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## Graphical Review

## Innate immunity and cell death in alcoholic liver disease: Role of cytochrome P4502E1

Mark A. Barnes<sup>a,b</sup>, Sanjoy Roychowdhury<sup>b</sup>, Laura E. Nagy<sup>a,b,c,\*</sup><sup>a</sup> Department of Molecular Medicine, Case Western Reserve University, Cleveland, OH, United States<sup>b</sup> Center for Liver Disease Research, Department of Pathobiology, Cleveland Clinic, Cleveland, OH, United States<sup>c</sup> Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, OH, United States

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## ABSTRACT

Ethanol-induced liver injury is a complex process dependent upon the interaction of multiple cell types in the liver, as well as activation of the innate immune response. Increased expression of CYP2E1 in response to high concentrations of ethanol leads to greater production of cytotoxic ethanol metabolites, which in turn contribute to production of reactive oxygen species, oxidative stress, and ultimately, cell death. Necroptotic hepatocyte cell death in response to ethanol is mediated via a CYP2E1-dependent expression of receptor-interacting protein kinase 3 (RIP3), a key component of the necrosome. In response to alarmins released during ethanol-induced necroptosis, the innate immune response is activated. Macrophage migration inhibitory factor (MIF), a pro-inflammatory multikine involved in many disease processes, is an essential component to this response to injury. MIF expression is increased during ethanol exposure via a CYP2E1-dependent pathway, likely contributing to an exacerbated innate immune response and chronic inflammation after chronic ethanol. This review will discuss the complex interactions between CYP2E1-dependent expression of RIP3 and MIF in the pathophysiology of chronic ethanol-induced liver injury.

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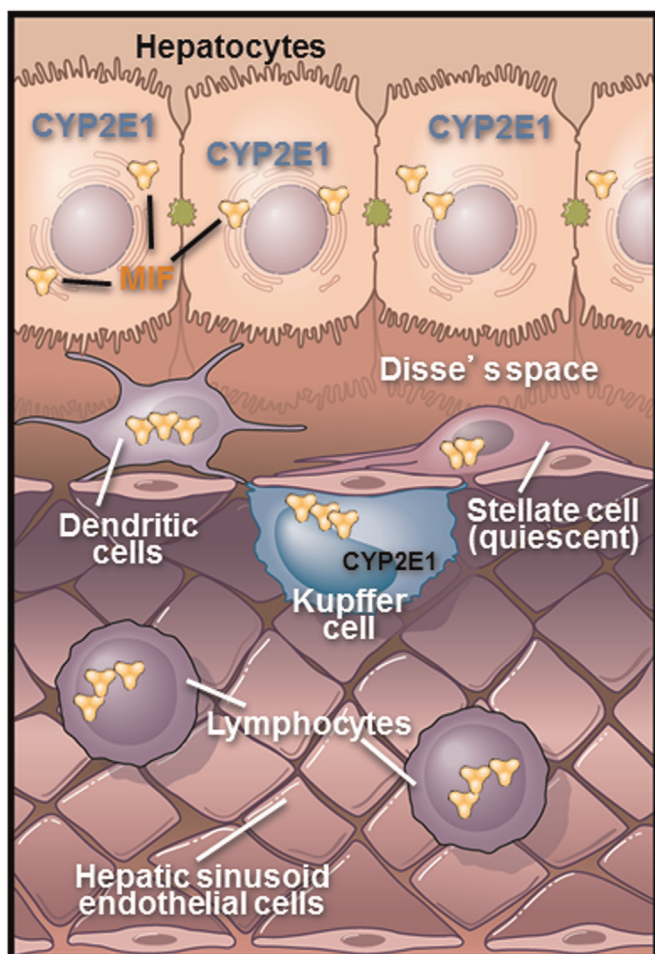
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## Overview: alcoholic liver disease

Long-term excessive alcohol consumption can lead to alcoholic liver disease (ALD) [1]. Heavy alcohol consumption is considered 40–80 g/day for males and 20–40 g/day for females; development

\* Correspondence to: Cleveland Clinic, Lerner Research Institute/NE40, 9500 Euclid Avenue, Cleveland, OH 44120, United States.

E-mail address: [nagyL3@ccf.org](mailto:nagyL3@ccf.org) (L.E. Nagy).



**Fig. 1.** Dynamic interactions between parenchymal and non-parenchymal cells in the liver during the progression of ALD. Parenchymal cells/hepatocytes carry out the primary metabolic functions of the liver. One particularly important function of hepatocytes is to metabolize ethanol. In the context of chronic ethanol exposure, expression of CYP2E1 is increased in hepatocytes and Kupffer cells. Ethanol metabolism via this pathway increases redox stress within the liver, resulting in a condition of “organelle stress” whereby the hepatocytes exhibit impaired metabolic function. Although hepatocytes are the most abundant cell type in the liver, normal physiologic function depends on interaction between hepatocytes and non-parenchymal cells (NPCs). NPCs include liver sinusoidal endothelial cells, natural killer cells, natural killer T cells, and Kupffer cells, the liver resident macrophage. Kupffer cells are an important site for the production of inflammatory mediators, such as  $\text{TNF}\alpha$ , during ethanol exposure. Kupffer cells and hepatocytes are also important sources of chemokines, such as MCP-1 and MIF, which serve to recruit peripheral leukocytes to the liver in response to hepatocellular injury and inflammation.

of ALD can result after such levels of consumption over an extended period of time, usually years [2]. ALD is one of the leading causes of preventable death and accounts for approximately 4% of global mortality [3]. Clinical manifestations of ALD patients include hepatic steatosis, steatohepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma. The majority of chronic, heavy drinkers develop steatosis, which is characterized by accumulation of triglyceride droplets in the liver. However, this initial stage of ALD is usually symptomless and reversible if the person reduces alcohol intake [4]. However, further consumption of alcohol may lead to the progression of liver disease. The development of steatohepatitis, characterized by infiltration of leukocytes into the liver, may occur at any stage of ALD and has a high risk of mortality.

The progression of ALD involves direct effects of ethanol on hepatocytes, resulting in hepatocellular injury and death, as well

as indirect effects of ethanol on innate immune pathways active in the liver (Fig. 1). Innate immune signaling is activated in response to bacterial derived signals, termed pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and danger-associated molecular patterns (DAMPs), which serve as alarmins that indicate the presence of cellular injury and debris. Unresolved activation of innate immune pathways then contributes to the acceleration of hepatocellular injury, with an eventual loss of hepatic function. Understanding the molecular and cellular mechanisms linking ethanol metabolism to hepatocellular injury and death to dysregulated innate immune activity is of critical importance for developing rationally-designed therapeutic interventions to prevent and treat ALD. Here we will provide an overview of one such pathway linking ethanol metabolism to a specific pathway of cytochrome P4502E1 (CYP2E1)-dependent, RIP3-mediated necroptotic cell death and subsequent activation of innate immune signaling via the chemokine/cytokine macrophage inhibitory factor (MIF) that contributes to the progression of ALD.

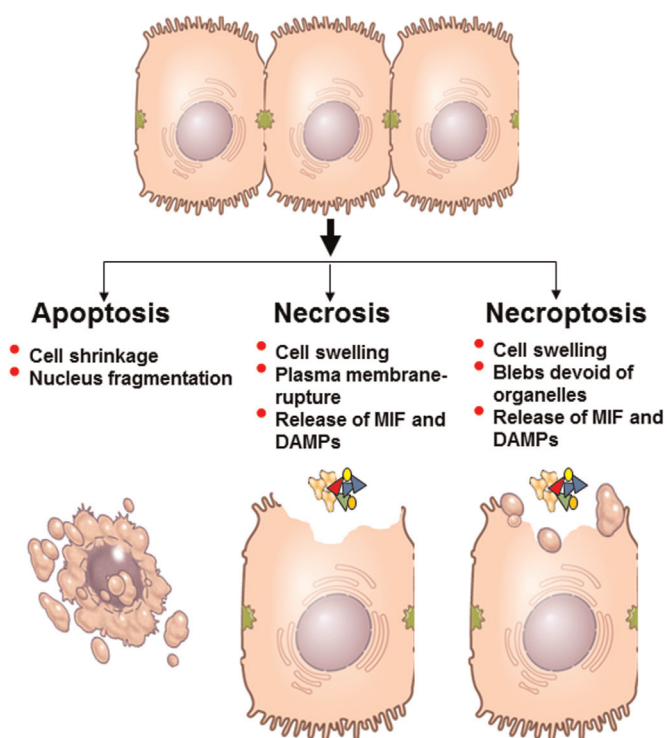
### Ethanol metabolism

Ethanol is metabolized in the liver parenchymal cells, hepatocytes, primarily in the area near the central vein [5]. Alcohol dehydrogenase metabolizes ethanol to acetaldehyde, and subsequently, acetaldehyde is converted to acetate via acetaldehyde dehydrogenase. Ethanol is also metabolized by cytochrome P450 2E1 (CYP2E1) to acetaldehyde; CYP2E1 expression is induced in response to high concentrations of ethanol. Hepatocytes are the primary site of CYP2E1 expression; however, CYP2E1 is induced in Kupffer cells, the resident macrophage in the liver, in response to chronic, heavy ethanol consumption (Fig. 1) [6]. Acetaldehyde is a highly reactive aldehyde; therefore, production of this metabolite can lead to oxidation of lipids and nucleic acids, as well as the formation of protein adducts. Additionally, ethanol metabolism via CYP2E1 leads to the generation of reactive oxygen species (ROS) [2,7]. The resultant oxidative stress has multiple deleterious effects on hepatocytes, including dysregulation of fatty acid synthesis and oxidation, changes in the turnover of macromolecules, as well as mitochondrial dysfunction and cellular stress, which can lead to hepatocyte death [8,9].

### Mechanisms of ethanol-induced hepatocyte death

Multiple mechanisms of cell injury are associated with hepatocyte death during progression of ALD (Fig. 2). Apoptosis, or programmed cell death, is implicated as one of the major modes of cell injury in the liver during ALD. Apoptosis is a caspase-dependent cell death mechanism, mediated by two distinct pathways called the extrinsic (death-receptor-mediated) or the intrinsic (mitochondria-mediated) pathway [10]. In addition to apoptosis, non-apoptotic cell death pathways, including necrosis, are implicated in ethanol-induced liver injury; however, little is known about the molecular mechanisms of alcohol-induced necrosis [11].

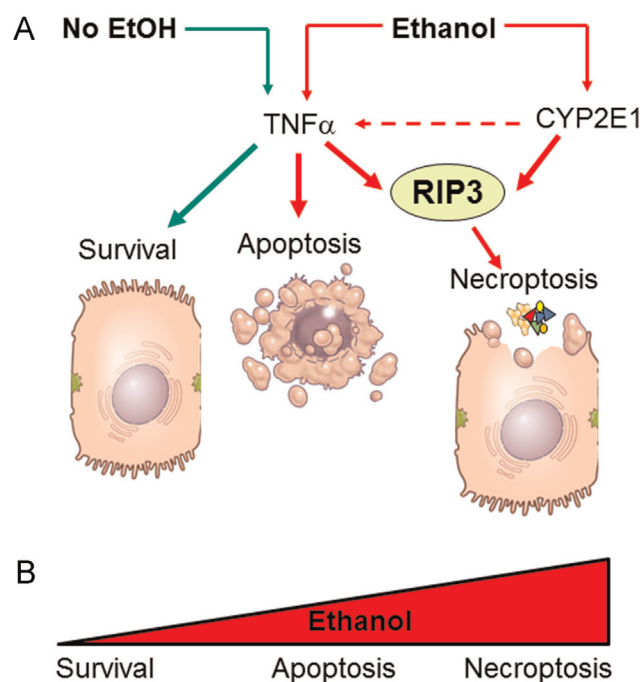
Recently, another mode of non-apoptotic cell death, necroptosis, has been characterized in a variety of cell types [12] and implicated in the development of chronic ethanol-induced liver injury in mice [13]. Necroptosis, like apoptosis, is a highly regulated pathway of cell death; however, morphologically, the process resembles necrosis. Exposure to death ligands, including  $\text{TNF}\alpha$  or CD95, initiates necroptotic signaling cascades that are initially similar to pathways initiating apoptosis; however, the metabolic status of the cell determines the subsequent steps of cell



**Fig. 2.** Mechanisms of cell death. Three potential pathways of hepatocellular cell death are illustrated: apoptosis, necrosis and necroptosis. Apoptosis is characterized by cell shrinking and blebbing, as well as fragmentation of the nucleus and DNA. Apoptosis proceeds under regulation from mitochondria and/or receptor signaling, particularly the receptors for  $\text{TNF}\alpha$  and Fas. Apoptosis is considered to be the least inflammatory mode of cell death, as there is little release of cellular contents (DAMPs) that would activate inflammatory and chemotactic responses. Necrotic cell death is due to acute injury; cells swell and burst, leading to a release of cytoplasmic contents into the extracellular space. These DAMPs trigger pathways termed sterile inflammation that can lead to further hepatocellular injury [41]. MIF, which is present in pre-formed pools in cells, can also be released by necrotic cells, likely contributing to the sterile inflammatory response evoked by necrotic cells. Necroptosis, or programmed necrosis, morphologically resembles necrosis; however, it is RIP3-mediated process and has been indicated as a mediator of ethanol-induced liver injury in mice [13].

death that ultimately lead to apoptosis or necroptosis [12]. If apoptosis is inhibited, exposure to the Fas ligand or  $\text{TNF}\alpha$  can even exacerbate cell death via necroptosis. Receptor-interacting protein (RIP) kinases, in particular RIP3, are key activators of necroptosis (Fig. 3). RIP3, an inducible protein, is implicated in  $\text{TNF}\alpha$ -induced non-apoptotic cell death in a variety of cell types [14,15]. RIP3 has a receptor homotypic interacting motif (RHIM) domain at its C-terminus that mediates interaction with RIP1, while the N-terminal domain contains the kinase activity [12]. Interaction of RIP1 with the RHIM-containing C-terminal domain of RIP3 initiates signals necessary for execution of necroptosis [15]. Under certain conditions, RIP3 can also execute  $\text{TNF}\alpha$ -induced cell death in a RIP1-independent manner [16].

Chronic exposure to ethanol in mice increases the expression of RIP3 in hepatocytes and RIP3 expression is increased in the livers of patients with alcoholic liver disease [13]. While the mechanisms for the induction of RIP3 in response to chronic, heavy ethanol exposure are not well understood, importantly, this induction is dependent on the expression of CYP2E1. Taken together, these data link ethanol metabolism in the liver to RIP3-dependent cell death (Fig. 3). This shift to the more injurious/pro-inflammatory pathway of RIP3-mediated necroptosis contributes to the exacerbation of chronic inflammation in the liver during ethanol exposure.



**Fig. 3.** Ethanol-induced necroptosis is RIP3-dependent. (A) In a healthy hepatocyte, under physiologic conditions,  $\text{TNF}\alpha$ -activated signaling is cytoprotective.  $\text{TNF}$  receptor activation results in the activation  $\text{NF}\kappa\text{B}$ , which promotes cell survival. Cell survival is also promoted by  $\text{TNF}\alpha$ -mediated inhibition of p53-dependent apoptosis and induction of anti-apoptotic Bcl-2 family of proteins. In contrast, under conditions of cellular stress, such as during chronic ethanol exposure, there is a shift in the cellular response to  $\text{TNF}\alpha$  from cytoprotection to cell death. Initially, this response shift to apoptosis, the less inflammatory mode of cell death. However, when expression of CYP2E1 is increased, there is an induction of RIP3, as well as loss of mitochondrial integrity. This combination likely shifts the mode of cell death to the more inflammatory necroptotic pathway. This shift in hepatocyte response to  $\text{TNF}\alpha$ , coupled with an increase in  $\text{TNF}\alpha$  expression in the liver, leads to an increase in RIP3-mediated cell death [13]. This shift in the continuum of hepatocellular response to  $\text{TNF}\alpha$  results in a switch from  $\text{TNF}\alpha$  being cell protective to cell killing, first to the less inflammatory mode of apoptosis, but ultimately to the more injurious necroptotic mode of cell death (B).

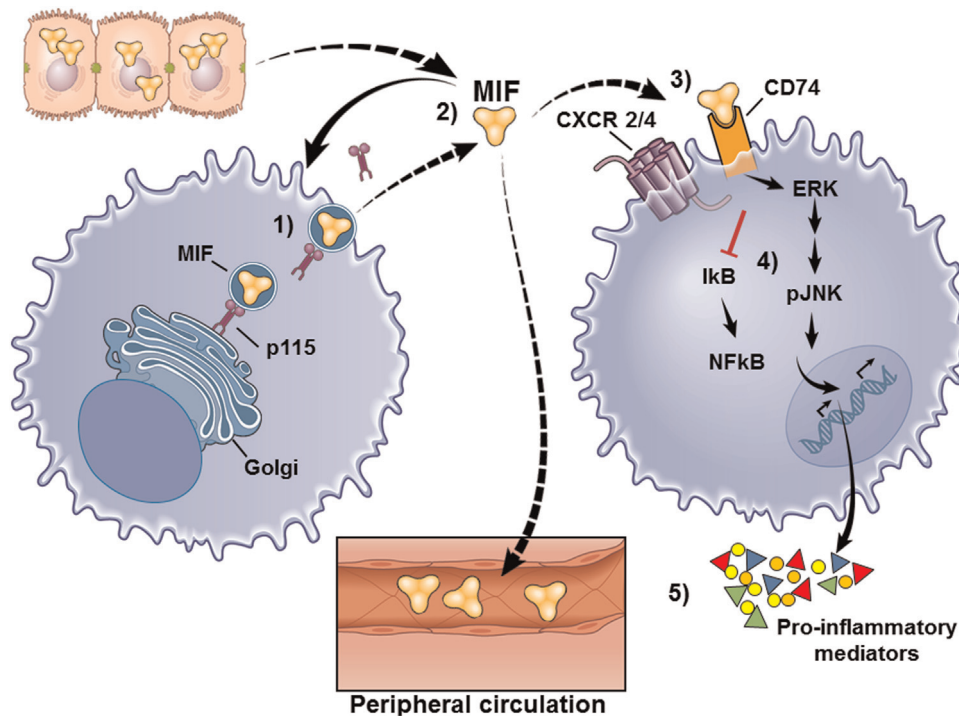
### Innate immune responses in alcoholic liver disease

The liver acts an important innate and adaptive immune organ with a broad range of actions, involving both cellular and soluble factors. This complex, dynamic organ houses a large population of natural killer, natural killer T cells and Kupffer cells (Fig. 1). The liver is also the principal site of production of complement proteins, as well as other molecules important for orchestrating an immune response [17]. Many cells types contribute to ALD pathogenesis and the essential role of Kupffer cell activation and sensitization in ALD has been well characterized [4] (Fig. 1).

In addition to contribution of the cellular components of the innate immune system to ALD, soluble innate immune factors have been identified as key factors in the progression of ALD. The role of soluble mediators, particularly pro-inflammatory cytokines [18], in early progression of ALD has been well documented. However, additional innate immune mediators, e.g. complement anaphylatoxins and chemokines, also contribute to promotion of the pathogenesis of ALD, but are much less understood.

### Cytokines and alcoholic liver disease

A variety of cytokines are upregulated in the liver in response to ethanol exposure; in particular,  $\text{TNF}\alpha$  is a critical mediator of ALD.  $\text{TNF}\alpha$  is produced by many cells types; however, in the liver it is primarily produced by Kupffer cells [19].  $\text{TNF}\alpha$  production is an



**Fig. 4.** MIF signaling. (1) MIF is stored in intracellular pools in unstimulated cells, associated with Golgi-associated protein p115. (2) Upon stimulation of a cell, a macrophage in this illustration, MIF is released into the extracellular environment, where it dissociates from p115. MIF can act in a paracrine, endocrine or autocrine fashion. (3) Once it reaches its target cell, MIF engages its cognate receptor complex, CD74–CXCR2/4. To date, only CD74, also known as major histocompatibility class II (MHC II) invariant chain, has been identified as a cognate receptor for MIF. As a part of the MHC II antigen presenting complex, the invariant chain prevents MHC II from binding proteins in the endoplasmic reticulum (ER) and acts as a protein chaperone in the ER. Though most CD74 is associated with MHC II, a small proportion of protein is located at the cell surface, where it is expressed as a transmembrane protein. (4) Interaction with the receptor complex initiates intracellular signaling events, including MAP kinase signal transduction. CD74's short cytoplasmic tail is not believed to possess direct signaling capacity; instead CD74 forms signaling complexes with CD44 and chemokine receptors CXCR2/4 [42]. CD44 is a multifunctional surface protein that acts as a receptor for ECM proteins and is involved with leukocyte activation. CD74 alone was able to bind MIF; however, activation of MAP kinase signaling required association and phosphorylation of CD44 to the signaling complex. Additionally, MIF signaling activates MAP kinases via interaction with CXCR4 [43]. CD74–CXCR4 complex is also important for endocytosis of MIF and subsequent intracellular signaling [44]. (5) Once MIF-dependent MAP kinase signaling is activated numerous cellular responses are possible, including production of cytokines and other pro-inflammatory mediators, such as TNF $\alpha$  and TLR4, and adhesion molecules, as well as inhibition of p53-dependent apoptosis. MIF can also lead to sustained MAP kinases signaling, which is usually transient [45,46]. Much less is known about the direct chemotactic properties of MIF, but they are likely linked to its ability to bind CXCR2/4. Interaction between MIF and CXCR2/4 mediates monocyte chemotaxis by promoting expression of integrins that assist in cell migration, arrest and extravasation [47].

important mediator for initiating injury in response to ethanol. Furthermore, LPS–TLR-4 signaling, which leads to production of TNF $\alpha$ , is important for development of ALD [20]. Ethanol exhibits a sensitizing effect on monocytes, as monocytes from patients with ALD, as well as rodents after heavy, chronic ethanol exposure, produce increased amounts of TNF $\alpha$  in response to LPS challenge compared to monocytes from healthy individuals [18,21]. IL-1 $\beta$  is another potent pro-inflammatory mediator and is elevated in patients with ALD, as well as in animal models after heavy, chronic ethanol exposure [22]. Clinical trials are currently underway to test the efficacy of IL1 receptor antagonists (Anakinra) in the treatment of alcoholic hepatitis [23].

### Complement and alcoholic liver disease

Complement is an important mediator of innate and adaptive immune responses. Complement proteins exhibit opsonizing, chemotactic and cell lytic properties. In the context of ALD, complement components C1q, C3, and C5 are known to contribute to the progression of ethanol-induced liver injury in mice [24,25].

### Chemokines and alcoholic liver disease

While the role of cytokines in ALD, particularly TNF $\alpha$ , has been extensively studied, the role of chemokines is not well understood.

Chemokines are a subset of cytokines that specialize in recruitment of cells during homeostasis and inflammation through chemotaxis, movement of cells toward a chemical signal or gradient [26]. Mandrekar et al. explored the role of MCP-1 (CCL2), a potent chemoattractant for monocytes, in ethanol-induced liver injury. Ethanol feeding to mice increased MCP-1 in both Kupffer cells and hepatocytes. Increased expression of MCP-1 was associated with increased liver injury, as well as, increased expression of pro-inflammatory mediators. Additionally, MCP-1 promotes hepatic triglyceride accumulation by inhibiting action of peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ), a protein that promotes oxidation of fatty acids [27]. This was the first study to establish a role for chemokines in promoting liver injury, and surprisingly, promoting hepatic steatosis.

### Macrophage migration inhibitory factor (MIF)

Macrophage migration inhibitory factor (MIF) is a soluble factor, initially described as a T cell-derived mediator that inhibited movement of macrophages, which is involved in many disease processes [28–30]. Since its original discovery, MIF has been shown to be a multipotent innate immune mediator that is involved in many diseases, including multiple liver diseases [31]. Although MIF is known to contribute to ALD [32], its precise role (s) in the progression of ALD is still under continued investigation.

## MIF expression and function

Determining the exact cellular source of MIF under specific pathophysiological conditions has remained elusive because there are many sources of MIF. MIF is released by many cell types including macrophages, which are considered a major source of MIF, other leukocytes, endothelial cells, epithelial cells, as well as the pituitary gland. It is likely that multiple cell types contribute to increased MIF in the systemic circulation [31,33].

One of the first described functions of MIF was its ability to inhibit glucocorticoid (GC) action. GCs exhibit potent anti-inflammatory properties, such as suppression of inflammation and leukocyte recruitment. Because MIF is induced by glucocorticoids and acts as a negative regulator to glucocorticoid mediated responses, it helps maintain normal immune function despite increases in anti-inflammatory signals. One method GCs utilize to reduce inflammation involves activating MAP kinase phosphatase (MKP). MKPs dephosphorylate MAP kinases, which are essential signaling proteins during immune responses, thus inactivating them and suppressing pro-inflammatory immune functions [34]. MIF counteracts GCs by activating MAP kinase signaling and promoting leukocyte recruitment [35,36].

## MIF and alcoholic liver disease

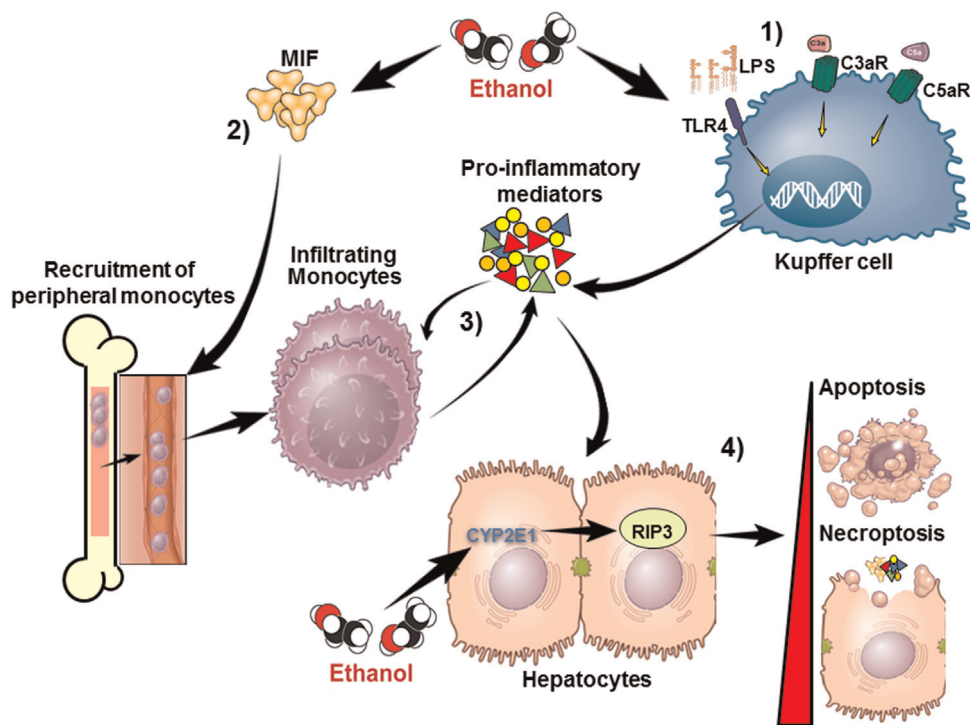
Expression of MIF in liver is increased in patients with alcoholic hepatitis or cirrhosis, with expression increased in both hepatocytes

and monocytes. Further, circulating concentrations of MIF are correlated with plasma aspartate aminotransferase in patients with alcoholic hepatitis or cirrhosis [32].

The underlying mechanisms by which ethanol promotes expression and release of MIF are likely complex, but it is clear that this response is at least partially dependent on CYP2E1 [37]. While regulation of MIF expression and secretion is not well understood, recent studies have provided insights into possible mechanisms of control. One mechanism involves microRNA-451 directly interacting with the 3' UTR of MIF and regulates MIF expression. Golgi-Associated Protein p115 was shown to be essential for releasing MIF from cytoplasmic pools (Fig. 4). When p115 was depleted in human monocytes, cells exhibited reduced ability to release MIF into the extracellular environment [38]. Cells stimulated with LPS in vitro secreted both MIF and p115 into their supernatant, further demonstrating the association between MIF and p115 in the control of MIF secretion [39].

## Summary

Hepatocellular injury in response to chronic ethanol exposure is dependent on expression and activity of CYP2E1. Metabolism of ethanol via this pathway is associated with a shift in the mode of hepatocyte cell death in response to inflammatory mediators, from the relatively homeostatic pathway of apoptosis to the more injurious pathway of necroptosis (Fig. 3). Multiple DAMPs are



**Fig. 5.** The role of MIF in ethanol-induced liver injury. (1) Ethanol exposure leads to activation of Kupffer cells. (2) Ethanol exposure also leads to increased expression of MIF. Studies modeling chronic ethanol feeding to mice have revealed that ethanol exposure increased the expression of MIF mRNA in the liver, as well as the concentration of MIF in the circulation. MIF production appears to be dependent on CYP2E1-mediated ethanol metabolism. CYP2E1<sup>-/-</sup> mice do not induce the expression of MIF mRNA after chronic ethanol feeding compared to wild-type mice, suggesting that induction of MIF is CYP2E1 dependent. Another contributor to induction of MIF during ethanol exposure may be related to localized hypoxia in the liver during ethanol metabolism [48,49]. Ischemia–reperfusion, which similar to ethanol exposure, results in hypoxia and is associated with increased serum MIF, as well as increased expression of MIF mRNA and protein [50]. MIF also enhances its own expression under hypoxic conditions [48]. Taken together, it is likely that ethanol metabolism via CYP2E1, in conjunction with localized ethanol-induced hypoxia in the liver, contributes to increased MIF expression during chronic ethanol feeding. (3) Activated Kupffer cells and MIF contribute to increased production of pro-inflammatory mediators. MIF additionally acts as a chemokine and recruits peripheral monocytes to the liver. MIF is involved in both the early and chronic innate immune responses in the liver after ethanol feeding and is critical for recruitment of infiltrating monocytes after chronic ethanol feeding [37]. During ethanol-induced liver injury, expression of MCP-1 mRNA was increased in a MIF-dependent fashion. These observations indicate that MIF directly, and/or indirectly via MCP-1, recruits monocytes from peripheral immune reservoirs. (4) It is not yet clear which happens first, but a complex feedback loop is formed between infiltrating monocytes and pro-inflammatory mediators. Ethanol-mediated infiltration of monocytes and increased pro-inflammatory mediators in the liver leads to death of hepatocytes.

released from necroptotic hepatocytes, and as a result, there are multiple innate immune pathways activated to respond to this injury.

One key pathway activated in response to ethanol-induced injury is MIF. MIF has been implicated in the pathogenesis of many inflammatory diseases including sepsis, colitis, metabolic disorders, and multiple types of arthritis [31]. MIF is an important for modulation of expression of pro-inflammatory mediators, including TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , TLR4, and is produced by and exerts its effects upon many different cells types, including directly promoting chemotaxis (Fig. 5). MIF contributes to the progression of ethanol-induced liver injury at multiple stages in the pathophysiology of ALD and ASH. The primary role of MIF is associated with regulation of expression of pro-inflammatory and chemotactic mediators, and recruitment of peripheral monocytes. MIF contributes to the pathophysiology of ethanol-induced liver injury in mice and suggest that inhibition of MIF activity with small molecule inhibitors of MIF, such as ISO-1 [40], may be an important therapeutic approach to the treatment of ALD.

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