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Review

Signalling pathways in alcohol-induced liver inflammation☆

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The pathogenesis of alcoholic liver injury involves interactions of several intracellular signalling pathways in different cell types of the liver. Alcohol-induced sensitization of liver macrophages to portal endotoxin/lipopolysaccharide (LPS) is considered a hallmark of alcoholic liver disease (ALD). Intracellular mechanisms associated with LPS-induced signalling play a crucial role in the initiation and progression of alcoholic liver injury, and are being extensively explored. LPS recognition by Toll-like receptor 4 (TLR4) on macrophages and other cell types in the liver, activation of downstream signalling pathways culminating in activation of transcription factors such as NFκB, AP-1 leads to increased inflammatory cytokine production in ALD. In addition, LPS-induced MAPK such as ERK and p38 also contribute to liver injury. The importance of alcohol-induced reactive oxygen species and interactions with TLR pathways in macrophages leading to inflammation is becoming increasingly evident. Collectively, these signalling pathways induce pro- and anti-inflammatory cytokines that play an important role in ALD. In this review we describe the key signalling intermediates leading to alcohol-induced inflammation in alcoholic liver disease.

***Keywords*: Macrophages; Kupffer cells; TLRs; MAP kinases; Transcription factors**

1. Introduction

Alcohol consumption is associated with a spectrum of diseases in the liver ranging from steatosis, steatohepatitis to cirrhosis and hepatocellular carcinoma. The pathogenesis of acute and chronic alcohol consumption is multi-factorial with diverse consequences in different cell types. Alcohol-induced injury occurs at multiple levels ranging from the innate immune cells to the liver parenchymal cells, hepatocytes. The innate immune cells including hepatic macrophages (Kupffer cells) play a pivotal role in early alcohol-induced liver injury via recognition of endotoxin/lipopolysaccharide in the portal circulation. The progression of alcohol-induced liver damage involves parenchymal cells and macrophages through the direct effects of alcohol as well as indirect effects of metabolites, oxidative stress, immunologic and inflammatory events. In macrophages, alcohol directly induces oxidative stress and sensitizes to LPS-induced inflammatory cytokine production. Inflammatory cytokines particularly TNFα, contributes to the development of alcoholic liver disease (ALD). Alcohol sensitizes hepatocytes to TNFα-induced apoptosis. A complete understanding of alcohol-mediated intracellular signalling mechanisms leading to inflammatory cytokine induction in macrophages will provide new insights into the development of new potential targets for therapeutic intervention.

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Abbreviations: ALD, alcoholic liver disease; Egr-1, early growth response factor-1; HSF, heat shock transcription factor; IRF, interferon-responsive factor; NFkB, nuclear factor kappa B; MAPK, mitogen activated protein kinase; MyD88, myeloid differentiation primary response gene (88); STAT, signal transducers and activators of transcription; SOCS, suppressor of cytokine signalling; TLR, Toll-like receptor.

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The goal of this concise article is to review the alcohol-mediated signalling pathways, particularly Toll-like receptors and their adaptors in macrophages. The importance of transcription factors such as NFκB, AP-1, Egr-1 and STATs, intracellular kinases such as MAP kinases and pro- and anti-inflammatory cytokines in ALD will also be reviewed.

2. Interactions of immune and parenchymal cells of the liver in ALD

Innate immune responses activated in the resident liver macrophages, Kupffer cells play a key role in the early pathogenesis of alcohol-induced liver injury [1]. Increased levels of circulating LPS in alcoholic patients have been shown [2]. The currently accepted model of alcoholic liver injury elucidates that LPS promotes hepatic injury via induction of Kupffer cell activation resulting in production of TNFα and other inflammatory mediators. The Kupffer cells respond to stimulation by gut-derived endotoxins and apoptotic dead cells in the tissue resulting in increased inflammatory responses. Circulating TNFα is increased in chronic alcoholics as well as in mouse chronic alcohol feeding models [3,4]. In addition to hepatocytes, abnormal stellate cell activity and induction of fibrosis is also dependent on Kupffer cells via production of reactive oxygen species and pro-inflammatory cytokines [5]. Liver natural killer (NK) cells exposed to alcohol contribute to fibrosis and inflammation via inhibition of NK cell accumulation and reduced NK cell killing of hepatic stellate cells [6]. Thus, modulation of the innate immune system is an important mechanism contributing to liver inflammation, hepatocyte death and liver fibrosis (Fig. 1).

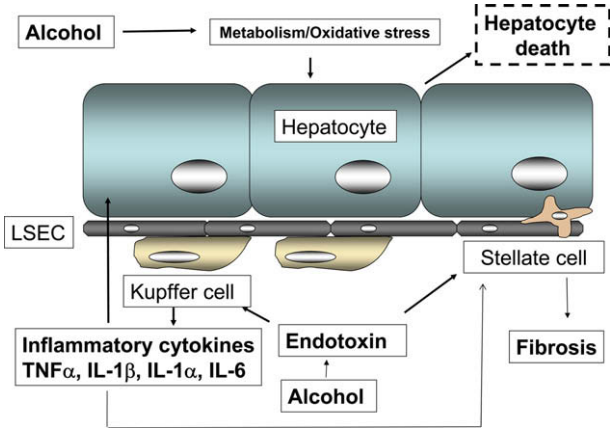

Fig. 1. Cells involved in alcoholic liver injury. Alcohol-mediated increase in gut-derived endotoxin with oxidative stress mechanisms sensitizes hepatic macrophages to release inflammatory cytokines such as TNFα, IL-1β, IL-1α and IL-6 that affects stellate cells and hepatocyte functions. Endotoxin also affects stellate cell and endothelial cell activation and contributes to liver injury.

3. Receptor-mediated signalling pathways affected by alcohol in liver inflammatory cells

3.1. Toll-like receptors, adapters and signalling

Recent discoveries of pattern recognition receptors focused attention on Toll-like receptors (TLRs) that sense pathogen-derived molecules as well as host-derived damage signals [7,8]. Among 10 different TLRs described in humans, the functional significance of TLR4 and its downstream signalling in alcoholic liver disease is extensively elucidated (Fig. 2). TLR4 recognizes the lipid A motif of the lipopolysaccharide (LPS), a suggested cofactor in the pathogenesis of ALD [9]. TLR4 is a major component of the LPS recognition receptor complex, which also involves the co-receptors CD14 and MD-2, and LPS binding protein (LBP) [10,11]. LBP is a soluble shuttle protein that directly binds LPS and facilitates the association between LPS and CD14 [12,13].

CD14 is a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form. CD14 facilitates the transfer of LPS to the TLR4/MD2 receptor complex and modulates LPS recognition [14]. CD14 facilitates TLR4 induced responses [7,15] and appears to be required for MyD88-independent signalling [16].

MD2 is a soluble protein that non-covalently associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs [17–19]. Although no evidence suggests that TLR4 can bind LPS directly, TLR4 can enhance the binding of LPS to MD2 [20].

In the liver, TLR4 is expressed not only on innate immune cells such as Kupffer cells and recruited macrophages, but also on hepatocytes, sinusoidal endothelial cells, biliary epithelial cells and stellate cells. Indeed, LPS activation was shown to result in functional changes in all of these cell types in different liver diseases [21,22].

A major role for LPS-induced, TLR4 mediated signalling via its ligand, endotoxin, in alcoholic liver disease (ALD) was established by studies of Thurman and colleagues [23,24]. Studies in knockout mouse models have shown that chronic alcohol feeding in mice deficient of CD14, TLR4 and LPS-binding protein (LBP) results in alleviation of alcohol-induced liver injury indicating an important role for the TLR4 pathway [24–27]. Recent studies suggested that LPS recognition by TLR4 expressed on hepatic stellate cells and sinusoidal epithelial cells may further contribute to the progression of ALD [28,29].

Alcohol sensitizes Kupffer cells and monocytes/macrophages to produce increased TNFα in response to endotoxin [30]. Studies investigating mechanisms of alcohol-induced sensitization of Kupffer cells to endotoxin focused on intermediates of the TLR4 induced signalling pathway. Reports on the effects of alcohol on

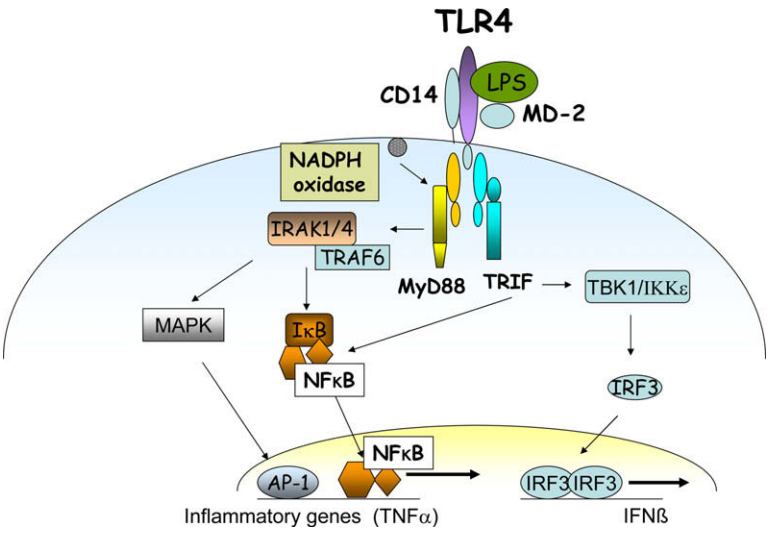

Fig. 2. TLR4 mediated signalling in alcohol-exposed macrophages. Alcohol alters functions of Toll-like receptors TLR4 in the liver resulting in regulation of MyD88 dependent activation of downstream signalling molecules such as IRAK kinase, IKK and NFκB. MyD88-independent, TRIF-dependent activation of IRF3 is also regulated alcohol exposure. Alcohol also induces NADPH oxidase via reactive oxygen species leading to inflammation.

membrane proximal events using mutant and knock out mice have shown an important role for CD14 [26] and TLR4 [24]. In another report, hepatic expression of TLR1, 2, 4, 6, 7, 8 and 9 mRNA was increased in wild-type mice using the Leiber–DeCarli chronic alcohol feeding model [31]. Alcohol feeding also resulted in sensitization to liver damage and inflammation because administration of TLR1, 2, 4, 6, 7, 8 and 9 ligands resulted in increased expression of TNFα mRNA [31]. Other investigations found that deficiency in TLR2 had no protective effect on ALD in a mouse model of chronic alcohol feeding [32].

TLR4 triggers signalling from the cytoplasmic TIR domain via recruitment of different intracellular adaptors and culminate in the production of pro-inflammatory cytokines or Type I IFN to activate innate immune responses [33,34] (Fig. 2). The myeloid differentiation factor 88 (MyD88) is a common adapter to all known TLRs except for TLR3 which exclusively utilizes TIR-domain containing adaptor inducing TGFß (TRIF) [35]. However, TLR4 is unique in utilization of both MyD88 and TRIF (MyD88-independent) downstream signalling [36]. Upon activation of TLR4, signalling intermediates IRAK1 and 4 are recruited to the TLR4 complex via interaction with MyD88 leading to IKK kinase activation and induction of pro-inflammatory cytokines. Recruitment of the TRIF adapter activates IRF3 phosphorylation that results in Type I IFN production [35,36]. Whether chronic alcohol affects activation and recruitment of the IRAK family members is currently not known. However, acute alcohol-induced tolerance and sensitization has been attributed to changes in IRAK-1 expression [37] and kinase activity in *in vitro* models in macrophages [38]. The role of MyD88, the common TLR adaptor molecule was recently evaluated in chronic alcohol-induced liver injury in a mouse model [32]. These studies showed that MyD88 knockout mice were highly susceptible to alcohol-induced fatty liver [32], indicating a role for a MyD88-independent pathway in alcoholic liver injury. TLR4-induced MyD88-independent signalling leads to activation of IKKε, NFκB as well as interferon regulatory factor 3 (IRF3) and downstream Type I IFN activation [33,34]. While the nuclear levels of IRF3, that would indicate activation, were not significantly increased in the livers of alcohol-fed mice, other investigators found that acute alcohol administration suppressed TLR3 downstream signalling in macrophages [39]. Recent investigations suggest that TRIF-regulated IRF3 binds to the promoter region of the TNFα gene and upregulates transcription in chronic alcohol-exposed macrophages contributing to alcohol-induced steatosis [40].

In contrast to chronic alcohol consumption, acute alcohol exposure inhibited TLR4 signalling in monocytes and macrophages after *in vitro* as well as *in vivo* alcohol treatment in mice leading to decreased LPS-induced TNFα production [41,42]. *In vitro* studies show that acute ethanol exposure leads to decreased LPS-induced IRAK-1 phosphorylation [38]. The role of membrane and endosomal TLRs and their intracellular adapter molecules is increasingly evident with TLR4 playing a "gate-keeper" role in ALD.

3.2. Alcohol, TLRs and ROS: an emerging link

Oxidative stress-induced cellular responses play an important role in innate immune cell activation. In the liver, Kupffer cells produce reactive oxygen species (ROS) in response to chronic alcohol exposure as well as endotoxin [43,44]. Alcohol-induced sensitization to

LPS has been recently attributed to ROS production. Evidence shows that direct interaction of NADPH oxidase isozyme 4 with TLR4 is involved in LPS-mediated ROS generation and NFκB activation in neutrophils [45]. Furthermore, NADPH oxidase induces TLR2 and TLR4 expression in human monocytic cells [46]. In chronic alcohol fed rats, pretreatment with diphenyliodonium (DPI), which inhibits NADPH oxidase, normalized ROS production, decreased LPS-induced ERK1/2 phosphorylation and inhibited increased TNFα production in Kupffer cells [43,44]. In a recent study, inhibition of NADPH oxidase prevented steatosis, upregulation of TLR2, 4, 6 and 9 mRNA, and sensitization to respective ligand-induced liver injury [31], indicating a cross talk between oxidative stress and TLR pathways in ALD. Previous reports indicate that p47 phox −/− mice are resistant to alcohol-induced liver injury, further suggesting an important role for NADPH oxidase in not only inflammatory responses but also liver injury [43]. In another study, dilinoleoylphosphotidylcholine (DPC), an anti-oxidant, prevented LPS-induced NFκB and ERK1/2 activation and TNFα production in Kupffer cells of chronic alcohol-fed rats [47]. It is now widely accepted that ROS not only plays a critical role in direct hepatocyte injury but also contributes to increased inflammatory responses contributing to liver injury.

4. Transcription factors in alcoholic liver injury

4.1. NFkB

The nuclear regulatory factor κB (NFκB) is a central regulator of cellular stress in all cell types in the liver. The family of NFκB proteins such as RelA/p65, RelB, c-Rel and p50, reside in the cytosol of resting cells as dimers in a complex with inhibitory κB molecules [48]. These dimers are activated and translocated to the nucleus upon stress signals that could be pathogen-derived, oxidative stress, etc. Danger signals lead to activation of the IKK kinase complex consisting of IKK/IKKß/NEMO and phosphorylation of IκB. Phosphorylated IκB is then ubiquitinated and degraded by the proteosomal pathway [48]. Dissociation of IκB exposes the nuclear translocation sites of NFκB allowing nuclear translocation and DNA binding. NFκB forms p65/p50 heterodimers in macrophages and binds to the promoter region of various pro-inflammatory genes to result in gene transactivation.

Hepatic macrophage expression of pro-inflammatory mediators is largely regulated by NFκB. Chronic alcohol-mediated liver injury is associated with activation of TLR4 by circulating LPS on liver macrophages culminating in NFκB activation and pro-inflammatory cytokine production. Murine models of chronic alcohol administration show increased NFκB DNA binding in the liver [49]. It is believed that chronic alcohol primes the liver by sustained NFκB activation and induction of basal and LPS-stimulated TNFα [50]. Similar to resident liver macrophages, monocytes from chronic alcoholic patients also showed increased NFκB activation compared to controls [51].

In contrast to increased NFκB activation by chronic alcohol, acute alcohol exposure decreased nuclear translocation and activation of NFκB p65/p50 heterodimer [52] whereas NFκB p50 homodimer binding to DNA was increased by acute alcohol in human monocytic cells [53]. It is evident from these studies that differential regulation of NFκB is based on the length of alcohol exposure and is pivotal in the regulation of TNFα induction in monocytes and macrophages in ALD.

4.2. AP-1

Transcription factors AP-1 are homodimers and heterodimers composed of basic region-leucine zipper (hZIP) protein that belongs to the Jun (c-Jun, Jun B and JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Jun dimerization partners (JDP1, JDP2) and the closely related activating transcription factors (ATFs) [54]. AP-1 regulates cellular proliferation and death through induction of cell cycle modulators such as cyclin D1 and p53 [55]. While AP-1 is activated by pro-inflammatory cytokines, oxidative stress, growth factors and endotoxin, the MAPK kinase cascade system seems to be the most important intracellular mediator [55]. Acute alcohol-induced increased sensitization of macrophages was associated with an upregulation of AP-1 activity, increased CD14 mRNA and pro-inflammatory cytokine production [56]. Inhibition of ROS production using a recombinant adenovirus overexpressing anti-oxidant Cu,Zn superoxide dismutase (SOD) in the liver abrogated both AP-1 activity and CD14 expression [56]. Furthermore, acute alcohol-induced AP-1 activation in human monocytes is dependent on Src kinase activity and regulates IL-10 production [57]. On the other hand, chronic alcohol-induced AP-1 mediated transcription through a PKC-dependent pathway via increased expression of c-jun and c-fos in hepatocytes, thus influencing proliferative activity [58] and possibly contributing to malignant transformation. Interestingly, LPS-stimulated activation of AP-1 binding to DNA was not increased in isolated Kupffer cells by chronic ethanol feeding [59].

4.3. EGR-1

Early growth response-1 (Egr-1) is a transcription factor regulated by the MAPK signalling cascade and induced by LPS [60]. Egr-1, an immediate early gene/zinc-finger transcription factor is required for induction of TNFα, adhesion molecules, basic fibroblast growth factor, transforming growth factor-β, MCP-1 and

MIP-2 [61]. Studies have shown that chronic alcohol feeding increases Egr-1 binding to the promoter of the TNFα promoter and this is MAPK-ERK dependent in hepatic macrophages [62]. In fact, it has been suggested that Egr-1 contributes to the increased sensitivity of macrophages to LPS-stimulated TNFα production after chronic alcohol exposure [59]. Furthermore, the requirement for Egr-1 in chronic alcohol-induced liver injury was shown by a lack of steatosis or increased ALT and TNFα levels in the liver of chronic alcohol-fed Egr-1 knockout mice [63]. Whether Egr-1 contributes to induction of other inflammatory cytokine genes such as MCP-1, MIP-2 and ICAM-1, all enhanced during alcoholic liver injury, requires further investigation.

4.4. STAT

The Janus-kinase (JAK) associated signal transducer and activator transcription factor (STAT) is activated by cytokines and growth factors and has been implicated in immune and hepatic cell functions [64,65]. Activation of STATs via interleukin-6 (IL-6) and interferons (IFN) play an important role in hepatic regeneration, anti-viral activity and gene expression [66]. Acute alcohol activates LPS-induced Src-dependent STAT3 activation to modulate IL-10 production in monocytes [57]. On the other hand, IL-6- activated STAT3 and IFN-induced STAT1 was blocked by acute alcohol exposure in monocytes [67] and freshly isolated but not in cultured hepatocytes [68]. Immunoinhibitory molecules such as SOCS1 and SOCS3 were upregulated in acute alcohol-exposed monocytes, resulting in down-modulation of cytokine-induced STAT1/3 signalling [67]. Activation of STAT3 was reportedly decreased in alcoholic liver disease patients with cirrhosis [69]. Recent studies suggest that chronic alcohol-fed hepatocyte-specific STAT3 knockout mice are more susceptible to alcohol-induced fatty liver injury with less pronounced inflammatory cytokine induction whereas macrophage-specific STAT3 knockout mice exhibit reduced liver inflammation [70]. Thus, the pro- or anti-inflammatory role of STAT3 after chronic alcohol feeding is dependent on the cell-type involved in the liver. Chronic alcohol consumption attenuates IFN-induced STAT1 activation and results in loss of NK cell activity in the liver leading to acceleration of hepatic fibrosis [6]. It appears that while STAT1 activity is abrogated by acute and chronic alcohol exposure, chronic alcohol enhances STAT3 and modulates alcohol-induced steatosis and inflammation based on the cell-type in which it is expressed.

5. MAPK signalling

LPS recognition activates the MAPK family members including extracellular receptor activated kinases 1/2 (ERK1/2), p38 and c-jun-N-terminal kinase (JNK) resulting in TNFα production [71]. It has been shown that chronic alcohol induces LPS-induced ERK1/2 activation and subsequent transcription of Egr-1, an immediate early gene transcription factor, which contributes to TNFα expression in murine hepatic macrophages [59,72]. Similarly, *in vitro* LPS stimulation of Kupffer cells exposed to chronic alcohol showed increased p38 activity whereas decreased JNK activity as observed in liver after chronic alcohol feeding [73]. Activation of p38 MAPK by LPS contributes to TNFα mRNA stability via interaction with tristetraprolin (TTP) [74]. Inhibition of p38 activation completely abrogated alcohol-mediated stabilization of TNFα mRNA [73]. On the other hand, ERK1/2 inhibition did not alter TNFα mRNA stability but affected its transcription in chronic alcohol-exposed macrophages [59]. LPS stimulation of JNK leads to phosphorylation of c-jun and subsequent binding of c-jun to CRE/AP-1 site in the TNFα promoter [71]. Although chronic alcohol feeding decreased JNK activity without any effect on TNFα mRNA, acute alcohol exposure increased JNK phosphorylation as well as AP-1 binding in the presence of combined TLR4 plus TLR2 stimulation [38] in human monocytes. Furthermore, LPS-induced ERK1/2 phosphorylation was decreased in acute *in vitro* alcohol-exposed monocytes [38], whereas p38 MAPK activity was increased contributing to anti-inflammatory mediators such as IL-10 after acute alcohol exposure in monocytes [75]. A single dose of *in vivo* alcohol exposure inhibited IL-6 and TNFα-induced by TLR2, TLR4 and TLR9 ligands due to impaired p38 and ERK 1/2 activation [76]. Thus, chronic and acute alcohol modulate MAP kinases differentially depending on the cell type and duration of alcohol exposure and ERK regulation appears to contribute to ALD (Fig. 3).

6. Cytokines and cytokine receptors in ALD

6.1. TNFα and pro-inflammatory cytokines

Among the pro-inflammatory cytokines produced during alcoholic liver injury, TNFα has been well characterized in animal models and human studies [4,77–80]. The most compelling evidence documenting the pivotal role of TNFα in alcohol-induced liver injury came from experiments that used anti-TNFα antibody to prevent liver injury in alcohol-fed rats [81,82] and the observation that mice lacking TNF type I receptor do not develop alcoholic liver injury [83]. The role of TNFα in human alcoholic hepatitis is evident from elevated serum levels of TNFα and increased immunohistochemical staining of TNFα in the liver [82,84,85]. Chronic alcohol not only increases TNFα in resident hepatic macrophages but also circulating monocyte/macro-

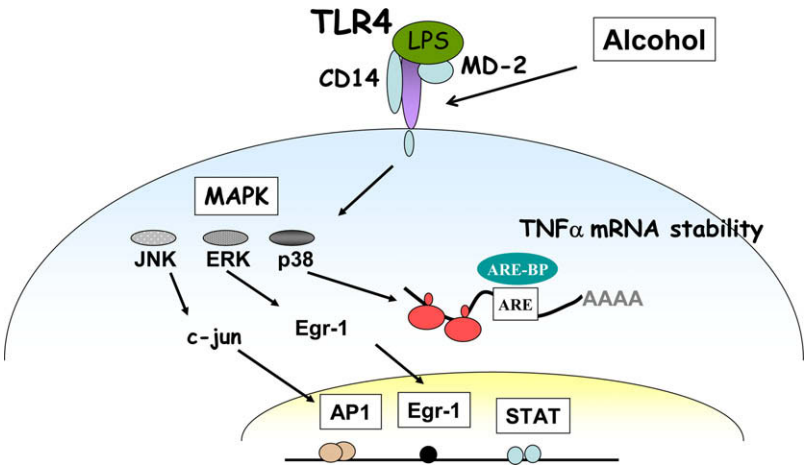

Fig. 3. TLR4 mediated alcohol-exposed macrophages signalling in alcohol-exposed macrophages. Alcohol alters functions of Toll-like receptors TLR4 in the liver resulting in activation of MAP kinases culminating in alteration of binding to transcription factors such as Egr-1, AP-1 and STAT1. Alcohol also affects cytokine mRNA stability via modulation of MAP kinase activity.

phages contributing to development and progression of liver disease. Recent studies have reported that higher bioactive TNFα and increased expression of Th1-type cytokines IL-6, IFN-γ and IL-12 was observed in the liver of alcohol of fed rats and contributes to alcohol-induced steatosis [85].

6.2. Anti-inflammatory cytokines and negative regulators

Resolution of inflammation is a hallmark to dampen the increased inflammatory gene expression and curb the inflammatory response. Various cytokines such as IL-10, prostaglandins, TGF-beta [86,87] and intracellular signalling molecules such as IRAK-M, ST2, PI3-K, SOCS1, A20 and SIGIRR [88] have been shown to contribute to the anti-inflammatory pathway. Increased injury in chronic alcohol fed IL-10 knockout mice indicate a role for IL-10 in alcohol-induced liver sensitization to LPS, [89] due to increase in pro-inflammatory cytokines. It is thus tempting to speculate that the anti-inflammatory cytokine, IL-10, could counter-regulate the sustained pro-inflammatory activation in the chronic alcoholic liver. However, increased expression of IL-10 by adiponectin, a adipokine with potent anti-inflammatory properties, suppressed TLR4-mediated signalling and TNFα production in Kupffer cells [90,91]. In contrast to chronic alcohol, acute alcohol exposure increases IL-10 and TGF-beta production in monocytes [92,93]. Acute alcohol-mediated augmentation of IL-10 was regulated by heme-oxygenase-1 (HO-1) via increase p38 MAP kinase activity in human monocytes [75]. Furthermore, it was shown that acute alcohol activates Src/STAT3, AP-1 and Sp-1 pathways to modulate IL-10 production in monocytes [57]. Immunoinhibitory molecules such as SOCS1 and SOCS3 were also upregulated in acute alcohol-exposed monocytes, resulting in modulation of cytokine-induced STAT1/3 signalling [67]. Additional studies on specific anti-inflammatory mechanisms will provide a better understanding of the contribution of negative regulation of inflammation to alcohol-induced liver injury.

7. Conclusion

In this review, we have discussed the signalling molecules affected during acute and chronic alcohol exposure of immune cells in the liver. Interactions of parenchymal and the non-parenchymal cells of the liver are mediated by inflammatory cytokines and alcohol-induced oxidative stress. Emerging evidence suggests a pivotal role for various signalling molecules including the Toll-like receptors, transcription factors such as NFκB, AP-1, Egr-1 and STATs. Studies so far indicate that a number of signalling pathways are involved in alcoholic liver disease. An integrative systems biology approach to characterize disease-specific pathways and their coordination within each cell type and between cells in the liver will provide a better understanding of the disease-phenotype and aid in drug discovery for treatment of alcoholic liver damage.

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