above, causation studies *in vivo* are

limited by the paucity of available mouse models of HIV infection.

### Efferocytosis in cholestatic liver disease

Cholestatic liver disease, which is caused by blockage of bile flow, is characterised by the elevation of alkaline phosphatase in the blood, with milder elevations of transaminases, associated with apoptotic and necrotic hepatocyte death, liver inflammation, and liver fibrosis.<sup>105</sup> In one study, KCs were isolated from a mouse model of cholestatic liver disease caused by experimental bile duct ligation (BDL) and then incubated *ex vivo* with hepatocyte-derived apoptotic bodies.<sup>106</sup> The KCs engulfed the material, leading to expression of the death receptor activator Fas ligand, hepatocyte apoptosis, and subsequent KC inflammatory activation. Uptake of apoptotic bodies by these KCs could be blocked by the KC toxicant gadolinium chloride, which, when administered to BDL mice, dampened liver inflammation and reduced the levels of HSC activation markers. Another study focusing on the uptake of apoptotic bodies by HSCs *in vitro* suggested that efferocytosis-induced HSC activation (above) may contribute to liver fibrosis in a rat BDL model.<sup>10</sup> As further evidence for the possible pathologic role of HSC efferocytosis in BDL mice, galectin-3 was shown to mediate apoptotic body uptake by HSCs *in vitro*, and BDL-induced liver fibrosis was decreased in galectin-3 knockout mice.<sup>107</sup> While these findings raise the possibility that KC- or HSC-mediated efferocytosis could drive inflammation and fibrosis in cholestatic liver disease, a direct link to efferocytosis *in vivo* is lacking.

A beneficial role of efferocytosis by hepatic macrophages in cholestatic liver disease has been suggested by a study examining the resolution of liver fibrosis in a reversible BDL model. In this model, BDL-induced liver fibrosis in mice was reversed by surgery that restored bile flow. During reversal of fibrosis, hepatic macrophages surround apoptotic cholangiocytes and there is an increase in the activity of collagen-degrading matrix metalloproteinases.<sup>108</sup> *In vitro*, engulfment of apoptotic cholangiocytes by rat peritoneal macrophages increased the expression of matrix metalloproteinase-3, -8, and -9. Pending causation studies *in vivo*, these findings suggest that efferocytosis by hepatic macrophages can promote the resolution of liver fibrosis after reversal of bile duct blockage.

### Efferocytosis in ALD

Following excessive alcohol consumption, the conversion of alcohol to acetaldehyde in the liver causes liver injury due to protein adduct formation and oxidative stress, as well as promoting hepatic steatosis due to increased fatty acid synthesis.<sup>109</sup> In addition, alcohol uptake affects immune cell activity in a manner that contributes to hepatic inflammation.<sup>110,111</sup> There are *in vitro* data to suggest that defective efferocytosis by hepatic macrophages may contribute to liver injury in ALD. For example, exposure of macrophages to ethanol impaired their ability to engulf dead cells.<sup>112</sup> This effect was associated with decreased expression of the efferocytosis bridging molecule MFGE8 and increased expression of HMGB1 (high mobility group box-1), which was shown to suppress efferocytosis in ethanol-treated macrophages. These effects could be prevented by treating the macrophages with the antioxidant N-acetylcysteine, suggesting a link between oxidative stress and defective efferocytosis in macrophages. Moreover, as in CCl<sub>4</sub>-treated mice (above), the livers of ethanol-treated mice contain two populations of infiltrating macrophages, pro-resolving Ly6C<sup>low</sup> macrophages

and pro-inflammatory Ly6C<sup>high</sup> macrophages.<sup>113</sup> When Ly6C<sup>high</sup> macrophages were incubated with apoptotic hepatocytes *ex vivo* to enable efferocytosis, the macrophages converted into a more resolving, Ly6C<sup>low</sup>-type phenotype, which is consistent with the concept of efferocytosis-induced resolution (above). There may also be defective efferocytosis by hepatocytes in ALD, as apoptotic cell phagocytosis was suppressed in primary hepatocytes isolated from ethanol-fed rats, which may be caused by impaired asialoglycoprotein receptor function in hepatocytes upon ethanol administration.<sup>12</sup> However, whether efferocytosis by macrophages or hepatocytes is blocked in the livers of mice or humans following excessive alcohol consumption remains to be determined.

Neutrophils and their pro-inflammatory product neutrophil extracellular traps (NETs) are elevated in the livers of humans with ALD and contribute to liver inflammation in ALD.<sup>114</sup> The accumulation of both neutrophils and NETs in the livers of individuals with ALD may be due to impaired clearance by hepatic macrophages.<sup>115,116</sup> *In vitro*, a particular subset of neutrophils that accumulates in ALD and NETs were poorly cleared by macrophages,<sup>116</sup> which may be due to increased expression of the "do-not-eat-me" molecule CD47 and to decreased expression of the "eat-me" molecule PS on these neutrophils.<sup>114</sup> Moreover, treatment of macrophages with ethanol blocked their ability to engulf NETs.<sup>115</sup> Thus, pending *in vivo* studies, it is possible that impairment of neutrophil and NET clearance by hepatic macrophages contributes to inflammation in ALD, which could be additive or synergistic with the resolution-suppressing effect of impaired efferocytosis.

### Efferocytosis in MASH

MASH is emerging as the leading cause of liver disease worldwide owing to the epidemic of obesity.<sup>117</sup> MASH is characterised by lipid accumulation in hepatocytes and multiple insults that cause liver inflammation, hepatocellular death, and fibrosis, which is the factor that correlates best with clinical outcomes.<sup>118,119</sup> The death of hepatocytes is a key feature of MASH<sup>6,120,121</sup> and has been linked to liver fibrosis via the activation of HSCs by dead cell debris.<sup>6,23,122,123</sup> Until recently, knowledge about dead hepatocyte clearance in MASH has been limited to mostly descriptive rather than causation studies. Earlier work showed that KCs form "crown-like structures" that surround and appear to process remnant lipid droplets of dead hepatocytes in MASH livers,<sup>124,125</sup> but the role of these crown-like structures in efferocytosis remains unproven. Other studies showed that the phagocytic activity of KCs was reduced in the livers of MASH rats vs. control rats.<sup>126,127</sup> In another report, macrophage mTORC1 activity, which is decreased in human MASH vs. control livers, was shown to play a protective role in experimental MASH.<sup>128</sup> *In vitro* studies revealed that in macrophages engulfing apoptotic hepatocytes, there was an increase in mTORC1 activity and subsequent release of calcium from mitochondria, which contributed to efferocytosis-induced polarisation of the macrophages toward a resolving-type phenotype. Macrophage mTORC1-deficient MASH mice exhibited increased hepatic inflammation, but a direct, causative link to an impaired efferocytosis-mediated resolution response remains to be demonstrated.

Several recent studies have suggested that the macrophage efferocytosis receptor TREM2 plays a role in efferocytosis in MASH. One group reported that *Trem2* mRNA is increased as

non-alcoholic fatty liver disease develops in mice fed a high-fat/high-cholesterol diet but that ADAM17-mediated cleavage of TREM2 during MASH progression partially lowered TREM2 protein expression on monocyte-derived hepatic macrophages.<sup>20</sup> Experimental deletion of macrophage TREM2 in this model further accelerated the development of MASH, including liver fibrosis, which was associated with an increase in apoptotic hepatocytes. Moreover, macrophages isolated from the livers of the *Trem2*-knockout mice were less able to engulf apoptotic hepatocytes than macrophages from wild-type mice, which was confirmed in another study.<sup>129</sup> Although efferocytosis was not measured *in situ* in the livers of these mice, the data from this study support the notion that TREM2 on hepatic macrophages can mediate the clearance of dead hepatocytes and that impaired efferocytosis due to TREM2 cleavage may be a factor in MASH progression. Consistent with this idea, soluble TREM2, the product of TREM2 cleavage, is increased in the plasma of humans with MASH.<sup>130,131</sup>

If the clearance of apoptotic cells is indeed impaired in MASH livers, how might it contribute to liver fibrosis? One obvious mechanism is HSC activation by the debris of uncleared dead cells.<sup>6,23,122,123</sup> For example, DAMPs released from the mitochondria of dead hepatocytes (mito-DAMPs) are increased in the plasma of individuals with MASH, and correlations were shown among mito-DAMPs, liver fibrosis, poor resolution of necrotic debris, and decreased uptake of injected apoptotic thymocytes in a non-MASH liver injury model.<sup>123</sup> Other HSC-activating DAMPs released from uncleared apoptotic cells may also be involved, such as molecules that activate the HSC purinergic receptor P2Y14.<sup>122</sup> However, further work is needed to demonstrate impaired efferocytosis of dead liver cells by macrophages in MASH livers and then, if found, to link this impairment directly and causatively to DAMP-mediated HSC activation. An additional idea based on the aforementioned studies in acute liver injury models (above) is that defective efferocytosis in MASH impairs the conversion of pro-inflammatory macrophages to pro-resolving macrophages and thereby impedes resolution of MASH-induced liver inflammation and injury.

In addition to apoptosis, hepatocytes die by necroptosis in MASH.<sup>6</sup> A recent study from our group showed that necroptotic hepatocytes were poorly cleared by hepatic macrophages in both human and experimental MASH due to the upregulation of CD47 on necroptotic hepatocytes and SIRP $\alpha$  on MASH macrophages.<sup>39</sup> Most importantly, blockade of either CD47 or SIRP $\alpha$  in mice with established diet-induced steatohepatitis improved the uptake of necroptotic hepatocytes by hepatic macrophages, as measured using an *in situ* efferocytosis assay, and lessened subsequent HSC activation and the progression to MASH-related fibrosis. Although the mechanisms linking impaired clearance of necroptotic hepatocytes to MASH fibrosis remain to be elucidated, these findings raise the possibility of a therapeutic approach to blocking steatosis-to-fibrosis progression in MASH, *i.e.*, by blocking the CD47-SIRP $\alpha$  axis to improve the clearance of necroptotic hepatocytes.

### Conclusions and future directions

There are several themes that emerge from this review. First, the results of most studies suggest that the clearance of dead cells by hepatic macrophages in various types of acute and chronic liver diseases protect against or help resolve liver injury and/or liver



fibrosis. However, there have been other reports suggesting that efferocytosis by hepatic macrophages can promote a fibrotic response in certain experimental liver disease models, e.g., those induced by CCl<sub>4</sub> or BDL. This may not be surprising given that resolution-induced tissue repair can involve a scarring response and that increased production of TGFβ is a well-known consequence of efferocytosis.<sup>14,132</sup> Moreover, dead cell clearance by HSCs may contribute to HSC activation and liver fibrosis in viral hepatitis, but further proof of the importance of this mechanism *in vivo* is needed. In this context, how the type of cell death, e.g. apoptosis vs. necrosis, affects the roles of efferocytosis in liver disease deserves future study. Second, in most studies showing a protective role of efferocytosis, the most common proposed mechanism is efferocytosis-induced macrophage reprogramming to a pro-resolving phenotype, particularly in ALI, although, as noted above, some studies suggest that benefit is also conferred by the decrease in inflammatory debris. Third, despite the importance of this topic and the presentation of important *in vitro*, *ex vivo*, and correlative *in vivo* data, only a few studies include robust molecular-genetic causation experiments that causatively link defective hepatic macrophage efferocytosis to liver injury or fibrosis. Although some studies report on apoptotic cell accumulation in the liver, quantification of dead cell uptake by hepatic macrophages *in situ* is usually lacking. Fourth, while some studies present correlative data with human liver specimens or plasma, genetic links that could begin to suggest causation in humans are lacking. Thus, while numerous studies suggest that dead cell clearance or lack thereof may play important roles in determining the outcome of both acute and chronic liver diseases, more work is needed to bolster these ideas.

The impact of future studies in this area is likely to be substantial in terms of providing pathophysiologic insight and suggesting new therapeutic ideas, especially related to liver

fibrosis. Studies in MASH have particular potential given the importance of dead liver cells and various macrophage phenotypes in this disease, in the context of MASH emerging as the leading cause of chronic liver disease worldwide.<sup>117</sup> In liver diseases in which impaired clearance of dead hepatocytes by hepatic macrophages likely contributes to liver fibrosis, therapies to boost efferocytosis warrant consideration. For example, as noted, blocking either CD47 or SIRPα boosts the clearance of necrotic hepatocytes in experimental MASH and thereby dampens the progression to liver fibrosis.<sup>39</sup> In this context, antibodies that block the CD47-SIRPα axis are in clinical trials for cancer. Another strategy is to leverage the tissue-repair capabilities of pro-resolving mediators, which not only boost resolution but can also enhance efferocytosis.<sup>16,17,133</sup> As one example, administration of resolvin D1 was shown to improve liver repair in experimental models of both ALI<sup>95,134</sup> and ALD.<sup>135</sup> Additional pharmacological strategies to enhance efferocytosis are being investigated.<sup>136</sup> Finally, exciting advances in developing therapeutic “designer” macrophages may lead to new types of efferocytosis-based therapies for liver disease, e.g. by infusing or generating *in vivo* “super-eater” macrophages that home to the liver. For example, a recent study described the design of macrophages that express a “chimeric receptor for efferocytosis” (CHEF) by fusing a specific signalling domain within the cytoplasmic adapter protein ELMO1 to the extracellular PS-recognition domains of the efferocytosis receptors BAI1 or TIM4.<sup>137</sup> These macrophages were shown to boost efferocytosis and improve tissue repair in several *in vivo* models, including a model of diethylnitrosamine-induced hepatotoxicity.<sup>137</sup> Such designer macrophages could be tailored to specific types of liver disease based on ongoing efforts, such as those highlighted in this review, to understand mechanisms and consequences of dead cell clearance in these diseases.

## Abbreviations

ADAM17, A disintegrin and metalloprotease 17; ALD, alcohol-associated liver disease; ALI, acute liver injury; APAP, acetaminophen; BDL, bile duct ligation; CCl<sub>4</sub>, carbon tetrachloride; DAMPs, damage-associated molecular patterns; DILI, drug-induced liver injury; FABP7, fatty acid binding protein 7; HSC, hepatic stellate cells; IL-, interleukin-; IRI, ischaemia-reperfusion injury; KCs, Kupffer cells; MASH, metabolic dysfunction-associated steatohepatitis; MoMΦs, monocyte-derived macrophages; NETs, neutrophil extracellular traps; PS, phosphatidylserine; SAMs, scar-associated macrophages; SIRPα, signal regulatory protein-α; TGFβ, transforming growth factor-β; TIM4, transmembrane; immunoglobulin, and mucin 4; TREM2, triggering receptor expressed on myeloid cells 2.

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## Conflict of interest

The authors declare no conflicts of interest relevant to the topic of this review.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

HS and IT: concept and design, writing of the article, and approval of the final manuscript. MPM and XW: writing of the article and approval of the final manuscript.

## Supplementary data

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*Author names in bold designate shared co-first authorship*

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