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Danger signals in liver injury and restoration of homeostasis

Hui Han[1], **Romain Desert**[1,†], **Sukanta Das**[1,†], **Zhuolun Song**[1,†], **Dipti Athavale**[1,†], **Xiaodong Ge**[1,†], **Natalia Nieto**[1,2,*]

[1]Department of Pathology, University of Illinois at Chicago, 840 S. Wood St., Suite 130 CSN, MC 847, Chicago, IL 60612, USA

[2]Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois at Chicago, 840 S. Wood St., Suite 1020N, MC 787, Chicago, IL 60612, USA

Summary

Damage-associated molecular patterns are signalling molecules involved in inflammatory responses and restoration of homeostasis. Chronic release of these molecules can also promote inflammation in the context of liver disease. Herein, we provide a comprehensive summary of the role of damage-associated molecular patterns as danger signals in liver injury. We consider the role of reactive oxygen species and reactive nitrogen species as inducers of damage-associated molecular patterns, as well as how specific damage-associated molecular patterns participate in the pathogenesis of chronic liver diseases such as alcohol-related liver disease, non-alcoholic steatohepatitis, liver fibrosis and liver cancer. In addition, we discuss the role of damage-associated molecular patterns in ischaemia reperfusion injury and liver transplantation and highlight current studies in which blockade of specific damage-associated molecular patterns has proven beneficial in humans and mice.

Keywords

Alcohol-related liver disease; Hepatocellular carcinoma; Liver fibrosis; Liver transplantation; Non-alcoholic steatohepatitis; Oxidative stress

*Corresponding author. Address: Department of Pathology, University of Illinois at Chicago, 840 S. Wood St., Suite 130 CSN, MC 847, Chicago, IL 60612, USA. Tel.: +1 (312) 996-7316, fax: +1 (312) 355-0774. nnieto@uic.edu (N. Nieto).

†These authors contributed equally.

Authors' contributions

H. H. coordinated editing of the manuscript and wrote the section on NASH; R. D. wrote the section on HCC; S. D. wrote the section on ALD; Z. S. wrote the section on IRI and LT; D. A. wrote the section on oxidative stress; X. G. wrote the section on fibrosis and obtained financial support; and N. N. wrote the introduction and concluding remarks and obtained financial support. All authors edited the manuscript and approved the final version for submission.

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Introduction

Detection of threats such as pathogens and cellular damage is critical to organismal survival. One mechanism of detection is the secretion of endogenous molecules to the extracellular environment, which cell-surface receptors recognise as a danger signals or “alarmins”, requiring initiation and persistence of innate immune responses. These relocated host cell-derived activators, called damage-associated molecular patterns (DAMPs), are a key aspect of inflammation.[1]

Dying cells passively release DAMPs following injury, trauma, ischaemia or infection-induced necrosis. In the liver, passive release occurs mostly in lipid-laden, damaged, apoptotic, necroptotic or necrotic hepatocytes.[2–4] DAMPs are also actively released via secretory lysosomes in immune cells[5–7] or in stressed parenchymal and non-parenchymal cells.[8] These molecules are sensed via pattern recognition receptors (PRRs) and the NOD-like receptor protein 3 (NLRP3) or inflammasome, all of which trigger release of chemokines and other mediators to provoke initial proinflammatory responses that fight infection and cellular damage.[2,9–17] While this response can be beneficial (*i.e.*, resolving danger), sustained release of DAMPs has adverse effects in chronic liver disease.

Indeed, further injury can result when DAMPs are activated by reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are also released in response to injury and inflammation.[18–23] The second wave of cell injury and death enhances release of second-line DAMPs, triggering a more complex and pronounced reaction. In the liver, inflammatory responses such as activation of Kupffer cells (KCs) and extravasation and activation of monocyte-derived macrophages (MFs) and neutrophils[24] prompt release of tumour necrosis factor-α (TNFα) and other proinflammatory cytokines that activate the NF-κB pathway in hepatocytes to exacerbate damage.[25] Thus, while physiological levels of ROS, RNS and DAMPs contribute to liver homeostasis, their uncontrolled production and release activate signalling cascades that, if left unchecked, exacerbate liver damage.

The rapid increase in circulating levels of DAMPs reflects the severity of liver injury; therefore, these molecules could be promising biomarkers and/or potential therapeutic targets to prevent liver damage. However, the number of clinical trials targeting DAMPs, some of which are disease-specific, is still very limited; hence, a careful review of the main DAMPs that contribute to chronic liver disease is warranted.

ROS and RNS induce DAMPs and events involved in chronic liver disease

ROS and RNS are typically generated by healthy cells during biological and metabolic processes.[26] The liver generates and is exposed to free radicals via mitochondrial metabolism[27] and activation of membrane-bound NADPH oxidase (NOX),[28–30] cytoplasmic inducible nitric oxide synthase (iNOS)[31,32] and microsomal cytochrome P450.[33,34] Maintaining a balance between free radical production and antioxidant defence is crucial in the regulation of cellular homeostasis.[26,35] Likewise, physiological levels of free radicals are indispensable to preserve the immune response against pathogens and to regulate cell proliferation in response to growth factors.[26,35]

Yet, the production of free radicals can also promote inflammatory disease. In the liver, excessive oxidative and nitrosative stress not only contributes to increased production of DAMPs, but also correlates with pathogenesis of chronic liver diseases such as alcohol-related liver disease (ALD), non-alcoholic steatohepatitis (NASH), fibrosis and hepatocellular carcinoma (HCC)[36–49] (Table 1 and Fig. 1). Thus, an initial injury response can promote subsequent chronic inflammatory processes and further cell and tissue damage.

Mitochondrial dysfunction is a key factor in the pathogenesis of fatty liver diseases.[43,50,51] Acetaldehyde, the end-product of alcohol metabolism, causes structural and functional alterations in mitochondria that lower production of ATP and increase generation of ROS.[52] Further, diets enriched in fructose and fat, together with insulin resistance, enhance flux of free fatty acids (FFAs) to the mitochondria for β-oxidation. This flux increases mitochondrial membrane permeability, proton leakage and ROS production, lowering ATP levels.[53] In NASH, $CD4^+$ T cells have increased mitochondrial mass, facilitating production of mitochondrial ROS (mtROS), although treatment with antioxidants increases $CD4^+$ T cells, delaying the progression of NAFLD and HCC.[54]

ROS increase production of DAMPs, such as osteopontin (OPN)[55] and high-mobility group box 1 (HMGB1), and induce oxidative modifications that enhance immunostimulatory properties.[56,57] In KCs and MFs, membrane-bound NOX is the major source of ROS and NOX-deficient ($p47^{phox-/-}$) mice are protected from ALD.[58,59] Alcohol stimulates cytoplasmic iNOS, the major source of RNS, and increases production of peroxynitrite ($ONOO^-$) and, thus, oxidative and nitrosative stress.[60,61] Indeed, $iNos^{-/-}$ mice are protected from ALD,[59] while lack of iNOS decreases carbon tetrachloride (CCl_4)-induced fibrosis.[62,63]

Alcohol is oxidised in hepatocytes by the microsomal cytochrome P450 2E1 (CYP2E1) and generates 1-hydroxyethyl radical, a major driver of alcohol-induced liver injury.[64,65] Mice lacking *Cyp2e1* display less alcohol-induced liver injury.[64,66] Moreover, ROS and RNS bind to proteins and generate neo-antigens that elicit immune responses.[52,67] In NASH and ALD, lipid peroxidation end-products such as 4-hydroxynonenal and malondialdehyde bind DNA and proteins[68,69] to form carcinogenic exocyclic etheno-DNA adducts[70,71] and protein-adducts,[67,72,73] both of which enhance injury. ROS also regulate proangiogenic and profibrogenic responses in hepatic stellate cells (HSCs).[20,74–78]

Importantly, peroxisomal ROS and kinases are implicated in HCC. The deacetylase sirtuin 5 suppresses activity of peroxisomal acetyl-CoA oxidase-1 (ACOX1), lowers generation of H_2O_2 and reduces oxidative DNA damage in *in vivo* models of HCC.[79] In addition, liver-specific ablation of the stress-activated protein kinase *p38α* enhances ROS, whereas its re-introduction prevents fibrosis and HCC by limiting ROS.[80]

ROS and RNS do not bind receptors; instead, most cells react to them by transmitting signals to organelles including the nucleus.[81–85] In MFs, the adaptor Kelch ECH associating protein 1 (KEAP1) senses ROS and transduces signals to nuclear factor erythroid 2-related factor-2 (NRF2) to regulate production of cytokines.[81,84] In myeloid and lymphoid cells, I-kappa-B kinase (IKK) senses ROS and transduces signals to activate NF-κB and regulate the

inflammatory response.[85,86] The NLRP3 inflammasome, a key player in chronic liver disease, is also stimulated by ROS.[82]

Pre-clinical and clinical trials have investigated the efficacy of antioxidants in acute and chronic liver disease, as the antioxidant defence is usually depleted.[87,88] Vitamin E alone or in combination with the lipid-lowering agent atorvastatin alleviates progression of steatosis to NASH in animal models.[89–91] However, in a randomised clinical trial of patients with alcoholic hepatitis (AH), vitamin E alone or in combination with corticosteroids failed to confer a benefit.[92,93] Nonetheless, a clinical trial (NCT01792115) is currently evaluating the most effective dose of vitamin E for the treatment of NAFLD. N-acetylcysteine, a precursor of glutathione, is the only FDA-approved antioxidant for treatment of acetaminophen-induced hepatotoxicity.[94] Another option to reduce oxidative stress is dietary restriction, as high-calorie intake is associated with increased mtROS and reduced activity of antioxidant enzymes.[95]

DAMPs in alcohol-related liver disease

In ALD, the type of cell death determines the release of DAMPs.[96] Apoptosis is the most common and is associated with the release of DAMPs from hepatocytes.[97] Necrosis is typically observed in severe acute AH,[98] where hepatocytes undergo swelling, autolysis and death without significant signal transduction.[99] Necroptosis, which resembles necrosis, is the regulated version of necrotic cell death through the RIPK1-RIPK3 heterodimer scaffold complex that leads to the release of intracellular contents.[100] In both necrosis and necroptosis, multiple DAMPs are secreted into the extracellular space and initiate an inflammatory response[100,101] (Table 2 and Fig. 2).

Mitochondrial DAMPs

Mitochondrial DNA (mtDNA) and ATP maintain the mitochondrial structure and aid in energy metabolism.[102,103] Chronic alcohol abuse increases mtROS and causes mtDNA oxidation.[104,105] Moreover, alcohol depolarises mitochondria, disrupts mitophagy and leads to the release of mitochondrial DAMPs (mtDAMPs) into the cytosol, before they are eventually secreted from hepatocytes into the extracellular space.[105] Once released, mtDAMPs promote proinflammatory and profibrotic events that lead to ALD progression.[105]

Metabolic DAMPs

Alcohol-induced hepatocyte damage leads to the release of metabolic DAMPs, such as uric acid (following the degradation of nucleic acids) and ATP.[106] Uric acid acts as an antioxidant by scavenging ROS and $ONOO^-$ in the plasma.[107–109] Uric acid and ATP levels are elevated in serum and liver tissue from alcoholic patients and alcohol-fed mice[15]; both uric acid and ATP mediate cross-talk between hepatocytes and immune cells, enhancing inflammation.[15] Further, pharmacological depletion of uric acid and blockade of ATP protect against ALD,[110] suggesting they are candidate targets to prevent disease progression.

Stress-induced DAMPs

Cellular stress increases expression of heat shock proteins (HSPs), which act as chaperones for refolding, disaggregation and degradation of polypeptides.[111] When the chaperone activity of HSP90 is abnormal, it promotes alcohol-induced injury by enhancing hepatic lipid accumulation, MF-mediated inflammation and cellular stress.[112–114] Pharmacological inhibition of HSP90 promotes reversal of alcohol-induced liver injury.[115]

HMGB1 is an architectural protein that plays a physiological role. It binds chromatin to facilitate bending and participates in nucleosome formation, DNA replication and DNA repair.[116,117] HMGB1 also acts as a DAMP, serving as a ligand for the receptor for advanced glycation end-products (RAGE) and for toll-like receptor 4 (TLR4).[8,118] Liver biopsies from alcoholic patients show a robust increase in HMGB1 expression and translocation, which correlate with disease stage. Similar findings are observed in chronic ethanol-fed mice.[8] Further, ablation of *Hmgb1* in hepatocytes protects mice from alcohol-induced liver injury by elevating LDL and VLDL export and increasing the levels of carnitine palmitoyltransferase-1, phosphorylated 5′ AMP-activated protein kinase-α and phosphorylated peroxisome proliferator-activated receptor-α.[8]

Non-parenchymal cells also release DAMPs in ALD. For instance, hyaluronic acid (HA) produced by HSCs and hepatocytes is abundant in the extracellular matrix (ECM) of alcoholic patients.[119,120] Individuals with ALD have increased serum HA levels, which correlate with progression of ALD and fibrosis.[121,122] In addition, lipocalin-2 (LCN2), an acute-phase protein increased in patients with AH,[123] acts as an alarmin by recruiting neutrophils to the liver.[124–126]

Prostaglandin E2 (PGE2) is a potent vasodilator. In patients with advanced AH, upregulation of cyclooxygenase-2 (COX2) in MFs and KCs elevates plasma levels of PGE2, which causes immunosuppression and thus increased susceptibility to infection.[127–129] Moreover, KC-derived PGE2 increases cAMP in hepatocytes and triglyceride accumulation in livers from alcoholic patients.[130]

DAMPs in non-alcoholic steatohepatitis

NASH is characterised by increased steatosis, lobular inflammation and the presence of chicken-wire fibrosis.[131,132] During NASH progression, excessive lipid accumulation, ROS generation and endoplasmic reticulum (ER) stress damage hepatocytes. This damage triggers regulated cell death primarily through apoptosis and pyroptosis, which involves the formation of plasma membrane pores by the gasdermin family of proteins, largely induced by activation of proinflammatory caspases.[36,99,133,134] Regulated cell death results in secondary necrosis and release of intracellular materials into the extracellular space, where they act as DAMPs recognised by PRRs.[135] TLRs and NLRs sense multiple DAMPs (Table 2 and Fig. 3) that mediate inflammation and fibrosis during NASH progression.[136–140]

Intrahepatic DAMPs

Mitochondrial damage and subsequent cell death release immunogenic mtDNA.[141] TLR9, a mtDNA receptor, is internalised in intracellular organelles such as endosomes and

recognises phagocytosed unmethylated CpG DNA fragments,[138,141] which are rare in host genomic DNA but abundant in mtDNA.[141] Unmethylated CpG DNA fragments are elevated in serum from obese patients, together with upregulated TLR9 expression.[141] Mice with global or myeloid cell-specific ablation of *Tlr9* fed either a high-fat (HF) diet or a high-fat, fructose and cholesterol (HFHC) diet show reduced liver steatosis, inflammation and fibrosis.[138,141] Likewise, treatment with the TLR9 antagonist IRS954 attenuates NASH, suggesting a possible therapeutic avenue.[141] Further, single-stranded RNA (ssRNA) binds TLR7 and triggers an inflammatory response in MFs and dendritic cells.[142] Ablation of *Tlr7* attenuates progression of NASH in a methionine and choline-deficient diet mouse model by suppressing TNFα and interferon-γ (IFNγ) production and $CD4^+$ T cell recruitment.[142,143]

mtROS also act as DAMPs and contribute to NASH progression. Hepatocyte-specific ablation of *Nox4* attenuates inflammation and fibrosis in the HF and choline-deficient L-amino acid-defined (CDAA) murine models.[144] In MFs, NOX4 accelerates β-oxidation of long-chain FFAs causing oxidative stress and polarisation toward a more proinflammatory phenotype.[145] NLRP3 is the intracellular PRR that responds to these ROS[145]; it is upregulated in the livers of patients with NASH and ablation of *Nlrp3* prevents NASH progression in mice.[140] Likewise, treating mice with GKT137831, a NOX1/4 inhibitor currently being tested in clinical trials, reduces ROS and activation of NLRP3 in palmitate-treated bone marrow-derived MFs and decreases inflammation in the CDAA murine model of NASH.[144,145] Notably, MCC950, an NLRP3 inhibitor, improves NAFLD and fibrosis in obese diabetic mice.[140]

ECM-derived DAMPs

The ECM is dynamic and supports tissue homeostasis.[146,147] Active ECM remodelling is observed in both patients with NASH and mouse models of NASH.[146,148] The deposition of fibrin and fibrinogen into the ECM occurs in the liver of patients with NASH and mice fed a HF diet. Additionally, mice overexpressing mutated fibrinogen are protected from fatty liver disease.[149] Although no functional studies were performed, proteomics analysis revealed a sustained increase in biglycan, a potential ligand for TLRs, in hepatic ECM from mouse models of NASH.[146] Further, galectin-3, a secreted lectin regulating matrix-to-cell interactions, promotes progression of NASH by interacting with the interleukin-33 (IL33)/ST2 axis.[150] Although a clinical trial (NCT02462967) of belapectin, an inhibitor of galectin-3, did not improve fibrosis in patients with NASH, a significant decrease in hepatocyte ballooning was observed.[151]

Extrahepatic DAMPs

Cholesterol species act as surfactants to maintain the plasma membrane and excessive cholesterol intake and hypercholesterolemia are risk factors for NASH.[152] Cholesterol crystals are delivered by oxidised LDLs through CD36 and activate the NLRP3 inflammasome in MFs.[153] Moreover, the cholesterol-lowering drugs ezetimibe and atorvastatin suppress NLRP3 expression and inflammation in an HFHC mouse model of NASH, while targeting CD36 protects mice from NASH.[154,155]

Advanced glycation end-products (AGEs) are generated via the non-enzymatic Amadori reaction between a reducing sugar (e.g., glucose) and proteins, lipids or nucleic acids.[156] Diabetic patients have increased AGEs due to hyperglycaemia.[156] In addition, population genetics suggest that a polymorphism in the AGE receptor (RAGE) gene and circulating soluble RAGE (encoded by *AGER*) are associated with the risk of NASH.[157] In addition, a HFHC mouse model shows that dietary supplementation with AGEs aggravates inflammation and ROS production in KCs, exacerbating NASH-induced liver injury.[158] However, global knockout of *Ager* in *Ldlr*$^{-/-}$ mice minimally affects progression of NASH under short- or long-term HFHC diet feeding.[159] The role of RAGE in NASH remains inconclusive as these studies used mice of different sex.[158,159]

DAMPs in liver fibrosis

Chronic liver injury leads to pathological scarring and fibrosis.[160,161] DAMPs such as HMGB1, OPN, HSPs, IL33 and ATP activate HSCs, the main source of fibrillar collagen, the main ECM component in fibrosis[162,163] (Table 2 and Fig. 4).

Hepatic expression and serum levels of HMGB1 correlate with fibrosis stage in patients with chronic HCV or HBV infection, primary biliary cirrhosis and AH, as well as in mouse models of fibrosis based on administration of CCl_4 or thioacetamide and in the bile duct ligation model.[118,164,165] HMGB1 activates HSCs[166] and induces ER stress (unpublished observations). Our laboratory demonstrated that ablation of *Hmgb1* in hepatocytes and myeloid cells as well as neutralisation of HMGB1 and RAGE protects mice from fibrosis.[118] In addition, HMGB1 signals through RAGE in HSCs to upregulate collagen type I expression via the pMEK1/2/pERK1/2/pc-Jun signalling pathway. We showed that pMEK1/2 is upstream of pAkt and enhances collagen type I as well.[55] In addition, nilotinib, a tyrosine kinase inhibitor, ameliorates CCl_4-induced fibrosis in rats by attenuating *Hmgb1/Rage* expression and oxidative stress.[167]

OPN, a matrix-bound protein sensitive to oxidant stress and highly induced upon liver damage emerges as a key DAMP in the pathogenesis of fibrosis by increasing HMGB1 and collagen type I expression in HSCs through RAGE.[55] OPN itself upregulates collagen type I through integrin $\alpha_v\beta_3$ engagement and PI3K/pAkt/NFκB signalling. Moreover, OPN drives ductular reaction and contributes to periportal scarring and fibrosis via TGFβ signalling.[168]

HSP90 is involved in the activation and survival of HSCs.[169,170] The HSP90 inhibitor 17-AAG induces apoptosis and reduces activation of HSCs in a thioacetamide model of fibrosis.[171] HSP47, a collagen-specific chaperone, plays a key role in the deposition of collagen around fibrotic areas and is thus involved in fibrosis.[172,173] Moreover, inhibitors of HSP47 such as lactoferrin and silymarin prevent HSC activation.[174] Overexpression of heat shock factor 1 (HSF1) in HSCs activates them and increases cell proliferation by inducing HSP47 and upregulating the TGFβ/SMAD4 signalling pathway. Notably, miR-455-3p alleviates HSC activation and fibrosis by suppressing its target gene, *Hsf1*.[175]

IL33 is constitutively present in the nucleus and binds DNA.[176] Hepatic IL33 expression is increased in mice with portal fibrosis and in liver biopsies from fibrotic patients.[177,178] In

chronic liver injury, IL33 binds the IL33 receptor (IL33R) and activates NF-κB and MAPKs to enhance profibrogenic responses.[179] IL33 binding to its receptor also produces proinflammatory and T helper 2 (Th2) cytokines. Recombinant IL33 increases hepatic inflammation and activates HSCs – an effect abrogated by ablation of *Il33r* or pharmacological inhibition of MAPK signalling.[177,180]

To fuel various processes, cells transport ATP into the extracellular space via pannexin-1, converting ATP to AMP and adenosine. Extracellular ATP activates MFs through the P2X7 receptor; activated MFs release IL1β and HMGB1 that trigger inflammation and fibrogenesis.[181] Extracellular adenosine interacts with the A_{2A} (A2AR) or A_{2B} (A2BR) G-coupled protein receptors to directly stimulate fibroblast production of ECM and increase fibrosis.[182] Deletion of *Cd73* or *Cd9,* involved in adenosine production and blockade of A_{2A} or A_{2B} prevents fibrosis in mice.[183] In addition, mice lacking adenosine deaminase have a marked increase in extracellular adenosine and develop fibrosis, which is prevented by blockade of A_{2A} and A_{2B}.[184]

DAMPs in liver cancer

Liver cancer represents the common end-stage of chronic liver disease. About 90% of HCCs arise from cirrhosis[185] and mouse models of liver cancer show greater tumour incidence when exposed to chemically induced fibrosis.[186,187] DAMPs participate in both initiation and progression of liver cancer (Table 2 and Fig. 5).

Initiation of HCC

HMGB1 is increased in the liver[188–190] and serum[191] in human HCC and is associated with tumour stage and poor outcome (meta-analysis in[192]). In the diethylnitrosamine (DEN) murine model of HCC, HMGB1 expression correlates with tumourigenesis,[193] yet hepatocyte-specific ablation of *Hmgb1* only reduces tumour burden when combined with CCl_4-induced liver injury[194] or in the early stages of tumourigenesis.[195,196] This effect is mediated by activation of Yes-associated protein 1 (YAP), a key driver of hepatocellular carcinogenesis, as HMGB1 binds to the transcription factor GABPα and enhances YAP signalling *in vivo* and *in vitro.*[195]

Further, OPN expression is significantly increased in patients with HCC, correlating with tumour stage and survival.[197,198] However, the role of OPN in HCC initiation is not fully understood, as global ablation of *Opn* in the DEN model provided inconsistent results.[199–201]

Proteins of the S100 family act as intracellular Ca^{2+} sensors and extracellular DAMPs that bind RAGE[202] and are frequently dysregulated in various cancers.[203] Ablation of *S100a9* decreases tumour burden in the DEN model,[204] whereas ablation of *S100a4* does not prevent HCC caused by hepatic deletion of *Pten.*[205]

To date, the role of RAGE in HCC initiation remains unknown but truncated soluble isoforms of RAGE negatively correlate with HCC risk in human HBV and HCV infection.

[206] In addition to RAGE, HMGB1 also interacts with TLR4. This receptor has a protective role in HCC initiation, as *Tlr4* ablation increases tumour burden in the DEN model.[207]

Progression of HCC

HMGB1 induces proliferation, migration and invasion in HCC cells.[190,208,209] In an orthotropic model, *Hmgb1* ablation decreases tumour growth.[190,195] Mechanistically, under hypoxic conditions HMGB1 translocates from the nucleus to the cytosol and binds TLR9[208–210]; in a mtDNA-mediated fashion.[210] TLR9 activation helps tumour cells adapt to hypoxia, leading to mitochondrial biogenesis, tumour-associated MF invasion, tumour growth and metastasis.[190,196,210–212] Two studies suggest that HMGB1 induces HCC progression by activating RAGE.[208,209] In HCC cell lines, RAGE signalling triggers proliferation,[213,214] angiogenesis,[215] tolerance to hypoxia[216] and migration.[217]

OPN induces tumour proliferation, invasion and metastasis *in vivo* and *in vitro* activating integrins and CD44.[218,219] Importantly, OPN is associated with PDL1 levels in human and mouse HCC, suggesting a role in immune escape.[200] Thus, targeting OPN in human HCC could be a promising approach as a second line of treatment after immunotherapy.

Among S100 proteins, S100A1 is upregulated in human HCC and correlates with poor survival and reduced apoptosis.[220] S100A4 secretion by mesenchymal stromal cells induces HCC proliferation, invasion, epithelial-to-mesenchymal transition and metastasis in humans.[221,222] Further, S100A8 and S100A9 trigger ROS production and promote cell survival in HCC cells *in vitro*.[223] S100A8 induces cell proliferation, migration, invasion and tumour growth in vivo and the extent of methylation decreases in human HCC and correlates with patient survival.[224] S100A9 also induces cell proliferation and invasion through RAGE signalling.[225]

New emerging DAMPs are also thought to play a role in HCC progression. Extracellular ATP induces HCC cell migration through activation of the purinergic 2 (P2) receptor, whose expression correlates with worse patient outcome.[226] Ablation of calreticulin decreases HCC cell growth and invasion.[227] Histones, found in the nuclei of eukaryotic cells, are involved in gene regulation but can be released into the circulation under stress conditions and act as DAMPs.[228] Histone secretion and subsequent activation of TLR4 induce HCC metastasis in an orthotopic mouse model.[229]

DAMPs in other liver cancers

Little is known about the role of DAMPs in other liver cancers, although HMGB1 is increased and associated with poor survival in intrahepatic cholangiocarcinoma[230] and perihilar cholangiocarcinoma.[231]

DAMPs in ischaemia reperfusion injury and liver transplantation

Patients who progress to end-stage liver disease may require liver transplantation (LT); multiple steps during LT induce the release of DAMPs, which mediate graft injury. Damage to the liver graft results in early allograft dysfunction,[232] rejection[233] and even recurrence of HCC.[234] Unfortunately, all these events negatively affect recipient outcomes and limit the

use of marginal organs that could increase the donor source. Consequently, DAMPs are not only early markers of graft injury but also potential therapeutic targets to prevent graft dysfunction. A list of DAMPs involved in ischaemia reperfusion injury (IRI) and LT is provided in Table 2 and Fig. 6.

Donor livers release DAMPs

Although IRI is the most common cause of injury during LT,[235] release of DAMPs occurs before organ procurement and the IRI insult.[236] The majority of deceased organs in the western world are donations after brain death.[237] Experimental studies on the response to brain death show that DAMPs are released and stimulate secretion of proinflammatory cytokines by activating TLRs[238]; consequently, DAMPs affect distant organs and act as the first insult to the liver graft.[239,240]

Liver graft preservation releases DAMPs

LT involves cold ischaemia and warm IRI. Damage due to cold ischaemia occurs during organ perfusion and cold storage. This step is designed to protect parenchymal cells by slowing metabolism and stabilising them.[241] However, KCs and MFs are more sensitive to cold ischaemia and release DAMPs when activated.[242] For instance, clinical studies show high levels of HMGB1 in liver graft effluent after cold storage,[243] which correlates with post-operative early allograft dysfunction.[244]

Normothermic machine perfusion aims to provide a more physiological environment to preserve liver grafts before implantation.[245] However, despite promising clinical trial results, a recent study shows that HMGB1 and extracellular DNA increase during normothermic machine perfusion under different temperature conditions and correlate with TLR activation, suggesting that DAMPs act as inflammatory mediators during machine perfusion.[16]

Effects of DAMPs during liver graft reperfusion

Liver graft implantation requires a period of portal flow occlusion to allow anastomosis of vessels. Warm ischaemia arises when the liver graft returns to normothermic conditions.[246] When blood flow is re-established, the subsequent oxidative burst directly damages hepatocytes that then release DAMPs, which are also secreted by KCs and MFs.[247] As the major player in graft injury, IRI is inevitable during LT. DAMPs, such as HMGB1,[248] HSP,[249] extracellular ATP[250] and extracellular DNA,[16] are involved in IRI and mediate graft injury.

IRI in mice increases HMGB1 levels after 1 hour and lasts for 24 hours, indicating that HMGB1 is an early biomarker of graft injury.[251,252] Indeed, neutralizing antibodies against HMGB1 or TLR4 lessen IRI,[252,253] whereas $Hmgb1^{\Delta Hep}$ show aggravated hepatic IRI and DNA damage.[254] These findings indicate that HMGB1 is essential for intracellular homeostasis and acts as a danger signal when it is released into the circulation.

IL33 is a nuclear protein released into the extracellular space during cell injury. IL33 promotes neutrophil infiltration, migration and formation of neutrophil extracellular traps (NETs) by binding to its receptor, suppression of tumorigenicity 2 (ST2).[255] Although it was

reported that NETs formation is beneficial for the host defence against pathogens,[256] recent studies found that IL33 secreted by liver sinusoidal endothelial cells promotes NETs formation and eventually exacerbates inflammation and liver injury.[257] Other DAMPs such as HMGB1 and histones induce NETs through TLR signalling. In addition, HMGB1 and histones reside in NETs and can recruit more neutrophils to further aggravate IRI.[258]

Further, ATP released from injured or stressed cells acts as a DAMP by binding to P2, activating the inflammasome in MFs via pannexin-1 and contributing to liver damage during IRI.[259]

Circulating histones significantly elevate and exacerbate liver damage following IRI signalling through TLR9. However, histone neutralisation and *Tlr9* ablation ameliorate injury *in vivo*.[260] During IRI, histones activate the NLRP3 inflammasome in KCs by generating ROS in a TLR9-dependent manner.[261]

Although HSP90 and HSP47 participate in the pathogenesis of ALD and fibrosis, HSP70 protects rat livers from IRI by reducing hepatic inflammatory and oxidative damage.[262] Overexpression of HSP27 in mice protects from hepatic IRI by reducing necrosis, apoptosis and neutrophil infiltration.[263] Likewise, PGE2 levels are significantly higher in the plasma of LT recipients with good graft function.[264] Although HSP70 and PGE2 are considered danger signals, their protective effects against liver IRI and graft injury suggest a re-evaluation of their role as DAMPs in the setting of LT [265,266]

Role of DAMPs in immune rejection

DAMPs induce innate and adaptive immune responses that result in immune rejection.[258] After organ reperfusion, in addition to accumulated DAMPs generated by cold storage and IRI, alloantibodies from recipients lead to non-infectious injury and release of DAMPs, which persist after resolution of IRI.[236] Many of these DAMPs bind to TLRs and drive the immune reaction toward the allograft. Further, lung and heart transplantation demonstrate a link between the release of DAMPs and acute rejection.[267,268] Although the liver is an immunotolerant organ, the effect of DAMPs in LT rejection has been reported.[269]

CD39 is essential to maintain homeostatic levels of ATP. *Cd39*$^{-/-}$ mice exhibit increased liver-infiltrating CD8+ T cells, stronger response to donor alloantigens and reduced recipient survival rates after major histocompatibility complex mismatched LT.[269] This outcome reinforces the involvement of extracellular ATP in post-transplant rejection.

HSPs also protect against LT rejection. Indeed, a retrospective clinical analysis shows significantly lower *HSP70* mRNA levels in graft biopsy samples from LT recipients who developed graft dysfunction caused by rejection.[270]

Resolving the effects of DAMPs

Reducing release, promoting clearance and inhibiting DAMP signalling have been proposed to reduce graft injury and improve recipient outcomes. Treatment with recombinant soluble thrombomodulin attenuates liver graft injury by binding to HMGB1 and preventing the proinflammatory response.[271] A similar effect is achieved by inhibiting TLR4, an HMGB1

receptor.[252,253] In addition, preconditioning with low concentrations of HMGB1 before LT protected against hepatic IRI.[272] Enhancement of extracellular ATP clearance by activating the P1 receptor A2A on bone marrow-derived cells also protects livers from IRI.[273] Further, since NETs play a role in IRI, studies have examined inhibition of NETs during IRI. Peptidylarginine-deiminase-4 (PAD4) is required for formation of NETs and inhibition of PAD4 alleviates liver IRI in mice.[274] However, considering the role of NETs in host defence, inhibition of NETs should be given special consideration due to the risk of infection in LT recipients.

Along with reducing harmful DAMPs, enhancing protective DAMPs holds promise for reducing hepatic IRI. Activation of HSP70 protects against hepatic IRI,[249] an effect attributed to iNOS. Although nitrosative stress promotes ALD, fibrosis and HCC,[22,32,59] it protects against hepatic IRI by activating HSP70. While specific HSPs are pathogenic in some liver diseases, the protective effect of HSP70 reported in these studies[262] suggests that the role of HSPs as DAMPs during hepatic IRI should be re-evaluated. HSPs could provide a potential target to attenuate liver injury after LT.

Concluding remarks

Overall, these studies suggest that DAMPs induced by ROS and RNS, as well as DAMPs that signal through receptors and are produced by injured hepatocytes or non-parenchymal cells during ALD, NASH, fibrosis and HCC drive liver injury by increasing oxidative stress, lipid accumulation, inflammation and fibrosis. To our knowledge, there are not many existing clinical trials successfully targeting DAMPs to prevent onset and progression of chronic liver diseases. Blocking specific DAMPs alone or in combination could be a promising strategy to improve patient survival in the future, as HCC is the second leading cause of cancer-related deaths worldwide.[275,276] While LT aims to rescue patients with end-stage liver disease, this surgical procedure is associated with significant release of DAMPs along with DAMP-induced graft injury and immune rejection. A thorough understanding of the role of each DAMP in LT is essential to improve graft quality and recipient outcomes, which could eventually be achieved by targeting specific DAMPs or controlling their signalling.

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Abbreviations

A2AR	adenosine A_{2A} receptor
A2BR	adenosine A_{2B} receptor

ACOX1	acetyl-CoA oxidase
AGE	advanced-glycation end-products
AH	alcoholic hepatitis
ALD	alcoholic liver disease
CCl_4	carbon tetrachloride
CDAA	choline-deficient amino acid-defined
COX2	cyclooxygenase-2
CYP2E1	cytochrome P450 2E1
DAMP(s)	damage-associated molecular pattern(s)
DEN	diethylnitrosamine
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FFA(s)	free fatty acid(s)
GABPα	GA binding protein transcription factor subunit-α
HA	hyaluronic acid
HCC(s)	hepatocellular carcinoma(s)
HF	high-fat
HFHC	high-fat, fructose and cholesterol
HMGB1	high-mobility group box-1
HSC(s)	hepatic stellate cell(s)
HSF1	heat shock factor-1
HSP	heat shock protein
IFNγ	interferon-γ
IKK	I-κappa-B kinase
IL33R	IL33 receptor
iNOS	inducible nitric oxide synthase
IRI	ischaemia reperfusion injury
KEAP1	Kelch ECH associating protein-1

KC(s)	Kupffer cell(s)
LCN2	lipocalin-2
LT	liver transplantation
MAPK	mitogen-activated protein kinase
MF(s)	macrophage(s)
mtDAMP(s)	mitochondrial DAMPs
mtDNA	mitochondrial DNA
mtROS	mitochondrial ROS
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NET(s)	neutrophil extracellular trap(s)
NLRP3	NOD-like receptor protein-3
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
NOX	NADPH oxidase
NRF2	nuclear factor erythroid 2-related factor-2
$ONOO^-$	peroxynitrite
OPN	osteopontin
P2	purinergic-2
P2RX7	purinergic receptor P2X7
PAD4	peptidyl-arginine-deiminase-4
pAKT	phosphorylated protein kinase-B
pc-Jun	phosphorylated c-Jun
pERK1/2	phosphorylated extracellular signal-regulated kinase
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
pMEK	phosphorylated mitogen-activated protein kinase
PRR(s)	pattern recognition receptor(s)
RAGE	receptor for advanced glycation end-products

RNS	reactive nitrogen species
ROS	reactive oxygen species
ssRNA	single-stranded RNA
ST2	suppression of tumourigenicity-2
TGFβ	transforming growth factor-β
Th2	T helper-2
TLR(s)	toll-like receptor(s)
TNFα	tumor necrosis factor-α
YAP1	Yes-associated protein-1

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Author names in bold designate shared co-first authorship

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Key point

Damage-associated molecular patterns are signalling molecules involved in inflammatory responses and restoration of homeostasis.

Chronic release of these molecules promotes inflammation in the context of liver disease.

Reactive oxygen species and reactive nitrogen species induce damage-associated molecular patterns.

Specific damage-associated molecular patterns participate in pathogenesis of chronic liver diseases such as alcohol-related liver disease, non-alcoholic steatohepatitis, liver fibrosis and liver cancer.

Damage-associated molecular patterns play a role in ischaemia reperfusion injury and liver transplantation.

Blockade of specific damage-associated molecular patterns has proven beneficial in humans and mice.

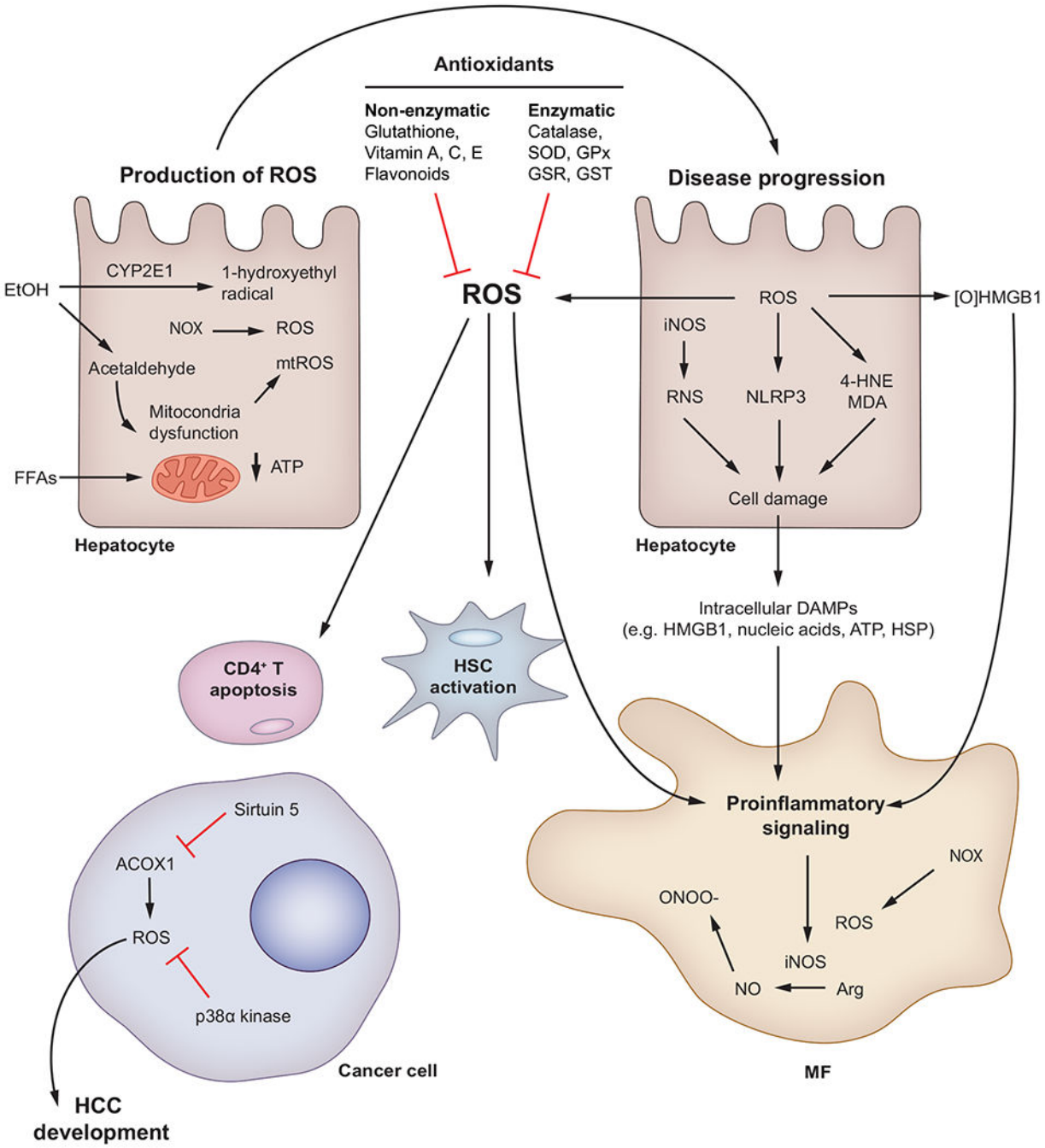

Fig. 1. ROS and RNS induce DAMPs and events involved in chronic liver disease.

ROS are produced mostly in hepatocytes and MFs by CYP2E1, mitochondrial injury and NOX. ROS participate in progression of chronic liver disease, causing hepatocyte damage, inflammation, HSC activation and $CD4^+$ T cell apoptosis. Peroxisomal ROS and kinases contribute to HCC development and resolution, respectively. RNS are generated in hepatocytes and MFs due to activation of iNOS. Excess NO reacts with ROS to generate damaging RNS such as $ONOO^-$. Enzymatic and non-enzymatic antioxidant defence systems balance the generation of ROS and play an important role in resolution of liver disease. 4-

HNE, 4-hydroxynonenal; ACOX1, acetyl-CoA oxidase; CYP2E1, cytochrome P450 2E1; DAMP(s), damage-associated molecular pattern(s); EtOH, ethanol; FFAs, free fatty acids; GPx, glutathione peroxidase; GSR, glutathione-disulfide reductase; GST, glutathione S-transferase; HCC, hepatocellular carcinoma; HSC(s), hepatic stellate cell(s); iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; MF(s), macrophages; mtROS, mitochondrial ROS; NLRP3, NOD-like receptor protein-3; NO, nitric oxide; NOX, NADPH oxidase; [O]HMGB1, disulfide High-mobility group box-1; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.

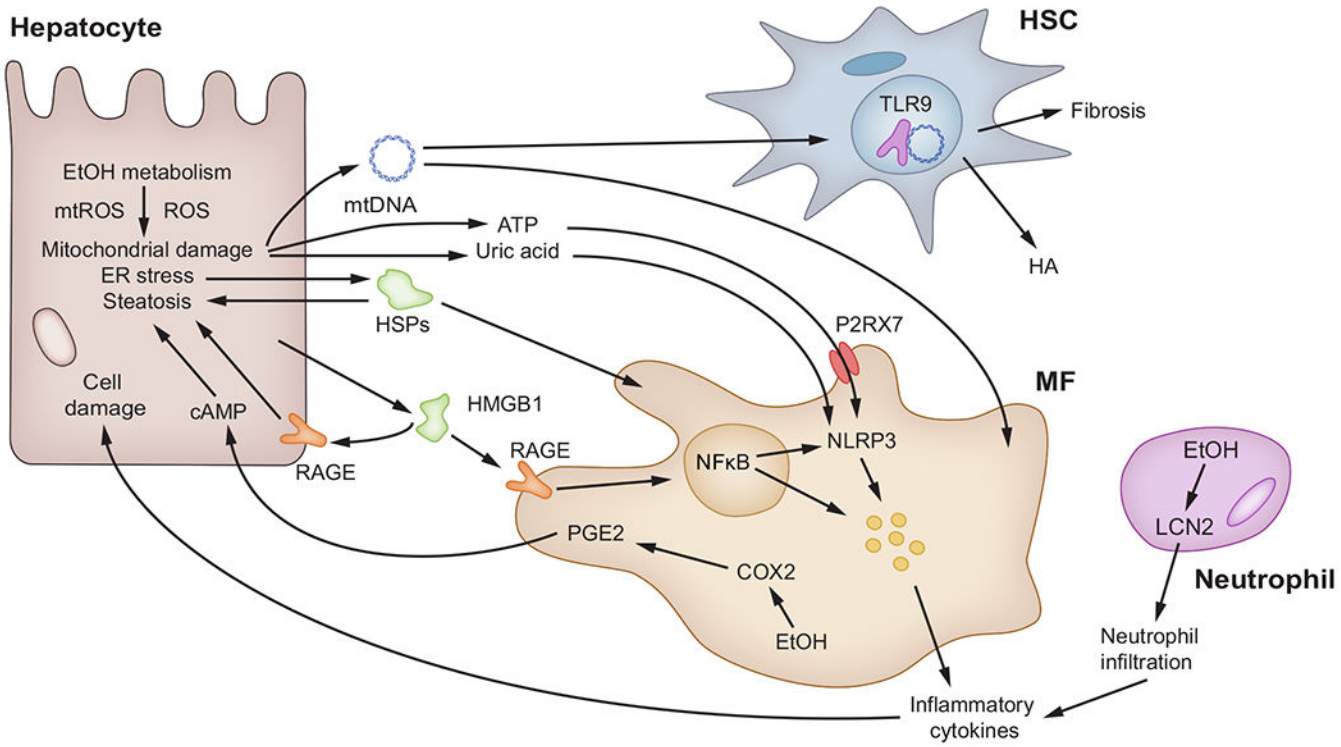

Fig. 2. DAMPs promote inflammation, steatosis and hepatocyte injury in ALD.

Ethanol-induced hepatocyte injury causes release of DAMPs, including mitochondrial DAMPs (mtDNA and ATP), uric acid, HSPs and HMGB1 from damaged hepatocytes. Most of these DAMPs are recognised by MFs through RAGE, TLRs and P2RX7 and activate NF-κB and the NLRP3 inflammasome. These result in release of proinflammatory cytokines that trigger cellular injury and steatosis. HSCs release HA and are responsive to mtDNA which activates them. MFs produce PGE2 that causes steatosis via cAMP activation. Neutrophils produce LCN2 and respond to it by infiltrating the liver to exacerbate cellular injury by releasing proinflammatory cytokines. cAMP, cyclic adenosine monophosphate; COX2, cyclooxygenase-2; DAMP(s), damage-associated molecular pattern(s); EtOH, ethanol; HA, hyaluronic acid; HMGB1, high-mobility group box-1; HSC(s), hepatic stellate cell(s); HSPs, heat shock proteins; LCN2, lipocalin-2; MF(s), macrophage(s); mtDNA, mitochondrial DNA; mtROS, mitochondrial ROS; NFκB, nuclear factor kappa B; NLRP3, NOD-like receptor protein-3; P2RX7, purinergic receptor P2X7; PGE2, prostaglandin E2; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; TLR9, toll-like receptor 9.

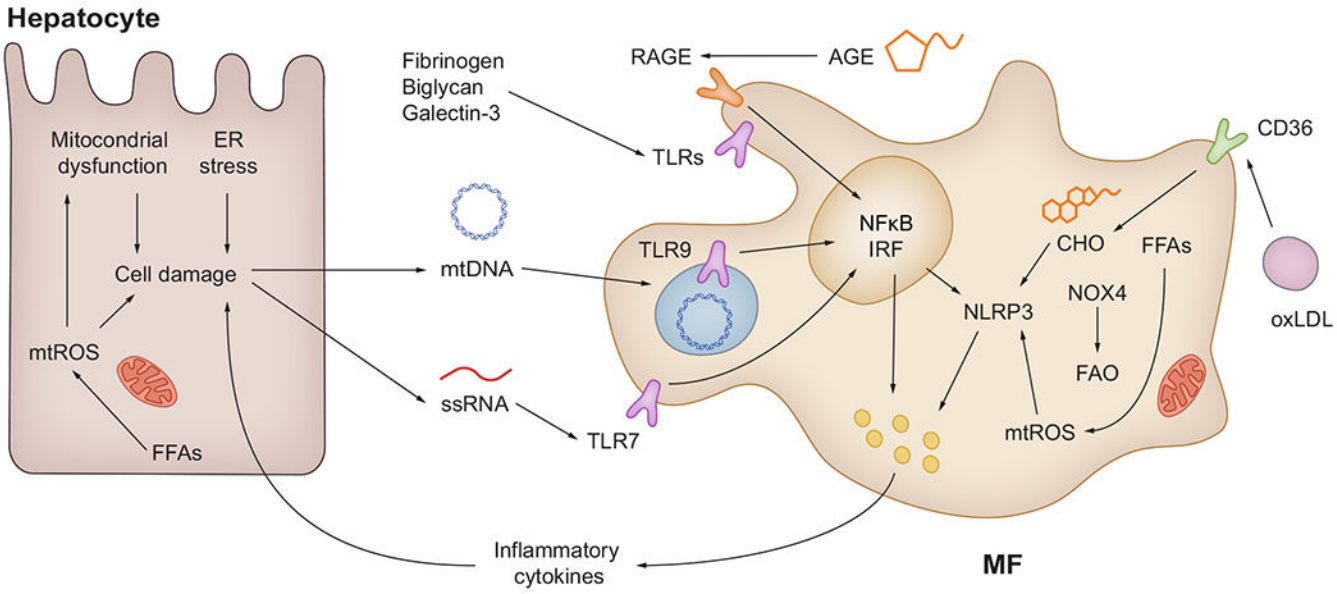

Fig. 3. Intrahepatic and extrahepatic DAMPs contribute to NASH.

Damaged hepatocytes are the major source of intrahepatic DAMPs (mtDNA and ssRNA). ECM components such as biglycan, fibrinogen and galectin-3 can also act as DAMPs to active TLRs. MFs and dendritic cells recognise DAMPs through RAGE, TLRs and NLRP3 signalling. Extrahepatic DAMPs (AGE, FFAs and oxidised LDLs) are delivered via circulation and can bind RAGE and CD36, contributing to steatohepatitis. AGE, advanced glycation end-products; CHO, cholesterol; DAMP(s), damage-associated molecular pattern(s); ER endoplasmic reticulum; FAO, fatty acid oxidation; FFA(s), free fatty acid(s); IRF, interferon-regulatory factor; MF(s), macrophage(s); mtDNA, mitochondrial DNA; mtROS, mitochondrial ROS; NFkB, nuclear factor kappa B; NLRP3, NOD-like receptor protein-3; NOX4, NADPH oxidase 4; oxLDL, oxidized low-density lipoproteins; ssRNA, single-stranded RNA; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; TLR(s), Toll-like receptor(s).

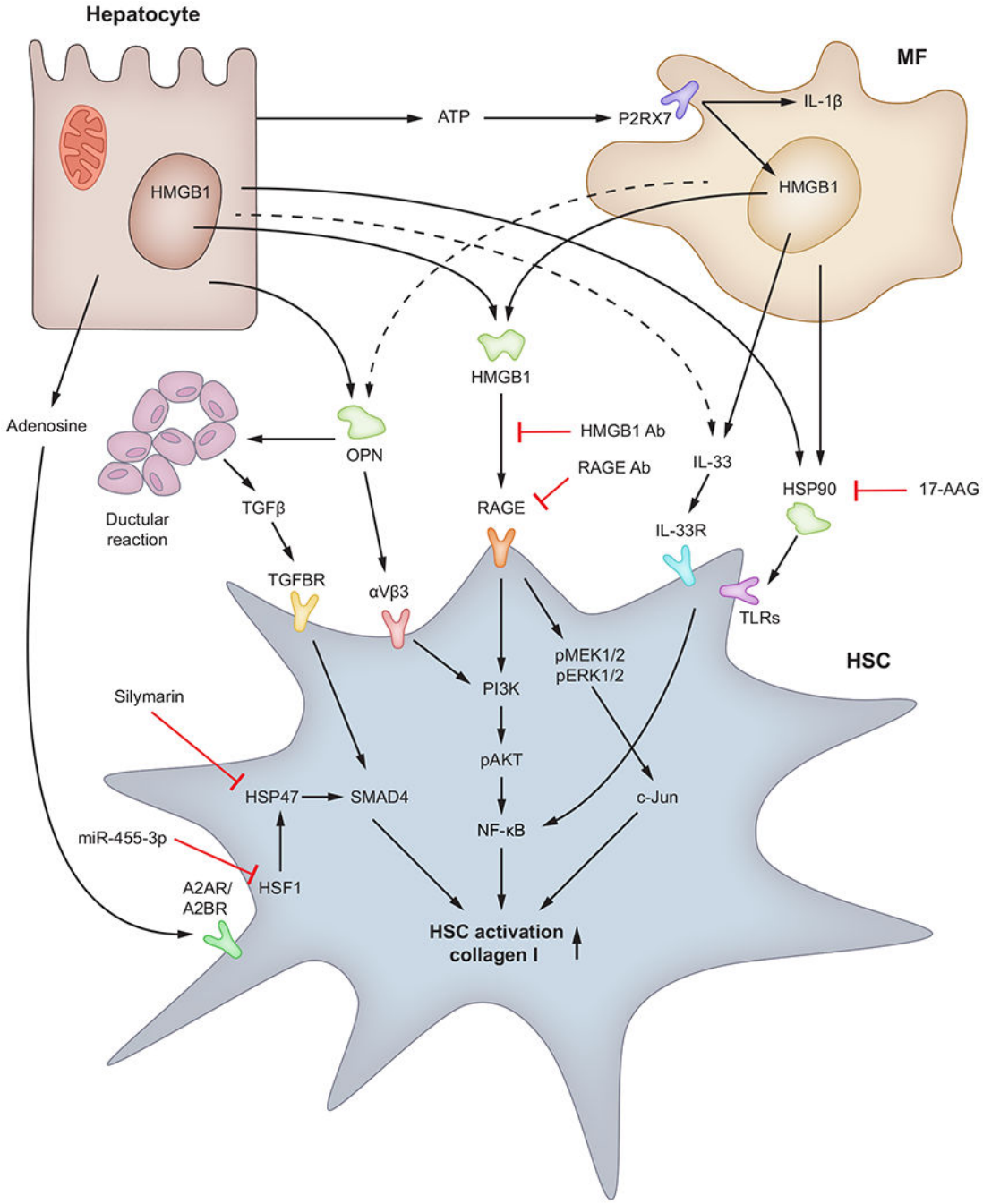

Fig. 4. DAMPs activate HSCs and contribute to fibrosis.

In addition to being a significant source of ROS, hepatocytes produce adenosine, OPN and HMGB1, which target HSCs through A2AR/A2BR, $\alpha_v\beta_3$ integrin and RAGE, respectively and activate HSCs to promote scar deposition. MFs are also a significant source of ROS due to NOX activation and they produce HMGB1, OPN, IL33 and HSPs, which signal through RAGE, $\alpha_v\beta_3$ integrin, IL33R and TLRs, respectively, in HSCs to magnify the fibrogenic response. The contribution of biliary epithelial cells to HSC activation is significant as they produce TGFβ, which enhances collagen type I synthesis. HMGB1, OPN, IL-33, HSPs, ATP

and adenosine, through interaction with their receptors on HSCs, signal via MEK1/2/c-Jun, PI3k/pAKT/NF-κB and TGFBR/Smad4 pathways to enhance collagen type I. Ab, antibody; DAMP(s), damage-associated molecular pattern(s); HMGB1, high-mobility group box-1; HSC(s), hepatic stellate cell(s); HSF, heat shock factor; HSPs, heat shock proteins; IL1, interleukin-1; IL33R, IL33 receptor; MF(s), macrophage(s); NOX, NADPH oxidase; OPN, osteopontin; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; TGFβ, transforming growth factor β; TGFBR, transforming growth factor beta receptor; TLR(s), Toll-like receptor(s).

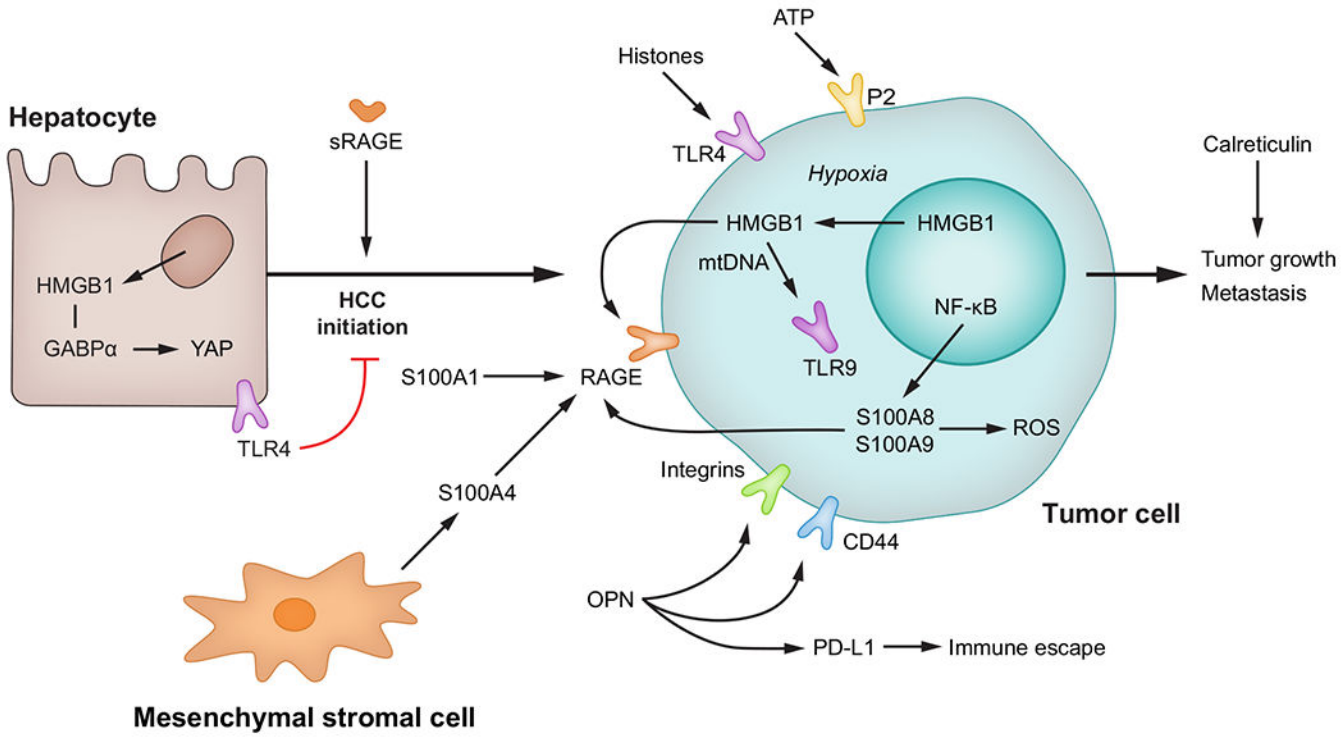

Fig. 5. Role of DAMPs in initiation and progression of HCC.

HMGB1 participates in initiation of HCC by GABPα-mediated activation of YAP signalling, while TLR4 represses it. In tumour cells, intracellular HMGB1, through mtDNA and TLR9 signalling as well as S100A8 and S100A9 via ROS production, contributes to tumour growth and metastasis. Extracellular DAMPs such as HMGB1, OPN, S100A1, S100A4, ATP, histones and calreticulin contribute to tumour progression. DAMP(s), damage-associated molecular pattern(s); GABPα, GA binding protein transcription factor subunit-α; HCC, hepatocellular carcinoma; HMGB1, high-mobility group box-1; mtDNA, mitochondrial DNA; OPN, osteopontin; RAGE, receptor for advanced-glycation end-products; ROS, reactive oxygen species; sRAGE, soluble RAGE; TLR(s), Toll-like receptor(s); YAP, Yes-associated protein.

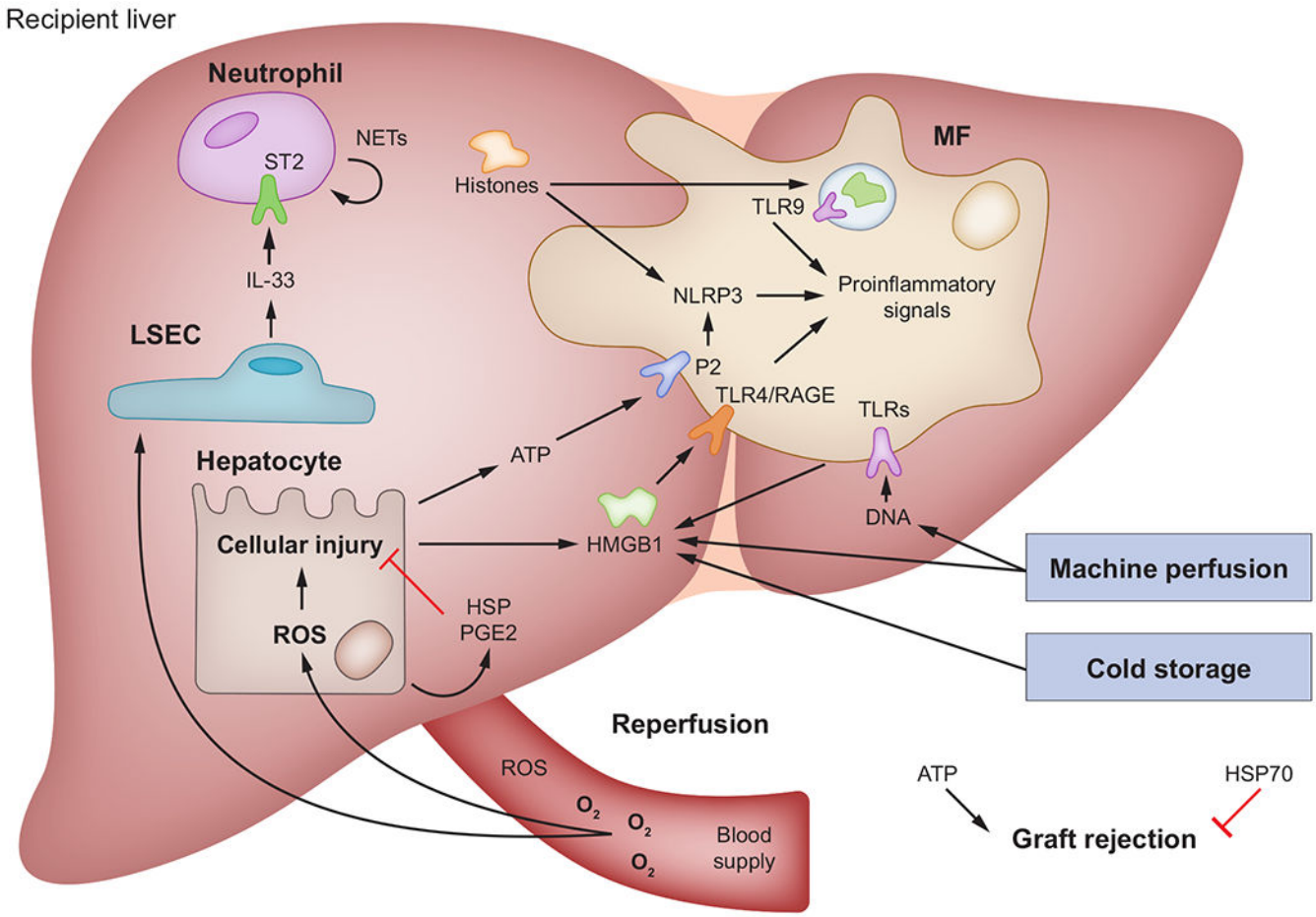

Fig. 6. The role of HMGB1 in hepatic IRI and LT.

Multiple steps during LT release DAMPs that, in turn, are involved in graft injury and immune rejection. First, methods for preserving liver grafts, such as cold storage and machine perfusion, induce release of DAMPs into the perfusate, which then become flushed into circulation after perfusion. Second, the oxidative burst during graft reperfusion damages hepatocytes and actives Kupffer cells and MFs to release various DAMPs, which mediate graft injury and immune response through selective receptors. Third, in addition to DAMPs that induce a harmful response, HSP and PGE2 protect the liver graft from injury and inhibit immune rejection. DAMP(s), damage-associated molecular pattern(s); HMGB1, high-mobility group box-1; HSP(s), heat shock proteins; IL, interleukin; IRI, ischaemia reperfusion injury; LSEC, liver sinusoidal endothelial cell: LT, liver transplantation; MF(s), macrophages; NETs, neutrophil extracellular traps; NLRP3, NOD-like receptor protein-3; PGE2, prostaglandin E2; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; ST2, suppression of tumorigenicity 2; TLR(s), Toll-like receptor(s).

Table 1.

ROS and RNS induce events involved in chronic liver disease.

	Effect(s)	Reference(s)
ALD		
ROS	Mitochondrial dysfunction; Proinflammatory; Profibrogenic	51,76,77
RNS	$ONOO^-$ induced liver injury	22,60,61
NASH		
ROS	Lipid peroxidation; Proinflammatory	43,44,69,71
RNS	*De novo* lipogenesis; Proinflammatory	45
Fibrosis		
ROS	TGFβ signalling; HSC activation	46,78
RNS	iNOS induces MMP9; DNA damage; Profibrogenic	62,63
HCC		
ROS	Oxidative DNA damage; DNA adducts; Proinflammatory; Oncogenic; Increase telomerase activity, telomere length and HCC tumour growth; Protein oxidation	47–49
RNS	iNOS promotes HCC stem cell phenotype	32

ALD, alcohol-related liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma.

Table 2.

DAMPs are involved in chronic liver injury and restoration of homeostasis.

DAMP	Receptor(s)	Effect(s)	Reference(s)
ALD			
mtDNA	TLR9	Proinflammatory; Profibrogenic	105
Uric acid	NLR	Proinflammatory	15
ATP	P2RX7	Proinflammatory	110
HSP90		Oxidative stress; Prosteatotic; Proinflammatory	115
HMGB1	RAGE/TLR4	Prosteatotic	118
Hyaluronic acid	TLRs	Profibrogenic	122
LCN2	LCN2R	Proinflammatory	125
PGE2	PGE2 receptor	Immunosuppression; Prosteatotic	128
NASH			
mtDNA	TLR9	Prosteatotic; Profibrogenic	2,138
ssRNA	TLR7	Monocyte-derived macrophage activation; IFNγ and TNFα production; T cell recruitment	142,143
AGE	RAGE	Increase during NASH progression	157
mtROS	NLRP3	Prosteatotic; Profibrogenic	144,145
Biglycan	TLR2/4	Increase during NASH progression	146
Galectin-3	TLR2/4	Promote fibrosis and hepatocyte ballooning	150,151
Fibrinogen	TLR4	Form deposits to promote fatty liver disease	149
Cholesterol crystals	NLRP3	Prosteatotic	153,154
Fibrosis			
HMGB1	RAGE	Hepatic stellate cell activation; Collagen type I production; Endoplasmic reticulum stress	55,118,166
OPN	Integrin $\alpha V\beta_3$	Increases HMGB1; HSC activation via PI3K/pAkt/NF-κB; Ductular reaction	55,168
HSP90	TLR2/TLR4	HSC activation	169,170
HSP47	TLRs	Profibrogenic	172,173
IL-33	IL-33R	Profibrogenic via NF-κB and MAPKs; Proinflammatory (Th2 cytokines)	176–179
ATP adenosine	P2rX7, A2AR, A2BR	MF release of IL1β and HMGB1; Profibrogenic	181,182
HCC			

DAMP	Receptor(s)	Effect(s)	Reference(s)
HMGB1	RAGE, TLR9	Tumour initiation and progression	190,209,210
OPN	Integrins, CD44	Tumour growth, metastasis and immune escape	200,219
S100A1	RAGE	Increases in HCC and correlates with poor survival	220
S100A4	RAGE	Tumour growth and metastasis	221,222
S100A8	RAGE	Tumour growth and metastasis	223,224
S100A9	RAGE	Tumour initiation and progression	204,223
mtDNA	TLR9	HMGB1 binding to TLR9	210
Extracellular ATP	P2	HCC cell migration	226
Calreticulin	N/A	Tumour growth and invasion	227
Histones	TLR4	HCC metastasis	229
IRI&LT			
ATP	P1, P2	IRI; Graft rejection	259,269
DNA	TLRs	Accumulates following machine perfusion	16
Histones	TLR9, NLRP3	IRI	260,261
HMGB1	TLR4, RAGE	Circulating HMGB1 exacerbates hepatic IRI; Intracellular HMGB1 protects from IRI	
HSP 70 HSP 27	TLRs, LOX-1	Protects from hepatic IRI; Inhibits graft rejection	262,263,270
IL-33	ST2	IRI; NETs	255,257
PGE2	PG receptor	Induced in recipients with good graft function	264

ALD, alcohol-related liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; IRI, ischaemia reperfusion injury; LT, liver transplantation; AGE, advanced glycation end-products.