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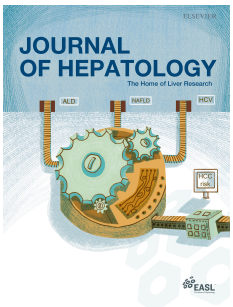

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The role of neutrophils in alcohol-related hepatitis

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Author 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.

Abstract:

Alcohol-related liver disease (ALD) is a major cause of liver disease associated mortality, with inpatient care being a major contributor to clinical and economic burden. Alcohol-related hepatitis (AH) is an acute inflammatory form of ALD. Severe AH is associated with a 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 alcoholic hepatitis. 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 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 the development of animal models that accurately reproduce human disease are important areas for facilitating translation of research in this important field.

Key words: alcoholic hepatitis, alcohol-related liver disease, neutrophils, immune cell recruitment, sepsis.

Key points:

- Alcohol-related hepatitis (AH) is frequently associated with increased neutrophil counts in the circulation and in the liver.
- Neutrophils in AH are activated at baseline, predisposing them to spontaneous NETosis, release of reactive oxygen species and proteases.
- Neutrophils in AH have impaired phagocytosis 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 inducing conversion of macrophages to a 'pro-resolution' phenotype.

- Neutrophil mir-223 protects against oxidative stress, but its expression is reduced in AH.

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, of which 18.4% had 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 one year [2-4]. Infection is a common cause of mortality in AH, and in the landmark STOPAH trial infections were responsible for 25% of all deaths [5]. Traditionally, steroids, pentoxiphylline 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 (MELD) 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 polymorphonuclar cells that encompass 70% of all leukocytes. They are made in large numbers (>1 x 10^{11} 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 the 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 stimulating factor (GM-CSF) promote neutrophil egress from the bone marrow as well as promoting neutrophil proliferation, survival, and differentiation. Alcohol excess and liver 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 (PRRs) that scan the extracellular milieu and endosomal compartments (Figure 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 PRRs, such as TLR2 and TLR9, that can detect structural motifs on invading pathogens (pathogen-associated molecular patterns, PAMPS), and endogenous danger signals indicating host injury or stress (danger-associated molecular patterns, DAMPs). 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 which kill microorganisms, and they can release neutrophil extracellular traps (NETS).

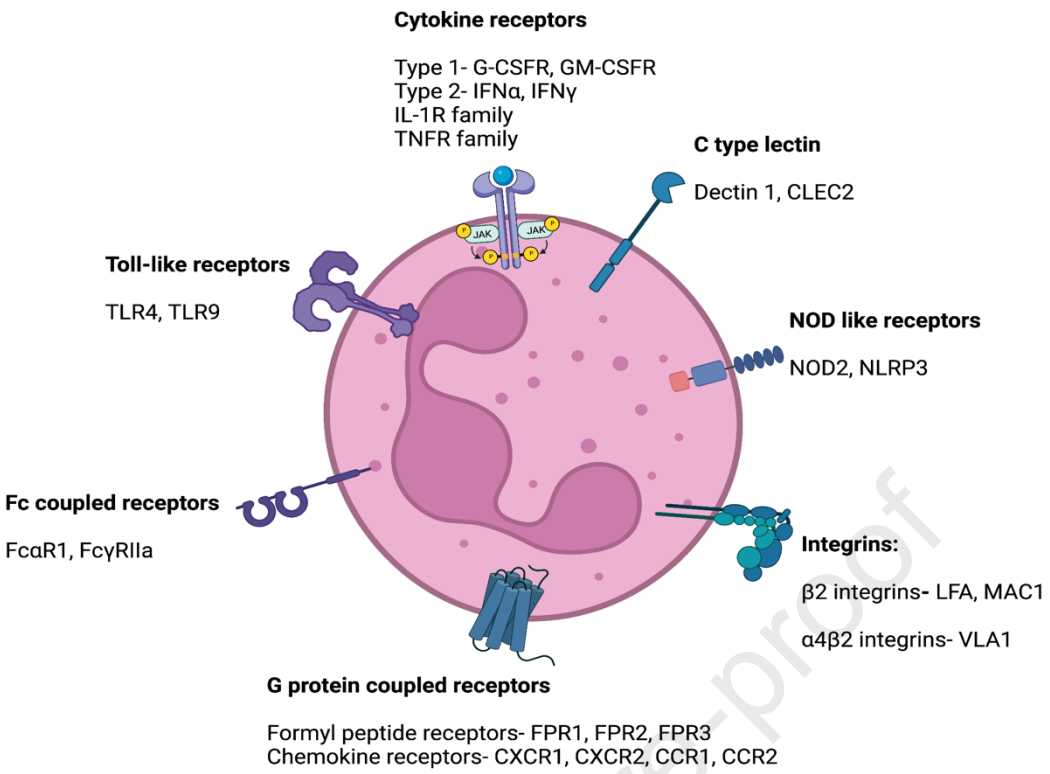

Figure 1. Key receptors found on mature neutrophils. *CCR1, C-C motif chemokine receptor 1; CCR2, C-C motif chemokine receptor 2; CLEC2; C-type lectin-like type 2; CXCR1, chemokine C-X-C motif chemokine receptor 1; CXCR2, chemokine C-X-C motif chemokine receptor 2; FcαR1, Fc alpha receptor 1; FcγRIIa, Fc gamma receptor 2a; G-CSFR; granulocyte colony stimulating factor receptor; GM-CSFR, granulocyte monocyte colony stimulating factor receptor; IFNα, interferon alpha; IFNβ, interferon beta; 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; TLR2, toll-like receptor 2; TLR4, toll like receptor 4; TLR9, toll like receptor 9. TNFR, tumour necrosis factor receptor; FPR1, formyl peptide receptor 1; FPR2, formyl peptide receptor 2; FPR3, formyl peptide receptor 3; VLA-1, very late antigen 1.*

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

Whilst there is heterogeneity in neutrophil phenotype and function, it is controversial 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 [16]. On density

gradient centrifugation, the bulk of neutrophils are dense and separate at the bottom of the gradient. These 'high density' neutrophils (HDN) have classic morphological features of a lobulated nucleus and a granular cytoplasm. In the upper part of the density gradient, where peripheral blood mononuclear cells are typically found, are 'low density' neutrophils (LDN). These comprise immature neutrophils and mature cells with immunosuppressive properties, known as 'myeloid derived suppressor cells' (MDSC). Studies of MDSC have predominantly been in the context of cancer, in which they promote tumour progression [17]. MDSC share the panel of surface markers that is used to define adult mature neutrophils (CD14-CD15+CD16+CD66b+) [18]. However, MDSC 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 had reduced reactive oxygen species production (ROS) at rest and upon lipopolysaccharide (LPS) stimulation, and reduced phagocytic capacity. They were also less susceptible to macrophage efferocytosis, impeding hepatic clearance.

Neutrophil recruitment in alcoholic hepatitis:

Neutrophil recruitment in AH is a complex process (summarized in **Figure 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 ALD 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 that a close association between upregulation of neutrophil activation markers in mice and stable accumulation of neutrophils in murine liver [23]. In the absence of neutrophil activation, CXCL1 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 **(Figure 2)**. Activated Kupffer cells can directly influence neutrophil recruitment through secretion of CXCL2, which binds CXCR2 on neutrophils. Cytokines produced by activated Kupffer cells can also promote neutrophil recruitment indirectly through stimulation of other cell types. For example, IL-1 and IL-6 activate Th17 cells to release IL-17, which stimulates hepatic stellate cell production of CXCL1 and CXCL8 [25]. TNF-alpha produced by activated Kupffer cells contributes to endothelial cell activation, resulting in further CXCL1 production and CXCL8 [26]. However, in a murine model of AH, there was upregulation of CXCL1 from hepatocytes and hepatic stellate cells, 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 had 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 by a neutralising antibody or genetic deletion, was associated with amelioration of neutrophil infiltration [28]. Dominguez et al. found increased levels of CXCL1 and CXCL8 expression in liver biopsy specimens from patients with AH, both at 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 shown in this study 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 (NAFLD) and those with cirrhosis without AH [31]. Compared to wild-type mice, ethanol-fed $LCN2^{-/-}$ mice had reduced serum transaminitis, and reduced histological evidence of inflammation and

steatosis, despite the presence of similar hepatic cytokine profiles. These results were also reproduced by using antibodies to LCN2 in wild type mice. The underlying mechanism of this effect is unknown but it is speculated that LCN2 has proinflammatory 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 (HSEC). 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 HSEC depends on surface interactions with adhesion molecules on endothelial cells. ICAM-1 can bind Mac1 integrin on neutrophils, and VCAM1 binds alpha-4-beta 1 integrin (VLA-4) 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 ICAM1 were increased in patients with AH [36-39]. Blaya et al. found that levels of plasma VCAM1 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 VCAM1 despite an increase in sICAM1 [38]. Further, in the study by Blaya et al., whilst qPCR revealed increased hepatic gene expression for ICAM1 in this study, there was no difference in hepatic VCAM1 gene expression in patients with AH and healthy controls [36]. The predominant role of ICAM1 in neutrophil infiltration in AH is further supported by animal studies; in rats

given intragastric ethanol to induce AH, there was increased hepatic expression of ICAM1, corresponding with increased neutrophil infiltration [40]. Further, ICAM1 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 of 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 subjects, patients with ALD cirrhosis who were actively drinking, had higher levels of CD44 expression in the liver, which reduced after abstinence [42]. Mcdonald et al. found that, in mice, the presence of endotoxaemia did not increase avidity of neutrophil CD44 for hyalorunan. However, TLR4 activation on HSEC 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 lipopolysaccharide- 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 alcoholic hepatitis 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]. In human liver however, E-selectin is hardly expressed in normal endothelium, although studies suggest some upregulation in inflammatory liver diseases [47]. Nevertheless, in n=15 human liver biopsy samples from patients with AH, Adams et al found that prominent E selectin staining in the sinusoids in only one case [47]. Thus it is possible that non-selectin mediated capture mechanisms can predominate in the human liver sinusoid in the context of AH. Once cells are firmly bound to the HSEC however, 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 (FPR1) on neutrophils [48]. At this stage, platelets also play a role in their migration by binding to HSEC and allowing the neutrophils to ‘crawl’ over them using Mac1 integrin [48]. In order to perform their anti-microbial 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 migration capacity towards CXCL8, in comparison to patients with ALD cirrhosis alone, and healthy controls [49]. They hypothesised that this was due to downregulation of CXCR2 on neutrophils, in response to endotoxinaemia. Administration of IL-33 was able to partially restore CXCR2 expression on neutrophils and enhance migration towards CXCL8 [49].

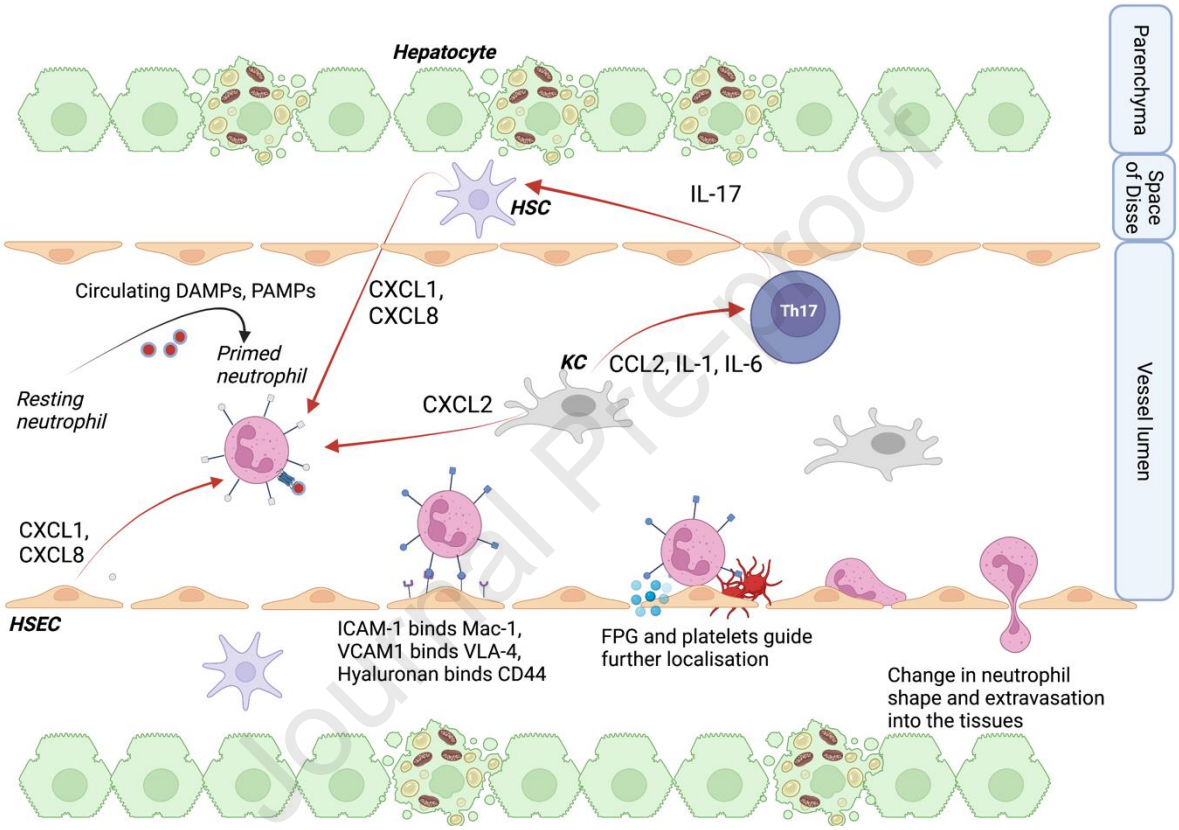

Figure 2. Neutrophil recruitment in the hepatic sinusoids in alcohol-induced hepatitis. *Neutrophil recruitment towards areas of liver injury occurs due to multiple cytokines: (a) CXCL2, released by activated Kupffer cells, (b) CXCL1 and CXCL8, released by activated hepatic sinusoidal endothelial cells, (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 TLR receptors for subsequent activation. Upon activation, there are conformational changes in the integrins on neutrophils allowing their binding to ICAM-1 (to Mac1), VCAM-1 (to VLA-4), which are upregulated on activated HSEC. Activated HSEC produce SHAP, which binds hyaluronan in the extra-cellular 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, chemokine C-C motif chemokine ligand 2; CXCL1, chemokine C-X-C motif ligand 1; CXCL2, chemokine C-X-C motif ligand 2; CXCL8, chemokine C-X-C motif ligand 8; DAMPs, damage associated molecular pattern molecules; FPG, formyl peptide gradient; HSEC, hepatic sinusoidal endothelial cell; HSC, hepatic stellate cell; ICAM1, intracellular adhesion molecule-1; IL-1, interleukin 1; IL-6, interleukin 6; IL-17, interleukin 17; KC, Kupffer cell; Mac-1, macrophage-1 antigen; PAMPs, pathogen associated molecular pattern molecules; SHAP, serum associated hyaluronan associated protein; TLR, Toll-like receptor; VCAM-1, vascular cell adhesion protein.

Neutrophil anti-microbial function in alcoholic 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 (FcR) and the complement receptors (CR), 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 reactive oxygen species (ROS) for 'oxidative burst' is mediated by the nicotinamide adenine dinucleotide (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 by inhibiting bacterial growth and through 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 alpha and MIP-2 [50]. The importance of neutrophil oxidative burst 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, reactive oxygen species can contribute to tissue injury.

Several studies have suggested that in AH the capacity of neutrophils to perform phagocytosis and oxidative burst is altered. Patients with AH frequently have liver 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 be responsible for the impaired phagocytosis because studies have demonstrated that patients with liver cirrhosis have impaired phagocytosis of opsonised bacteria, in comparison to healthy volunteers [8, 51-54]. Tritto et al. found that clinical disease severity in patients with stable liver cirrhosis of varying aetiologies, correlated with the degree of neutrophil phagocytic dysfunction [54]. Interestingly the impairment of function may not be system-wide. Fiuza et al. reported that the reduction in phagocytic capacity of patients with liver cirrhosis was more pronounced for neutrophils in ascitic fluid samples compared with blood, which may explain their particularly high risk for developing spontaneous bacterial peritonitis [53]. Similarly, patients with stable cirrhosis who had normal phagocytic function in neutrophils obtained from peripheral blood, were demonstrated to have impaired phagocytic capacity in neutrophils emigrating into skin blisters [54]. The impaired 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-alpha was associated with defects in subsequent phagocytic activity that was comparable to that seen in patients with liver cirrhosis [51].

There is some evidence that neutrophils are hyper-responsive in AH, as shown by data that they produce more TNF-alpha and CXCL8 upon LPS stimulation [55]. Mookerjee et al. found that patients with ALD cirrhosis and AH had an increased baseline oxidative burst activity in neutrophils compared with healthy controls but the groups did not differ in 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 ALD 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 liver 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 liver 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 liver cirrhosis and AH [8]. Albumin is a potent scavenger of reactive oxygen species 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]. Levels of serum bile acids were elevated in patients with cirrhosis and had a different composition in comparison to that of healthy volunteers, with a greater proportion of chenodeoxycholic acid (CDCA), and a lower proportion of ursodeoxycholic acid (UDCA). Using individual bile acids, the authors demonstrated that CDCA suppressed ROS production and phagocytosis, but UDCA 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 (MPO) and neutrophil gelatinase associated lipoprotein (NGAL) (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 pathogen at the site of injury. The NETs form a scaffold which can initiate thrombosis, sequester pathogens (preventing dissemination), and minimize 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-myrisate 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 TLR receptors on neutrophils in a process known as 'vital netosis'[61]. In vital netosis, NETs are released via vesicles, sparing the neutrophil outer membrane, allowing continued neutrophil function. Although NETosis may be a mechanism of antimicrobial defence, the presence of extracellular histones may activate TLR2 and TLR4 receptors, and contribute to cell death and hepatic tissue injury [62].

Neutrophils in AH have impaired NET formation in response to a toxic insult. 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 hours, 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 high-density to low-density neutrophils. The LDNs were cleared less effectively by macrophages, as compared to 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 one-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 three-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 alcoholic hepatitis. 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 beta, TNF alpha, 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 (HGF) production, a cytokine involved in liver regeneration [55].

Neutrophils may also impact on resolution of fibrosis. Data from murine models of liver fibrosis, induced by carbon tetrachloride, and acetaminophen induced acute liver failure show that liver-infiltrating neutrophils release matrix metalloproteinases (MMP) 8 and 9, which are anti-fibrotic [70]. This may be related to production of anti-inflammatory and anti-fibrotic miR-223 by neutrophils within the hepatic environment [66]. It is unknown if these anti-fibrotic 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 propose IL-6 usually 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 Sirtuin 1 (SIRT1) as the gene that controls miR-223 expression, via its effects of the transcription factor C/EBPalpha [72] . In chronic alcohol users with acute intoxication, the authors found a reduction in SIRT1gene expression, which correlated with the degree of miR-223 expression and serum ALT [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-seq analysis in explanted livers from patients with severe AH, showing that NCF1 was upregulated [10]. Using data generated from RNA-seq analysis, in conjunction with data from experiments with NCF1 knockout mice in a model of chronic plus binge

ethanol feeding, the authors concluded that NCF1 promotes AH by inhibition of 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 promoting myeloid progenitor proliferation [73]. This may potentially 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 (NLRP3) inflammasome expression in macrophages. The NLRP3 inflammasome is a multiprotein complex that acts as a central driver of inflammation and cell death in alcoholic hepatitis, 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-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 alpha, and suppression of neutrophil oxidative burst activity [22]. Steroids also exert pro-inflammatory effects, such as 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 Figure 3.

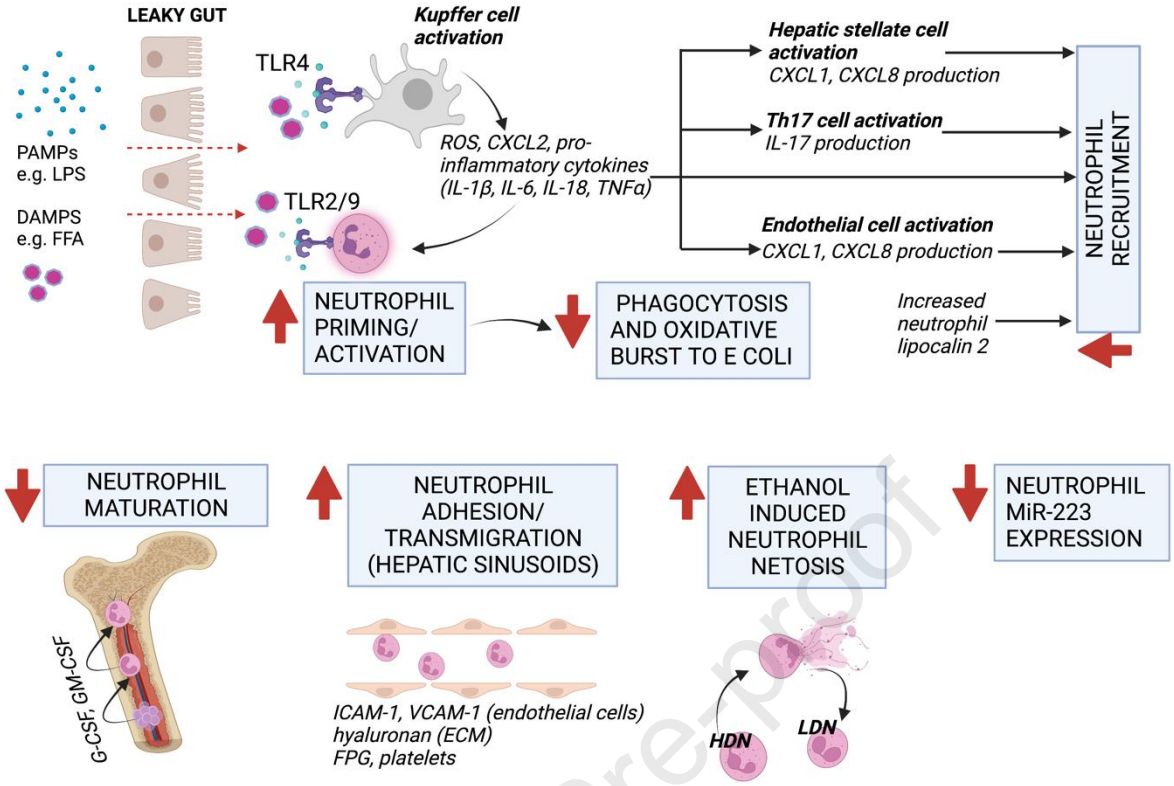

Figure 3. A summary of the potential mechanisms by which neutrophils may contribute to disease pathogenesis in alcohol-related hepatitis (AH). *In AH neutrophil maturation is impaired. Alcohol is associated with intestinal dysbiosis and increased gut permeability allowing the interaction of PAMPS and 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 AH 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 phagocytosis and oxidative burst response to E coli. Alcohol can directly induce neutrophil NETosis, resulting in the conversion of HDN to LDN, which have reduced functional capacity for phagocytosis and reduced susceptibility to phagocytic clearance by macrophages. Patients with AH have reduced expression of miR-223, which reduces its beneficial effects on reducing oxidative stress. CXCL1, C-X-C motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; CXCL8, C-X-C motif chemokine ligand 8; DAMP, damage associated molecular pattern; FFA, free fatty acids; G-CSF, granulonocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; HDN, high density neutrophil; ICAM1, intracellular adhesion*

molecule-1; IL-1β, interleukin-1 beta; IL-6, interleukin 6; IL-17, interleukin-17 ;interleukin-18, IL-18; 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; TNFa, tumour necrosis factor alpha; VCAM1, vascular cell adhesion protein 1

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 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 avoiding 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 anti-bacterial activity [49] but before it is trialled as a potential treatment, it would be important to assess its impact on neutrophil recruitment and hepatic injury. A further consideration is that 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 HSEC and injured hepatocytes. The choice of stimulant used in experiments can 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 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 inhibition of NETosis and promote clearance of the dysfunctional LDNs.

Strategies to limit hepatic neutrophil infiltration, through blockade of adhesion molecules ICAM1 [41], E-selectin [46] 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 in murine studies studying therapeutic interventions for AH [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 the studies [80]. There were five RCTs performed in Asia, which suggested that G-CSF produced a mortality benefit and significantly reduced 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 underlying mechanism of 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 n=118 patients with decompensated liver 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 an FXR receptor agonist, which works by suppressing bile salt production and improving biliary flow, is licensed for use in primary biliary cirrhosis. 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.

There are several clinical trials underway to treat AH patients with strategies to improve intestinal dysbiosis (antibiotics [NCT03157388], [NCT02116556], probiotics [NCT01922895], [NCT02335632], faecal microbial transplant ([NCT03091010], [NCT02458079]), or improve 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 this was 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-1β and CCL2 [86]. The discrepancy in the findings may be due to variation in the 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, generation of some mechanistic data specifically looking at the role of neutrophils in mediating the observed effects will be invaluable in furthering our understanding of neutrophils in AH.

Conclusions:

Neutrophils play a crucial role in innate defence against anti-microbial 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 **(Figure 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 determine enhance our understanding of the degree to which neutrophils mediate the pathogenesis of AH, and therefore determine their therapeutic potential.

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. As we progress in our understanding of the detailed transcriptional mechanisms of neutrophil dysfunction in AH, and our understanding of neutrophil subsets in health and disease, we can use them for 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.

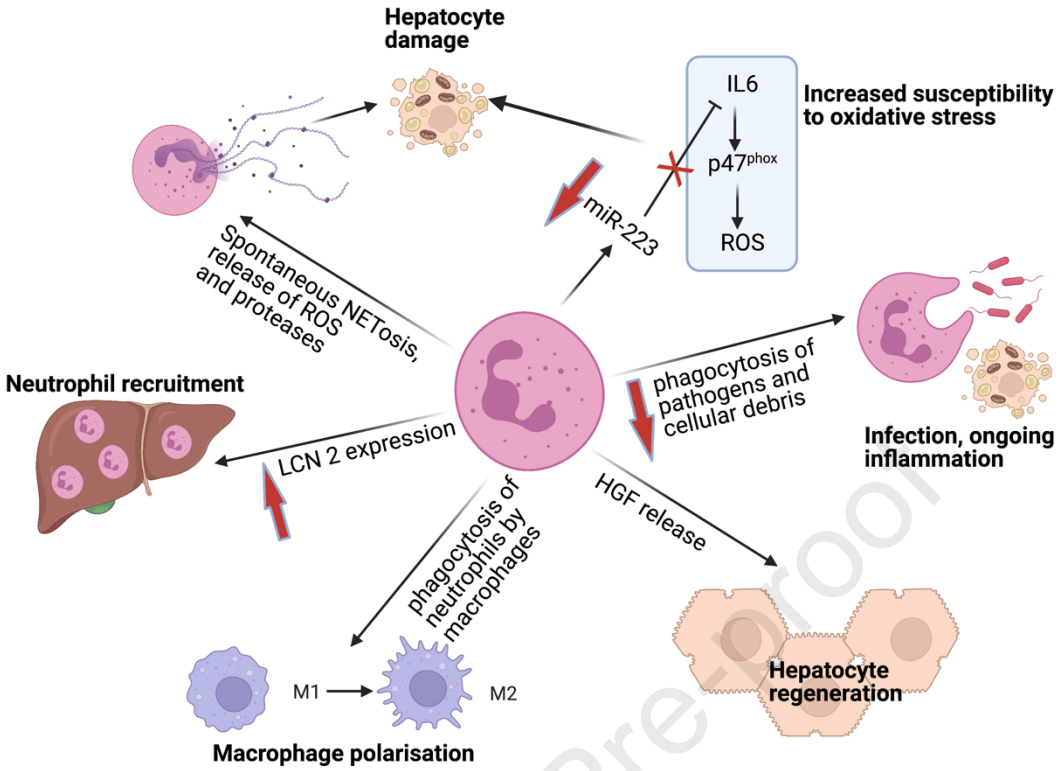

Figure 4. An illustration of the key roles that neutrophils play in alcohol-related hepatitis. *Neutrophils play a role in phagocytosis of pathogens and cellular debris, although their ability to do this is impaired in AH, predisposing to infection. Spontaneous NETosis, release of ROS and proteases results 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. Abbreviations: miR-223, microRNA 223; IL-6, interleukin-6; HGF, hepatocyte growth factor; LCN2, lipocalin 2; ROS, reactive oxygen species.*

REFERENCES

[1] Shirazi F, Singal AK, Wong RJ. Alcohol-associated Cirrhosis and Alcoholic Hepatitis Hospitalization Trends in the United States. J Clin Gastroenterol 2021;55:174-179.
[2] Singal AK, Kamath PS, Gores GJ, Shah VH. Alcoholic hepatitis: current challenges and future directions. Clin Gastroenterol Hepatol 2014;12:555-564; quiz e531-552.
[3] Crabb DW, Bataller R, Chalasani NP, Kamath PS, Lucey M, Mathurin P, et al. Standard Definitions and Common Data Elements for Clinical Trials in Patients With Alcoholic Hepatitis: Recommendation From the NIAAA Alcoholic Hepatitis Consortia. Gastroenterology 2016;150:785-790.
[4] Ventura-Cots M, Argemi J, Jones PD, Lackner C, El Hag M, Abraldes JG, et al. Clinical, histological and molecular profiling of different stages of alcohol-related liver disease. Gut 2022;71:1856-1866.
[5] Thursz MR, Richardson P, Allison M, Austin A, Bowers M, Day CP, et al. Prednisolone or pentoxifylline for alcoholic hepatitis. N Engl J Med 2015;372:1619-1628.
[6] Buzzetti E, Kalafateli M, Thorburn D, Davidson BR, Thiele M, Gluud LL, et al. Pharmacological interventions for alcoholic liver disease (alcohol-related liver disease): an attempted network meta-analysis. Cochrane Database Syst Rev 2017;3:CD011646.
[7] Mathurin P, Moreno C, Samuel D, Dumortier J, Salleron J, Durand F, et al. Early liver transplantation for severe alcoholic hepatitis. N Engl J Med 2011;365:1790-1800.
[8] Mookerjee RP, Stadlbauer V, Lidder S, Wright GA, Hodges SJ, Davies NA, et al. Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome. Hepatology 2007;46:831-840.
[9] Das S, Maras JS, Hussain MS, Sharma S, David P, Sukriti S, et al. Hyperoxidized albumin modulates neutrophils to induce oxidative stress and inflammation in severe alcoholic hepatitis. Hepatology 2017;65:631-646.
[10] Ma J, Guillot A, Yang Z, Mackowiak B, Hwang S, Park O, et al. Distinct histopathological phenotypes of severe alcoholic hepatitis suggest different mechanisms driving liver injury and failure. J Clin Invest 2022;132.
[11] Altamirano J, Miquel R, Katoonizadeh A, Abraldes JG, Duarte-Rojo A, Louvet A, et al. A histologic scoring system for prognosis of patients with alcoholic hepatitis. Gastroenterology 2014;146:1231-1239 e1231-1236.
[12] Vaz K, Little R, Majeed A, Kemp W, Roberts SK. Determinants of Short- and Long-Term Outcomes of an Australian Cohort of Patients Admitted with Alcoholic Hepatitis. Dig Dis Sci 2022;67:3356-3365.
[13] Forrest EH, Storey N, Sinha R, Atkinson SR, Vergis N, Richardson P, et al. Baseline neutrophil-to-lymphocyte ratio predicts response to corticosteroids and is associated with infection and renal dysfunction in alcoholic hepatitis. Aliment Pharmacol Ther 2019;50:442-453.
[14] Terstappen LW, Safford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. Leukemia 1990;4:657-663.
[15] Cheretakis C, Leung R, Sun CX, Dror Y, Glogauer M. Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. Blood 2006;108:2821-2826.
[16] Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front Physiol 2018;9:113.

[17] Keskinov AA, Shurin MR. Myeloid regulatory cells in tumor spreading and metastasis. Immunobiology 2015;220:236-242.
[18] Dumitru CA, Moses K, Trellakis S, Lang S, Brandau S. Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. Cancer Immunol Immunother 2012;61:1155-1167.
[19] Pillay J, Tak T, Kamp VM, Koenderman L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. Cell Mol Life Sci 2013;70:3813-3827.
[20] Cho Y, Bukong TN, Tornai D, Babuta M, Vlachos IS, Kanata E, et al. Neutrophil extracellular traps contribute to liver damage and increase defective low-density neutrophils in alcohol-associated hepatitis. J Hepatol 2023;78:28-44.
[21] Vergis N, Atkinson SR, Knapp S, Maurice J, Allison M, Austin A, et al. In Patients With Severe Alcoholic Hepatitis, Prednisolone Increases Susceptibility to Infection and Infection-Related Mortality, and Is Associated With High Circulating Levels of Bacterial DNA. Gastroenterology 2017;152:1068-1077 e1064.
[22] Taieb J, Mathurin P, Elbim C, Cluzel P, Arce-Vicioso M, Bernard B, et al. Blood neutrophil functions and cytokine release in severe alcoholic hepatitis: effect of corticosteroids. J Hepatol 2000;32:579-586.
[23] Bajt ML, Farhood A, Jaeschke H. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. Am J Physiol Gastrointest Liver Physiol 2001;281:G1188-1195.
[24] Lowe PP, Gyongyosi B, Satishchandran A, Iracheta-Vellve A, Ambade A, Kodys K, et al. Alcohol-related changes in the intestinal microbiome influence neutrophil infiltration, inflammation and steatosis in early alcoholic hepatitis in mice. PLoS One 2017;12:e0174544.
[25] Lemmers A, Moreno C, Gustot T, Marechal R, Degre D, Demetter P, et al. The interleukin-17 pathway is involved in human alcoholic liver disease. Hepatology 2009;49:646-657.
[26] Liu M, Cao S, He L, Gao J, Arab JP, Cui H, et al. Super enhancer regulation of cytokine-induced chemokine production in alcoholic hepatitis. Nat Commun 2021;12:4560.
[27] Roh YS, Zhang B, Loomba R, Seki E. TLR2 and TLR9 contribute to alcohol-mediated liver injury through induction of CXCL1 and neutrophil infiltration. Am J Physiol Gastrointest Liver Physiol 2015;309:G30-41.
[28] Chang B, Xu MJ, Zhou Z, Cai Y, Li M, Wang W, et al. Short- or long-term high-fat diet feeding plus acute ethanol binge synergistically induce acute liver injury in mice: an important role for CXCL1. Hepatology 2015;62:1070-1085.
[29] Swiatkowska-Stodulska R, Bakowska A, Drobinska-Jurowiecka A. Interleukin-8 in the blood serum of patients with alcoholic liver disease. Med Sci Monit 2006;12:CR215-220.
[30] Gonzalez-Quintela A, Campos J, Gude F, Perez LF, Tome S. Serum concentrations of interleukin-8 in relation to different levels of alcohol consumption. Cytokine 2007;38:54-60.
[31] Wieser V, Tymoszuk P, Adolph TE, Grander C, Grabherr F, Enrich B, et al. Lipocalin 2 drives neutrophilic inflammation in alcoholic liver disease. J Hepatol 2016;64:872-880.
[32] Moschen AR, Adolph TE, Gerner RR, Wieser V, Tilg H. Lipocalin-2: A Master Mediator of Intestinal and Metabolic Inflammation. Trends Endocrinol Metab 2017;28:388-397.
[33] Lee WY, Kubes P. Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. J Hepatol 2008;48:504-512.

[34] Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, et al. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. J Clin Invest 1997;99:2782-2790.
[35] Lomakina EB, Waugh RE. Adhesion between human neutrophils and immobilized endothelial ligand vascular cell adhesion molecule 1: divalent ion effects. Biophys J 2009;96:276-284.
[36] Blaya D, Rubio-Tomas T, Rodrigo-Torres D, Lozano J, Coll M, Argemi J, et al. Endothelial dysfunction markers predict short-term mortality in patients with severe alcoholic hepatitis. Hepatol Int 2021;15:1006-1017.
[37] Xia Y, Yang J, Sanyal AJ, Shah VH, Chalasani NP, Yu Q, et al. Persistent Hyperactivation of Endothelial Cells in Patients with Alcoholic Hepatitis. Alcohol Clin Exp Res 2020;44:1075-1087.
[38] Adams DH, Burra P, Hubscher SG, Elias E, Newman W. Endothelial activation and circulating vascular adhesion molecules in alcoholic liver disease. Hepatology 1994;19:588-594.
[39] Hill DB, Deaciuc IV, McClain CJ. Hyperhyaluronanemia in alcoholic hepatitis is associated with increased levels of circulating soluble intercellular adhesion molecule-1. Alcohol Clin Exp Res 1998;22:1324-1327.
[40] Iimuro Y, Frankenberg MV, Arteel GE, Bradford BU, Wall CA, Thurman RG. Female rats exhibit greater susceptibility to early alcohol-induced liver injury than males. Am J Physiol 1997;272:G1186-1194.
[41] Kono H, Uesugi T, Froh M, Rusyn I, Bradford BU, Thurman RG. ICAM-1 is involved in the mechanism of alcohol-induced liver injury: studies with knockout mice. Am J Physiol Gastrointest Liver Physiol 2001;280:G1289-1295.
[42] Urashima S, Tsutsumi M, Ozaki K, Tsuchishima M, Shimanaka K, Ueshima Y, et al. Immunohistochemical study of hyaluronate receptor (CD44) in alcoholic liver disease. Alcohol Clin Exp Res 2000;24:34S-38S.
[43] McDonald B, McAvoy EF, Lam F, Gill V, de la Motte C, Savani RC, et al. Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. J Exp Med 2008;205:915-927.
[44] de la Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. Am J Pathol 2003;163:121-133.
[45] Zhuo L, Kanamori A, Kannagi R, Itano N, Wu J, Hamaguchi M, et al. SHAP potentiates the CD44-mediated leukocyte adhesion to the hyaluronan substratum. J Biol Chem 2006;281:20303-20314.
[46] Bertola A, Park O, Gao B. Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury in mice: a critical role for E-selectin. Hepatology 2013;58:1814-1823.
[47] Adams DH, Hubscher SG, Fisher NC, Williams A, Robinson M. Expression of E-selectin and E-selectin ligands in human liver inflammation. Hepatology 1996;24:533-538.
[48] McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science 2010;330:362-366.

[49] Artru F, Bou Saleh M, Maggiotto F, Lassailly G, Ningarhari M, Demaret J, et al. IL-33/ST2 pathway regulates neutrophil migration and predicts outcome in patients with severe alcoholic hepatitis. J Hepatol 2020;72:1052-1061.
[50] Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. Front Cell Infect Microbiol 2017;7:373.
[51] Ono Y, Watanabe T, Matsumoto K, Ito T, Kunii O, Goldstein E. Opsonophagocytic dysfunction in patients with liver cirrhosis and low responses to tumor necrosis factor-alpha and lipopolysaccharide in patients' blood. J Infect Chemother 2004;10:200-207.
[52] Rajkovic IA, Williams R. Mechanisms of abnormalities in host defences against bacterial infection in liver disease. Clin Sci (Lond) 1985;68:247-253.
[53] Fiuza C, Salcedo M, Clemente G, Tellado JM. In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease. J Infect Dis 2000;182:526-533.
[54] Tritto G, Bechlis Z, Stadlbauer V, Davies N, Frances R, Shah N, et al. Evidence of neutrophil functional defect despite inflammation in stable cirrhosis. J Hepatol 2011;55:574-581.
[55] Taieb J, Delarche C, Paradis V, Mathurin P, Grenier A, Crestani B, et al. Polymorphonuclear neutrophils are a source of hepatocyte growth factor in patients with severe alcoholic hepatitis. J Hepatol 2002;36:342-348.
[56] Breitmeier D, Becker N, Weilbach C, Albrecht K, Scheinichen D, Panning B, et al. Ethanol-induced malfunction of neutrophils respiratory burst on patients suffering from alcohol dependence. Alcohol Clin Exp Res 2008;32:1708-1713.
[57] Stadlbauer V, Mookerjee RP, Wright GA, Davies NA, Jurgens G, Hallstrom S, et al. Role of Toll-like receptors 2, 4, and 9 in mediating neutrophil dysfunction in alcoholic hepatitis. Am J Physiol Gastrointest Liver Physiol 2009;296:G15-22.
[58] Balazs I, Horvath A, Leber B, Feldbacher N, Sattler W, Rainer F, et al. Serum bile acids in liver cirrhosis promote neutrophil dysfunction. Clin Transl Med 2022;12:e735.
[59] Li H, Feng D, Cai Y, Liu Y, Xu M, Xiang X, et al. Hepatocytes and neutrophils cooperatively suppress bacterial infection by differentially regulating lipocalin-2 and neutrophil extracellular traps. Hepatology 2018;68:1604-1620.
[60] Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 2007;176:231-241.
[61] Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ 2009;16:1438-1444.
[62] Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT. Extracellular histones are mediators of death through TLR2 and TLR4 in mouse fatal liver injury. J Immunol 2011;187:2626-2631.
[63] Jin L, Batra S, Jeyaseelan S. Diminished neutrophil extracellular trap (NET) formation is a novel innate immune deficiency induced by acute ethanol exposure in polymicrobial sepsis, which can be rescued by CXCL1. PLoS Pathog 2017;13:e1006637.
[64] Bukong TN, Cho Y, Iracheta-Vellve A, Saha B, Lowe P, Adejumo A, et al. Abnormal neutrophil traps and impaired efferocytosis contribute to liver injury and sepsis severity after binge alcohol use. J Hepatol 2018;69:1145-1154.
[65] Taylor NJ, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, et al. The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. Aliment Pharmacol Ther 2014;40:705-715.

[66] Calvente CJ, Tameda M, Johnson CD, Del Pilar H, Lin YC, Adronikou N, et al. Neutrophils contribute to spontaneous resolution of liver inflammation and fibrosis via microRNA-223. J Clin Invest 2019;129:4091-4109.
[67] Yang W, Tao Y, Wu Y, Zhao X, Ye W, Zhao D, et al. Neutrophils promote the development of reparative macrophages mediated by ROS to orchestrate liver repair. Nat Commun 2019;10:1076.
[68] Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 1989;83:865-875.
[69] Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature 1997;390:350-351.
[70] Saijou E, Enomoto Y, Matsuda M, Yuet-Yin Kok C, Akira S, Tanaka M, et al. Neutrophils alleviate fibrosis in the CCl4-induced mouse chronic liver injury model. Hepatol Commun 2018;2:703-717.
[71] Li M, He Y, Zhou Z, Ramirez T, Gao Y, Gao Y, et al. MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47(phox)-oxidative stress pathway in neutrophils. Gut 2017;66:705-715.
[72] Ren R, He Y, Ding D, Cui A, Bao H, Ma J, et al. Aging exaggerates acute-on-chronic alcohol-induced liver injury in mice and humans by inhibiting neutrophilic sirtuin 1-C/EBPalpha-miRNA-223 axis. Hepatology 2022;75:646-660.
[73] Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature 2008;451:1125-1129.
[74] Knorr J, Wree A, Tacke F, Feldstein AE. The NLRP3 Inflammasome in Alcoholic and Nonalcoholic Steatohepatitis. Semin Liver Dis 2020;40:298-306.
[75] Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, et al. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest 2012;122:3476-3489.
[76] Cavalcanti DM, Lotufo CM, Borelli P, Ferreira ZS, Markus RP, Farsky SH. Endogenous glucocorticoids control neutrophil mobilization from bone marrow to blood and tissues in non-inflammatory conditions. Br J Pharmacol 2007;152:1291-1300.
[77] Filep JG, Delalandre A, Payette Y, Foldes-Filep E. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. Circulation 1997;96:295-301.
[78] Liles WC, Dale DC, Klebanoff SJ. Glucocorticoids inhibit apoptosis of human neutrophils. Blood 1995;86:3181-3188.
[79] Shasthry SM, Sharma MK, Shasthry V, Pande A, Sarin SK. Efficacy of Granulocyte Colony-stimulating Factor in the Management of Steroid-Nonresponsive Severe Alcoholic Hepatitis: A Double-Blind Randomized Controlled Trial. Hepatology 2019;70:802-811.
[80] Marot A, Singal AK, Moreno C, Deltenre P. Granulocyte colony-stimulating factor for alcoholic hepatitis: A systematic review and meta-analysis of randomised controlled trials. JHEP Rep 2020;2:100139.
[81] Tayek JA, Stolz AA, Nguyen DV, Fleischman MW, Donovan JA, Alcorn JM, et al. A phase II, multicenter, open-label, randomized trial of pegfilgrastim for patients with alcohol-associated hepatitis. EClinicalMedicine 2022;54:101689.

[82] Cho Y, Joshi R, Lowe P, Copeland C, Ribeiro M, Morel C, et al. Granulocyte colony-stimulating factor attenuates liver damage by M2 macrophage polarization and hepatocyte proliferation in alcoholic hepatitis in mice. Hepatol Commun 2022;6:2322-2339.
[83] Spahr L, Lambert JF, Rubbia-Brandt L, Chalandon Y, Frossard JL, Giostra E, et al. Granulocyte-colony stimulating factor induces proliferation of hepatic progenitors in alcoholic steatohepatitis: a randomized trial. Hepatology 2008;48:221-229.
[84] Fernandez J, Angeli P, Trebicka J, Merli M, Gustot T, Alessandria C, et al. Efficacy of Albumin Treatment for Patients with Cirrhosis and Infections Unrelated to Spontaneous Bacterial Peritonitis. Clin Gastroenterol Hepatol 2020;18:963-973 e914.
[85] Stadlbauer V, Mookerjee RP, Hodges S, Wright GA, Davies NA, Jalan R. Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis. J Hepatol 2008;48:945-951.
[86] Horvath A, Leber B, Schmerboeck B, Tawdrous M, Zettel G, Hartl A, et al. Randomised clinical trial: the effects of a multispecies probiotic vs. placebo on innate immune function, bacterial translocation and gut permeability in patients with cirrhosis. Aliment Pharmacol Ther 2016;44:926-935.