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DOI:

[10.1016/j.jhep.2023.05.017](https://doi.org/10.1016/j.jhep.2023.05.017)

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Document Version

Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Khan, RS, Lalor, PF, Thursz, M & Newsome, P 2023, 'The role of neutrophils in alcohol-related hepatitis', *Journal of Hepatology*, vol. 79, no. 4, pp. 1037-1048. <https://doi.org/10.1016/j.jhep.2023.05.017>

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The role of neutrophils in alcohol-related hepatitis

Reenam S. Khan¹, Patricia F. Lalor¹, Mark Thursz², Philip N. Newsome^{1,*}

Summary

Alcohol-related liver disease is a major cause of liver disease-associated mortality, with inpatient care being a major contributor to its clinical and economic burden. Alcohol-related hepatitis (AH) is an acute inflammatory form of alcohol-related liver disease. Severe AH is associated with high short-term mortality, with infection being a common cause of death. The presence of AH is associated with increased numbers of circulating and hepatic neutrophils. We review the literature on the role of neutrophils in AH. In particular, we explain how neutrophils are recruited to the inflamed liver and how their antimicrobial functions (chemotaxis, phagocytosis, oxidative burst, NETosis) may be altered in AH. We highlight evidence for the existence of 'high-density' and 'low-density' neutrophil subsets. We also describe the potentially beneficial roles of neutrophils in the resolution of injury in AH through their effects on macrophage polarisation and hepatic regeneration. Finally, we discuss how manipulation of neutrophil recruitment/function may be used as a therapeutic strategy in AH. For example, correction of gut dysbiosis in AH could help to prevent excess neutrophil activation, or treatments could aim to enhance miR-223 function in AH. The development of markers that can reliably distinguish neutrophil subsets and of animal models that accurately reproduce human disease will be crucial for facilitating translational research in this important field.

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Introduction

Alcohol-related hepatitis (AH) is an acute inflammatory condition, that typically occurs on a background of chronic liver disease and is associated with features of liver failure. Based on National Inpatient Sample data in the US, between 2007-2014 there were 159,973 admissions for ALD, 18.4% of which involved a primary diagnosis of AH.¹

Patients with severe AH have a poor prognosis, with mortality rates of 20% at 28 days, up to 30% at 90 days, and 50% at 1 year.²⁻⁴ Infection is a common cause of mortality in AH; for example, in the landmark STOPAH trial, infections were responsible for 25% of all deaths.⁵ Traditionally, steroids, pentoxifylline and N-acetylcysteine have been given for the treatment of AH, but recent studies suggest no convincing long-term survival benefit.⁶ Whilst liver transplant has been associated with an unequivocal survival benefit in severe AH,⁷ this intervention is not widely available due to systemic stigmatisation, lack of social support, recurrent alcohol use, presence of sepsis and frequency of multi-organ failure. This highlights an unmet clinical need for new therapies in AH.

Patients with AH frequently have increased circulating neutrophil counts.^{8,9} According to a study by Ma *et al.*, there are two histological subtypes in AH, despite a similar clinical presentation, one with high intrahepatic neutrophils and low levels of CD8 T cells and vice versa. In their study, the degree

of hepatic neutrophil infiltration significantly correlated with the model for end-stage liver disease score.¹⁰ Other studies demonstrate an association between hepatic neutrophil infiltration and 90-day mortality.¹¹ In patients with severe AH, a high systemic neutrophil-to-lymphocyte ratio has been shown to be predictive of mortality,¹² acute kidney injury and infection.¹³ In this review, we summarise what is known about neutrophils in the pathogenesis of AH, to determine if and how they may be used as a therapeutic target.

Neutrophil production, maturation and classification

Neutrophils are polymorphonuclear cells that encompass 70% of all leukocytes. They are made in large numbers (>1x10¹¹ cells/day) from haematopoietic precursors in the bone marrow, in a process known as granulopoiesis, that takes around 14 days. During neutrophil maturation in humans, they start to express CD15 and CD11b, followed by CD16 and CD10 at a later stage.¹⁴ In health, only 1-2% of neutrophils in the body are found in the circulation due to tight regulation of neutrophil migration between the bone marrow, the blood, and the target tissues. Cytokines, such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor promote neutrophil egress from the bone marrow as well as neutrophil proliferation, survival, and differentiation.

Keywords: alcoholic hepatitis; alcohol-related liver disease; neutrophils; immune cell recruitment; sepsis.

Received 12 January 2023; received in revised form 24 May 2023; accepted 25 May 2023; available online 6 June 2023

* Corresponding author. Address: Institute of Biomedical Research, The Medical School, University of Birmingham, Birmingham, B15 2TT, United Kingdom.

Tel.: +44 (0) 121 415 8700; fax: +44 (0) 121 414 5614.

E-mail address: p.n.newsome@bham.ac.uk (P.N. Newsome).

<https://doi.org/10.1016/j.jhep.2023.05.017>



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Key points

- Alcohol-related hepatitis (AH) is frequently associated with increased neutrophil counts in the circulation and the liver.
- Neutrophils in AH are activated at baseline, predisposing them to spontaneous NETosis, and promoting the release of reactive oxygen species and proteases.
- Neutrophils in AH exhibit impaired phagocytotic and oxidative burst responses to *E. coli*, which may increase infection risk.
- Neutrophils may contribute to resolution of injury through release of hepatocyte growth factor and by inducing conversion of macrophages to a 'pro-resolution' phenotype.
- Neutrophil miR-223 protects against oxidative stress, but its expression is reduced in AH.

Alcohol excess and cirrhosis, both of which are commonly associated with AH, are associated with impaired granulopoiesis.

Neutrophils provide the first line of defence against bacterial infection. Mature neutrophils have a broad array of pattern recognition receptors that scan the extracellular milieu and endosomal compartments (Fig. 1). Circulating neutrophils are usually in a 'resting' state, characterised by non-adherence, a round morphology and limited capacity to respond to activating stimuli. There are neutrophil pattern recognition receptors, such as Toll-like receptor (TLR)2 and TLR9, that can detect structural motifs on invading pathogens (pathogen-associated molecular patterns), and endogenous danger signals indicating host injury or stress (danger-associated molecular patterns). These 'prime' the neutrophils for a more robust response to activation.

Neutrophil Fc receptors or complement receptors can also recognise opsonised microbes, which cause direct cell activation. Activated cells can ingest pathogens in a process

known as phagocytosis. They can also produce an 'oxidative burst' of superoxide ions and reactive oxygen species (ROS) which kill microorganisms, and they can release neutrophil extracellular traps (NETs).

Emerging evidence demonstrates that neutrophils play diverse roles beyond bacterial killing. Although circulating neutrophils only have a half-life of around 12 h, the half-life of tissue neutrophils is approximately 6-15-fold longer than this,¹⁵ allowing sufficient time for neutrophil plasticity.

Whilst there is heterogeneity in neutrophil phenotype and function, whether this is due to the presence of distinct neutrophil subsets, or whether this simply represents different stages of neutrophil activation in response to environmental cues remains controversial.¹⁶ On density gradient centrifugation, the bulk of neutrophils are dense and separate at the bottom of the gradient. These 'high-density' neutrophils (HDNs) have classic morphological features of a lobulated nucleus and a granular cytoplasm. In the upper part of the

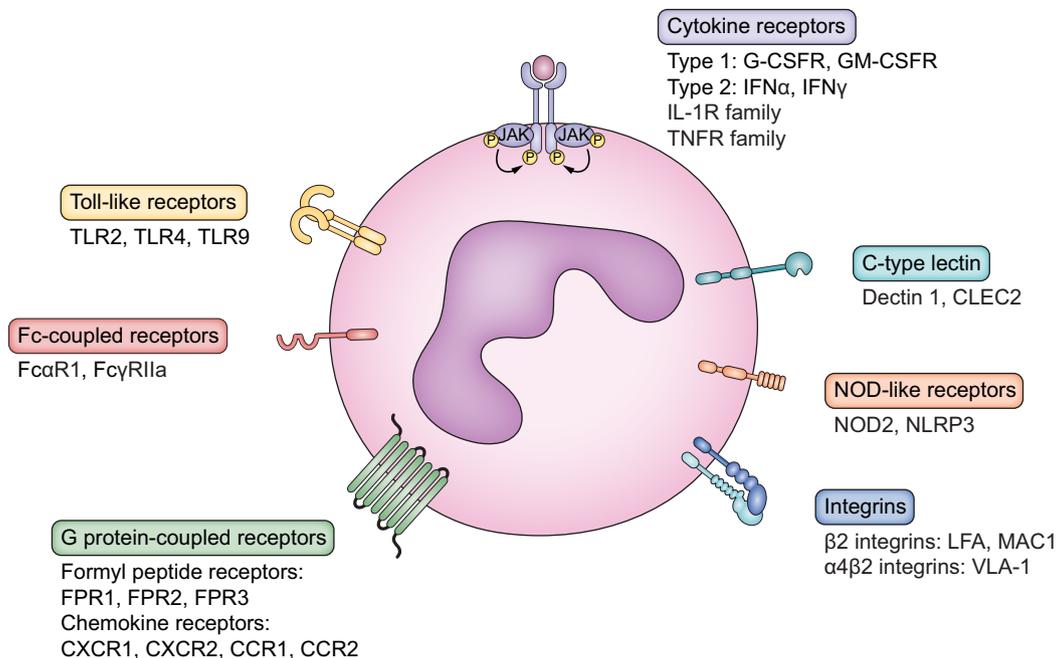


Fig. 1. Key receptors found on mature neutrophils. CCR, C-C motif chemokine receptor; CLEC2, C-type lectin-like type 2; CXCR, chemokine C-X-C motif chemokine receptor; Fc α R1, Fc alpha receptor 1; Fc γ RIIa, Fc gamma receptor 2a; FPR, formyl peptide receptor; G-CSFR, granulocyte colony stimulating factor receptor; GM-CSFR, granulocyte monocyte colony stimulating factor receptor; IFN, interferon; IL1R, interleukin 1 receptor; LFA, lymphocyte function-associated antigen; Mac-1, macrophage-1 antigen; NLRP3, NLR family pyrin domain containing 3; NOD, nucleotide-binding oligomerization domain; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor; VLA-1, very late antigen 1.

density gradient, where peripheral blood mononuclear cells are typically found, are ‘low-density’ neutrophils (LDNs). These comprise immature neutrophils and mature cells with immunosuppressive properties, known as ‘myeloid-derived suppressor cells’ (MDSCs). Studies of MDSCs have predominantly been in the context of cancer, in which they promote tumour progression.¹⁷ MDSCs share the panel of surface markers that is used to define adult mature neutrophils (CD14-CD15+CD16+CD66b+).¹⁸ However, MDSCs are morphologically immature, with band-like nuclei,¹⁹ yet exhibit an immunosuppressive phenotype that is not seen in immature neutrophils. Cho *et al.* reported an increased population of LDNs in patients with AH.²⁰ In comparison to HDNs, these LDNs exhibited reduced ROS production at rest and upon lipopolysaccharide (LPS) stimulation, as well as reduced phagocytic capacity. They were also less susceptible to macrophage efferocytosis, impeding hepatic clearance.

Neutrophil recruitment in alcohol-related hepatitis

Neutrophil recruitment in AH is a complex process (summarised in Fig. 2), which depends on neutrophil priming, chemokines, and activation of the endothelial cells lining the hepatic sinusoids.

Neutrophil priming and/or activation

Neutrophil priming and/or activation may be a pre-requisite for their efficient hepatic recruitment. In AH, gut dysbiosis and increased gut permeability results in an increased bacterial load in the enterohepatic circulation,²¹ which can contribute to neutrophil priming and/or activation. Taieb *et al.* found that, in comparison to patients with alcohol-related cirrhosis and healthy volunteers, patients with AH had increased systemic neutrophil activation on flow cytometry, as evidenced by increased beta integrin expression and reduced L-selectin expression.²² Bajt *et al.* demonstrated the close association between upregulation of neutrophil activation markers in mice and stable accumulation of neutrophils in the murine liver.²³ In the absence of neutrophil activation, C-X-C motif chemokine ligand (CXCL)1 administration was not associated with hepatic recruitment in this study.²³ Lowe *et al.* found that, in a murine model of AH, treatment with antibiotics was associated with reduced neutrophil infiltration, reduced hepatic injury and reduced hepatic steatosis.²⁴

Neutrophil chemotaxis

In AH, several chemokines, including CXCL1, CXCL2 and CXCL8 promote neutrophil recruitment (Fig. 2). Activated

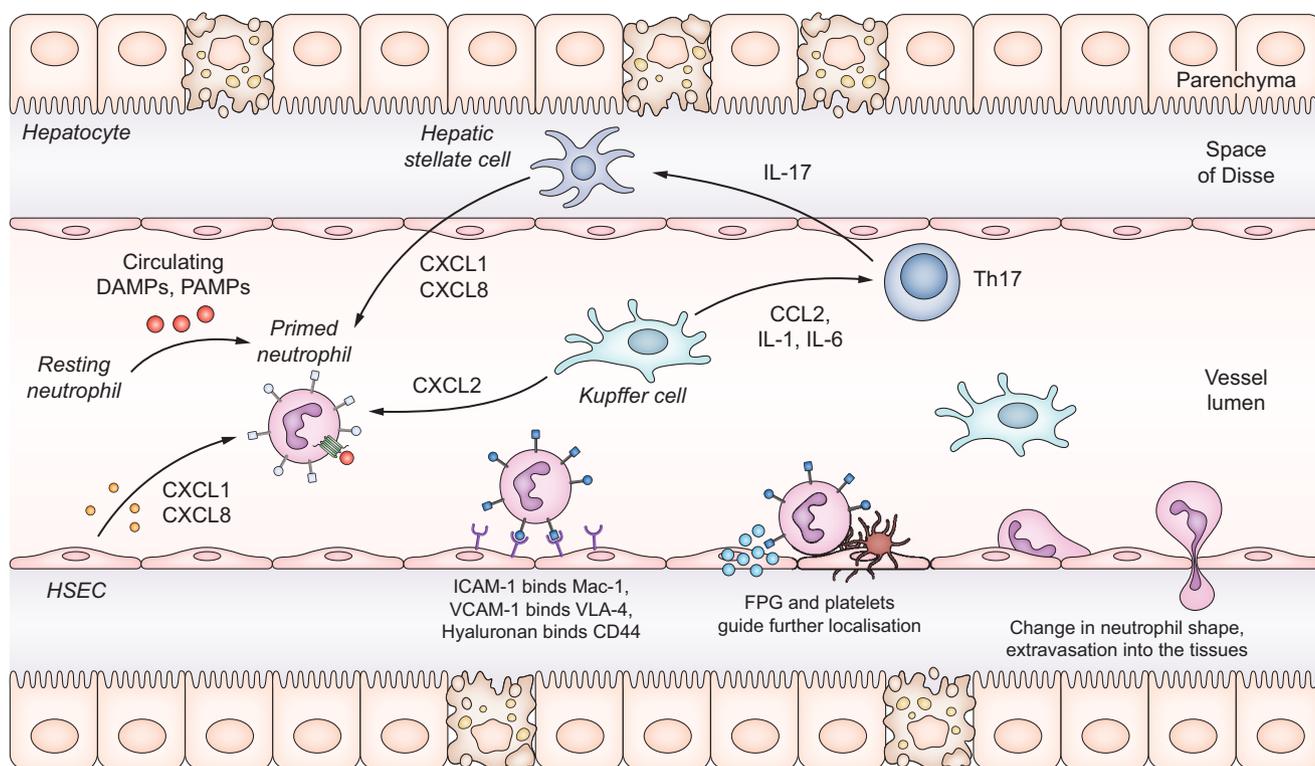


Fig. 2. Neutrophil recruitment in the hepatic sinusoids in alcohol-related hepatitis. Neutrophil recruitment towards areas of liver injury occurs due to multiple cytokines: (A) CXCL2, released by activated Kupffer cells, (B) CXCL1 and CXCL8, released by activated HSECs, (C) CXCL1 and CXCL8, released by activated hepatic stellate cells. CCL2 released by Kupffer cells plays an indirect role, through the recruitment of Th17 cells, which release IL-17 to activate the hepatic stellate cells. Circulating DAMPs and PAMPs prime neutrophils via TLRs for subsequent activation. Upon activation, there are conformational changes in the integrins on neutrophils allowing their binding to ICAM-1 (to Mac-1), VCAM-1 (to VLA-4), which are upregulated on activated HSECs. Activated HSECs produce SHAP, which binds hyaluronan in the extracellular matrix, promoting its interaction with CD44 on neutrophils. These interactions result in cell arrest and adhesion, following which neutrophils further localise to the site of tissue injury through their interaction with platelets and chemotactic peptides (FPG) released by activated endothelium. The neutrophils then extravasate into the tissues. CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand; DAMPs, damage-associated molecular patterns; FPG, formyl peptide gradient; HSEC, hepatic sinusoidal endothelial cell; ICAM1, intracellular adhesion molecule-1; IL-, interleukin; Mac-1, macrophage-1 antigen; PAMPs, pathogen-associated molecular patterns; SHAP, serum-associated hyaluronan-associated protein; TLR, Toll-like receptor; VCAM1, vascular cell adhesion protein.

Kupffer cells can directly influence neutrophil recruitment through secretion of CXCL2, which binds C-X-C motif chemokine receptor 2 (CXCR2) on neutrophils. Cytokines produced by activated Kupffer cells can also promote neutrophil recruitment indirectly through stimulation of other cell types. For example, interleukin (IL)-1 and IL-6 activate T helper 17 cells, prompting the release of IL-17, which stimulates hepatic stellate cell production of CXCL1 and CXCL8.²⁵ Tumour necrosis factor α (TNF α) produced by activated Kupffer cells contributes to endothelial cell activation, resulting in further CXCL1 and CXCL8 production.²⁶ However, in a murine model of AH, CXCL1 production by hepatocytes and hepatic stellate cells was upregulated, even when Kupffer cells were depleted using clodronate.²⁷ The authors proposed that the increased CXCL1 production was mediated by TLR2 and TLR9 activation. Supporting this hypothesis, they showed, in a murine model of AH, that *Tlr2* and *Tlr9* knockout mice exhibited reduced hepatic CXCL1 protein, reduced serum CXCL1 and reduced neutrophil infiltration compared with wild-type mice.²⁷

In a murine model of AH, CXCL1 blockade with a neutralising antibody or its genetic deletion were associated with amelioration of neutrophil infiltration.²⁸ Dominguez *et al.* observed increased levels of CXCL1 and CXCL8 expression in liver biopsy specimens from patients with AH, both at the gene and protein level. Hepatic expression of these chemokines correlated with neutrophil infiltration. Elevated hepatic CXCL8 levels, but not CXCL1 levels, were found to be associated with an adverse prognosis. Serum CXCL8 was also shown to be elevated in patients with AH, a finding supported by other studies.^{29,30}

The ability of neutrophils to perform chemotaxis for transmigration is dependent on a neutrophil secondary granule protein called lipocalin 2 (LCN2). Using biopsy samples, Wieser *et al.* demonstrated increased hepatic expression of LCN2+ neutrophils in patients with AH, compared with patients with non-alcoholic fatty liver disease and those with cirrhosis without AH.³¹ Compared to wild-type mice, ethanol-fed *Lcn2*^{-/-} mice exhibited reduced serum transaminitis, and reduced histological evidence of inflammation and steatosis, despite the presence of similar hepatic cytokine profiles. These results were also reproduced using antibodies to LCN2 in wild-type mice. The underlying mechanism of this effect is unknown but it is speculated that LCN2 has pro-inflammatory properties and influences macrophage polarisation.³²

Neutrophil adhesion and transmigration

Typically, neutrophils are captured from blood flow and 'tether and roll' on the vascular endothelium, before firmly binding to it and emigrating out of the vasculature into the target tissues. In most organs this occurs in post-capillary venules. However, the liver is unique in that it receives a dual blood supply from the hepatic artery and the portal vein, both of which drain into hepatic sinusoids, lined by hepatic sinusoidal endothelial cells (HSECs). Within the liver, around 70-80% of neutrophil recruitment occurs across the hepatic sinusoidal endothelium, rather than in the post-capillary venules.³³ The sinusoidal endothelium is discontinuous, fenestrated, and lacks basal lamina and tight junctions. Blood flow rates through the hepatic sinusoids are also relatively slow compared to other vascular beds. This means that in the hepatic sinusoids, initial

capture and leukocyte adhesion may occur without a 'rolling' step.³⁴

In conditions of inflammation, when neutrophils are activated and less deformable, there may be some physical trapping in the narrow sinusoidal vessels. Initial adherence of neutrophils to HSECs depends on surface interactions with adhesion molecules on endothelial cells. Intercellular adhesion molecule 1 (ICAM-1) can bind Mac1 integrin on neutrophils, and vascular cell adhesion molecule 1 (VCAM-1) binds VLA-4 ($\alpha 4\beta 1$ integrin) on neutrophils.³⁵ Evidence suggests that endothelial cells are activated in AH, with accompanying upregulation of surface adhesion molecules, some of which may be shed to yield soluble forms of adhesion molecules. However, the relative importance of key soluble and membrane bound receptors varies between different studies. In four studies, circulating levels of ICAM-1 were increased in patients with AH.³⁶⁻³⁹ Blaya *et al.* found that levels of plasma VCAM-1 were also raised in patients with AH and correlated with 90-day mortality in this group.³⁶ However, Adams *et al.* found no change in circulating VCAM-1 despite an increase in soluble ICAM-1.³⁸ Further, in the study by Blaya *et al.*, whilst qPCR revealed increased hepatic gene expression of *ICAM1*, there was no difference in hepatic *VCAM1* gene expression in patients with AH and healthy controls.³⁶ The predominant role of ICAM-1 in neutrophil infiltration in AH is further supported by animal studies; in rats given intragastric ethanol to induce AH, hepatic expression of ICAM-1 was increased and corresponded with increased neutrophil infiltration.⁴⁰ Further, ICAM-1 deficiency decreased alcohol-induced liver injury and hepatic migration of neutrophils in mice fed a high-fat diet with ethanol.⁴¹

Hyaluronan is a molecule found in the extracellular matrix that is secreted by multiple cell types, including endothelial cells. It is highly expressed in the liver sinusoids, and binds CD44 on the neutrophil surface, providing an additional mechanism of neutrophil recruitment. Serum hyaluronan levels were found to be elevated in patients with AH, with levels reducing after cessation of alcohol consumption.³⁹ Urashima *et al.* demonstrated that, in comparison to healthy individuals, patients with alcohol-related cirrhosis who were actively drinking had higher levels of CD44 expression in the liver, which decreased after abstinence.⁴² McDonald *et al.* found that, in mice, the presence of endotoxaemia did not increase avidity of neutrophil CD44 for hyaluronan. However, TLR4 activation on HSECs triggered increased deposition of serum-associated hyaluronan-associated protein, which covalently binds hyaluronan.⁴³ The formation of this complex results in alteration of the hyaluronan configuration in a way that potentiates its binding to CD44.⁴³⁻⁴⁵ Thus, McDonald *et al.* demonstrated that LPS-induced hepatic injury and neutrophil infiltration could be significantly ameliorated by blocking hyaluronan-CD44 interactions using an anti-CD44 antibody.⁴³

The role of E-selectin in neutrophil recruitment in AH is less clear. In a murine model of AH, Bertola *et al.* demonstrated that hepatic expression of E-selectin was increased 10-fold, and that genetic depletion of E-selectin was associated with reduced neutrophil infiltration and hepatic injury.⁴⁶ However, in the human liver, E-selectin is hardly expressed in normal endothelium, although studies suggest some upregulation in inflammatory liver diseases.⁴⁷ Nevertheless, in 15 human liver biopsy samples from patients with AH, Adams *et al.* found

prominent E-selectin staining in the sinusoids in only one case.⁴⁷ Thus, it is possible that non-selectin-mediated capture mechanisms predominate in the human liver sinusoid in the context of AH. However, once cells are firmly bound to HSECs, they are directed towards their targets in tissue by chemotaxis. Peptides released by activated hepatic endothelium can also provide a stimulus for neutrophil chemotaxis by binding their specialised receptors (formyl peptide receptor 1) on neutrophils.⁴⁸ At this stage, platelets also play a role in their migration by binding to HSECs and allowing the neutrophils to 'crawl' over them using Mac1 integrin.⁴⁸ In order to perform their antimicrobial activities, neutrophils must effectively migrate to the site of infection. Artru *et al.* used transwell assays to demonstrate that neutrophils from patients with severe AH have a reduced capacity to migrate towards CXCL8, in comparison to patients with alcohol-related cirrhosis alone, or healthy controls.⁴⁹ They hypothesised that this was due to downregulation of CXCR2 on neutrophils, in response to endotoxaemia. Administration of IL-33 was able to partially restore CXCR2 expression on neutrophils and enhance migration towards CXCL8.⁴⁹

Neutrophil antimicrobial function in alcohol-related hepatitis

Enriched pre-made granular proteins enable neutrophils to mount rapid, potent antimicrobial responses. For phagocytosis, neutrophils have 'opsonic' and 'non-opsonic' receptors. Opsonic receptors, such as such as Fc receptors and the complement receptors, can detect host-derived proteins (opsonins) bound to target particles. Non-opsonic receptors recognise distinct molecular patterns on the target particle. Upon receptor activation, signalling pathways are activated that result in internalisation of particles within a distinctive organelle known as a phagosome. This fuses with internal lysosomes to form a phagolysosome.

Production of ROS for oxidative bursts is mediated by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. The NADPH oxidase complex has cytoplasmic (p47^{phox} and p67^{phox}) and membrane components (gp91^{phox} and p22^{phox}), which are disassembled in unstimulated cells.⁵⁰ ROS exert direct antimicrobial effects through inhibition of bacterial growth and destruction of the cellular membrane.⁵⁰ Further, ROS production may trigger signalling pathways that result in release of neutrophil cytoplasmic granules, NET generation, and production of neutrophil pro-inflammatory cytokines, such as TNF α and CXCL2.⁵⁰ The importance of neutrophil oxidative bursts in antimicrobial defence is highlighted by the fact that patients with genetic deficiencies in NADPH oxidase develop chronic granulomatous disease, characterised by life-threatening infections.⁵⁰ However, in the presence of sterile injury and trauma, ROS can contribute to tissue injury.

Several studies have suggested that the capacity of neutrophils to perform phagocytosis and to generate an oxidative burst is altered in AH. Patients with AH frequently have cirrhosis, which is associated with a reduction in serum complement proteins and consequently a reduced capacity to opsonise bacteria.⁵¹ However, difficulties in opsonisation alone cannot explain impaired phagocytosis in these patients, as studies have demonstrated impaired phagocytosis of

opsonised bacteria in patients with cirrhosis compared to healthy volunteers.^{8,51–54} Tritto *et al.* found that clinical disease severity in patients with stable cirrhosis of varying aetiologies correlated with the degree of neutrophil phagocytic dysfunction.⁵⁴ Interestingly the impairment of function may not be systemic. Fiuza *et al.* reported that the reduction in phagocytic capacity observed in patients with cirrhosis was more pronounced in neutrophils from ascitic fluid than blood, which may explain the particularly high risk of spontaneous bacterial peritonitis.⁵³ Similarly, in patients with stable cirrhosis, the phagocytic function of neutrophils obtained from peripheral blood was normal, while the phagocytic capacity of neutrophils that had emigrated to skin blisters was impaired.⁵⁴ The impairment in phagocytosis may be at least partially related to prior neutrophil priming, as evidenced by a study in which exposure of neutrophils from healthy volunteers to LPS or TNF α was associated with defects in subsequent phagocytic activity that were comparable to those seen in patients with cirrhosis.⁵¹

There is some evidence that neutrophils are hyper-responsive in AH, as shown by data that they produce more TNF α and CXCL8 upon LPS stimulation.⁵⁵ Mookerjee *et al.* found that baseline oxidative burst activity was increased in neutrophils in patients with alcohol-related cirrhosis and AH compared with healthy controls, but the groups did not differ in terms of their total oxidative burst responses to *E. coli* stimulation, which they attributed to neutrophil priming.⁸ In contrast, Rajkovic *et al.* found that in patients with alcohol-related cirrhosis and AH, there was reduced superoxide production and degranulation, which they attributed to reduced levels of the anti-oxidant glutathione in neutrophils.⁵² In the absence of cirrhosis, alcohol dependence has been associated with impairment of burst activity in response to *E. coli*.⁵⁶

Several studies suggest that neutrophil dysfunction in AH is mediated by soluble factors. For example, incubation of healthy volunteer neutrophils with plasma from patients with cirrhosis reproduced the abnormalities seen in patients with AH, including defective phagocytosis and increased oxidative burst.^{8,57} Mookerjee *et al.* reported that that addition of endotoxin to patient serum reduced phagocytic capacity by 20% and removal of endotoxin from plasma was associated with restoration of neutrophil function in patients with cirrhosis and AH.⁸ Albumin is a potent scavenger of ROS and endotoxin. Stadlbauer *et al.* showed that incubation of neutrophils with albumin could prevent plasma-induced neutrophil dysfunction.⁵⁷ However, oxidation of albumin, which is associated with AH, can prevent it from functioning as a scavenger.⁹ Other studies have proposed a link between cirrhosis-associated neutrophil dysfunction and bile acid composition,⁵⁸ with one study showing that levels of serum bile acids were elevated in patients with cirrhosis – in whom the proportion of chenodeoxycholic acid was higher and the proportion of ursodeoxycholic acid was lower – compared to healthy volunteers. Using individual bile acids, the authors demonstrated that chenodeoxycholic acid suppressed ROS production and phagocytosis, but ursodeoxycholic acid had no effect, and that these effects were reversible after washing out the bile acids.

Neutrophils can also kill microbes independently of their phagocytic uptake, through the release of extracellular web-like structures comprised of DNA, cationic proteins (elastase, cathepsin G, lactoferrin, myeloperoxidase and neutrophil

gelatinase-associated lipoprotein), chromatin and serine proteases, which are known as 'neutrophil extracellular traps' (NETs). NETs retain a high local concentration of antimicrobial substances which capture and inactivate pathogens at the site of injury. NETs form a scaffold which can initiate thrombosis, sequester pathogens (preventing dissemination), and minimise blood loss.⁵⁹ It is controversial whether NET release represents a specific antimicrobial defence mechanism, or if it is simply a consequence of cellular rupture due to toxins or trauma. In response to chemical stimulation with phorbol 12-myristate 13-acetate (PMA), neutrophils undergo 'suicidal NETosis', which is an NADPH oxidase-dependent process, and results in neutrophil lysis.⁶⁰ However, cell lysis following pathogen capture would result in release of the pathogens from the cell. Therefore, it has been suggested that in response to bacteria or LPS, NETosis is induced by activation of TLRs on neutrophils in a process known as 'vital netosis'.⁶¹ In vital netosis, NETs are released via vesicles, sparing the neutrophil outer membrane and allowing for continued neutrophil function. Although NETosis may be a mechanism of antimicrobial defence, the presence of extracellular histones may activate TLR2 and TLR4, and contribute to cell death and hepatic tissue injury.⁶²

NET formation in response to a toxic insult is impaired in AH. Jin *et al.* reported that in murine caecal ligation injury, superimposed acute alcohol challenge was associated with increased bacterial growth,⁶³ reduced neutrophil recruitment, impaired oxidative burst and reduced NETosis. Bukong *et al.* performed a study in which mice were given a binge dose of alcohol, and an intraperitoneal dose of LPS.⁶⁴ This was associated with reduced LPS-induced NET formation (decreased citrullinated histone H3, neutrophil elastase and neutrophil myeloperoxidase). However, at 15 h, in the efferocytosis phase, there was evidence of decreased clearance of NETs. The authors also isolated neutrophils from healthy volunteers after a binge drink of vodka. They showed that alcohol alone could induce NETosis, but the presence of alcohol was associated with reduced NET formation in response to PMA. Similarly in patients with AH, Cho *et al.*, confirmed that alcohol could directly induce NETosis.²⁰ Additionally, they showed that after extrusion of nuclear material, the neutrophils remained intact, causing their conversion from a high- to low-density state. The LDNs were cleared less effectively by macrophages than the HDNs and were functionally defective.

Whilst *in vitro* studies suggest impaired neutrophil function in AH, it is important to consider the clinical significance of these findings. In a study by Taylor *et al.* which included patients with cirrhosis of various aetiologies, baseline circulating neutrophil dysfunction was a predictor of 90-day and 1-year survival.⁶⁵ Mookerjee *et al.* found that a resting oxidative burst >55% and a reduced phagocytic capacity of <42% was associated with increased risk of infection, organ failure and mortality,⁸ although Tritto *et al.* found no significant association between impaired phagocytic capacity and 3-month survival.⁵⁴ This may reflect patient demographics since Tritto *et al.* studied patients with stable cirrhosis rather than AH.

Neutrophils in injury resolution

Whilst the mechanisms above suggest that neutrophils can contribute to end organ damage in alcohol-related injury, emerging evidence also shows the importance of neutrophils in resolution of injury after AH. For example, neutrophil

depletion in mice during the resolution phase of liver injury was associated with ongoing hepatic inflammation and early fibrosis.⁶⁶ Neutrophils can also play a direct role in resolution of liver injury. They can phagocytose pathogens and cellular debris that would otherwise prolong inflammation. There is evidence to suggest that ROS release by neutrophils can promote polarisation of monocytes/macrophages from a pro-inflammatory (Ly6C^{hi} CX3CR1^{lo}) to an anti-inflammatory (Ly6C^{lo} CX3CR1^{hi}) phenotype.⁶⁷ Moreover, apoptotic neutrophils in the liver are phagocytosed by macrophages,⁶⁸ after which macrophage production of IL-1 β , TNF α , and IL-8 is reduced.⁶⁹ This suggests that neutrophil apoptosis is a cue for initiation of reparative mechanisms after injury. Further, in patients with AH, neutrophils are the main source of hepatocyte growth factor production, a cytokine involved in liver regeneration.⁵⁵

Neutrophils may also impact on the resolution of fibrosis. Data from murine models of carbon tetrachloride-induced liver fibrosis and acetaminophen-induced acute liver failure show that liver-infiltrating neutrophils release matrix metalloproteinases 8 and 9, which are antifibrotic.⁷⁰ This may be related to production of anti-inflammatory and antifibrotic miR-223 by neutrophils within the hepatic environment.⁶⁶ It is unknown if these antifibrotic effects are also present in alcohol-induced liver injury. MiR-223 is the most abundant miRNA in neutrophils. In a murine model of AH, miR-223 deletion was associated with enhanced liver injury and increased ROS production.⁷¹ The authors demonstrate that IL-6 promotes expression of p47phox, which is a component of NADPH oxidase, and thus results in ROS generation. MiR-223 inhibits IL-6, thereby reducing ROS generation. Although patients with AH may have neutrophil infiltration, the protective effect of miR-223 may be diminished. Excessive drinking has also been associated with reduction in miR-223 levels in peripheral neutrophils of humans.⁷¹ In another study, immunoprecipitation experiments implicated neutrophilic *SIRT1* (sirtuin 1) as the gene that controls miR-223 expression, via its effects on the transcription factor C/EBP α .⁷² In acutely intoxicated chronic alcohol users, the authors observed a reduction in *SIRT1* gene expression, which correlated with the degree of miR-223 expression and serum alanine aminotransferase.⁷²

A recent study highlighted the key role played by the p47phox gene, also known as neutrophil cytosolic factor 1 (*NCF1*), in regulating neutrophil function.¹⁰ Ma *et al.*, performed RNA-sequencing analysis in explanted livers from patients with severe AH and showed that *NCF1* was upregulated.¹⁰ Using data generated from RNA-sequencing analysis, in conjunction with data from experiments with *Ncf1* knockout mice subjected to chronic plus binge ethanol feeding, the authors concluded that *NCF1* promotes AH by inhibiting miR-223 and AMP-activated protein kinase (a key regulator of lipid metabolism).

Neutrophil miR-223 may also have additional protective effects independently of ROS. For example, it negatively regulates Mef2c, a transcription factor that promotes myeloid progenitor proliferation.⁷³ This may explain observations of increased levels of circulating neutrophils and exacerbated liver injury in miR-223 knockout mice.⁷¹ MiR-223 is also a negative regulator of (NLR family, pyrin containing 3) inflammasome expression in macrophages. The NLRP3 inflammasome is a multiprotein complex that acts as a central driver of inflammation and cell death in AH, via activation of caspase 1, and

maturation and release of pro-inflammatory cytokines including IL-1B and IL-18.^{74,75}

Can neutrophils be used as a therapeutic target in AH?

Corticosteroids are the only recommended medical treatment proven to reduce mortality in severe AH, although the benefits are short-lived.⁵ The neutrophil-to-lymphocyte ratio is predictive of steroid responsiveness in AH,¹³ suggesting that at least some of their effects may be mediated through transcriptional regulation of gene production in neutrophils. Corticosteroids promote mobilisation of neutrophils from the bone marrow.⁷⁶ However, they also cause shedding of surface integrins, such as L-selectin, on neutrophils,^{22,77} thereby restricting tissue infiltration. Steroid treatment has been associated with a reduction in levels of pro-inflammatory cytokines, such as TNF α , and suppression of neutrophil oxidative burst activity.²² Steroids also exert pro-inflammatory effects, e.g. inhibition of neutrophil apoptosis.⁷⁸ However, as steroids exert their effects

on multiple cell types, it is unclear to what degree their effects in AH are directly mediated by neutrophils. In a clinical trial by Shastry *et al.* only 26% of hospitalised patients with severe AH were deemed suitable for steroid treatment and, of those, 25% did not respond to steroids,⁷⁹ highlighting an urgent need to explore alternative therapeutic options. A summary of the key mechanisms by which neutrophils may contribute to AH, and therefore potential therapeutic targets, are shown in Fig. 3.

Studies exploring the potential of modulating neutrophil action in the treatment of AH are limited. One of the main reasons for this is likely to be that neutrophils are a double-edged sword in AH. Whilst pro-inflammatory cytokines and excess NET production by neutrophils are considered to contribute to inflammatory injury in AH, they also play a role in injury resolution, and neutrophil dysfunction in AH may contribute to impaired antimicrobial defences against infection. Thus, it is unclear whether the desired therapeutic goal is enhancement or attenuation of neutrophil recruitment and neutrophil function. In steroid non-responders, without any

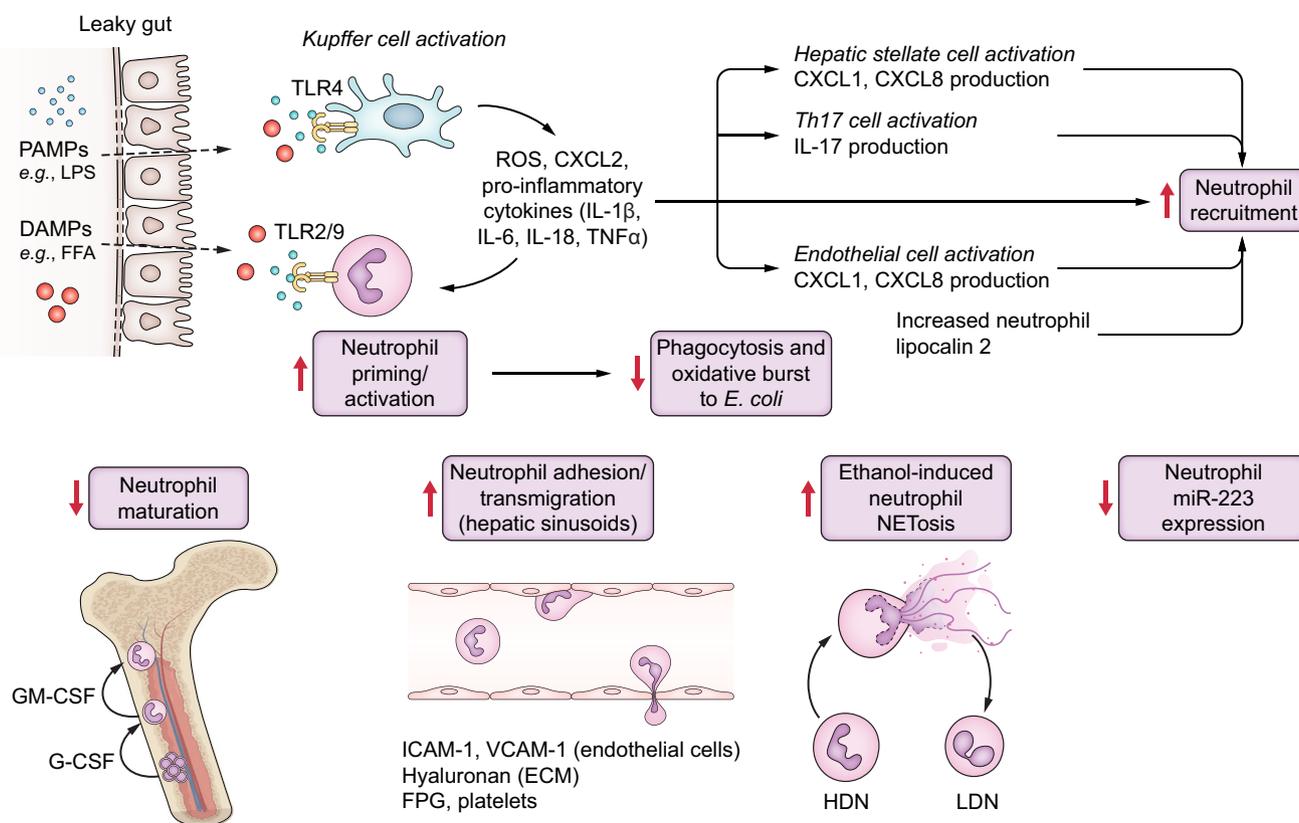


Fig. 3. A summary of the potential mechanisms by which neutrophils may contribute to disease pathogenesis in alcohol-related hepatitis. Neutrophil maturation is impaired in alcohol-related hepatitis. Alcohol is associated with intestinal dysbiosis and increased gut permeability allowing the interaction of PAMPs and DAMPs with TLR4 on Kupffer cells/macrophages, resulting in their activation. ROS and inflammatory cytokines released by activated macrophages promote (A) recruitment of further monocytes and neutrophils (B) activation of stellate cells and endothelial cells, causing them to upregulate CXCL8 and CXCL1 production and (C) activation of Th17 cells, which release the chemokine IL17. Neutrophils in alcohol-related hepatitis have increased granular lipocalin 2, which further promotes neutrophil recruitment to tissues. Neutrophil adhesion to sinusoidal endothelial cells and subsequent transmigration into the liver is mediated by several molecules including ICAM-1, VCAM-1, hyaluronan, FPG and platelets. Neutrophils may be activated by cytokines released by Kupffer cells, or directly activated by PAMPs and DAMPs. The increased baseline activation may be associated with impaired phagocytotic and oxidative burst responses to *E. coli*. Alcohol can directly induce neutrophil NETosis, resulting in the conversion of HDNs to LDNs, which have reduced functional capacity for phagocytosis and reduced susceptibility to phagocytic clearance by macrophages. Patients with alcohol-related hepatitis have reduced expression of miR-223, which reduces its beneficial effects on oxidative stress. AH, alcohol-related hepatitis; CXCL, C-X-C motif chemokine ligand; DAMP, damage-associated molecular pattern; FFA, free fatty acid; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HDN, high-density neutrophil; ICAM-1, intracellular adhesion molecule-1; IL-, interleukin-; LDN, low-density neutrophil; LPS, lipopolysaccharide; miR-223, microRNA 223; PAMP, pathogen-associated molecular pattern; PD1 programmed cell death 1; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; TNF α , tumour necrosis factor- α ; VCAM-1, vascular cell adhesion protein 1.

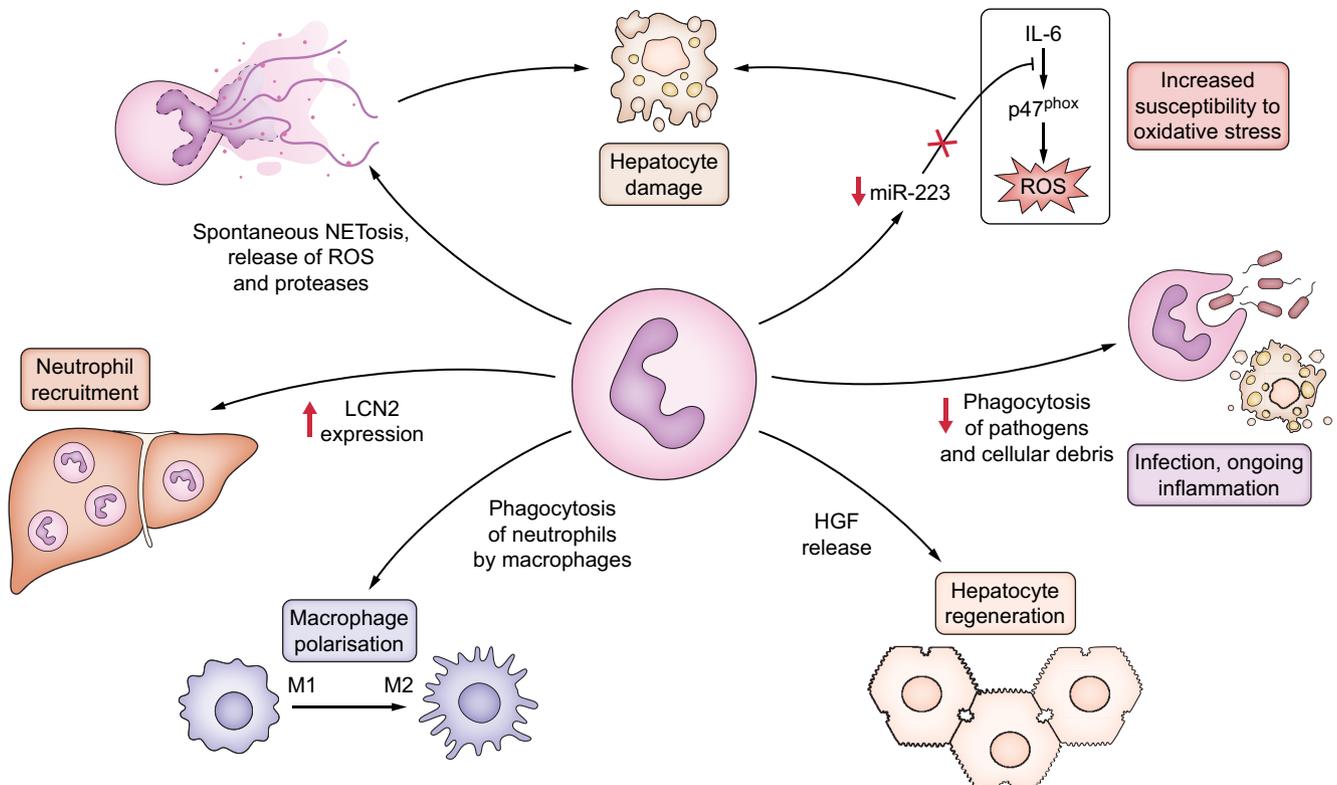


Fig. 4. An illustration of the key roles that neutrophils play in alcohol-related hepatitis. Neutrophils play a role in phagocytosis of pathogens and cellular debris, although their ability to do this is impaired in AH, predisposing to infection. Spontaneous NETosis, release of ROS and proteases results in hepatocyte damage. Neutrophils have increased LCN2, which promotes hepatic infiltration. Neutrophils in AH have reduced miR-223 expression; this reduces their inhibitory effect on IL-6, contributing to p47^{phox}-mediated ROS production, which causes hepatocyte damage. Neutrophils may aid in resolution of injury through release of HGF, which facilitates hepatocyte regeneration. Phagocytosis of neutrophils by macrophages may trigger their polarisation towards a pro-resolution 'M2' phenotype. miR-223, microRNA 223; IL-6, interleukin-6; HGF, hepatocyte growth factor; LCN2, lipocalin 2; ROS, reactive oxygen species.

evidence of infection, treatments that primarily reduce hepatic infiltration may still be a therapeutic option for patients with AH.

Whilst *in vitro* studies have enhanced our understanding of neutrophils in AH pathogenesis, neutrophils do not act in isolation but rather have complex interactions with other cell types, including endothelial cells, hepatocytes, Kupffer cells and T cells for example. Caution needs to be exercised to avoid a reductionist approach to experimental design. For example, *in vitro* data showed the benefit of IL-33 in restoring neutrophils' ability to perform chemotaxis toward CXCL8 in order to enhance antibacterial activity;⁴⁹ however, before it is trialled as a potential treatment, it will be important to assess its impact on neutrophil recruitment and hepatic injury. A further consideration is the importance of rigorous experimental methodology to ensure valid results. *In vitro* experiments are often performed using neutrophils isolated from the peripheral blood, which may be phenotypically and functionally different from neutrophils that have entered the liver after interaction with HSECs and injured hepatocytes. The choice of stimulant used in experiments can also influence the outcome. For example, in comparison with healthy controls, patients with AH have been shown to have reduced responses to PMA in NETosis assays, but increased responses to ethanol.²⁰

The finding that neutrophils can exist in both 'high-density' and 'low-density' states, each with different functional characteristics may at least partially explain their paradoxical

effects.²⁰ Further studies are needed to establish the clinical significance of increased LDNs in patients with AH; specifically, whether this correlates with disease severity in AH and whether it can predict the clinical outcomes of mortality and infection. Thereafter, in assessing the effects of therapeutic interventions on neutrophil function, it is important that effects on both types of neutrophils are considered. Ideally, a treatment would selectively prevent conversion of HDNs to LDNs, by inhibiting NETosis, and promote clearance of the dysfunctional LDNs.

Strategies to limit hepatic neutrophil infiltration, through blockade of adhesion molecules ICAM-1,⁴¹ E-selectin⁴⁶ and CD44,⁴³ have been associated with reduced liver injury in animal models, but such interventions have not yet been tested in patients. One of the issues with translation of these findings is that, unfortunately, there is no single animal model that accurately reproduces the features of human AH. The NIAAA 'acute on chronic' model is commonly used to study therapeutic interventions for AH in mice.⁴⁶ However, this model results in a variable degree of hepatic inflammation, making it difficult to assess the effects of interventions. Further, the model does not result in significant hepatic fibrosis or hepatocyte senescence, which are typically present in humans with AH.

In clinical trials, administration of G-CSF, which promotes mobilisation of neutrophils from the bone marrow, has produced mixed results in patients with AH, which may be due to the high heterogeneity amongst studies.⁸⁰ Five RCTs

performed in Asia suggested that G-CSF produced a mortality benefit and significantly reduced the risk of infection ($p < 0.001$).⁸⁰ However, European and American trials have shown no significant difference in mortality or infection risk in treated patients.^{80,81} The mechanism(s) underlying the benefit observed in some studies is unclear. Proposed mechanisms include neutrophil-induced polarisation of macrophages towards an anti-inflammatory phenotype,⁸² and enhanced migration of CD34+ bone marrow stem cells that promote hepatic regeneration and repair.⁸³

Other clinical studies have focused on addressing the soluble factors that may contribute to neutrophil activation or dysfunction. Supporting the findings by Stadlbauer *et al.* showing the utility of albumin in preventing plasma-induced neutrophil dysfunction *in vitro*,⁵⁷ the albumin INFECIR 2 study was a phase IV multi-centre randomised-controlled trial that used samples from 118 patients with decompensated cirrhosis and bacterial infection (excluding spontaneous bacterial peritonitis).⁸⁴ Patients were treated with either antibiotics alone, or antibiotics and 20% albumin. At 90-day follow-up, whilst there was no difference in mortality between the groups, patients treated with albumin had a lower incidence of nosocomial infections (6.6% vs. 24.6%, $p = 0.007$). However, this study was not specific for patients with AH. *In vitro* data suggests that manipulation of bile acid composition may improve neutrophil function in patients with cirrhosis. Obeticholic acid is a farnesoid X receptor agonist licensed for use in primary biliary cholangitis, which works by suppressing bile salt production and improving biliary flow. Whilst a phase II clinical trial was started to investigate the efficacy of obeticholic acid in AH [NCT02039219], the trial was terminated early due to concerns about hepatotoxicity.

Several ongoing clinical trials are assessing different treatments for patients with AH aimed at improving intestinal dysbiosis (antibiotics [NCT03157388], [NCT02116556], probiotics [NCT01922895], [NCT02335632], faecal microbial transplant [NCT03091010], [NCT02458079]) or gut barrier function ([NCT03775109], [NCT01809132]). This could potentially reduce bacterial endotoxin load, with consequences for neutrophil priming and/or activation. In clinical trials of patients with stable cirrhosis, administration of probiotics has shown mixed results on neutrophil function. For example, Stadlbauer *et al.* found that probiotics were associated with restoration of neutrophil phagocytic capacity and reduced neutrophil TLR4 expression.⁸⁵ Hovarth *et al.* reported no effects on phagocytosis, but an increase in neutrophil oxidative burst capacity.⁸⁶ McNaughtan *et al.* found no effect on neutrophil function, but

a reduction in levels of pro-inflammatory cytokines IL-1 β and CCL2 (C-C motif chemokine ligand 2).⁸⁶ Differences in the findings may be due to variations in study design and choice of probiotics. However, whether the results of studies in patients with stable cirrhosis can be extrapolated to those with AH is questionable. In ongoing and future trials in AH, the generation of some mechanistic data specifically looking at the role of neutrophils will be invaluable in furthering our understanding.

Conclusions

Neutrophils play a crucial role in innate defence against pathogens. However, this is not their only purpose. Neutrophils have a diverse repertoire of functional responses. Neutrophil recruitment and excess hepatic infiltration contributes to inflammatory injury in AH, and experimental strategies to limit neutrophil recruitment/infiltration are associated with reduced liver injury, although such findings are limited to animal models. Neutrophils are also important for timely resolution of tissue damage in AH, particularly through cross-talk with activated Kupffer cells. Thus, they act as a double-edged sword in AH (Fig. 4). Therapeutic interventions using neutrophils are unlikely to involve simple strategies to promote or inhibit neutrophil mobilisation from the bone marrow but rather may need to manipulate key functions within tissue. There are several clinical trials already underway for AH, which are testing therapies that may influence neutrophil recruitment and function through alteration of their microenvironment. These trials should be supplemented by mechanistic studies to enhance our understanding of the degree to which neutrophils mediate the pathogenesis of AH and, thus, to determine the therapeutic potential of neutrophil-targeting approaches.

Despite the increase in circulating and hepatic neutrophils that are observed in AH, patients have increased susceptibility to infection due to impaired neutrophil function. Whilst increasing evidence suggests the importance of neutrophil miR-223 in beneficially regulating the effects of neutrophils in liver disease, this is again dysfunctional in patients with AH. Advances in our understanding of the detailed transcriptional mechanisms of neutrophil dysfunction in AH, and of neutrophil subsets in health and disease, will hopefully guide the development of targeted therapies to correct neutrophil dysfunction in AH. Effective translation of this work is likely to require development of animal models that better reproduce features of human disease.

Affiliations

¹Centre for Liver and Gastrointestinal Research, Institute of Immunology and Inflammation, and National Institute for Health Research (NIHR) Birmingham Biomedical Research Centre, The Medical School, University of Birmingham, Birmingham, B15 2TT, UK; ²Hepatology Unit, Imperial College School of Medicine, St. Mary's Hospital, London W21NY, England, UK

Abbreviations

AH, alcohol-related hepatitis; ALD, alcohol-related liver disease; CXCL, C-X-C motif chemokine ligand; CXCR2, C-X-C motif chemokine receptor 2; *E coli*, *Escherichia coli*; G-CSF, granulocyte colony stimulating factor; HDNs, high-density neutrophils; HSECs, hepatic sinusoidal endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-, interleukin-; LDNs, low-density neutrophils; LCN2, lipocalin 2; LPS, lipopolysaccharide; MDSCs, myeloid-derived suppressor cells; miR-223, microRNA 223; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NCF1, neutrophil cytosolic factor 1; NETs, neutrophil

extracellular traps; NLRP3, NLR family pyrin domain containing protein 3; PMA, phorbol myristate acetate; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF α , tumour necrosis factor- α ; VCAM-1, vascular cell adhesion protein 1.

Financial support

PNN and PFL are supported by the NIHR Birmingham Biomedical Research Centre, hosted by University Hospitals Birmingham and University of Birmingham. The NIHR Birmingham BRC grant code is NIHR203326. RK is supported by a Medical Research Council Clinical Research Training Fellowship grant. The

MRC grant code is MR/S001581/1. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

Conflict of interest

The authors declare no conflicts of interest that pertain to this work. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

PNN and RK had the original concept. RK wrote the first draft of the manuscript and all authors reviewed the final version. PNN is guarantor.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2023.05.017>.

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Author names in bold designate shared co-first authorship

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