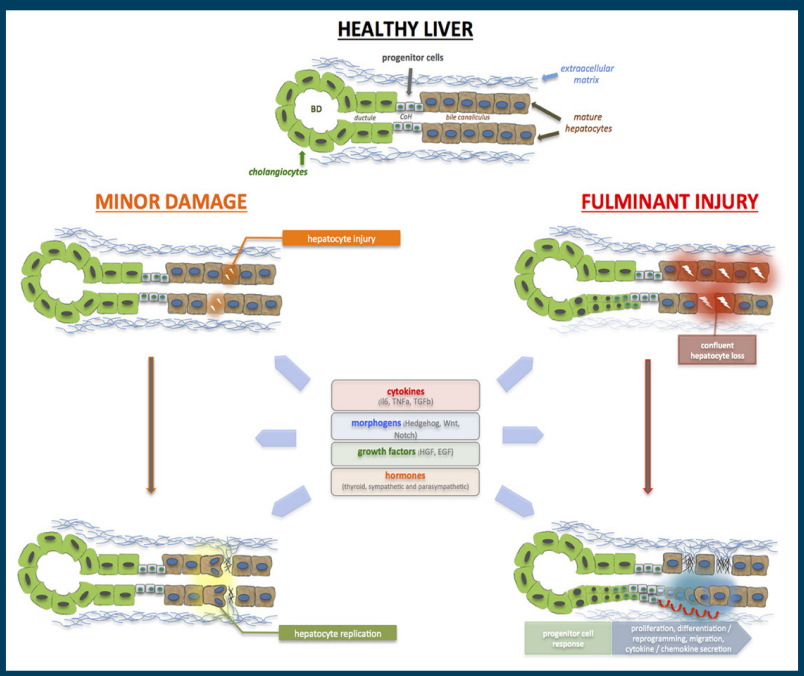
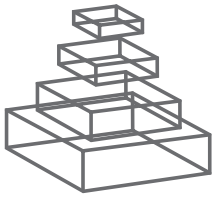
frontiers
RESEARCH TOPICS



MOLECULAR MECHANISMS AND THERAPEUTIC INTERVENTIONS IN ACUTE LIVER INJURY

Topic Editor
Ali Canbay

frontiers in PHYSIOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-237-3
DOI 10.3389/978-2-88919-237-3

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MOLECULAR MECHANISMS AND THERAPEUTIC INTERVENTIONS IN ACUTE LIVER INJURY

Topic Editor:
Ali Canbay, University Hospital Essen, Germany

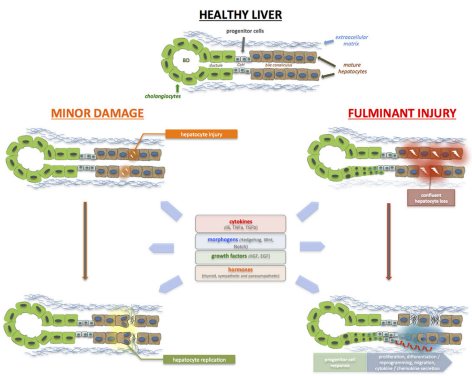


Figure taken from: Best J, Dollé L, Manka P, Coombes J, van Grunsven LA and Syn W-K (2013) Role of liver progenitors in acute liver injury. *Front. Physiol.* 4:258. doi: 10.3389/fphys.2013.00258

The owner of the image is Paul Manka

Acute liver injury is a rather rare but often fatal condition. While the mechanisms leading to liver injury for certain etiologies (drug induced liver injury, acetaminophen poisoning) have been elucidated in detail, it remains unclear, what causes a fatal course in many types of liver damage. Similarly, little is known about the mechanisms that account for the diversity of clinical outcomes in acute liver failure, which range from spontaneous recovery to death or the need for orthotopic liver transplantation. Contribution of different hepatocyte cell death mechanisms, immune-competent cell populations, other non-parenchymal cell types and the metabolic status influence the regeneratory capacity of the damaged liver. Thus, wound healing processes including endothelial to mesenchymal transdifferentiation are in the focus of translational studies. New approaches for the treatment of certain etiologies and of acute liver failure in general could be derived from molecular targets or mechanisms involved in these processes. In addition, improved prognosis of patient outcome or accelerated identification of the underlying etiology may be facilitated by more detailed knowledge of effectors and targets related to acute liver failure. With this special topic edition, expert researchers working on the frontiers of hepatology will be asked to share their most current insights on the pathogenesis, underlying mechanisms, prediction of clinical outcome and therapeutic management of acute liver injury. Expert reviews should include state-of-the-art aspects of liver replacement therapy, hepatocyte transplantation, endothelial-to-mesenchymal transdifferentiation and metabolic pathways involved in liver regeneration. Reviewers are encouraged to discuss translational research, including cell culture data, in vivo models of acute liver injury and liver regeneration as well as clinical data.

Table of Contents

frontiers in
PHYSIOLOGY

EDITORIAL
published: 30 January 2014
doi: 10.3389/fphys.2014.00018

From bedside to bench and back again—molecular mechanisms in acute liver failure

Jan-Peter Sowa, Guido Gerken and Ali Canbay*

Department of Gastroenterology and Hepatology, University Hospital Essen, University Duisburg-Essen, Essen, Germany
**Correspondence: ali.canbay@uni-due.de*

Edited by:
Stephen J. Pandol, University of California, Los Angeles, USA

Keywords: acute liver failure, cell death, ethiology, molecular mechanisms, therapeutic options

A major challenge for medical science is the ability to relate findings in cell cultures and animal models back to the patient. In the current setting of technological advance, we now have the ability to over-express or knockdown the expression of specific genes, and we are able to challenge cells with an array of putative factors, but are we able to translate these findings back to the patient? This collection of reviews (which includes unpublished data) aims to summarise our current understanding of acute liver failure (ALF). This has been a daunting task for authors, as ALF is a highly variable condition, whose outcome is determined by a variety of inter-related factors. As such, we are unable to present an exhaustive review of all putative cellular and molecular processes. Nevertheless, all authors have concisely and critically appraised the available literature on the different aspects of ALF, which include ALF pathogenesis, the role of immunity, the diagnostic and treatment strategies, and the role of prognostic algorithms in use today.

ALF can be caused by toxins, infections, metabolic and genetic diseases, but irrespective of etiology, ALF is characterized by the massive and confluent loss of functioning hepatocytes. In their review, Bantel and Schulze-Osthoff (2012) presented putative mechanisms of hepatocyte cell death, and discussed their relevance in patients with ALF. They propose that the degree of hepatocyte cell death may be a surrogate biomarker of ALF severity, and may be utilized as a predictor of ALF outcomes. This is supported findings of etiology dependent modes of hepatocyte cell death in ALF (Bechmann et al., 2010).

While the excessive consumption of alcohol is generally associated with the development of chronic liver disease, the acute intoxication of alcohol can also lead to ALF, or predispose an individual to ALF (from other etiology). It remains unclear how acute alcohol consumption could lead to ALF, although Massey and Arteel (2012) have presented novel data on the possible contributions by PAI-1, fibrins, and integrins.

The importance and role of miRNAs in the development or progression of ALF is only coming to fore. Recent studies show that miRNAs are differentially expressed in liver diseases, and may have a direct pathogenic role in ALF. Elfimova et al. (2012) presented an elegant review of currently identified miRNAs in liver disease, and highlighted the over-expression of miR-122 in patients with acute liver injury. It would be interesting to evaluate if miR-122 could serve as a prognostic biomarker in patients with ALF.

ALF is associated with a massive immune response, with recruitment of inflammatory cells from the peripheral circulation into the liver, the activation of stress and death receptors, and the clearance of apoptotic/necrotic debris, that lead to the perpetuation of hepatic inflammation and injury. In their review, Zimmermann et al. (2012) described the importance of resident macrophages (Kupffer cells) and recruited monocytes in the pathogenesis of ALF. When activated by danger signals, these cells secrete pro-inflammatory cytokines TNFa and upregulate expression of FasL, which in concert, enhance hepatocyte death. Activated immune cells, as well as dying hepatocytes and stromal cells are capable of secreting chemokines that lead to the further recruitment and retention of effector T and NK cells that amplify the inflammatory response. Saiman and Friedman (2012) discussed the role of putative chemokines in ALF, which remains to be fully elucidated as studies have demonstrated apparently conflicting results. This could be related to differences in underlying etiology, and/or to the role of other immune subsets such as regulatory T cells. The use of chemokine inhibitors in treating ALF is attractive, but remains out of reach of clinical applications at this stage, as we have yet to fully understand the roles of specific chemokines and/or immune subsets in the patient.

The liver exhibits a remarkable ability to regenerate itself after an acute insult. For example, after liver resection in man, or partial hepatectomy in rodents, the liver is capable of efficient regeneration that leads to the spontaneous restoration of liver mass and function. During fulminant ALF, however, the loss of hepatocyte mass is just too massive and outweighs the intrinsic ability of residual hepatocytes to regenerate sufficiently. As such, an alternative source of hepatocytes and cholangiocytes is necessary to help restore liver function. Best et al. (2013) reviewed the role of the liver progenitor cell population (LPC) during acute liver injury. They provided examples from preclinical models to show that the LPC is an active participant of liver regeneration, and discussed putative signaling pathways considered important for LPC responses. One such signaling pathway is the Hedgehog pathway that plays an important role tissue development, but has recently been reported to be critical for liver regeneration after partial hepatectomy in mice. Recent data from man confirm that the LPC population is expanded during ALF, and may be useful in predicting the outcome of ALF. Another cell type, which may be involved in ALF are hepatic stellate cell. These could also contribute to liver regeneration by alteration of extracellular matrix (Dechêne et al., 2010).

Whether LPC or stem cells (such as mesenchymal stem cells, MSC or hematopoietic stem cells) may be effective in treating ALF remains to be seen. Hepatocyte transplantation has been successful in rodents, while pre-differentiated MSC have been shown by Christ and Brückner (2012) to ameliorate acetaminophen-induced liver injury. MSC exhibits additional anti-inflammatory properties, thus, may lead to an attenuated inflammatory response. Chamulitrat et al. (2012) suggest a derivative of ursodeoxycholic acid as possible novel treatment option. For the majority of individuals with fulminant ALF, however, a liver transplant (LTx) remains the only curative option. Despite the availability of numerous scoring systems, they are generally poor at predicting survival (i.e., those who do not need a liver transplant). These difficult issues faced by transplant physicians are reviewed extensively by Hadem et al. (2012). Unknown reasons for ALF further complicate clinical handling. Drebber et al. (2013) identified hepatitis E virus as cause for some previously unclear ALF in cases in Europe. Thus, this neglected etiology should also be considered in Europe and not only in Asian or African countries.

In summary, ALF is a challenging disease with many etiologies and mechanisms of injury. A better understanding of the complex pathogenic mechanisms involved is necessary for us to identify new targets for therapy, and/or better predictors of outcome. Studies will be needed to dissect the contributions of individual cell types (recruited vs. resident; parenchymal vs. non-parenchymal) during ALF and regeneration. Future research should focus on novel treatment strategies, including the use of stem cells and LPCs.

REFERENCES

Bantel, H., and Schulze-Osthoff, K. (2012). Mechanisms of cell death in acute liver failure. *Front. Physiol.* 3:79. doi: 10.3389/fphys.2012.00079

Bechmann, L. P., Jochum, C., Kocabayoglu, P., Sowa, J. P., Kassalik, M., Gieseler, R. K., et al. (2010). Cytokeratin 18-based modification of the MELD score improves prediction of spontaneous survival after acute liver injury. *J. Hepatol.* 53, 639–647. doi: 10.1016/j.jhep.2010.04.029

Best, J., Dollé, L., Manka, P., Coombes, J., van Grunsven, L. A., and Syn, W. K. (2013). Role of liver progenitors in acute liver injury. *Front. Physiol.* 4:258. doi: 10.3389/fphys.2013.00258

Chamulitrat, W., Zhang, W., Xu, W., Pathil, A., Setchell, K., and Stremmel, W. (2012). Hepatoprotectant ursodeoxycholyl lysophosphatidylethanolamide increasing phosphatidylcholine levels as a potential therapy of acute liver injury. *Front. Physiol.* 3:24. doi: 10.3389/fphys.2012.00024

Christ, B., and Brückner, S. (2012). Rodent animal models for surrogate analysis of cell therapy in acute liver failure. *Front. Physiol.* 3:78. doi: 10.3389/fphys.2012.00078

Dechêne, A., Sowa, J. P., Gieseler, R. K., Jochum, C., Bechmann, L. P., El Fouly, A., et al. (2010). Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 52, 1008–1016. doi: 10.1002/hep.23754

Drebber, U., Odenthal, M., Aberle, S. W., Winkel, N., Wedemeyer, I., Hemberger, J., et al. (2013). Hepatitis E in liver biopsies from patients with acute hepatitis of clinically unexplained origin. *Front. Physiol.* 4:351. doi: 10.3389/fphys.2013.00351

Elfimova, N., Schlattjan, M., Sowa, J.-P., Dienes, H. P., Canbay, A., and Odenthal, M. (2012). Circulating microRNAs: promising candidates serving as novel biomarkers of acute hepatitis. *Front. Physiol.* 3:476. doi: 10.3389/fphys.2012.00476

Hadem, J., Strassburg, C. P., and Manns, M. P. (2012). Prediction of outcome and selection of the liver transplantat candidate in acute liver failure. *Front. Physiol.* 3:340. doi: 10.3389/fphys.2012.00340

Massey, V. L., and Arteel, G. E. (2012). Acute alcohol-induced liver injury. *Front. Physiol.* 3:193. doi: 10.3389/fphys.2012.00193

Saiman, Y., and Friedman, S. L. (2012). The role of chemokines in acute liver injury. *Front. Physiol.* 3:213. doi: 10.3389/fphys.2012.00213

Zimmermann, H. W., Trautwein, C., and Tacke, F. (2012). Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Front. Physiol.* 3:56. doi: 10.3389/fphys.2012.00056

Received: 23 August 2013; accepted: 09 January 2014; published online: 30 January 2014.

Citation: Sowa J-P, Gerken G and Canbay A (2014) From bedside to bench and back again—molecular mechanisms in acute liver failure. Front. Physiol. ***5****:18. doi: 10.3389/fphys.2014.00018*

This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 02 April 2012
doi: 10.3389/fphys.2012.00079

Mechanisms of cell death in acute liver failure

Heike Bantel*[1] *and* ***Klaus Schulze-Osthoff*[2]**

[1] *Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany*
[2] *Interfaculty Institute for Biochemistry, Eberhard Karls University Tübingen, Tübingen, Germany*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Eileen F. Grady, University of California at San Francisco, USA
Claus Hellerbrand, University of Regensburg, Germany
Hermann Wasmuth, University Hospital Aachen, Germany

****Correspondence:***
Heike Bantel, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany.
e-mail: Bantel.Heike@mh-hannover.de

Acute liver failure (ALF) can be the consequence of various etiologies, that might vary between different geographic regions. Most frequent are intoxications with acetaminophen, viral hepatitis, or liver damage of unknown origin. ALF occurs when the extent of hepatocyte death exceeds the regenerative capacity of the liver. The mode of liver cell death that is predominantly induced in ALF, i.e., apoptosis or necrosis, is still controversial and presumably determined by the etiology, duration, and magnitude of liver injury. Severe liver damage involves oxidative stress and depletion of ATP resulting in necrosis. In contrast, maintenance of ATP stores is required for the execution of apoptosis. Recent data suggest that necrosis resulting from severe liver damage is associated with poor outcome of ALF patients. Discrimination between apoptosis and necrosis might be therefore useful for the identification of ALF patients requiring liver transplantation. Identification of the molecular cell death mechanisms remains an important issue not only for early prediction of ALF outcome, but also for therapeutic interventions. In view of the pleiotropic functions of critical mediators of cell death and tissue regeneration, a particular challenge will be to reduce hepatocellular death without inhibiting the regenerative capacity of the liver. Here, we review the molecular mechanisms of hepatocyte injury and the pathways leading to apoptosis and necrosis, which might represent potential diagnostic and therapeutic targets in ALF.

Keywords: acute liver failure, apoptosis, ATP, caspases, death receptors, necrosis, oxidative stress, tumor necrosis factor

INTRODUCTION

Acute liver failure (ALF) can occur as a result of various etiologies including hepatic injury by drugs and poison, viral hepatitis, ischemia, or other causes. The mechanisms by which liver cells are destroyed as well as the processes mediating liver regeneration, remain largely unknown. It has become evident that liver cell death can occur via distinct biochemical pathways and morphological alterations, including apoptosis, autophagic cell death, and necrosis. Apoptosis is defined by chromatin condensation, nuclear fragmentation, cell shrinkage, blebbing of the plasma membrane, and formation of apoptotic bodies that contain nuclear or cytoplasmic material (Kerr et al., 1972; Russell et al., 1972). Autophagic cell death, on the other hand, is characterized by a massive accumulation of double-membrane containing vacuoles, called autophagosomes, that subsequently fuse with lysosomes. Necrotic cell death is often negatively defined as a form of cell death that lacks signs of apoptosis or autophagy. Typically, necrotic cells show cytoplasmic swelling, dilation of organelles, and mechanical rupture of the plasma membrane. Although necrosis has been deemed to be a mainly passive process, the initiation, and modulation of necrotic cell death are currently under intense investigation at the molecular level.

The relative contribution of apoptosis or necrosis to organ dysfunction in ALF remains controversial (Schulze-Osthoff and Bantel, 2011). Necrosis is typically the consequence of acute metabolic perturbation with ATP depletion, whereas apoptosis represents an ATP-dependent cell death program. Furthermore, in several cases, the nature and duration of cellular injury determine if cells die by apoptosis, necrosis, or other mechanisms. At low doses, a variety of injurious stimuli often induce apoptosis, but the same stimuli can result in necrosis at higher doses. Therefore, in many situations cell death might be not executed as a clear-cut form of cell death, but as a continuum with intermediate features of both apoptosis and necrosis. A distinction of different cell death forms is therefore not only relevant for semantical reasons, but has important clinical implications when considering the therapeutic targeting of cell death processes. Thus, an understanding of the cell death processes is most important for development of effective interventions to prevent hepatocellular death in acute liver damage (Fischer and Schulze-Osthoff, 2005).

DEATH RECEPTOR SIGNALING IN ACUTE LIVER FAILURE

Apoptosis represents a programmed form of cell death that is required for the maintenance of tissue homeostasis by counterbalancing cell proliferation and eliminating damaged, infected, or transformed cells. This process is particularly important in the liver as an organ that is naturally exposed to toxins, drugs, and viruses. However, excessive apoptosis can result in tissue destruction and organ failure.

Apoptosis results from a collapse of cellular infrastructure through internal proteolytic digestion, which leads to cytoskeletal disintegration, metabolic derangement, and genomic fragmentation. Members of the caspase family of proteases form the core engine of apoptosis and are involved in initiation, execution,

and regulatory phases of the pathway. Caspases are cysteine proteases that cleave substrates after aspartate residues within specific peptide recognition sequences. To preclude unwarranted cell death, caspases are expressed as inactive zymogens consisting of a prodomain followed by two subunits with the catalytic domain. Caspases operate in hierarchical cascades that serve to amplify the apoptotic signal (Los et al., 1999). Based on their structure and order in cell death pathways, caspases can be divided into upstream initiators and downstream effectors of apoptosis. Effector caspases such as caspase-3, -6, and -7 cleave diverse cellular substrates including structural proteins such as cytokeratin-18 and many others (Fischer et al., 1998; Leers et al., 1999; Bantel et al., 2000, 2001a). In contrast, initiator caspases, such as caspase-8, -9, and -10, exert regulatory roles by activating downstream effector caspases.

Caspases are activated by two major signaling routes, namely the extrinsic death receptor and the intrinsic mitochondrial pathway, that both depend on the formation of large multi-protein complexes (Schulze-Osthoff et al., 1998; Yoon and Gores, 2002). Initiator caspase-8 is the key mediator of the extrinsic pathway. In a simplified model, binding of death ligands such as TRAIL or CD95L or tumor necrosis factor (TNF)-α to their respective death receptors leads to receptor oligomerization. This event then results in the recruitment of the adapter protein FADD and the initiator caspase-8 into a death-inducing signaling complex (DISC), wherein caspase-8 becomes activated by dimerization and autoproteolytic cleavage (**Figure 1**). Subsequently, caspase-8 cleaves and activates the effector caspase-3, culminating in the demise of so-called type I cells. In most cells including hepatocytes, however, only low amounts of initiator caspases are activated at the DISC, which is insufficient for cell death. In those type II cells, the extrinsic receptor pathway must be amplified by the intrinsic mitochondrial apoptotic pathway through the caspase-8-mediated cleavage of Bid, a pro-apoptotic Bcl-2 family protein, which subsequently initiates together with the Bcl-2 family members Bak and Bax the release of mitochondrial pro-apoptotic mediators (Schwerk and Schulze-Osthoff, 2005). Interestingly, CD95-induced hepatocyte apoptosis is delayed but not inhibited in Bak/Bax-deficient mice indicating that hepatocytes can act as type I cells in the absence of pro-apoptotic Bcl-2 proteins (Hikita et al., 2011).

Inappropriate activation of death receptors might lead to ALF. This has been impressively demonstrated in mice that died rapidly

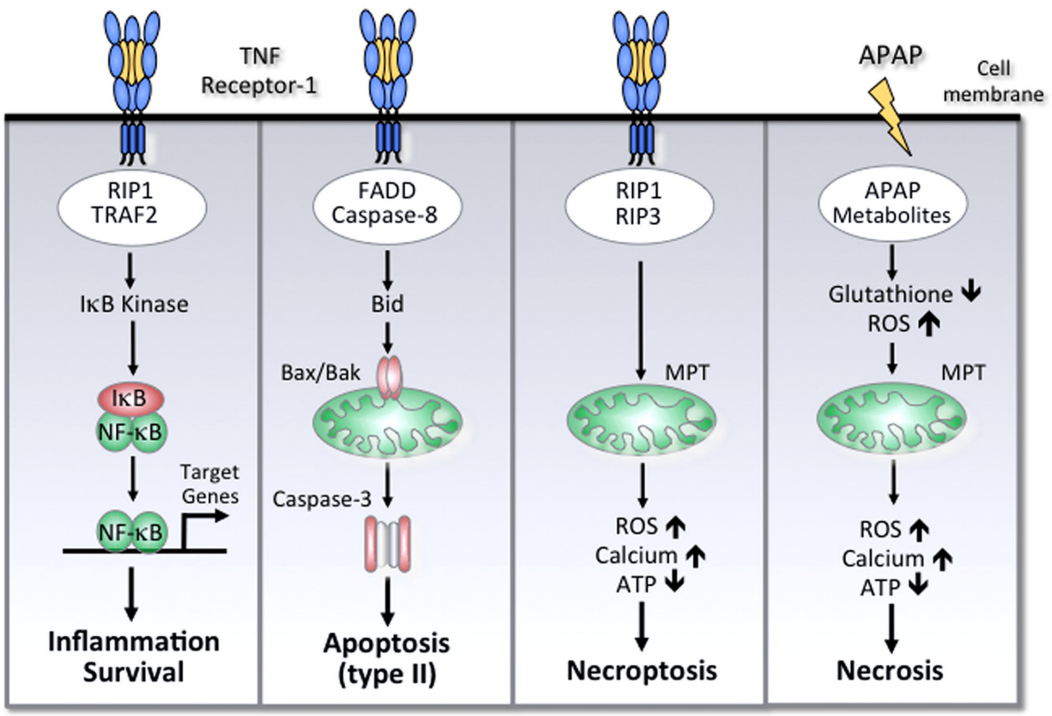


FIGURE 1 | Simplified scheme of cell death and survival pathways involved in ALF. Activation of TNF receptor-1 can mediate NF-κB activation, apoptosis, or necroptosis. The different outcomes are determined by distinct TNF receptor-associated signaling complexes. Activation of NF-κB is mediated by TRAF-2, RIP-1, and other signaling molecules that lead to activation of IκB kinase and subsequent activation of NF-κB target genes. FADD and caspase-8 are the essential adapter proteins involved in apoptosis, which in hepatocytes requires a mitochondrial amplification loop through caspase-8-mediated cleavage of Bid. The subsequent translocation of Bax and Bak results in mitochondrial outer membrane permeabilization, cytochrome *c* release, and effector caspase-3 activation. Under conditions of impaired apoptosis, TNF receptor-1 can induce necroptosis, which involves RIP-1 and RIP-3 kinases. Among other effects, RIP-3 can increase the production of reactive oxygen species (ROS) due to increased oxidative phosphorylation, resulting in intracellular calcium overload, mitochondrial membrane permeability transition (MPT), depletion of ATP, and necrosis. APAP-induced necrosis is essentially mediated by a toxic metabolite, which depletes glutathione and forms APAP protein adducts, triggering oxidative stress, compromised respiratory function, and ATP depletion. Although APAP treatment can instigate the mitochondrial pathway of apoptosis, high doses of APAP will ultimately mediate liver cell death by necrosis.

of liver failure with massive hepatocyte apoptosis when agonistic anti-CD95 antibody was injected (Ogasawara et al., 1993). Similarly, treatment of mice with TNF-α in combination with a transcription-blocking agent, such as D-galactosamine (D-GalN) or actinomycin D, induces lethal hepatitis (Leist et al., 1994, 1995; Libert et al., 1994). Another well established mouse model of ALF consists of a combined treatment with D-GalN and lipopolysaccharide (LPS), which induces TNF-α expression and an inflammatory response that is predominantly directed toward the liver (Galanos et al., 1979).

It has been suggested that the toxicity in the murine TNF-α and anti-CD95 models resembles viral forms of acute hepatic failure in patients (Keppler et al., 1968; El-Mofty et al., 1975). CD95/CD95L expression has been shown to be upregulated in viral hepatitis and to correlate with disease activity and hepatocyte apoptosis (Hiramatsu et al., 1994; Mita et al., 1994; Pianko et al., 2001; Lee et al., 2004). We have recently demonstrated that diseased, e.g., HCV-infected, livers show an upregulation of TRAIL receptors and increased susceptibility toward TRAIL-induced apoptosis (Volkmann et al., 2007). These data implicate that viral forms of ALF are associated with death receptor-induced cell death. Recent data show that the CD95 system is involved in human ALF caused not only by viral hepatitis but also by Wilson's disease (Strand et al., 1998; Rivero et al., 2002). Increased levels of death ligands or receptors such as CD95L, TNF-α, or TNF receptors (TNF-R) were found in blood of patients with ALF (Ryo et al., 2000; Streetz et al., 2000; Tokushige et al., 2000; Nakae et al., 2001; Volkmann et al., 2008). Particularly, high serum levels of soluble death receptor CD95 have also been found in drug-induced ALF (Tagami et al., 2003; Rutherford et al., 2007).

Silencing of CD95 or caspase-8 protected mice from ALF or fulminant hepatitis induced by agonistic CD95 antibody or concanavalin A, respectively (Song et al., 2003; Zender et al., 2003). However, CD95 and caspase-8 also promotes liver regeneration by inducing differentiation of stellate cells and possibly of other non-parenchymal liver cells (Desbarats and Newell, 2000; Canbay et al., 2003; Ben Moshe et al., 2007). Additionally, TNF-α plays a pivotal role in liver regeneration by activation of transcription factors such as NF-κB, which induces the transcription of a huge number of cytokines and growth-promoting target genes (Wullaert et al., 2007). The activation of NF-κB by TNF-α is mediated by distinct adapter proteins that are recruited to TNF-R-1 upon ligand binding (**Figure 1**). Whether increased levels of circulating death receptors or ligands in human ALF mirror apoptotic cell death or liver regeneration and whether death receptor-induced cell death depends on special ALF etiologies remains unknown.

ROLE OF MITOCHONDRIAL DAMAGE IN ACUTE LIVER FAILURE

In contrast to viral infection, drug-induced liver injury is mainly associated with signaling pathways triggered by mitochondrial damage (Chan et al., 2005). In the intrinsic pathway, apoptosis is mediated by translocation of pro-apoptotic Bcl-2 molecules, such as Bax and Bak, from the cytosol to mitochondria to form pores in the outer mitochondrial membrane (Los et al., 1999). This process is followed by the mitochondrial release of cytochrome *c* and other pro-apoptotic factors. Cytochrome *c* normally functions in electron transport processes of the respiratory chain to generate ATP. In the cytosol of apoptotic cells, however, it serves as a cofactor for the adapter protein Apaf-1. Upon binding of cytochrome *c* and dATP, Apaf-1 oligomerizes, and recruits the initiator caspase-9 to trigger the formation of the apoptosome. Thus, similar to the DISC, the apoptosome is a high-molecular weight complex that serves as a caspase activation platform. Once assembled in the apoptosome, caspase-9 becomes activated and subsequently triggers the caspase cascade (Schulze-Osthoff et al., 1998). As mentioned above, there is also considerable crosstalk between the extrinsic and intrinsic pathways. For example, during death receptor-mediated apoptosis caspase-8 can proteolytically activate the Bcl-2 protein Bid, which facilitates cytochrome *c* release and amplifies the apoptotic signal following death receptor activation.

In contrast to apoptosis, necrosis is mediated by opening of the mitochondrial membrane permeability transition (MPT) pore, which triggers the collapse of the membrane potential and cessation of ATP formation. The resulting mitochondrial swelling leads to the rupture of the outer mitochondrial membrane with the release of intermembrane proteins and subsequent nuclear DNA fragmentation. Other prominent features include massive energy depletion, formation of reactive oxygen species (ROS), and activation of non-apoptotic proteases. Furthermore, during necrosis a strong increase of intracellular calcium is observed. The elevated calcium levels in the cytosol trigger mitochondrial calcium overload, leading to depolarization of the inner mitochondrial membrane and a shut-down of ATP production. While depletion of ATP impedes the function of membrane channels, increased calcium activates calcium-dependent proteases, such as calpains. Calcium fluxes, ATP depletion, and oxidative stress involve complex and interactive feedback loops, which self-amplify and potentiate each other leading to exaggerated cell death. The relative amount of ATP might be an important factor that determines whether hepatocytes die by apoptosis or necrosis (Ferrari et al., 1998; Hinson et al., 2010). Another important distinguishing feature of apoptotic versus necrotic cell death relates to inflammation. When the necrotic cell ruptures, an inflammatory response follows due to the release of intracellular contents. In contrast, inflammation is not typical of apoptosis, because phagocytic cells rapidly engulf apoptotic cells and thereby prevent the release of noxious intracellular compounds.

CELL DEATH MECHANISMS INVOLVED IN ACETAMINOPHEN-INDUCED ACUTE LIVER FAILURE

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol; APAP) overdose represents one of the most common causes of ALF in developed countries (Larson et al., 2005). APAP-induced hepatotoxicity is due to the formation of the toxic metabolite *N*-acetyl-*p*-benzoquinone imine by the cytochrome P450 system, which causes glutathione depletion, oxidative stress, alterations of calcium homeostasis, resulting in MPT, loss of mitochondrial membrane potential, and ATP depletion (Hinson et al., 2010). Although necrosis has been thought to be the predominant mode of cell death in APAP-induced liver injury, conflicting *in vitro* and animal data have emerged suggesting a potential role of apoptosis in acetaminophen-induced hepatotoxicity (El-Hassan et al., 2003; Kon et al., 2004). Mice treated with a toxic dose of acetaminophen

showed 40% apoptotic and 60% necrotic hepatocytes (Ray et al., 1996). It was demonstrated that mice with defective CD95 receptor (lpr mice) or CD95 ligand (gld mice) were partially protected from APAP-induced liver injury (Liu et al., 2004). Moreover, increased circulating levels of CD95 have been found in humans with APAP intoxication (Tagami et al., 2003).

It has been recently suggested that APAP hepatotoxicity is caused by the mitochondrial apoptosis pathway and facilitated by chemokine (C-X-C motif) receptor 2 (CXCR2) signaling. In this study caspase inhibition prevented DNA fragmentation, although the authors did not investigate whether caspase inhibition was also associated with cell survival (Hu and Colletti, 2010; Schulze-Osthoff and Bantel, 2011). In contrast, in another study no caspase activation was observed and, accordingly, caspase inhibition did not protect from liver injury in APAP-treated mice (Jaeschke et al., 2006). Further data from animal models revealed also no evidence that apoptotic cell death contributes to APAP-induced liver injury. For instance, following the application of an APAP overdose in mice, less than 1% of the parenchymal cells revealed an apoptotic morphology (Gujral et al., 2002). Another study in mice showed that knockdown of the CD95 receptor protected against 300 mg/kg APAP overdose but not against 700 mg/kg overdose (Zhang et al., 2000), indicating that the mode of cell death might at least partially depend on the APAP dose. Indeed, several studies demonstrated that APAP induces mitochondrial dysfunction with ATP depletion which even interrupts initial CD95-induced mitochondrial signaling pathways (Lawson et al., 1999; Knight and Jaeschke, 2002). On the other hand, subliminal CD95 activation can also increase APAP-induced liver injury (Tinel et al., 2004), indicating that death receptor signaling might influence the extent of APAP-induced necrotic liver injury.

Conflicting data exist also about the role of TNF-α in APAP-induced liver injury. Increased TNF-α expression in liver and circulating TNF-α levels have been observed after APAP poisoning (Blazka et al., 1995, 1996). However, the role of TNF-α in APAP-induced necrosis remains controversial, as TNF-α inhibitors exerted either protection or no effect (Blazka et al., 1995; Simpson et al., 2000). In addition, TNF-α knockout mice showed similar sensitivity to acetaminophen compared to wildtype mice (Boess et al., 1998). It has been recently shown that inhibition of c-jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase family, reduced paracetamol-induced toxicity in mice by inhibiting hepatic TNF-α production (Henderson et al., 2007). Furthermore, APAP-induced JNK activation has been linked to activation of the pro-apoptotic Bcl-2 protein Bim. In line, APAP-induced necrotic liver injury was shown to be reduced in Bim knockout mice (Badmann et al., 2011). However, neither JNK1 nor JNK2 knockdown did protect mice from APAP-induced liver toxicity, raising concerns about a major role of JNK in APAP-induced liver injury (Gunawan et al., 2006; Henderson et al., 2007; Bourdi et al., 2008). Altogether, although apoptotic alterations can occur, profound energy depletion, and mitochondrial failure presumably divert cell death to necrosis as the principal mode of APAP-induced liver toxicity.

Autophagy represents another process that might influence the outcome of APAP-induced liver toxicity. Autophagy is a catabolic mechanism by which long-lived proteins and organelles are recycled in order to maintain energy and protein synthesis. It is characterized by the appearance of numerous cytosolic vacuole-like structures, called autophagosomes, which encapsulate cytosolic materials and fuse with lysosomes. Although the role of the autophagy in protection during nutrient starvation is accepted, its function in programmed cell death remains controversial. Under normal physiological conditions autophagy occurs at low basal levels, contributing to the turnover of cytoplasmic components and promoting cell survival during stress conditions. Excess autophagy, on the other hand, leads to autophagic cell death. Interestingly, it has been recently demonstrated that APAP induces autophagy in mouse liver and primary human hepatocytes and that activation of autophagy protects against APAP-induced hepatotoxicity (Ni et al., 2012). In this study it was suggested that the induction of oxidative stress might play an important role in APAP-induced autophagy. Moreover, it was shown that pharmacological inhibition of autophagy increased APAP-induced liver injury. Clearly, more work is needed to elucidate the role of autophagy in APAP-induced hepatotoxicity and the complex crosstalk with other cell death pathways.

CELL DEATH BIOMARKERS FOR MONITORING ACUTE LIVER FAILURE

In addition to measuring death ligands, several other cell death biomarkers have been proposed for monitoring the clinical outcome of ALF and other liver diseases (Volkmann et al., 2006; Rutherford et al., 2007; Bechmann et al., 2008; Joka et al., 2012). Caspases cleave the intermediate filament protein cytokeratin (CK)-18 into specific fragments that are released into circulating blood and can be detected by the M30 ELISA (Bantel et al., 2001b, 2004; Seidel et al., 2005). Moreover, when this assay is combined with a second ELISA that detects the total release of caspase-cleaved and uncleaved CK-18 (M65 ELISA), even different forms of cell death, such as necrosis and apoptosis, can be discriminated. Using these serological assays, it has been recently demonstrated that the predictive sensitivity of total CK-18 for lethal outcome was comparable to the model for end-stage liver disease (MELD) score at time of admission of ALF patients (Bechmann et al., 2010). Moreover, modification of the MELD score by substitution of bilirubin for total CK-18 significantly increased the prediction of ALF outcome.

Interestingly, we have demonstrated that ALF patients display considerable caspase activity and high levels of caspase-cleaved CK-18 in the serum, which was unexpectedly higher in spontaneous survivors than in patients that required transplantation or died (Volkmann et al., 2008). Nevertheless, despite a weaker activation of caspases, liver biopsies of patients without spontaneous recovery revealed extensive TUNEL reactivity, which detects both apoptotic and necrotic cell death. Moreover, sera from those patients contained increased levels of total CK-18, but reduced levels of its caspase-generated fragments as compared to patients with spontaneous recovery. These findings therefore indicate that necrosis but not apoptosis is the predominant cell death in those critically ill ALF patients, whereas in ALF patients with spontaneous recovery apoptotic cell death predominates. In contrast, in another study, detection of caspase-cleaved CK-18 could not adequately predict ALF outcome (Rutherford et al., 2007).

However, unlike in our study in which APAP-induced ALF only played a minor role, in this study a relevant number of patients with APAP-induced ALF was included. As mentioned above, in those patients necrotic cell death might predominate irrespective of the outcome. In line with this observation, patients with APAP-induced ALF showed higher levels of total CK-18 compared to caspase-cleaved CK-18 levels (Bechmann et al., 2008; Volkmann et al., 2008; Craig et al., 2011; Bantel and Schulze-Osthoff, 2012).

Caspase activation might also play a role in liver regeneration and this might explain our observation of higher caspase activity in spontaneous survivors. Caspases can cleave different cytokine precursors to generate active cytokines and thus create an environment that could be essential for liver regeneration. Experiments in mice showed that caspase activation is associated with chemokine production and inflammation in the liver, whereas caspase-3 inhibition strongly reduced activity of pro-inflammatory transcription factors and chemokines (Faouzi et al., 2001). A role of caspases in liver regeneration is best exemplified by a recent report showing that a hepatocyte-specific knockout of caspase-8 attenuates hepatocyte proliferation after partial hepatectomy (Ben Moshe et al., 2007).

In addition to cell death biomarkers, circulating levels of nucleosomes and high-mobility group box 1 (HMBG1) protein, both of which are released during hepatocyte death, have been shown to be elevated in patients with ALF (Roth et al., 2009; Craig et al., 2011). However, those biomarkers were not able to predict ALF outcome, and no significant differences in HMBG1 or nucleosome levels between paracetamol- and non-paracetamol-induced liver injury were found (Craig et al., 2011). One explanation for the lacking correlation of those biomarkers with ALF outcome might be that the release of HMBG1 and nucleosomes are not strictly related to necrotic cell death, but also occur during apoptosis (Bell et al., 2006; Jaeschke et al., 2012). Certainly, further larger cohort studies are required to evaluate the predictive value of cell death markers in patients with paracetamol- or non-paracetamol-induced ALF.

POSSIBLE THERAPEUTIC STRATEGIES FOR CELL DEATH INHIBITION IN ACUTE LIVER FAILURE

An admixture of necrosis and apoptosis occurs in ALF and might therefore open up novel strategies for therapeutic intervention (Fischer and Schulze-Osthoff, 2005). More extreme injury leads to necrotic killing, whereas milder injury may result in apoptosis. Whether inhibition of apoptosis using available pharmacological caspase inhibitors can indeed prevent liver cell death or will just simply shift the mode of cell death to necrosis, remains to be shown (Los et al., 2002). As caspases not only play a role in apoptosis but also in processes of liver regeneration, the possibility of adverse effects should not be ignored when considering the therapeutic use of caspase inhibitors in ALF treatment. Death receptor-blocking agents, such as CD95-Fc or TRAIL-R-Fc proteins, might be promising candidates in virus-induced ALF (Bantel and Schulze-Osthoff, 2003). MicroRNAs (miRNAs) are non-coding RNAs which have been implicated in the posttranscriptional regulation of various cellular pathways. It was recently demonstrated that overexpression of miRNA-221 delays CD95-induced fulminant liver failure in mice (Sharma et al., 2011). Thus, miRNAs involved in cell death signaling pathways might serve as potential therapeutic targets in ALF.

Due to the pleiotropic effects of TNF-α, inhibitors of this cytokine might not only influence liver cell death but also immune response and liver regeneration. Treatment of APAP intoxicated mice with anti-TNF-α partially prevented hepatotoxicity (Blazka et al., 1995). Other studies showed no alterations of APAP toxicity in mice treated either with anti-TNF-α antibody or soluble TNF receptor (Simpson et al., 2000). Whether the observed discrepancy is due to the variant experimental conditions, such as different mouse strains, remains to be elucidated (Jaeschke et al., 2012). There is increasing evidence suggesting that TNF-α triggers cell death not only by apoptosis, but also by a necrosis-like process, which has been recently called necroptosis (Schulze-Osthoff et al., 1994; Vandenabeele et al., 2010). Whereas TNF-α-induced apoptosis involves caspase-8, TNF-α-induced necroptosis is essentially mediated by the kinases RIP-1 and RIP-3 that are recruited to TNF-R1 (**Figure 1**). Interestingly, specific inhibitors of RIP-1, called necrostatins, have been recently designed as novel cytoprotective agents that might be a promising tool to positively influence ALF outcome, at least in conditions in which death receptor-mediated necrosis predominates.

Therapeutic targets to improve APAP-induced ALF outcome might be JNK or other members of the mitogen-activated protein kinase family, such as p38. JNK inhibition by SP600125, a small-molecule reversible ATP-competitive inhibitor, or by D-JNKII, a peptide inhibitor that inhibits the interaction of JNK with substrates, markedly reduced mortality in murine paracetamol-induced hepatotoxicity, with a significant reduction of hepatic apoptosis and necrosis (Bennett et al., 2001; Borsello et al., 2003; Henderson et al., 2007). Since glutathione depletion and subsequent oxidative stress formation are key pathogenic mechanisms of APAP-induced hepatocyte death, application of *N*-acetylcysteine, a pro-drug for glutathione synthesis, which has been shown to reduce APAP-induced liver necrosis, is the current standard therapy in APAP-induced ALF (Corcoran et al., 1985; Saito et al., 2010). In addition, other drugs with anti-oxidant effects might improve ALF outcome. In this respect, application of cyclooxygenase inhibitors exerted protective effects in an experimental ALF mouse model with decreased oxidative stress formation and marked reduction of hepatic necrosis (Chang et al., 2011; Liong et al., 2012). A novel therapeutic target in ALF that connects the immune system with cell death might be cyclophilin A. This intracellular protein exerts pro-inflammatory and hepatotoxic activity when released from necrotic liver cells in APAP-induced liver injury. Conversely, inhibition of cyclophilin reduced inflammatory response to necrotic liver (Dear et al., 2011). Thus, targeting of cyclophilin might represent an opportunity for a novel therapeutic approach in acetaminophen poisoning. As mentioned above, pharmacological inhibition of autophagy exacerbated APAP-induced liver toxicity. Vice versa, induction of autophagy by rapamycin was shown to inhibit APAP-induced hepatotoxicity (Ni et al., 2012). Thus, autophagy induction might be a further strategy to improve ALF outcome in APAP intoxicated patients.

Taken together, there are many promising therapeutic approaches for inhibition of APAP- and non-APAP-induced ALF. However, it should be kept in mind that most of the described targets exert a pleiotropic role and might interfere not only with cell death but also with survival pathways.

REFERENCES

Badmann, A., Keough, A., Kaufmann, T., Bouillet, P., Brunner, T., and Corazza, N. (2011). Role of TRAIL and pro-apoptotic Bcl2 homolog Bim in acetaminophen-induced liver damage. *Cell Death Dis.* 2, e171.

Bantel, H., Lügering, A., Heidemann, J., Volkmann, X., Poremba, C., Strassburg, C. P., Manns, M. P., and Schulze-Osthoff, K. (2004). Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. *Hepatology* 40, 1078–1087.

Bantel, H., Lügering, A., Poremba, C., Lügering, N., Held, J., Domschke, W., and Schulze-Osthoff, K. (2001a). Caspase activation correlates with the degree of inflammatory liver injury in chronic hepatitis C virus infection. *Hepatology* 34, 758–767.

Bantel, H., Ruck, P., Gregor, M., and Schulze-Osthoff, K. (2001b). Detection of elevated caspase activation and early apoptosis in liver diseases. *Eur. J. Cell Biol.* 80, 230–239.

Bantel, H., Ruck, P., and Schulze-Osthoff, K. (2000). In situ monitoring of caspase activation in hepatobiliary diseases. *Cell Death Differ.* 7, 504–505.

Bantel, H., and Schulze-Osthoff, K. (2003). Apoptosis in hepatitis C virus infection. *Cell Death Differ.* 10, 48–58.

Bantel, H., and Schulze-Osthoff, K. (2012). Can cell death biomarkers predict the outcome of acute liver failure? *Liver Int.* 32, 346.

Bechmann, L. P., Jochum, C., Kocabayoglu, P., Sowa, J. P., Kassalik, M., Gieseler, R. K., Saner, F., Paul, A., Trautwein, C., Gerken, G., and Canbay, A. (2010). Cytokeratin 18-based modification of the MELD score improves prediction of spontaneous survival after acute liver injury. *J. Hepatol.* 53, 639–647.

Bechmann, L. P., Marquitan, G., Jochum, C., Saner, F., Gerken, G., and Canbay, A. (2008). Apoptosis versus necrosis rate as a predictor in acute liver failure following acetaminophen intoxication compared with acute-on-chronic liver failure. *Liver Int.* 28, 713–716.

Bell, C. W., Jiang, W., Reich, C. F. III, and Pisetsky, D. S. (2006). The extracellular release of HMGB1 during apoptotic cell death. *Am. J. Physiol. Cell Physiol.* 291, 1318–1325.

Ben Moshe, T., Barash, H., Kang, T. B., Kim, J. C., Kovalenko, A., Gross, E., Schuchmann, M., Abramovitch, R., Galun, E., and Wallach, D. (2007). Role of caspase-8 in hepatocyte response to infection and injury in mice. *Hepatology* 45, 1014–1024.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13681–11386.

Blazka, M. E., Elwell, M. R., Holladay, S. D., Wilson, R. E., and Luster, M. I. (1996). Histopathology of acetaminophen-induced liver changes: role interleukin 1 alpha and tumor necrosis factor alpha. *Toxicol. Pathol.* 24, 181–189.

Blazka, M. E., Wilmer, J. L., Holladay, S. D., Wilson, R. E., and Luster, M. I. (1995). Role of proinflammatory cytokines in acetaminophen hepatotoxicity. *Toxicol. Appl. Pharmacol.* 133, 43–52.

Boess, F., Bopst, M., Althaus, R., Polsky, S., Cohen, S. D., Euster, H. P., and Boelsterli, U. A. (1998). Acetaminophen hepatotoxicity in tumor necrosis factor/lymphotoxin-alpha gene knockout mice. *Hepatology* 27, 1021–1029.

Borsello, T., Clarke, P. G., Hirt, L., Vercelli, A., Repici, M., Schorderet, D. F., Bogousslavsky, J., and Bonny, C. (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat. Med.* 9, 1180–1186.

Bourdi, M., Korrapati, M. C., Chakraborty, M., Yee, S. B., and Pohl, L. R. (2008). Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury. *Biochem. Biophys. Res. Commun.* 374, 6–10.

Canbay, A., Higuchi, H., Bronk, S. F., Taniai, M., Sebo, T. J., and Gores, G. J. (2003). Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. *Gastroenterology* 123, 1323–1330.

Chan, K., Truong, D., Shangari, N., and O'Brien, P. J. (2005). Drug-induced mitochondrial toxicity. *Expert Opin. Drug Metab. Toxicol.* 4, 655–669.

Chang, C. C., Wang, S. S., Huang, H. C., Chan, C. Y., Lee, F. Y., Lin, H. C., Nong, J. Y., Chuang, C. L., and Lee, S. D. (2011). Selective cyclooxygenase inhibition improves hepatic encephalopathy in fulminant hepatic failure of rat. *Eur. J. Pharmacol.* 666, 226–232.

Corcoran, G. B., Racz, W. J., Smith, C. V., and Mitchell, J. R. (1985). Effects of N-acetylcysteine on acetaminophen covalent binding and hepatic necrosis in mice. *J. Pharmacol. Exp. Ther.* 232, 864–872.

Craig, D. G., Lee, P., Pryde, E. A., Masterton, G. S., Hayes, P. C., and Simpson, K. J. (2011). Circulating apoptotic and necrotic cell death markers in patients with acute liver injury. *Liver Int.* 31, 1127–1136.

Dear, J. W., Simpson, K. J., Nicolai, M. P., Catterson, J. H., Street, J., Huizinga, T., Craig, D. G., Dhaliwal, K., Webb, S., Bateman, D. N., and Webb, D. J. (2011). Cyclophilin A is a damage-associated molecular pattern molecule that mediates acetaminophen-induced liver injury. *J. Immunol.* 187, 3347–3352.

Desbarats, J., and Newell, M. K. (2000). Fas engagement accelerates liver regeneration after partial hepatectomy. *Nat. Med.* 6, 920–923.

El-Hassan, H., Anwar, K., Macanas-Pirard, P., Crabtree, M., Chow, S. C., Johnson, V. L., Lee, P. C., Hinton, R. H., Price, S. C., and Kass, G. E. (2003). Involvement of mitochondria in acetaminophen-induced apoptosis and hepatic injury: roles of cytochrome c, bax, bid, and caspases. *Toxicol. Appl. Pharmacol.* 191, 118–129.

El-Mofty, S. K., Scrutton, M. C., Serroni, A., Nicolini, C., and Farber, J. L. (1975). Early, reversible plasma membrane injury in galactosamine-induced liver cell death. *Am. J. Pathol.* 79, 579–596.

Faouzi, S., Burckhardt, B. E., Hanson, J. C., Campe, C. B., Schrum, L. W., Rippe, R. A., and Maher, J. J. (2001). Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-kappa B-independent, caspase-3-dependent pathway. *J. Biol. Chem.* 276, 49077–49082.

Ferrari, D., Stepzynska, A., Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1998). Differential regulation and ATP requirement for caspase-8 and -3 activation during CD95- and anticancer drug-induced apoptosis. *J. Exp. Med.* 188, 979–984.

Fischer, U., Jänicke, R. U., and Schulze-Osthoff, K. (1998). Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.* 10, 76–100.

Fischer, U., and Schulze-Osthoff, K. (2005). New approaches and therapeutics targeting apoptosis in disease. *Pharmacol. Rev.* 57, 187–215.

Galanos, C., Freudenberg, M. A., and Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 76, 5939–5943.

Gujral, J. S., Knight, T. R., Farhood, A., Bajt, M. L., and Jaeschke, H. (2002). Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? *Toxicol. Sci.* 67, 322–328.

Gunawan, B. K., Liu, Z. X., Han, D., Hanawa, N., Gaarde, W. A., and Kaplowitz, N. (2006). C-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 131, 165–178.

Henderson, N. C., Pollock, K. J., Frew, J., Mackinnon, A. C., Flavell, R. A., Davis, R. J., Sethi, T., and Simpson, K. J. (2007). Critical role of c-jun (NH2) terminal kinase in paracetamol-induced acute liver failure. *Gut* 56, 982–990.

Hikita, H., Takehara, T., Kodama, T., Shimizu, S., Shigekawa, M., Hosui, A., Miyagi, T., Tatsumi, T., Ishida, H., Li, W., Kanto, T., Hiramatsu, N., Shimizu, S., Tsujimoto, Y., and Shimizu, N. (2011). Delayed-onset caspase-dependent massive hepatocyte apoptosis upon Fas activation in Bak/Bax-deficient mice. *Hepatology* 54, 240–251.

Hinson, J. A., Roberts, D. W., and James, L. P. (2010). Mechanisms of acetaminophen-induced liver necrosis. *Handb. Exp. Pharmacol.* 196, 369–405.

Hiramatsu, N., Hayashi, N., Katayama, K., Mochizuki, K., Kawanishi, Y., Kasahara, A., Fusamoto, H., and Kamada, T. (1994). Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. *Hepatology* 19, 1354–1359.

Hu, B., and Colletti, L. M. (2010). CXC receptor-2 knockout genotype increases X-linked inhibitor of apoptosis protein and protects mice from acetaminophen hepatotoxicity. *Hepatology* 52, 691–702.

Jaeschke, H., Cover, C., and Bajt, M. L. (2006). Role of caspases in acetaminophen-induced liver injury. *Life Sci.* 78, 1670–1676.

Jaeschke, H., Williams, C. D., Ramachandran, A., and Bajt, M. L. (2012). Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int.* 32, 8–20.

Joka, D., Wahl, K., Moeller, S., Schlue, J., Vaske, B., Bahr, M. J., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2012). Prospective biopsy-controlled evaluation of cell death biomarkers for prediction of liver fibrosis and nonalcoholic steatohepatitis. *Hepatology* 55, 455–464.

Keppler, D., Lesch, R., Reutter, W., and Decker, K. (1968). Experimental hepatitis induced by D-galactosamine. *Exp. Mol. Pathol.* 9, 279–290.

Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26, 239–257.

Knight, T. R., and Jaeschke, H. (2002). Acetaminophen-induced inhibition of Fas receptor-mediated liver cell apoptosis: mitochondrial dysfunction versus glutathione depletion. *Toxicol. Appl. Pharmacol.* 181, 133–141.

Kon, K., Kim, J. S., Jaeschke, H., and Lemasters, J. J. (2004). Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology* 40, 1170–1179.

Larson, A. M., Polson, J., Fontana, R. J., Davern, T. J., Lalani, E., Hynan, L. S., Reisch, J. S., Schiødt, F. V., Ostapowicz, G., Shakil, A. O., and Lee, W. M. (2005). Acute Liver Failure Study Group. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 42, 1364–1372.

Lawson, J. A., Fisher, M. A., Simmons, C. A., Farhood, A., and Jaeschke, H. (1999). Inhibition of Fas receptor (CD95)-induced hepatic caspase activation and apoptosis by acetaminophen in mice. *Toxicol. Appl. Pharmacol.* 156, 179–186.

Lee, J. Y., Chae, D. W., Kim, S. M., Nam, E. S., Jang, M. K., Lee, J. H., Kim, H. Y., and Yoo, J. Y. (2004). Expression of FasL and perforin/granzyme B mRNA in chronic hepatitis B virus infection. *J. Viral Hepat.* 11, 130–135.

Leers, M. P. G., Kölgen, W., Björklund, V., Bergmann, T., Gordon, T., Persson, B., Björklund, P., Ramaekers, F. C., Björklund, B., Nap, M., Jörnvall, H., and Schutte, B. (1999). Immunohistochemical detection and mapping of cytokeratin 18 neo-epitope exposed during early apoptosis. *J. Pathol.* 187, 567–572.

Leist, M., Gantner, F., Bohlinger, I., Germann, P. G., Tiegs, G., and Wendel, A. (1994). Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. *J. Immunol.* 153, 1778–1788.

Leist, M., Gantner, F., Bohlinger, I., Tiegs, G., Germann, P. G., and Wendel, A. (1995). Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am. J. Pathol.* 146, 1220–1234.

Libert, C., Brouckaert, P., and Fiers, W. (1994). Protection by alpha 1-acid glycoprotein against tumor necrosis factor-induced lethality. *J. Exp. Med.* 180, 1571–1575.

Liong, E. C., Xiao, J., Lau, T. Y., Nanji, A. A., and Tipoe, G. L. (2012). Cyclooxygenase inhibitors protect d-galactosamine/lipopolysaccharide induced acute hepatic injury in experimental mice model. *Food Chem. Toxicol.* 50, 861–866.

Liu, Z. X., Govindarajan, S., and Kaplowitz, N. (2004). Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology* 127, 1760–1774.

Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, Z. Q., and Schulze-Osthoff, K. (2002). Activation and caspase-mediated inhibition of PARP: a molecular switch between necrosis and apoptosis in death receptor signaling. *Mol. Biol. Cell* 13, 978–988.

Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999). The role of caspases in development, immunity and apoptotic signal transduction: lessons from knock-out mice. *Immunity* 10, 629–639.

Mita, E., Hayashi, N., Iio, S., Takehara, T., Hijioka, T., Kasahara, A., Fusamoto, H., and Kamada, T. (1994). Role of Fas ligand in apoptosis induced by hepatitis C virus infection. *Biochem. Biophys. Res. Commun.* 204, 468–474.

Nakae, H., Narita, K., and Endo, S. (2001). Soluble Fas and soluble Fas ligand levels in patients with acute hepatic failure. *J. Crit. Care* 16, 59–63.

Ni, H. M., Bockus, A., Boggess, N., Jaeschke, H., and Ding, W. X. (2012). Activation of autophagy protects against acetaminophen-induced hepatotoxicity. *Hepatology* 55, 222–232.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T, Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. *Nature* 364, 806–809.

Pianko, S., Patella, S., Ostapowicz, G., Desmond, P., and Sievert, W. (2001). Fas-mediated hepatocyte apoptosis is increased by hepatitis C virus infection and alcohol consumption, and may be associated with hepatic fibrosis: mechanisms of liver cell injury in chronic hepatitis C virus infection. *J. Viral Hepat.* 8, 406–413.

Ray, S. D., Mumaw, V. R., Raje, R. R., and Fariss, M. W. (1996). Protection of acetaminophen-induced hepatocellular apoptosis and necrosis by cholesteryl hemisuccinate pretreatment. *J. Pharmacol. Exp. Ther.* 279, 1470–1483.

Rivero, M., Crespo, J., Fabrega, E., Casafont, F., Mayorga, M., Gomez-Fleitas, M., and Pons-Romero, F. (2002). Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. *J. Viral Hepat.* 9, 107–113.

Roth, G. A., Lubsczyk, B. A., Pilz, J., Faybik, P., Hetz, H., and Krenn, C. G. (2009). Nucleosome serum levels in acute hepatic failure and MARS treatment. *Transplant. Proc.* 41, 4207–4210.

Russell, S. W., Rosenau, W., and Lee, J. C. (1972). Cytolysis induced by human lymphotoxin. *Am. J. Pathol.* 69, 103–118.

Rutherford, A. E., Hynan, L. S., Borges, C. B., Forcione, D. G., Blackard, J. T., Lin, W., Gorman, A. R., Obaid, S. S., Reuben, A., Harrison, E., Reddy, K. R., Le, W. M., Chung, R. T., and for the ALF Study Group. (2007). Serum apoptosis markers in acute liver failure: a pilot study. *Clin. Gastroenterol. Hepatol.* 5, 1477–1483.

Ryo, K., Kamogawa, Y., Ikeda, I., Yamauchi, K., Yonehara, S., Nagat, S., and Hayashi, N. (2000). Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. *Am. J. Gastroenterol.* 95, 2047–2055.

Saito, C., Zwingmann, C., and Jaeschke, H. (2010). Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. *Hepatology* 51, 246–254.

Schulze-Osthoff, K., and Bantel, H. (2011). Necrosis versus apoptosis in acetaminophen-induced hepatotoxicity. *Hepatology* 53, 1070.

Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., and Peter, M. E. (1998). Apoptosis signaling by death receptors. *Eur. J. Biochem.* 254, 439–459.

Schulze-Osthoff, K., Krammer, P. H., and Dröge, W. (1994). Divergent signaling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO J.* 13, 4587–4596.

Schwerk, C., and Schulze-Osthoff, K. (2005). Regulation of apoptosis by alternative pre-mRNA splicing. *Mol. Cell* 19, 1–13.

Seidel, N., Volkmann, X., Länger, F., Flemming, P., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2005). The extent of liver steatosis in chronic hepatitis C virus infection is mirrored by caspase activity in serum. *Hepatology* 42, 113–120.

Sharma, A. D., Narain, N., Händel, E. M., Iken, M., Singhal, N., Cathomen, T., Manns, M. P., Schöler, H. R., Ott, M., and Cantz, T. (2011). MicroRNA-221 regulates FAS-induced fulminant liver failure. *Hepatology* 53, 1651–1661.

Simpson, K. J., Lukas, N. W., McGregor, A. H., Harrison, D. J., Strieter, R. M., and Kunkel, S. L. (2000). Inhibition of tumor necrosis factor alpha does not prevent experimental paracetamol-induced hepatic necrosis. *J. Pathol.* 190, 489–494.

Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., and Lieberman, J. (2003). RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* 3, 347–351.

Strand, S., Hofmann, W. J., Grambihler, A., Hug, H., Volkmann, M., Otto, G., Wesch, H., Mariani, S. M., Hack, V., Krammer, P. H., and Galle, P. R. (1998). Hepatic failure and liver cell damage in acute Wilson's disease involve CD95 (APO-1/Fas) mediated apoptosis. *Nat. Med.* 4, 588–593.

Streetz, K., Leifeld, L., Grundmann, D., Ramakers, J., Eckert, K., Spengler, U., Brenner, D., Manns, M., and Trautwein, C. (2000). Tumor necrosis factor alpha in the pathogenesis of human and murine fulminant hepatic failure. *Gastroenterology* 119, 446–460.

Tagami, A., Ohnishi, H., and Huges, R. D. (2003). Increased serum soluble Fas in patients with acute liver failure due to paracetamol overdose. *Hepatogastroenterology* 5, 1477–1483.

Tinel, M., Berson, A., Vadrot, M., Descatoire, V., Grodet, A., Feldmann, G., Thénot, J. P., and Pessayre, D. (2004). Subliminal Fas stimulation increases the hepatotoxicity of acetaminophen and bromobenzene in mice. *Hepatology* 39, 655–666.

Tokushige, K., Yamaguchi, N., Ikeda, I., Hashimoto, E., Yamauchi, K., and Hayashi, N. (2000). Significance of soluble TNF receptor I in acute type fulminant heaptitis. *Am. J. Gastroenterol.* 95, 2040–2046.

Vandenabeele, P., Galluzzi, L., Berghe, T. V., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat. Rev. Mol. Cell Biol.* 11, 700–714.

Volkmann, X., Ansteatt, M., Hadem, J., Stiefel, P., Bahr, M. J., Lehner,

F., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2008). Caspase activation is associated with spontaneous recovery from acute liver failure. *Hepatology* 47, 1624–1633.

Volkmann, X., Cornberg, M., Wedemeyer, H., Lehner, F., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2006). Caspase activation is required for antiviral treatment response in chronic hepatitis C virus infection. *Hepatology* 43, 1311–1316.

Volkmann, X., Fischer, U., Bahr, M. J., Ott, M., Lehner, F., Macfarlane, M., Cohen, G. M., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2007). Increased hepatotoxicity of tumor necrosis factor-related apoptosis-inducing ligand in diseased human liver. *Hepatology* 46, 1498–1508.

Wullaert, A., van Loo, G., Heynick, K., and Beyaert, R. (2007). Hepatic tumor necrosis factor signaling and nclear factor-kappaB: effects on liver homeostasis and beyond. *Endocr. Rev.* 28, 365–386.

Yoon, J. H., and Gores, G. J. (2002). Death receptor-mediated apoptosis and the liver. *J. Hepatol.* 37, 400–410.

Zender, L., Hutker, S., Liedtke, C., Tillmann, H. L., Zender, S., Mundt, B., Waltemathe, M., Gosling, T., Flemming, P., Malek, N. P., Trautwein, C., Manns, M. P., Kuhnel, F., and Kubicka, S. (2003). Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9053–9058.

Zhang, H., Cook, J., Nickel, J., Yu, R., Stecker, K., Myers, K., and Dean, N. M. (2000). Reduction of liver Fas expression by antisense oligonucleotide protects mice from fulminant hepatitis. *Nat. Biotechnol.* 18, 862–867.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 February 2012; accepted: 16 March 2012; published online: 02 April 2012.

Citation: Bantel H and Schulze-Osthoff K (2012) Mechanisms of cell death in acute liver failure. Front. Physio. ***3****:79. doi: 10.3389/fphys.2012.00079*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 20 June 2012
doi: 10.3389/fphys.2012.00213

The role of chemokines in acute liver injury

Yedidya Saiman and Scott L. Friedman*

Division of Liver Diseases, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Frank Tacke, University Hospital Aachen, Germany
Leo A. Van Grunsven, Vrije Universiteit Brussel, Belgium

***Correspondence:**
Scott L. Friedman, Division of Liver Diseases, Mount Sinai School of Medicine, Box 1123, 1425 Madison Avenue, Room 11-70C, New York, NY 10029-6574, USA.
e-mail: scott.friedman@mssm.edu

Chemokines are small molecular weight proteins primarily known to drive migration of immune cell populations. In both acute and chronic liver injury, hepatic chemokine expression is induced resulting in inflammatory cell infiltration, angiogenesis, and cell activation and survival. During acute injury, massive parenchymal cell death due to apoptosis and/or necrosis leads to chemokine production by hepatocytes, cholangiocytes, Kupffer cells, hepatic stellate cells, and sinusoidal endothelial cells. The specific chemokine profile expressed during injury is dependent on both the type and course of injury. Hepatotoxicity by acetaminophen for example leads to cellular necrosis and activation of Toll-like receptors while the inciting insult in ischemia reperfusion injury produces reactive oxygen species and subsequent production of pro-inflammatory chemokines. Chemokine expression by these cells generates a chemoattractant gradient promoting infiltration by monocytes/macrophages, NK cells, NKT cells, neutrophils, B cells, and T cells whose activity are highly regulated by the specific chemokine profiles within the liver. Additionally, resident hepatic cells express chemokine receptors both in the normal and injured liver. While the role of these receptors in normal liver has not been well described, during injury, receptor up-regulation, and chemokine engagement leads to cellular survival, proliferation, apoptosis, fibrogenesis, and expression of additional chemokines and growth factors. Hepatic-derived chemokines can therefore function in both paracrine and autocrine fashions further expanding their role in liver disease. More recently it has been appreciated that chemokines can have diverging effects depending on their temporal expression pattern and the type of injury. A better understanding of chemokine/chemokine receptor axes will therefore pave the way for development of novel targeted therapies for the treatment of liver disease.

Keywords: chemokines, acute liver injury

INTRODUCTION

Chemokines are small molecular weight proteins (8–13 kDa) initially identified by their ability to provide migratory cues to inflammatory cells (Wasmuth et al., 2010). Their expression is up-regulated in nearly all forms of injury in all tissues, leading to infiltration by immune cells. To date, more than 50 different chemokines and 20 chemokine receptors have been identified (Bromley et al., 2008). Most chemokine receptors engage more than one ligand leading to redundancy in chemokine signaling and divergent outcomes following signaling through a single receptor (Bromley et al., 2008).

Chemokines are subdivided into two broad categories based on their amino acid sequences. *CC chemokines* contain two adjacent N-terminus cysteine residues while the N-terminal cysteines in *CXC chemokines* are separated by a single amino acid. These cysteine residues form disulfide bonds with additional internal cysteines providing tertiary structure to the protein. Two additional categories, *C-* and *CX_3C-chemokines* have either a single N-terminus cysteine or two cysteine residues separated by three amino acids, respectively. Additionally, *CXC chemokines* can be further subdivided based on specific sequence motifs (ELR positive/negative) that impart specific functional properties to the chemokine (Bajetto et al., 2002; Fernandez and Lolis, 2002; Oo et al., 2010; Wasmuth et al., 2010).

The field of chemokine biology has rapidly progressed and additional functions are recognized well beyond immune cell migration. Chemokine receptors have been identified on non-immune cells, and as a result, their roles have expanded to include organ homeostasis and non-inflammatory aspects of injury (Rossi et al., 1999; Shibuta et al., 2002; Zlotnik et al., 2011). The functions of chemokines have therefore expanded to include: cellular differentiation, survival, proliferation, and apoptosis in addition to the ability to modulate development, organ fibrogenesis, vascular angiogenesis, and tumor metastasis (Shibuta et al., 2002; Hong et al., 2009; Zlotnik et al., 2011; Mukaida and Baba, 2012).

For the purposes of this review we discuss chemokine responses in both immune cells and liver parenchymal cells. Although infiltrating immune cells secrete chemokines, the primary sources in the liver are hepatocytes, Kupffer cells, stellate cells, sinusoidal endothelial cells, and biliary epithelial cells. Together, these cells secrete an array of chemokines that drive immune cell infiltration, development of chronic inflammation, liver injury and regeneration, and progression and resolution of fibrosis (Karlmark et al., 2008; Oo et al., 2010; Wasmuth et al., 2010). The numerous and often disparate functions of chemokines in the liver reflect

the divergent temporal expression of these molecules and their receptors by immune and resident hepatic cells.

The regulation of both chemokine and receptor expression is modulated by a range of stimuli including growth factors (Gerritsma et al., 1998), cytokines (Harvey et al., 2003), cellular stressors (bile acids, ROS, etc.; Friedman, 2008a,b; Steib et al., 2010), cellular activation by apoptotic bodies (Zernecke et al., 2009), and release of cellular debris from necrotic cells (Jaeschke et al., 2002). Chemokines function as paracrine signals and in autocrine loops, with both positive and negative feedback elements. The complexity of these networks is immense and therefore we highlight herein those aspects that are most instructive in clarifying chemokine biology in acute liver injury (ALI; **Table 1**).

CCL2 PROMOTES MONOCYTE/MACROPHAGE RECRUITMENT AND CYTOKINE PRODUCTION DURING ACUTE LIVER INJURY

CCL2 (MCP-1) is among the most extensively studied chemokines in liver injury. Its primary role is the recruitment of monocytes and macrophages via its receptor, CCR2, but NK cells and lymphocytes also express CCR2 and migrate in response to CCL2 (Hokeness et al., 2005; Karlmark et al., 2008). Intrahepatic and serum levels of CCL2 are increased in patients with fulminant hepatic failure and also in murine models of acute liver failure (Possamai et al., 2010). Injured hepatocytes and activated Kupffer cells are thought to be the primary sources of hepatic CCL2, however, hepatic stellate cells and liver sinusoidal endothelial cells can also secrete this molecule (Leifeld et al., 2003; Friedman, 2008b; Kolios et al., 2008; Chen et al., 2010; **Figure 1**).

The role of CCL2 in liver injury is twofold. First, as CCL2 levels in the serum rise, it stimulates monocytic hematopoiesis and increased differentiation and production of the monocyte/macrophage lineages (Leifeld et al., 2003; Friedman, 2008b; Kolios et al., 2008; Chen et al., 2010). Furthermore, CCL2 levels in the liver are greater than in the blood, establishing a gradient driving macrophage egress from the bone marrow (Dambach et al., 2002; Tsou et al., 2007; Karlmark et al., 2009). Moreover, it has been shown that it predominantly recruits the inflammatory Gr1 high monocyte-derived macrophage subpopulation (Karlmark et al., 2009). Macrophages have distinctive roles in initiation, propagation, and resolution of ALI. Their involvement is critical during the initial inflammatory stages by phagocytosing necrotic cells, secreting cytokines and growth factors, and recruiting inflammatory cells (Duffield, 2003). Additionally, macrophages assist in the reparative phase leading to hepatic remodeling and the return to normal liver function (Duffield et al., 2005).

Macrophage derived chemokines, cytokines, and growth factors can influence hepatocyte function and recruitment of additional immune cell populations. TNF-α which leads to hepatocyte cell death, is increased early in acetaminophen (APAP) injury and is secreted predominantly by macrophages but also hepatocytes and other immune cells (Hassan et al., 2007). Additionally, IFN-γ, a pro-inflammatory cytokine released by macrophages as well as CCR2-dependent T cells and NKT cells, may promote injury by stimulating hepatic inflammation and amplifying liver damage (Hogaboam et al., 2000; Dambach et al., 2002). Loss of IFN-γ signaling in both KO mice or mice treated with IFN-γ neutralizing

Table 1 | Cellular expression and function of chemokines in acute liver injury.

Chemokine	Receptor	Cellular production of ligand	Hepatic expression of receptor	Immune cell expression of receptor	Types of injury leading to chemokine expression	Function of chemokine axis
CCL2	CCR2	Hepatocytes, Kupffer cells, stellate cells, LSECs, BECs	Kupffer cells, stellate cells	Monocyte/macrophage, lymphocyte, NK cells	APAP, ConA, CCl_4, I/R	The axis promotes infiltration by macrophages generally leading to increased injury. Differences in injury model, macrophage depletion method, and timing of analysis after injury leads to different results
CXCL9, CXCL10, CXCL11	CXCR3	Hepatocytes, stellate cells, LSECs	Kupffer cells, stellate cells, LSECs	Th1, Th2, TH17, Tregs Cells, CD8+ T cells, Tregs, NK, NKT	APAP, ConA, I/R	CXCR3 promotes infiltration of T cells providing protective immune response, however this effect is model specific. CXCR3 also has indirect effect on hepatocyte survival
CXCL1, CXCL2, CXCL8	CXCR1, CXCR2	Hepatocytes, Kupffer cells, HSCs, LSECs, BECs	Hepatocytes	Neutrophils, monocytes, mast cells	APAP, I/R	Neutrophil infiltration to the liver. Disparate effects on hepatocyte survival based on type of injury and dose of chemokine ligand
CXCL12	CXCR4	Stellate cells, LSEC, BECs	Hepatic oval cells, HSCs, LSECs	T and B cells, monocytes, NKT cells, hematopoietic stem cells	Unknown	Role in immune cell infiltration not known. Appears important in stem cell recruitment to the liver

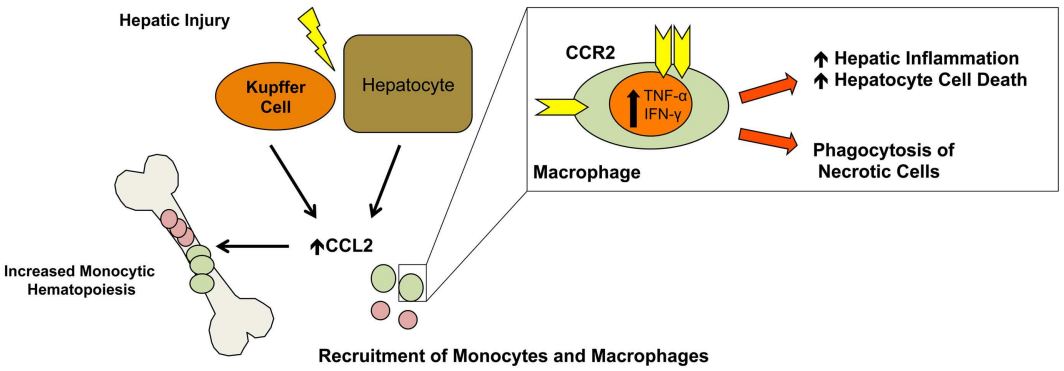


FIGURE 1 | CCL2 monocyte/macrophage recruitment. In response to injury, hepatocytes and Kupffer cells secrete CCL2 leading to liver infiltration by monocytes and macrophages from the periphery. Additionally, CCL2 promotes monocytic hematopoiesis in the bone marrow increasing the pool of circulating monocytes/macrophages. In the liver, macrophages secrete TNF-α and IFN-γ promoting inflammation and hepatocyte cell death while also removing necrotic cells which is important for liver remodeling and return to normal function.

antibodies demonstrates a protective effect in APAP or ConA induced ALI (Jaruga et al., 2004; Dong et al., 2005). Interestingly, IFN-γ is important in regulation of the CXCL9–11/CXCR3 chemokine axis, which is reviewed below.

Given the dependence of macrophage recruitment on the CCL2/CCR2 axis, many groups have utilized monocyte/macrophage depletion studies to establish the relevance of the CCL2/CCR2 axis. While the overall role of CCL2 recruited macrophages in ALI appears to be protective, conflicting reports have made it difficult to clearly define the role of CCL2 during injury (Laskin et al., 1995; Michael et al., 1999; Hogaboam et al., 2000; Dambach et al., 2002; Ju et al., 2002; Holt et al., 2008; Karlmark et al., 2009). The method of macrophage depletion (clodronate, gadolinium chloride, CD11b DTR), the type of injury (APAP, CCl_4, Ischemia reperfusion), and the extent of injury all influence the interpretation of how macrophages affect liver function.

One technical concern is that many macrophage depletion methods affect other cell lineages including dendritic cells and bone marrow hematopoietic cells. Additionally, as a result of strain differences and the dependence of bone marrow stem cells egress on CCL2/CCR2, global knockouts of this axis have failed to delineate the specific role of hepatic-derived CCL2 during injury. Overall, it appears that loss of CCL2 function perturbs monocyte/macrophage infiltration into the injured liver with a concomitant dampening of pro-inflammatory cytokine production. Therefore, future studies will need to utilize conditional CCL2 or CCR2 knockouts from specific hepatic and immune cell populations in order to determine the cellular populations involved in ALI.

Macrophages recruited by CCL2 are involved in both the initial stages of injury during ALI and the reparative stages. However, little data exists on the role of macrophages during liver regeneration or during restoration after acute injury. In murine models of chronic liver injury and fibrosis, there exist two waves of macrophage infiltration responsible for both disease progression and regression (although this is not CCL2-dependent; Duffield et al., 2005). Similar studies have not been undertaken in ALI models, which might shed additional light on the role of CCL2 and infiltrating macrophages in ALI.

The concept of multiple waves of macrophage has also been shown in patients with ALI. After APAP-induced acute liver failure there is an increase in hepatic CCL2 which directly correlated with clinical outcomes. Furthermore, there is a specific and marked reduction in circulating monocytes which negatively correlated with the hepatic CCL2 levels indicating that their recruitment into the inflamed liver is via CCR2. In areas of liver necrosis there was a predominance proliferating resident Kupffer cells. This study indicates that the appearance of macrophages early after injury are derived from circulating monocytes and resident proliferating Kupffer cells represent a second wave implicated in organ resolution (Antoniades et al., 2012). While not definitive, these results impressively mimic rodent models of macrophage recruitment in rodent models.

While the majority of studies of CCL2 have focused on its effect on monocytes and macrophages, it can also recruit/activate T cell and NKT cell populations, albeit to a lesser extent. Neutralization of the CCL2/CCR2 axis in the T cell-mediated ConA injury model leads to an increase in hepatic injury, indicating that, in this model, CCL2 may be anti-inflammatory (Ajuebor et al., 2003). Mice treated with CCR2 neutralizing serum showed a decrease in TNF-α and IFN-γ, but worse hepatitis and an unexpected and significant increase in IL-4, representing a shift from a Th1 to a Th2 response. ConA treatment increased CCR2 expression on resident hepatic NKT cells and receptor engagement dampened their secretion of IL-4. Therefore, blocking this axis actually increased the expression of IL-4, indicating that CCL2 may also function in an anti-inflammatory manner.

The only resident liver cells to express CCR2 are Kupffer cells and hepatic stellate cells (Friedman, 2008b; Krohn et al., 2009). As noted previously, Kupffer cells are an important source of CCL2 whose production is stimulated by ROS. The specific role of resident hepatic cell CCR2 in ALI has not been demonstrated, but in models of chronic liver injury, the stellate cell's response to CCL2 is important in the production of ROS, cellular chemotaxis, and the acquisition of a pro-fibrogenic phenotype. Given the limited

data regarding the role of hepatic stellate cells during ALI, additional work is required to determine the role of CCL2 on resident hepatic cell function during acute injury.

CXCL9–11 RECRUITS T CELL POPULATIONS INVOLVED IN IMMUNE MODULATION

The CXCL9–11/CXCR3 axis is unique based on the alternative regulation of its three ligands, which are all induced by IFN-γ. CXCR3 is primarily expressed on CD4+ Th1 helper cells, CD8+ cytotoxic lymphocytes, and innate lymphocytes including NK cells, NKT cells, and dendritic cell subsets. During activation by professional antigen presenting cells, CXCR3 is rapidly up-regulated on leukocyte populations (Groom and Luster, 2011; **Figure 2**).

Similar to other chemokine axes, CXCL9–11 signaling is characterized by significant redundancy, however each of the chemokines has a distinctive role. Expression of all three ligands is mediated by IFN-γ, but the identification of unique promoter elements for each ligand leads to a distinct spatial and temporal expression pattern. Briefly, CXCL9 is dependent solely on IFN-γ whereas CXCL11 can also be induced by IFN-β and CXCL10 by both IFN-α/IFN-β and NF-κB activation. Hepatocytes, stellate cells, sinusoidal endothelial cells, and activated infiltrating lymphocytes all secrete CXCR3 ligands in response to these stimuli. Differences in the sensitivity of each cell type to IFN-γ signaling along with the differences in the ligand promoters leads to the differential expression patterns seen in liver disease (Luster et al., 1985; Farber, 1990;Ohmori and Hamilton, 1993, 1997;Ohmori et al., 1993, 1997; Cole et al., 1998; Majumder et al., 1998; Tensen et al., 1999; Medoff et al., 2006; Yang et al., 2007).

In patients with chronic liver disease, hepatic levels of both CXCR3 and its ligands are increased, and CXCR3 ligand levels correlate with the severity of disease (Apolinario et al., 2002). Additionally, in mouse models of chronic disease, CXCR3 is protective through several mechanisms. While no human data exists on the role of CXCR3 in ALI, CXCR3-deficient mice point to its being hepatoprotective (Hokeness et al., 2007; Zaldivar et al., 2012). Th1-polarized T cells, which provide a protective immune response, are rapidly recruited to the liver after injury in a CXCR3-dependent manner. Additionally, a large subset of Foxp3+/CD25+ regulatory T cells (Tregs) found in the liver during injury are CXCR3+, and this population is diminished in CXCR3-deficient mice. Loss of CXCR3 therefore affects recruitment of both effector T cell populations and T regulatory cells (Erhardt et al., 2011). The balance between these populations influences the outcome following blockade of this axis and may explain the contrasting results seen in different experimental models.

Ischemia reperfusion (I/R) injury is a major concern following liver transplantation and is an important contributor to liver rejection in this setting. After I/R, levels of CXCL9–11 are increased early after injury leading to infiltration of CXCR3+ T cells. Blocking this interaction in rats decreases hepatocellular damage and increases survival. Similarly, studies of cardiac and islet cell allografts indicate that blocking CXCL10 and CXCL11 prolongs survival and prevents acute allograft rejection by inhibiting the recruitment of effector T cells (Horiguchi et al., 2002).

Tregs have important functions in controlling immune cell activation within the liver. Patients with autoimmune hepatitis, as well as autoimmune liver disease, have decreased numbers of Tregs. In the T cell-mediated ConA injury model, CXCL9–11 expression is increased and mice deficient for CXCR3 are significantly more susceptible to ConA injury. Similar to other models, the total number of CXCR3+ T cells was decreased as were the number of hepatic Tregs. However, in this model, the decrease in infiltrating CXCR3+ effector T cells does not compensate for the loss of hepatic Tregs, and nonetheless leads to increased injury and lethality. Consistent with this finding, the number of Tregs in the spleen and lymph nodes were increased, indicating that they failed to recruit to the liver in the absence of CXCR3 (Erhardt et al., 2011). Similarly, loss

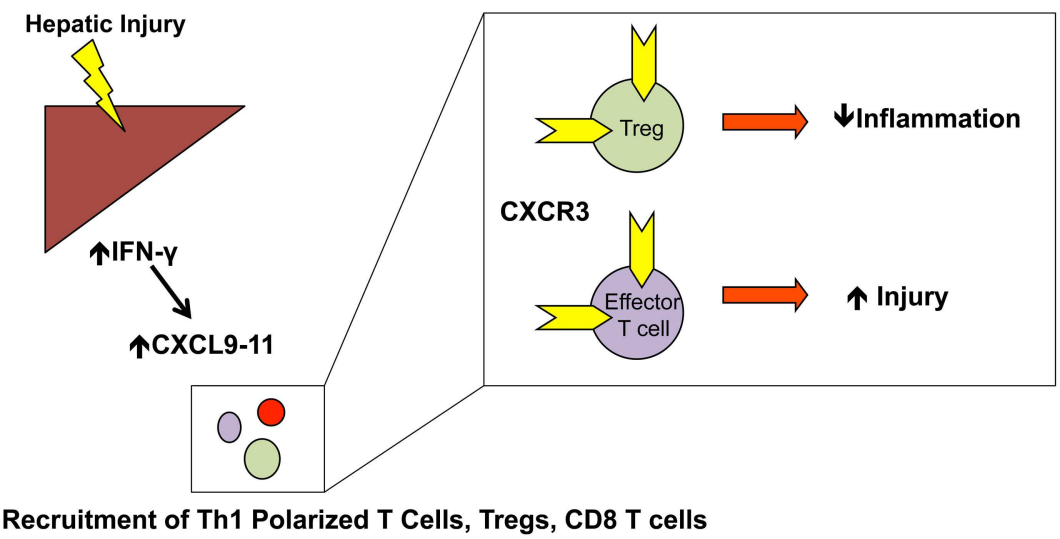


FIGURE 2 | CXCL9–11 T cell recruitment: CXCL9–11 expression is increased in an IFN-γ dependent manner. Numerous T cell populations, including Th1-polarized T cells, Tregs, and effector T cells are recruited to the liver in a CXCR3-dependent manner. The specific type of injury will determine the relative recruitment of Tregs vs. effector T cells and the protective/injurious role of the CXCL9–11/CXCR3 axis.

of IFN-γ (and subsequently CXCL9–11 up-regulation) or neutralization of CXCL10 leads to decreased hepatic Tregs and enhanced hepatic inflammation. This effect is partially mediated by resident liver NKT cells, which express high levels of IFN-γ in response to injury. Mice deficient in NKT cells therefore show a marked decrease in Treg recruitment, as do mice deficient for IFN-γ.

These results point to the difficulty of establishing whether a single chemokine is protective or injurious. Undoubtedly, the specific disease process and chemokine/cytokine signature will determine the relative contribution of each cell type, and whether inhibition of the axis will be protective or injurious, further highlighting the multidimensional approach required to understanding the function of any chemokine axis (Ajuebor et al., 2007).

In models of ALI, CXLC10, and CXCR3 are up-regulated upon APAP administration, whereas recombinant CXCL10 is hepatoprotective. The effect of CXCL10 is indirect by increasing hepatocyte expression of CXCR2, which is protective in certain injury models, and blocking CXCR2 abrogates the effect of exogenously administered CXCL10. These results are particularly interesting, as hepatocytes do not express CXCR3, and therefore the up-regulation of CXCR2 on hepatocytes must be regulated by an independent yet unidentified signaling axis (Bone-Larson et al., 2001).

Within the liver CXCR3 is expressed by hepatic stellate cells and liver sinusoidal endothelial cells, and is anti-fibrotic in chronic injury (Shields et al., 1999; Crosby et al., 2009; Wasmuth et al., 2009). CXCR3 ligands have seemingly opposing effects on hepatic stellate cells. CXCL9 decreases collagen mRNA expression leading to a less activated phenotype, while CXCL10 promotes hepatic stellate cell migration (Wasmuth et al., 2009). Such opposing effects may reflect different roles stellate cells play during liver injury or could categorize subpopulations of these cells with divergent functions. Furthermore, CXCL9–11 ligands are angiostatic, and administration of CXCL9 *in vivo* inhibits neoangiogenisis and prevents development of fibrosis (Sahin et al., 2012).

As a final note, it is important to recognize that there are mouse strain differences in CXCL9–11 expression. C57Bl/6 mice do not express CXCL11 due to a point mutation leading to an early stop codon. Bl/6 mice are still responsive to exogenous CXCL11, but because CXCL9 and CXLC10 knockout mice were backcrossed onto a Bl/6 background it is difficult to distinguish between the function of each ligand (Sahin et al., 2012).

CXCR1/CXCR2 LIGANDS PROMOTE NEUTROPHIL MIGRATION TO THE LIVER AND REGULATE HEPATOCYTE SURVIVAL AND PROLIFERATION

The CXCL1, CXCL2, and CXCL8 chemokine axis is especially important in acute injury given the dependence of neutrophil and macrophage chemotaxis on CXCR1/CXCR2 ligands (Chen et al., 2006; Ishida et al., 2006; Kobayashi, 2008). Several ligands signal through CXCR1/CXCR2, including CXCL8 (IL-8), found only in humans, and CXCL1 and CXCL2 (KC and MIP-2) present in both humans and mice. In patients, CXCL8 levels are increased in alcoholic hepatitis, as well as in APAP overdose, which are predictive of hepatocellular damage (James et al., 2001; Zimmermann et al., 2011). Additionally, post-liver transplantation, patients with elevated serum CXCL8 have higher serum transaminases (Ilmakunnas et al., 2010). In view of the direct correlation between CXCL8 levels and hepatic function after acute injury, much research has focused on the role of CXCR1/CXCR2 in I/R and APAP injury models. Furthermore, this axis is of great interest due to the expression of CXCR1/CXCR2 by hepatocytes, enabling these chemokines to directly affect both acute inflammation and hepatocyte survival/function (**Figure 3**).

During injury most hepatic cells express CXCR1/CXCR2 ligands including hepatocytes, Kupffer cells, stellate cells, endothelial cells, and biliary epithelial cells (Kuboki et al., 2008). After I/R injury, Kupffer cell activation leads to a release of ROS and subsequent activation of hepatocyte-derived chemokines CXCL1 and CXCL2 (Jaeschke et al., 1991; Jaeschke and Farhood, 2002; Kuboki et al., 2008). Hepatocyte injury due to exposure to ethanol, IL-1, and TNF-α has also been shown to result in CXCL8 expression by human hepatocytes (Stefanovic and Stefanovic, 2006).

Neutrophils express CXCR1 and CXCR2, and increased ligand expression after injury is associated with neutrophil infiltration (Kobayashi, 2008). CXCL8 binds with high affinity to CXCR1 and with lower affinity to CXCR2, which binds an additional six chemokines. Both receptors drive neutrophil chemotaxis, but a neutrophil respiratory burst occurs only through CXCR1, indicating distinct roles for each receptor. A feature of all chemokines, which has been extensively studied in CXCL8, is that they can exist as a monomer, dimer, or a mixture of the two under physiological conditions. These two forms of CXCL8 differentially regulate CXCR1 phosphorylation, desensitization, and receptor internalization, but not that of CXCR2. In general, monomeric CXCL8 shows increased activity via CXCR1, and that in models of lung injury the ability of CXCL8 to reversibly exist as both a monomer and dimer regulates neutrophil chemotaxis and function (Nasser et al., 2009).

The development of effective chemokine gradients is dependent on the interactions between tissue-expressed glycosaminoglycans (GAGs) and chemokines. Monomeric and dimeric chemokine forms have different binding affinities for GAGs, and changes in the monomer/dimer equilibrium will affect the chemokine gradients established within an organ. Chemokine dimerization therefore adds an additional level of regulation to chemokine axes (Gangavarapu et al., 2012).

In models of I/R, receptor inhibition by neutralizing antibodies leads to decreased neutrophil infiltration and less damage overall. Similarly, CXCR2 deficient mice exhibit decreased neutrophil accumulation at 24 h, but not at 96 h, indicating that CXCR2 might be important specifically in early neutrophil infiltration. Knockout mice also exhibit less injury (ALT/AST) and hepatic necrosis (Kuboki et al., 2008). While there is a decrease in neutrophil infiltration, expression levels of CXCR2 chemokines are increased, indicating a potential negative feedback loop through CXCR2. Similarly, in models of APAP-induced injury, loss of CXCR2 leads to decreased neutrophil and macrophage infiltration and less injury (Hogaboam et al., 1999a; Hu and Colletti, 2010). However, when only neutrophils are depleted using a neutrophil specific depleting antibody, the protective effect is even greater. The authors contend that production of iNOS by neutrophils leads to increased injury, but that macrophage derived heme-oxygenase

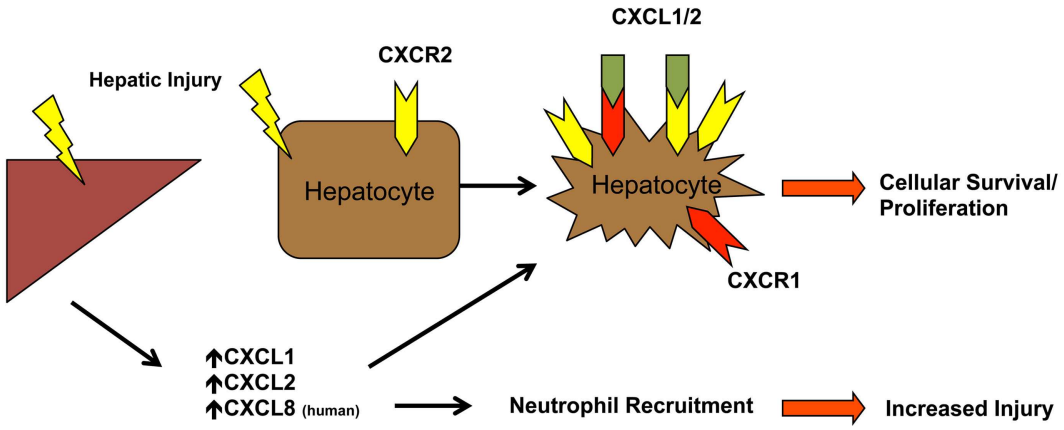


FIGURE 3 | CXCL1/CXCL2/CXCL8 neutrophil recruitment and hepatocyte proliferation. Neutrophils are recruited from the periphery via CXCR2 in response to increased hepatic levels of CXCL1, CXCL2, and CXCL8 (in humans). Hepatic injury also promotes expression of CXCR1 and increased expression of CXCR2 on hepatocytes. Receptor engagement leads to changes in hepatocyte survival and proliferation in a dose dependent mechanism.

(HO)-1 is protective. CXCR2 KO mice exhibit increased injury compared with the neutrophil depleted mice due to the additional loss of macrophage derived HO-1 (Ishida et al., 2006).

Among the most interesting aspects of this axis is CXCR1/CXCR2 expression by hepatocytes and their role in survival and proliferation. While CXCR2 is expressed under normal physiological conditions and is up-regulated with injury, hepatocyte expression of CXCR1 is detected only after injury. After I/R injury, signaling through CXCR2 is detrimental to hepatocyte proliferation and regeneration. Loss of CXCR2 function leads to an increase in STAT3 and NK-κB signaling which regulates liver regeneration. Furthermore, CXCR1 expression is increased during I/R injury, and while inhibition of CXCR1 does not mitigate early liver injury and neutrophil infiltration, it appears to be involved in the reparative and regenerative phase after I/R injury (Kuboki et al., 2008; Clarke et al., 2011).

Alternatively, in models of APAP injury and partial hepatectomy, CXCR2 activation by recombinant CXCL2 is mitogenic and promotes liver regeneration, while loss of CXCR2 leads to marked liver necrosis and hemorrhaging (Sakai et al., 2011). Currently, the only available treatment for APAP toxicity includes administration of *N*-acetylcysteine, however, the window of efficacy is only within 8 h after APAP ingestion. Conversely, administration of CXCL2 is only effective if administered 10 h after the initial injury, indicating that its role is to support hepatocyte survival and proliferation after the initial injury (Hu and Colletti, 2010). In another model, adenoviral administration of CXCL2 inhibits neutrophil influx while promoting hepatocyte proliferation after APAP, while CXCL2 inhibition increased liver injury and overall mortality (Hogaboam et al., 1999b).

One explanation for the discrepancies in assessing the effects of this axis on hepatocyte proliferation is that high doses of CXCR2 ligands are hepatotoxic while low doses are hepatoprotective. *In vitro* studies indicate that hepatocyte treatment with equal concentration of CXCR2 ligands, CXCL1 and CXCL2, may act synergistically and adjusting the relative concentration of each chemokine alters the overall effect (Kuboki et al., 2008). As numerous chemokines signal through CXCR2, differences in injury models may induce unique expression levels of CXCR2 binding chemokines leading to divergent outcomes.

CXCL12 PROMOTES HEMATOPOIETIC STEM CELL RECRUITMENT TO THE LIVER AND NEUTROPHIL EGRESS FROM THE BONE MARROW

CXCL12 (SDF-1α), which binds to the CXCR4 receptor, regulates organ homeostasis and several pathological responses (Nagasawa et al., 1996). CXCL12 is crucial in early embryogenesis, hematopoiesis, and angiogenesis, as well as maintenance of the bone marrow stem cell niche. A unique feature of CXCL12 is its high expression levels even in normal tissues that is further up-regulated with injury. In the uninjured liver, biliary epithelial cells constitutively express CXCL12 and in patients with chronic injury and fibrosis, CXCL12 levels increase in parallel with the extent of fibrotic injury (Wald et al., 2004). In distinction to many other chemokines in the liver, CXCL12 is not expressed by hepatocytes and Kupffer cells, but is localized to biliary epithelial cells, cells of the ductular reaction, hepatic stellate cells, and sinusoidal endothelial cells (Sawitza et al., 2009). Dissecting the specific role of the CXCL12/CXCR4 axis has proven difficult given the lack of a suitable animal model. As mentioned, CXCL12 is crucial for embryogenesis and mice deficient in either CXCL12 or its receptor CXCR4 are not viable due to a lack of cardiac development and a failure of hematopoiesis (Nagasawa et al., 1996; **Figure 4**).

Most inflammatory cells, including neutrophils, monocytes, and B and T lymphocytes express CXCR4 and locally produced CXCL12 recruits immune cells to the injured site and promotes angiogenesis (Liekens et al., 2010). Neutrophil regulation and egress from the bone marrow is predominantly CXCR4-dependent and in chronic liver injury over 50% of liver infiltrating cells are CXCR4 positive. Neutrophils contain large cytoplasmic stores of CXCR4, which can be rapidly expressed on the cell surface in response to cytokines, growth factors, or injurious stimuli (Link, 2005; Christopher and Link, 2007; Ramaiah and Jaeschke, 2007). During injury, bone marrow G-CSF levels increase leading to changes in the bone marrow CXCL12/CXCR4 axis and subsequent release of neutrophils (Lei et al., 2010). No study has directly

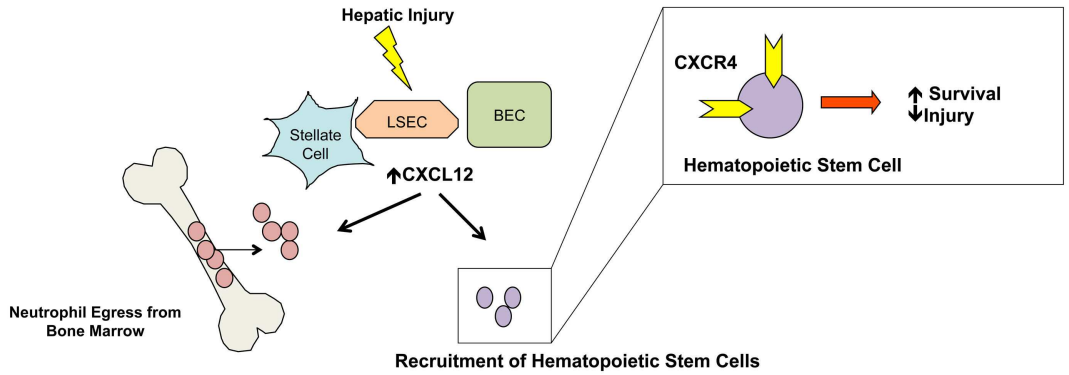


FIGURE 4 | CXCL12 stem cell mobilization and neutrophil egress. Increased expression of CXCL12 by stellate cells, endothelial cells, and biliary epithelial cells promotes migration of bone marrow stem cells to the liver. Additionally, neutrophil egress from the bone marrow is regulated by CXCL12 and increased levels of hepatic and serum levels promotes neutrophil egress. While not depicted, nearly 50% of liver infiltrating cells are CXCR4 positive and localized around CXCL12-rich periportal regions.

examined the effect of CXCR4-dependent neutrophil migration to the liver during acute injury, but based on studies of CXCR2-dependent neutrophil migration, the inhibition of CXCR4 driven neutrophil migration would be protective (Cao et al., 2000). Alternatively, inhibition of this axis alone may lead to an unfavorable outcome due to increased neutrophil egress from the bone marrow that migrate to the liver.

CXCL12 also plays a role in lymphocyte adhesion, migration, and extravasation into the liver parenchyma. Treatment of lymphocytes with CXCL12 increases ICAM-1 dependent tethering and can enhance tethering and firm adhesion required for transendothelial migration, suggesting a role for endothelial cell-derived CXCL12 (Campbell et al., 1998; Peled et al., 1999; Goddard et al., 2001). Additionally, liver sinusoidal endothelial cells can present abluminal CXCL12 on their luminal cell surface via transcytosis, which leads to T cell adhesion and migration across endothelial cell membranes (Schrage et al., 2008).

The role of bone marrow derived cells in ALI has been of great interest, with considerable progress made. It is unclear if bone marrow cells can directly replenish hepatocytes, however, injection of autologous bone marrow cells, or increased mobilization of bone marrow cells, are protective after ALI (Li et al., 2010; Possamai et al., 2010; Shi et al., 2010; Inagaki and Higashiyama, 2012; Takami et al., 2012; Zhao et al., 2012). In both animal models and clinical studies, delivery of bone marrow cells or agents that increase circulating bone marrow cells are advantageous. Whole bone marrow and different subpopulations of cells including CD34+, CD133+, mononuclear cells, mesenchymal stem cells, and endothelial stem cells are all reportedly beneficial. The specific bone marrow-derived cell type that is responsible for protection is controversial, but the CXCL12/CXCR4 axis is implicated in many of these models (Shafritz et al., 2006; Khurana and Mukhopadhyay, 2007; Oertel and Shafritz, 2008; Jin et al., 2009, 2010; Karlmark et al., 2009; Baldo et al., 2010).

Myeloid cells alone are sufficient to generate hepatocytes, but this only occurs in models of severe hepatic disease. In more mild models including chronic CCl_4 and bile duct ligation, these cells do not directly contribute to hepatocytes, but may be beneficial by secreting chemokines, cytokines, and growth factors thereby establishing a specific reparative niche (Kisseleva et al., 2010).

Rats administered a sublethal dose of acute CCl_4 along with G-CSF and AMD3100, have increased survival and accumulation of CD34+ cells around CXCL12-rich periportal areas (Mark et al., 2010). G-CSF releases bone marrow progenitor cells by decreasing expression of CXCL12 by bone marrow endothelium, and by activating osteoclasts, inducing their expression of proteolytic activity which cleaves cell surface CXCR4, further releasing hematopoietic stem cells into the periphery (Damon, 2009). Furthermore, in NOD/SCID mice injected with human CD34+ stem cells, liver engraftment is dependent on the CXCL12/CXCR4 axis. Neutralization of CXCR4 with a specific antibody inhibits engraftment, while hepatic administration of recombinant CXCL12 leads to increased engraftment. The location of the CD34+ cells around the CXCL12-rich periportal areas further demonstrates their dependence on CXCL12. Finally, induction of either acute or chronic liver injury with CCl_4 increases CXCR4 expression on human CD34+ cells and a greater degree of hepatic engraftment (Kollet et al., 2003).

CXCL12 can also bind the CXCR7 chemokine receptor. CXCR7, which also binds CXCL11, is classically thought to be a scavenging receptor capable of binding ligand, but unable to generate downstream signals, effectively sequestering the chemokine and preventing it from signaling through CXCR4 (Maksym et al., 2009; Luker et al., 2012; Sartina et al., 2012). Other receptors including DARC and D6 have similar scavenging properties, and their role in liver disease is apparent from mouse models deficient in the D6 receptor. D6 knockout mice exhibit prolonged liver damage after CCl_4 injury associated with increased hepatic levels of chemokines CCL2, CCL3, and CCL5 (Berres et al., 2009). Increased inflammatory chemokines resulted in greater inflammation highlighting the role of chemokine sequestration in the control of inflammation.

As discussed above, inflammatory cell recruitment by CXCR4 and other chemokine receptors is largely regulated by chemokine ligand expression. However, chemokine receptor expression on infiltrating cells is also tightly controlled and finely tunable. Cell surface expression of CXCR4 in particular is highly regulated

by factors including TGF-β, TNF-α, bacterial glycoproteins, and hypoxia.

Receptor expression can be controlled through transcriptional regulation and receptor internalization. TNF-α, IFN-γ, and LPS specifically reduces cell surface CXCR4 expression on neutrophils, but not lymphocytes, via receptor internalization in a time and dose dependent manner (Nagase et al., 2002; Kim et al., 2007). Paradoxically, however, CXCR4 mRNA levels are actually increased after stimulation, indicating that the change in cell surface CXCR4 expression is due to receptor internalization (Kim et al., 2007).

TGF-β is implicated in all forms of liver injury and has many functions in controlling immune cell differentiation and chemokine receptor expression. In both neutrophils and macrophages, TGF-β1 increases mRNA levels and cell surface expression of CXCR4 and enhances the effect of CXCL12 stimulation (Nagase et al., 2002; Chen et al., 2005). Furthermore, treatment of both immature and mature dendritic cells with TGF-β decreases the expression of CCR7 and increases expression of CCR1, CCR3, CCR5, and CXCR4 leading to a chemokine receptor profile that preferentially migrates toward sites of inflammation (Sato et al., 2000).

The liver with its dual arterial and venous blood supply has a low oxygen tension, which is worsened during injury, leading to a hypoxic environment. Monocytes and macrophages increase mRNA and cell surface expression of CXCR4 in response to hypoxic conditions in a HIF-1α-dependent mechanism, whereas transcript levels of CCR5 are unaffected (Schioppa et al., 2003). CXCL12 production by endothelial cells is induced by hypoxia, further increasing the effect of CXCL12/CXCR4 axis during hypoxia (Hitchon et al., 2002; Santiago et al., 2011).

ADDITIONAL CHEMOKINES IN ACUTE LIVER INJURY

Two additional chemokines whose expression is induced during ALI are CCL5 (RANTES) and CX_3CL1 (Fractalkine). CCL5 which binds to both CCR1 and CCR5 is of particular interest given the availability of maraviroc, a CCR5 small molecule inhibitor (Proudfoot et al., 2010). NK and NKT cells, CD4+ T cells, macrophages, and hepatic stellate cells all express CCR5 and in models of ConA induced hepatitis CCR5 deficiency promotes liver failure by preventing NKT cell activation-induced apoptosis (Ajuebor et al., 2006). Mice deficient for CCR5 show an unexpected increase in CCL5 ligand, perhaps due to loss of a negative feedback through CCR5, and an increase in CCR1-dependent NK cell recruitment and worse injury (Ajuebor et al., 2007). Within the liver CCR5 is expressed by stellate cells and is important in promoting their migration during chronic injury (Schwabe et al., 2003; Seki et al., 2009). The role of CCR5 on stellate cells has not been elucidated in models of acute injury.

CX_3CL1, the only member of the *CX_3C* family, is unique in that it is synthesized as a transmembrane protein and can be released by metalloproteinase cleavage. CX_3CR1 is increased in patients with either chronic or acute liver disease specifically in areas of inflammation and in regenerating bile duct epithelia (Efsen et al., 2002). Similar to CCR5, loss of CX_3CR1 leads to greater injury and delayed recovery after acute and chronic CCl_4. Loss of CX_3CR1 on macrophages promotes increased apoptosis and a more pro-inflammatory phenotype (Aoyama et al., 2010). As different waves of macrophages are important in injury and resolution of ALI, similar to CCL2, CX_3CL1 may be important in the different stages of macrophage recruitment to the liver.

THERAPEUTIC POTENTIAL OF CHEMOKINE RECEPTOR INHIBITION

The development of small molecule chemokine inhibitors and neutralizing antibodies provides promise for modulating immune cell infiltration during inflammatory diseases. A number of trials have examined the use of small molecule inhibitors in autoimmune and infectious diseases (Proudfoot et al., 2010); however, to date no clinical trial has examined the use of small molecule inhibitors directly in liver disease. Despite the large amount of data generated from knockout mice, inhibition of these axes in human disease states has not recapitulated pre-clinical data. Blocking CCR2 or inhibiting CCL2 in patients with rheumatoid arthritis (RA) did not achieve clinical endpoints (Haringman et al., 2006; Vergunst et al., 2008). Similarly, trials targeting CCR1 for RA, psoriasis, or multiple sclerosis have failed despite strong pre-clinical data (Trebst et al., 2001; Proudfoot et al., 2010).

There are numerous hurdles in the development of such inhibitors. Chemokine redundancy, with most receptors binding numerous ligands and receptor heterodimerization ensures that a single molecule will not achieve full inhibition of a given ligand. Additionally, the timing of chemokine inhibition during the disease process may be crucial and the window of effective inhibition narrow. For instance, the CXCR1/CXCR2 inhibitor, reparixin, has no effect on hepatic I/R injury when administered immediately after the injury, yet when administered 24 h after injury led to a worse outcome (Clarke et al., 2011). Similarly, in murine models of cardiac ischemia, AMD3100 can either increase or decrease the extent of injury based on the dosing protocol (Dai et al., 2010; Jujo et al., 2010). Finally, the choice of disease may determine if an inhibitor is successful. Mozobil (AMD3100), a CXCR4 inhibitor, is currently used for hematopoietic stem cell mobilization, yet failed as an HIV-entry inhibitor. Despite these difficulties successful chemokine inhibitors have been developed including maraviroc (CCR5 HIV-entry inhibitor) which may also show beneficial effects in patients with liver disease (Macias et al., 2012).

CONCLUSION

Chemokines are a large family of molecules whose function has expanded to now include immune cell infiltration, cellular survival and proliferation, vascular angiogenesis, organ fibrogenesis, and tumor metastasis. The interactions between chemokines, cytokines, and growth factors add additional levels of complexity to the networks and allow for spatial and temporal regulation. While most studies have focused on their adverse role in acute liver disease, a growing number of studies have revealed the beneficial role that they may play during injury as well. The availability of small molecule inhibitors for many chemokine axes makes them promising targets for therapeutics. However, given the many roles of chemokines during disease, a greater understanding of the underlying mechanisms is mandated.

ACKNOWLEDGMENTS

Work in the author's laboratory is supported by RO1-DK56621, NIDDK F30-DK090986 to Yedidya Saiman and MSTP Training Grant T32-GM007280.

REFERENCES

Ajuebor, M. N., Carey, J. A., and Swain, M. G. (2006). CCR5 in T cell-mediated liver diseases: what's going on? *J. Immunol.* 177, 2039–2045.

Ajuebor, M. N., Hogaboam, C. M., Le, T., and Swain, M. G. (2003). C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. *J. Immunol.* 170, 5252–5259.

Ajuebor, M. N., Wondimu, Z., Hogaboam, C. M., Le, T., Proudfoot, A. E., and Swain, M. G. (2007). CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. *Am. J. Pathol.* 170, 1975–1988.

Antoniades, C. G., Quaglia, A., Taams, L. S., Mitry, R. R., Hussain, M., Abeles, R., Possamai, L. A., Bruce, M., Mcphail, M., Starling, C., Wagner, B., Barnardo, A., Pomplun, S., Auzinger, G., Bernal, W., Heaton, N., Vergani, D., Thursz, M. R., and Wendon, J. (2012). Source and characterisation of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology*. doi: 10.1002/hep.25657. [Epub ahead of print].

Aoyama, T., Inokuchi, S., Brenner, D. A., and Seki, E. (2010). CX3CL1-CX3CR1 interaction prevents carbon tetrachloride-induced liver inflammation and fibrosis in mice. *Hepatology* 52, 1390–1400.

Apolinario, A., Majano, P. L., Alvarez-Perez, E., Saez, A., Lozano, C., Vargas, J., and Garcia-Monzon, C. (2002). Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. *Am. J. Gastroenterol.* 97, 2861–2870.

Bajetto, A., Bonavia, R., Barbero, S., and Schettini, G. (2002). Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. *J. Neurochem.* 82, 1311–1329.

Baldo, G., Giugliani, R., Uribe, C., Belardinelli, M. C., Duarte, M. E., Meurer, L., Da Silveira, T. R., and Matte, U. (2010). Bone marrow mononuclear cell transplantation improves survival and induces hepatocyte proliferation in rats after CCl(4) acute liver damage. *Dig. Dis. Sci.* 55, 3384–3392.

Berres, M. L., Trautwein, C., Zaldivar, M. M., Schmitz, P., Pauels, K., Lira, S. A., Tacke, F., and Wasmuth, H. E. (2009). The chemokine scavenging receptor D6 limits acute toxic liver injury in vivo. *Biol. Chem.* 390, 1039–1045.

Bone-Larson, C. L., Hogaboam, C. M., Evanhoff, H., Strieter, R. M., and Kunkel, S. L. (2001). IFN-gamma-inducible protein-10 (CXCL10) is hepatoprotective during acute liver injury through the induction of CXCR2 on hepatocytes. *J. Immunol.* 167, 7077–7083.

Bromley, S. K., Mempel, T. R., and Luster, A. D. (2008). Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat. Immunol.* 9, 970–980.

Campbell, J. J., Hedrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A., and Butcher, E. C. (1998). Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* 279, 381–384.

Cao, X., Zhang, W., Wan, T., He, L., Chen, T., Yuan, Z., Ma, S., Yu, Y., and Chen, G. (2000). Molecular cloning and characterization of a novel CXC chemokine macrophage inflammatory protein-2 gamma chemoattractant for human neutrophils and dendritic cells. *J. Immunol.* 165, 2588–2595.

Chen, S., Tuttle, D. L., Oshier, J. T., Knot, H. J., Streit, W. J., Goodenow, M. M., and Harrison, J. K. (2005). Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology* 114, 565–574.

Chen, X., Xu, C., Zhang, F., and Ma, J. (2010). Comparative analysis of expression profiles of chemokines, chemokine receptors, and components of signaling pathways mediated by chemokines in eight cell types during rat liver regeneration. *Genome* 53, 608–618.

Chen, Y. X., Sato, M., Kawachi, K., and Abe, Y. (2006). Neutrophil-mediated liver injury during hepatic ischemia-reperfusion in rats. *HBPD INT* 5, 436–442.

Christopher, M. J., and Link, D. C. (2007). Regulation of neutrophil homeostasis. *Curr. Opin. Hematol.* 14, 3–8.

Clarke, C., Kuboki, S., Sakai, N., Kasten, K. R., Tevar, A. D., Schuster, R., Blanchard, J., Caldwell, C. C., Edwards, M. J., and Lentsch, A. B. (2011). CXC chemokine receptor-1 is expressed by hepatocytes and regulates liver recovery after hepatic ischemia/reperfusion injury. *Hepatology* 53, 261–271.

Cole, K. E., Strick, C. A., Paradis, T. J., Ogborne, K. T., Loetscher, M., Gladue, R. P., Lin, W., Boyd, J. G., Moser, B., Wood, D. E., Sahagan, B. G., and Neote, K. (1998). Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J. Exp. Med.* 187, 2009–2021.

Crosby, H. A., Lalor, P. F., Ross, E., Newsome, P. N., and Adams, D. H. (2009). Adhesion of human haematopoietic (CD34+) stem cells to human liver compartments is integrin and CD44 dependent and modulated by CXCR3 and CXCR4. *J. Hepatol.* 51, 734–749.

Dai, S., Yuan, F., Mu, J., Li, C., Chen, N., Guo, S., Kingery, J., Prabhu, S. D., Bolli, R., and Rokosh, G. (2010). Chronic AMD3100 antagonism of SDF-1alpha-CXCR4 exacerbates cardiac dysfunction and remodeling after myocardial infarction. *J. Mol. Cell. Cardiol.* 49, 587–597.

Dambach, D. M., Watson, L. M., Gray, K. R., Durham, S. K., and Laskin, D. L. (2002). Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. *Hepatology* 35, 1093–1103.

Damon, L. E. (2009). Mobilization of hematopoietic stem cells into the peripheral blood. *Expert Rev. Hematol.* 2, 717–733.

Dong, Z., Zhang, C., Wei, H., Sun, R., and Tian, Z. (2005). Impaired NK cell cytotoxicity by high level of interferon-gamma in concanavalin A-induced hepatitis. *Can. J. Physiol. Pharmacol.* 83, 1045–1053.

Duffield, J. S. (2003). The inflammatory macrophage: a story of Jekyll and Hyde. *Clin. Sci.* 104, 27–38.

Duffield, J. S., Forbes, S. J., Constandinou, C. M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R., and Iredale, J. P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J. Clin. Invest.* 115, 56–65.

Efsen, E., Grappone, C., Defranco, R. M., Milani, S., Romanelli, R. G., Bonacchi, A., Caligiuri, A., Failli, P., Annunziato, F., Pagliai, G., Pinzani, M., Laffi, G., Gentilini, P., and Marra, F. (2002). Up-regulated expression of fractalkine and its receptor CX3CR1 during liver injury in humans. *J. Hepatol.* 37, 39–47.

Erhardt, A., Wegscheid, C., Claass, B., Carambia, A., Herkel, J., Mittrucker, H. W., Panzer, U., and Tiegs, G. (2011). CXCR3 deficiency exacerbates liver disease and abrogates tolerance in a mouse model of immune-mediated hepatitis. *J. Immunol.* 186, 5284–5293.

Farber, J. M. (1990). A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5238–5242.

Fernandez, E. J., and Lolis, E. (2002). Structure, function, and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.* 42, 469–499.

Friedman, S. L. (2008a). Hepatic fibrosis-overview. *Toxicology* 254, 120–129.

Friedman, S. L. (2008b). Hepatic stellate cells – protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172.

Gangavarapu, P., Rajagopalan, L., Kolli, D., Guerrero-Plata, A., Garofalo, R. P., and Rajarathnam, K. (2012). The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment. *J. Leukoc. Biol.* 91, 259–265.

Gerritsma, J. S., Van Kooten, C., Gerritsen, A. F., Van Es, L. A., and Daha, M. R. (1998). Transforming growth factor-beta 1 regulates chemokine and complement production by human proximal tubular epithelial cells. *Kidney Int.* 53, 609–616.

Goddard, S., Williams, A., Morland, C., Qin, S., Gladue, R., Hubscher, S. G., and Adams, D. H. (2001). Differential expression of chemokines and chemokine receptors shapes the inflammatory response in rejecting human liver transplants. *Transplantation* 72, 1957–1967.

Groom, J. R., and Luster, A. D. (2011). CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol. Cell Biol.* 89, 207–215.

Haringman, J. J., Gerlag, D. M., Smeets, T. J., Baeten, D., Van Den Bosch, F., Bresnihan, B., Breedveld, F. C., Dinant, H. J., Legay, F., Gram, H., Loetscher, P., Schmouder, R., Woodworth, T., and Tak, P. P. (2006). A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis Rheum.* 54, 2387–2392.

Harvey, C. E., Post, J. J., Palladinetti, P., Freeman, A. J., Ffrench, R. A., Kumar, R. K., Marinos, G., and Lloyd, A. R.

(2003). Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. *J. Leukoc. Biol.* 74, 360–369.

Hassan, M., Selimovic, D., Ghozlan, H., and Abdel-Kader, O. (2007). Induction of high-molecular-weight (HMW) tumor necrosis factor(TNF) alpha by hepatitis C virus (HCV) non-structural protein 3 (NS3) in liver cells is AP-1 and NF-kappaB-dependent activation. *Cell. Signal.* 19, 301–311.

Hitchon, C., Wong, K., Ma, G., Reed, J., Lyttle, D., and El-Gabalawy, H. (2002). Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. *Arthritis Rheum.* 46, 2587–2597.

Hogaboam, C. M., Bone-Larson, C. L., Steinhauser, M. L., Lukacs, N. W., Colletti, L. M., Simpson, K. J., Strieter, R. M., and Kunkel, S. L. (1999a). Novel CXCR2-dependent liver regenerative qualities of ELR-containing CXC chemokines. *FASEB J.* 13, 1565–1574.

Hogaboam, C. M., Simpson, K. J., Chensue, S. W., Steinhauser, M. L., Lukacs, N. W., Gauldie, J., Strieter, R. M., and Kunkel, S. L. (1999b). Macrophage inflammatory protein-2 gene therapy attenuates adenovirus- and acetaminophen-mediated hepatic injury. *Gene Ther.* 6, 573–584.

Hogaboam, C. M., Bone-Larson, C. L., Steinhauser, M. L., Matsukawa, A., Gosling, J., Boring, L., Charo, I. F., Simpson, K. J., Lukacs, N. W., and Kunkel, S. L. (2000). Exaggerated hepatic injury due to acetaminophen challenge in mice lacking C-C chemokine receptor 2. *Am. J. Pathol.* 156, 1245–1252.

Hokeness, K. L., Deweerd, E. S., Munks, M. W., Lewis, C. A., Gladue, R. P., and Salazar-Mather, T. P. (2007). CXCR3-dependent recruitment of antigen-specific T lymphocytes to the liver during murine cytomegalovirus infection. *J. Virol.* 81, 1241–1250.

Hokeness, K. L., Kuziel, W. A., Biron, C. A., and Salazar-Mather, T. P. (2005). Monocyte chemoattractant protein-1 and CCR2 interactions are required for IFN-alpha/beta-induced inflammatory responses and antiviral defense in liver. *J. Immunol.* 174, 1549–1556.

Holt, M. P., Cheng, L., and Ju, C. (2008). Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J. Leukoc. Biol.* 84, 1410–1421.

Hong, F., Tuyama, A., Lee, T. F., Loke, J., Agarwal, R., Cheng, X., Garg, A., Fiel, M. I., Schwartz, M., Walewski, J., Branch, A., Schecter, A. D., and Bansal, M. B. (2009). Hepatic stellate cells express functional CXCR4: role in stromal cell-derived factor-1alpha-mediated stellate cell activation. *Hepatology* 49, 2055–2067.

Horiguchi, K., Kitagawa-Sakakida, S., Sawa, Y., Li, Z. Z., Fukushima, N., Shirakura, R., and Matsuda, H. (2002). Selective chemokine and receptor gene expressions in allografts that develop transplant vasculopathy. *J. Heart Lung Transplant.* 21, 1090–1100.

Hu, B., and Colletti, L. M. (2010). CXC receptor-2 knockout genotype increases X-linked inhibitor of apoptosis protein and protects mice from acetaminophen hepatotoxicity. *Hepatology* 52, 691–702.

Ilmakunnas, M., Hockerstedt, K., Makisalo, H., Siitonen, S., Repo, H., and Pesonen, E. J. (2010). Hepatic IL-8 release during graft procurement is associated with impaired graft function after human liver transplantation. *Clin. Transplant.* 24, 29–35.

Inagaki, Y., Nemoto, T., Kushida, M., Sheng, Y., Higashi, K., Ikeda, K., Kawada, N., Shirasaki, F., Takehara, K., Sugiyama, K., Fujii, M., Yamauchi, H., Nakao, A., De Crombrugghe, B., Watanabe, T., and Okazaki, I. (2003). Interferon alfa down-regulates collagen gene transcription and suppresses experimental hepatic fibrosis in mice. *Hepatology* 38, 890–899.

Ishida, Y., Kondo, T., Kimura, A., Tsuneyama, K., Takayasu, T., and Mukaida, N. (2006). Opposite roles of neutrophils and macrophages in the pathogenesis of acetaminophen-induced acute liver injury. *Eur. J. Immunol.* 36, 1028–1038.

Jaeschke, H., Bautista, A. P., Spolarics, Z., and Spitzer, J. J. (1991). Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radic. Res. Commun.* 15, 277–284.

Jaeschke, H., and Farhood, A. (2002). Kupffer cell activation after no-flow ischemia versus hemorrhagic shock. *Free Radic. Biol. Med.* 33, 210–219.

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., and Lemasters, J. J. (2002). Mechanisms of hepatotoxicity. *Toxicol. Sci.* 65, 166–176.

James, L. P., Farrar, H. C., Darville, T. L., Sullivan, J. E., Givens, T. G., Kearns, G. L., Wasserman, G. S., Simpson, P. M., and Hinson, J. A. (2001). Elevation of serum interleukin 8 levels in acetaminophen overdose in children and adolescents. *Clin. Pharmacol. Ther.* 70, 280–286.

Jaruga, B., Hong, F., Kim, W. H., and Gao, B. (2004). IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G1044–G1052.

Jin, S. Z., Meng, X. W., Han, M. Z., Sun, X., Sun, L. Y., and Liu, B. R. (2009). Stromal cell derived factor-1 enhances bone marrow mononuclear cell migration in mice with acute liver failure. *World J. Gastroenterol.* 15, 2657–2664.

Jin, S. Z., Meng, X. W., Sun, X., Han, M. Z., Liu, B. R., Wang, X. H., Sun, L. Y., Huang, Q., Zhao, R. B., Ban, X., Yu, H. Y., and Yu, H. W. (2010). Granulocyte colony-stimulating factor enhances bone marrow mononuclear cell homing to the liver in a mouse model of acute hepatic injury. *Dig. Dis. Sci.* 55, 2805–2813.

Ju, C., Reilly, T. P., Bourdi, M., Radonovich, M. F., Brady, J. N., George, J. W., and Pohl, L. R. (2002). Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem. Res. Toxicol.* 15, 1504–1513.

Jujo, K., Hamada, H., Iwakura, A., Thorne, T., Sekiguchi, H., Clarke, T., Ito, A., Misener, S., Tanaka, T., Klyachko, E., Kobayashi, K., Tongers, J., Roncalli, J., Tsurumi, Y., Hagiwara, N., and Losordo, D. W. (2010). CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11008–11013.

Karlmark, K. R., Wasmuth, H. E., Trautwein, C., and Tacke, F. (2008). Chemokine-directed immune cell infiltration in acute and chronic liver disease. *Expert Rev. Gastroenterol. Hepatol.* 2, 233–242.

Karlmark, K. R., Weiskirchen, R., Zimmermann, H. W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C., and Tacke, F. (2009). Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 50, 261–274.

Khurana, S., and Mukhopadhyay, A. (2007). Characterization of the potential subpopulation of bone marrow cells involved in the repair of injured liver tissue. *Stem Cells* 25, 1439–1447.

Kim, H. K., Kim, J. E., Chung, J., Han, K. S., and Cho, H. I. (2007). Surface expression of neutrophil CXCR4 is down-modulated by bacterial endotoxin. *Int. J. Hematol.* 85, 390–396.

Kisseleva, T., Gigante, E., and Brenner, D. A. (2010). Recent advances in liver stem cell therapy. *Curr. Opin. Gastroenterol.* 26, 395–402.

Kobayashi, Y. (2008). The role of chemokines in neutrophil biology. *Front. Biosci.* 13, 2400–2407.

Kolios, G., Valatas, V., Manousou, P., Xidakis, C., Notas, G., and Kouroumalis, E. (2008). Nitric oxide and MCP-1 regulation in LPS activated rat Kupffer cells. *Mol. Cell. Biochem.* 319, 91–98.

Kollet, O., Shivtiel, S., Chen, Y. Q., Suriawinata, J., Thung, S. N., Dabeva, M. D., Kahn, J., Spiegel, A., Dar, A., Samira, S., Goichberg, P., Kalinkovich, A., Arenzana-Seisdedos, F., Nagler, A., Hardan, I., Revel, M., Shafritz, D. A., and Lapidot, T. (2003). HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. *J. Clin. Invest.* 112, 160–169.

Krohn, N., Kapoor, S., Enami, Y., Follenzi, A., Bandi, S., Joseph, B., and Gupta, S. (2009). Hepatocyte transplantation-induced liver inflammation is driven by cytokines-chemokines associated with neutrophils and Kupffer cells. *Gastroenterology* 136, 1806–1817.

Kuboki, S., Shin, T., Huber, N., Eismann, T., Galloway, E., Schuster, R., Blanchard, J., Edwards, M. J., and Lentsch, A. B. (2008). Hepatocyte signaling through CXC chemokine receptor-2 is detrimental to liver recovery after ischemia/reperfusion in mice. *Hepatology* 48, 1213–1223.

Laskin, D. L., Gardner, C. R., Price, V. F., and Jollow, D. J. (1995). Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology* 21, 1045–1050.

Lei, Y., Liu, Z., Han, Q., Kang, W., Zhang, L., and Lou, S. (2010). G-CSF enhanced SDF-1 gradient between bone marrow and liver associated with mobilization of peripheral blood CD34+ cells in rats with acute liver failure. *Dig. Dis. Sci.* 55, 285–291.

Leifeld, L., Dumoulin, F. L., Purr, I., Janberg, K., Trautwein, C., Wolff, M., Manns, M. P., Sauerbruch, T., and Spengler, U. (2003). Early

up-regulation of chemokine expression in fulminant hepatic failure. *J. Pathol.* 199, 335–344.

Li, N., Zhang, L., Li, H., and Fang, B. (2010). Human CD34+ cells mobilized by granulocyte colony-stimulating factor ameliorate radiation-induced liver damage in mice. *Stem Cell Res. Ther.* 1, 22.

Liekens, S., Schols, D., and Hatse, S. (2010). CXCL12-CXCR4 axis in angiogenesis, metastasis and stem cell mobilization. *Curr. Pharm. Des.* 16, 3903–3920.

Link, D. C. (2005). Neutrophil homeostasis: a new role for stromal cell-derived factor-1. *Immunol. Res.* 32, 169–178.

Luker, K. E., Lewin, S. A., Mihalko, L. A., Schmidt, B. T., Winkler, J. S., Coggins, N. L., Thomas, D. G., and Luker, G. D. (2012). Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells. *Oncogene.* doi: 10.1038/onc.2011.633. [Epub ahead of print].

Luster, A. D., Unkeless, J. C., and Ravetch, J. V. (1985). Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature* 315, 672–676.

Macias, J., Viloria, M. M., Rivero, A., De Los Santos, I., Marquez, M., Portilla, J., Di Lello, F., Camacho, A., Sanz-Sanz, J., Ojeda, G., Mata, R., Gomez-Mateos, J., and Pineda, J. A. (2012). Lack of short-term increase in serum mediators of fibrogenesis and in non-invasive markers of liver fibrosis in HIV/hepatitis C virus-coinfected patients starting maraviroc-based antiretroviral therapy. *Eur. J. Clin. Microbiol. Infect. Dis.* doi: 10.1007/s10096-012-1546-5. [Epub ahead of print].

Majumder, S., Zhou, L. Z., Chaturvedi, P., Babcock, G., Aras, S., and Ransohoff, R. M. (1998). p48/STAT-1alpha-containing complexes play a predominant role in induction of IFN-gamma-inducible protein, 10 kDa (IP-10) by IFN-gamma alone or in synergy with TNF-alpha. *J. Immunol.* 161, 4736–4744.

Maksym, R. B., Tarnowski, M., Grymula, K., Tarnowska, J., Wysoczynski, M., Liu, R., Czerny, B., Ratajczak, J., Kucia, M., and Ratajczak, M. Z. (2009). The role of stromal-derived factor-1–CXCR7 axis in development and cancer. *Eur. J. Pharmacol.* 625, 31–40.

Mark, A. L., Sun, Z., Warren, D. S., Lonze, B. E., Knabel, M. K., Melville Williams, G. M., Locke, J. E., Montgomery, R. A., and Cameron, A. M. (2010). Stem cell mobilization is life saving in an animal model of acute liver failure. *Ann. Surg.* 252, 591–596.

Medoff, B. D., Wain, J. C., Seung, E., Jackobek, R., Means, T. K., Ginns, L. C., Farber, J. M., and Luster, A. D. (2006). CXCR3 and its ligands in a murine model of obliterative bronchiolitis: regulation and function. *J. Immunol.* 176, 7087–7095.

Michael, S. L., Pumford, N. R., Mayeux, P. R., Niesman, M. R., and Hinson, J. A. (1999). Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. *Hepatology* 30, 186–195.

Mukaida, N., and Baba, T. (2012). Chemokines in tumor development and progression. *Exp. Cell Res.* 318, 95–102.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382, 635–638.

Nagase, H., Miyamasu, M., Yamaguchi, M., Imanishi, M., Tsuno, N. H., Matsushima, K., Yamamoto, K., Morita, Y., and Hirai, K. (2002). Cytokine-mediated regulation of CXCR4 expression in human neutrophils. *J. Leukoc. Biol.* 71, 711–717.

Nasser, M. W., Raghuwanshi, S. K., Grant, D. J., Jala, V. R., Rajarathnam, K., and Richardson, R. M. (2009). Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *J. Immunol.* 183, 3425–3432.

Oertel, M., and Shafritz, D. A. (2008). Stem cells, cell transplantation and liver repopulation. *Biochim. Biophys. Acta* 1782, 61–74.

Ohmori, Y., and Hamilton, T. A. (1993). Cooperative interaction between interferon (IFN) stimulus response element and kappa B sequence motifs controls IFN gamma- and lipopolysaccharide-stimulated transcription from the murine IP-10 promoter. *J. Biol. Chem.* 268, 6677–6688.

Ohmori, Y., and Hamilton, T. A. (1997). IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages. *J. Immunol.* 159, 5474–5482.

Ohmori, Y., Schreiber, R. D., and Hamilton, T. A. (1997). Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J. Biol. Chem.* 272, 14899–14907.

Ohmori, Y., Wyner, L., Narumi, S., Armstrong, D., Stoler, M., and Hamilton, T. A. (1993). Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines in vivo. *Am. J. Pathol.* 142, 861–870.

Oo, Y. H., Shetty, S., and Adams, D. H. (2010). The role of chemokines in the recruitment of lymphocytes to the liver. *Dig. Dis.* 28, 31–44.

Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. (1999). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. *J. Clin. Invest.* 104, 1199–1211.

Possamai, L. A., Antoniades, C. G., Anstee, Q. M., Quaglia, A., Vergani, D., Thursz, M., and Wendon, J. (2010). Role of monocytes and macrophages in experimental and human acute liver failure. *World J. Gastroenterol.* 16, 1811–1819.

Proudfoot, A. E., Power, C. A., and Schwarz, M. K. (2010). Anti-chemokine small molecule drugs: a promising future? *Expert Opin. Investig. Drugs* 19, 345–355.

Ramaiah, S. K., and Jaeschke, H. (2007). Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol. Pathol.* 35, 757–766.

Rossi, D. L., Hurst, S. D., Xu, Y., Wang, W., Menon, S., Coffman, R. L., and Zlotnik, A. (1999). Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. *J. Immunol.* 162, 5490–5497.

Sahin, H., Borkham-Kamphorst, E., Kuppe, C., Zaldivar, M. M., Grouls, C., Al-Samman, M., Nellen, A., Schmitz, P., Heinrichs, D., Berres, M. L., Doleschel, D., Scholten, D., Weiskirchen, R., Moeller, M. J., Kiessling, F., Trautwein, C., and Wasmuth, H. E. (2012). The chemokine Cxcl9 attenuates liver fibrosis-associated angiogenesis in mice. *Hepatology.* doi: 10.1002/hep.25545. [Epub ahead of print].

Sakai, N., Kuboki, S., Van Sweringen, H. L., Tevar, A. D., Schuster, R., Blanchard, J., Edwards, M. J., and Lentsch, A. B. (2011). CXCR1 deficiency does not alter liver regeneration after partial hepatectomy in mice. *Transplant. Proc.* 43, 1967–1970.

Santiago, B., Calonge, E., Del Rey, M. J., Gutierrez-Canas, I., Izquierdo, E., Usategui, A., Galindo, M., Alcami, J., and Pablos, J. L. (2011). CXCL12 gene expression is upregulated by hypoxia and growth arrest but not by inflammatory cytokines in rheumatoid synovial fibroblasts. *Cytokine* 53, 184–190.

Sartina, E., Suguihara, C., Ramchandran, S., Nwajei, P., Rodriguez, M., Torres, E., Hehre, D., Devia, C., Walters, M. J., Penfold, M. E., and Young, K. C. (2012). Antagonism of CXCR7 attenuates chronic hypoxia-induced pulmonary hypertension. *Pediatr. Res.* 71, 682–688.

Sato, K., Kawasaki, H., Nagayama, H., Enomoto, M., Morimoto, C., Tadokoro, K., Juji, T., and Takahashi, T. A. (2000). TGF-beta 1 reciprocally controls chemotaxis of human peripheral blood monocyte-derived dendritic cells via chemokine receptors. *J. Immunol.* 164, 2285–2295.

Sawitza, I., Kordes, C., Reister, S., and Haussinger, D. (2009). The niche of stellate cells within rat liver. *Hepatology* 50, 1617–1624.

Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., Bernasconi, S., Saccani, S., Nebuloni, M., Vago, L., Mantovani, A., Melillo, G., and Sica, A. (2003). Regulation of the chemokine receptor CXCR4 by hypoxia. *J. Exp. Med.* 198, 1391–1402.

Schrage, A., Wechsung, K., Neumann, K., Schumann, M., Schulzke, J. D., Engelhardt, B., Zeitz, M., Hamann, A., and Klugewitz, K. (2008). Enhanced T cell transmigration across the murine liver sinusoidal endothelium is mediated by transcytosis and surface presentation of chemokines. *Hepatology* 48, 1262–1272.

Schwabe, R. F., Bataller, R., and Brenner, D. A. (2003). Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285, G949–G958.

Seki, E., De Minicis, S., Gwak, G. Y., Kluwe, J., Inokuchi, S., Bursill, C. A., Llovet, J. M., Brenner, D. A., and Schwabe, R. F. (2009). CCR1 and CCR5 promote hepatic fibrosis in mice. *J. Clin. Invest.* 119, 1858–1870.

Shafritz, D. A., Oertel, M., Menthena, A., Nierhoff, D., and Dabeva, M. D. (2006). Liver stem cells and prospects for liver reconstitution by transplanted cells. *Hepatology* 43, S89–S98.

Shi, X. L., Gu, J. Y., Han, B., Xu, H. Y., Fang, L., and Ding, Y. T. (2010). Magnetically labeled mesenchymal stem cells after autologous transplantation into acutely injured

liver. *World J. Gastroenterol.* 16, 3674–3679.

Shibuta, K., Mori, M., Shimoda, K., Inoue, H., Mitra, P., and Barnard, G. F. (2002). Regional expression of CXCL12/CXCR4 in liver and hepatocellular carcinoma and cell-cycle variation during in vitro differentiation. *Jpn. J. Cancer Res.* 93, 789–797.

Shields, P. L., Morland, C. M., Salmon, M., Qin, S., Hubscher, S. G., and Adams, D. H. (1999). Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J. Immunol.* 163, 6236–6243.

Stefanovic, L., and Stefanovic, B. (2006). Mechanism of direct hepatotoxic effect of KC chemokine: sequential activation of gene expression and progression from inflammation to necrosis. *J. Interferon Cytokine Res.* 26, 760–770.

Steib, C. J., Bilzer, M., Hartl, J. M., Beitinger, F., Gulberg, V., Goke, B., and Gerbes, A. L. (2010). Kupffer cell activation by hydrogen peroxide: a new mechanism of portal pressure increase. *Shock* 33, 412–418.

Takami, T., Terai, S., and Sakaida, I. (2012). Stem cell therapy in chronic liver disease. *Curr. Opin. Gastroenterol.* 28, 203–208.

Tensen, C. P., Flier, J., Rampersad, S. S., Sampat-Sardjoepersad, S., Scheper, R. J., Boorsma, D. M., and Willemze, R. (1999). Genomic organization, sequence and transcriptional regulation of the human CXCL 11(1) gene. *Biochim. Biophys. Acta* 1446, 167–172.

Trebst, C., Sorensen, T. L., Kivisakk, P., Cathcart, M. K., Hesselgesser, J., Horuk, R., Sellebjerg, F., Lassmann, H., and Ransohoff, R. M. (2001). CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. *Am. J. Pathol.* 159, 1701–1710.

Tsou, C. L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A. M., Weisberg, S. P., Mack, M., and Charo, I. F. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* 117, 902–909.

Vergunst, C. E., Gerlag, D. M., Lopatinskaya, L., Klareskog, L., Smith, M. D., Van Den Bosch, F., Dinant, H. J., Lee, Y., Wyant, T., Jacobson, E. W., Baeten, D., and Tak, P. P. (2008). Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial. *Arthritis Rheum.* 58, 1931–1939.

Wald, O., Pappo, O., Safadi, R., Dagan-Berger, M., Beider, K., Wald, H., Franitza, S., Weiss, I., Avniel, S., Boaz, P., Hanna, J., Zamir, G., Eid, A., Mandelboim, O., Spengler, U., Galun, E., and Peled, A. (2004). Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. *Eur. J. Immunol.* 34, 1164–1174.

Wasmuth, H. E., Lammert, F., Zaldivar, M. M., Weiskirchen, R., Hellerbrand, C., Scholten, D., Berres, M. L., Zimmermann, H., Streetz, K. L., Tacke, F., Hillebrandt, S., Schmitz, P., Keppeler, H., Berg, T., Dahl, E., Gassler, N., Friedman, S. L., and Trautwein, C. (2009). Antifibrotic effects of CXCL9 and its receptor CXCR3 in livers of mice and humans. *Gastroenterology* 137, 309-319; 319.e301–319.e303.

Wasmuth, H. E., Tacke, F., and Trautwein, C. (2010). Chemokines in liver inflammation and fibrosis. *Semin. Liver Dis.* 30, 215–225.

Yang, C. H., Wei, L., Pfeffer, S. R., Du, Z., Murti, A., Valentine, W. J., Zheng, Y., and Pfeffer, L. M. (2007). Identification of CXCL11 as a STAT3-dependent gene induced by IFN. *J. Immunol.* 178, 986–992.

Zaldivar, M. M., Berres, M. L., Sahin, H., Nellen, A., Heinrichs, D., Schmitz, P., Gassler, N., Streetz, K. L., Trautwein, C., and Wasmuth, H. E. (2012). The chemokine receptor CXCR3 limits injury after acute toxic liver damage. *Lab. Invest.* 92, 724–734.

Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Koppel, T., Jahantigh, M. N., Lutgens, E., Wang, S., Olson, E. N., Schober, A., and Weber, C. (2009). Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci. Signal.* 2, ra81.

Zhao, W., Li, J. J., Cao, D. Y., Li, X., Zhang, L. Y., He, Y., Yue, S. Q., Wang, D. S., and Dou, K. F. (2012). Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. *World J. Gastroenterol.* 18, 1048–1058.

Zimmermann, H. W., Seidler, S., Gassler, N., Nattermann, J., Luedde, T., Trautwein, C., and Tacke, F. (2011). Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. *PLoS ONE* 6, e21381. doi:10.1371/journal.pone.0021381

Zlotnik, A., Burkhardt, A. M., and Homey, B. (2011). Homeostatic chemokine receptors and organ-specific metastasis. *Nat. Rev. Immunol.* 11, 597–606.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 March 2012; paper pending published: 09 April 2012; accepted: 30 May 2012; published online: 20 June 2012.

Citation: Saiman Y and Friedman SL (2012) The role of chemokines in acute liver injury. Front. Physio. ***3****:213. doi: 10.3389/fphys.2012.00213*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 19 October 2012
doi: 10.3389/fphys.2012.00056

Functional role of monocytes and macrophages for the inflammatory response in acute liver injury

Henning W. Zimmermann, Christian Trautwein and Frank Tacke*

Department of Medicine III, RWTH-University Hospital Aachen, Aachen, Germany

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Wing-Kin Syn, Foundation for Liver Research, UK
Gavin Arteel, University of Louisville, USA

****Correspondence:***
Frank Tacke, Department of Medicine III, RWTH-University Hospital Aachen, Pauwelsstrasse 30, D-52074 Aachen, Germany.
e-mail: frank.tacke@gmx.net

Different etiologies such as drug toxicity, acute viral hepatitis B, or acetaminophen poisoning can cause acute liver injury or even acute liver failure (ALF). Excessive cell death of hepatocytes in the liver is known to result in a strong hepatic inflammation. Experimental murine models of liver injury highlighted the importance of hepatic macrophages, so-called Kupffer cells, for initiating and driving this inflammatory response by releasing proinflammatory cytokines and chemokines including tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1beta, or monocyte-chemoattractant protein-1 (MCP-1, CCL2) as well as activating other non-parenchymal liver cells, e.g., endothelial or hepatic stellate cells. Many of these proinflammatory mediators can trigger hepatocytic cell death pathways, e.g., via caspase activation, but also activate protective signaling pathways, e.g., via nuclear factor kappa B (NF-κB). Recent studies in mice demonstrated that these macrophage actions largely depend on the recruitment of monocytes into the liver, namely of the inflammatory Ly6c+ (Gr1+) monocyte subset as precursors of tissue macrophages. The chemokine receptor CCR2 and its ligand MCP-1/CCL2 promote monocyte subset infiltration upon liver injury. In contrast, the chemokine receptor CX3CR1 and its ligand fractalkine (CX3CL1) are important negative regulators of monocyte infiltration by controlling their survival and differentiation into functionally diverse macrophage subsets upon injury. The recently identified cellular and molecular pathways for monocyte subset recruitment, macrophage differentiation, and interactions with other hepatic cell types in the injured liver may therefore represent interesting novel targets for future therapeutic approaches in ALF.

Keywords: liver injury, acute liver failure, macrophages, monocytes, TNF-alpha, chemokines, CCR2, review

INTRODUCTION

Acute liver injury (ALI) and acute liver failure (ALF) represent different severity stages of a sudden deterioration of liver function without evidence for prior chronic liver disease. It is a dreaded disease condition due to its tremendous morbidity and mortality without adequate treatment. Clinical hallmarks of ALF are coagulopathy (defined as an INR > 1.5) and mental alterations (i.e., hepatic encephalopathy of any degree) within a 26-weeks time-frame after the initial symptoms (Bernal et al., 2010). The latter clinical condition is absent in ALI in which coagulation abnormalities are predominant. The annual incidence of ALF is estimated at one to six cases per million in the developed world but may be higher in endemic regions of viral hepatitis (Bernal et al., 2010). Due to insufficient surveillance and reporting systems and lack of consistent diagnostic criteria accurate data concerning the global epidemiology of ALI are scarce. Drug-induced liver injury as a common underlying cause is estimated to affect 44,000 individuals in the US per year (Bell and Chalasani, 2009). Miscellaneous causes of acute liver deterioration exist, and etiology is the best predictor of clinical outcome (Ostapowicz et al., 2002). In major parts of the western hemisphere acute dose-dependent acetaminophen (paracetamol) toxicity is the most prevalent cause of ALF, whereas viral agents (mainly hepatitis A, B, or E virus) predominate in developing countries. In recent years, idiosyncratic, non-acetaminophen, drug-induced hepatotoxicity became a major etiology of ALF in Europe (Canbay et al., 2011). ALF is a systemic disease. Owing to its devastating nature implications of liver failure rapidly affect virtually all vital organs eventually leading to multi-organ failure. Despite remarkable progress in disease management and understanding of basic molecular mechanisms involved, disease-specific, targeted therapies cannot be provided in a considerable proportion of cases where liver transplantation constitutes the sole medical mean to prevent death.

Local and circulatory components of the innate immune system fundamentally shape the outcome of the immunological response to an acute hepatic insult. There is a robust body of evidence that hepatic macrophages (traditionally called "Kupffer cells," KCs) are essential players in the propagation of acute liver damage. These cells attracted much attention lately in the context of chronic

Abbreviations: ALF, acute liver failure; ALI, acute liver injury; APAP, acetaminophen; CCl_4, carbon tetrachloride; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; ConA, Concanavalin A; CXC, C-X-C motif chemokine; DC, dendritic cell(s); GalN, D-galactosamine; HSC, hepatic stellate cell(s); IFN, interferon; IL, interleukin; iNOs, induced nitric oxide synthetase; INR, international normalized ratio; I/R, ischemia/reperfusion; KC, Kupffer cell(s); LSEC, liver sinusoidal endothelial cell(s); LPS, lipopolysaccharide; MCP-1, monocyte-chemoattractant protein-1; NF-κB, nuclear factor kappa B; NK-cell, natural killer cell; TNF, tumor necrosis factor.

liver inflammation due to their dual pro- and antifibrotic qualities (Zimmermann and Tacke, 2011) but evidence for their critical involvement in fulminant hepatitis even date back several decades. Ever since, the evolvement of intriguing techniques to impact KC function has paved the way for a deepened knowledge and enabled us to decipher detrimental as well as beneficial aspects of KC activity. The present review intends to focus on hepatic macrophages in ALI as well as on monocytes, the bone-marrow-derived macrophage precursors that are vigorously recruited upon liver damage. Chemokine pathways governing this process will be a main focus in the subsequent sections, because interference with these pathways might perspectively allow developing novel and effective therapeutic approaches for ALF in the near future.

RESIDENT AND INFILTRATING HEPATIC MACROPHAGES DURING HOMEOSTASIS AND INJURY

GENERAL ASPECTS OF LIVER ANATOMY AND MICROVASCULATURE

The liver is not only the largest solid organ of the human body but also possesses the most extensive reticuloendothelial system (RES), thus playing a central role in the immune response against invading pathogens. It is unique in its property as an organ that encounters all the foreign material adsorbed from the intestine after digestion including food-derived antigens and environmental toxins (Gao et al., 2008). In addition, blood floating into the liver via the portal vein (accounting for ∼80% of total liver blood supply) contains microbial components even under steady state conditions with lipopolysaccharide (LPS) from gut-derived Gram-negative germs representing some of the most prominent bacterial constituents. Moreover, due to an arterial blood supply the liver also samples antigens from systemic circulation. The hepatic microvasculature is composed of liver sinusoids that are lined by highly specialized liver sinusoidal endothelial cells (LSEC) that tremendously differ from generic vascular endothelium in terms of phenotype, surface markers, and function (Lalor et al., 2006). Portal venous and arterial vessel branches supply the sinusoids with blood. Sinusoidal fenestrations and the lack of a basal membrane facilitate the delivery of solutes across the subendothelial space of Dissé to the hepatocytes which constitute the hepatic parenchyma. Signals evoked by invading macro-material and cellular effectors rely on active recruitment via sinusoidal cells or endocytosis/phagocytosis and cytokine-release of resident phagocytic cells. Following drainage into the central vein the "liver-modified" blood reaches systemic circulation through the vena cava inferior.

KUPFFER CELLS ARE RESIDENT MACROPHAGES AND FULFILL ESSENTIAL TASKS DURING STEADY STATE

Owing to the direct vascular connection to the splanchnic organs as a source of potential environmental and inherent threats, integral parts of the innate immune system are highly enriched in the liver. This renders the liver as an immunological organ with predominant innate immune functions (Racanelli and Rehermann, 2006; Gao et al., 2008). Apart from resident immune cells that respond to exterior and interior damaging influences, the liver is also source of a host of soluble factors encompassing acute-phase-proteins, complement factors, cytokines, and chemokines, which all contribute to the meticulous orchestration of immune response to various stimuli (Ishibashi et al., 2009). However, the liver is perpetually confronted with harmless nutrient-borne antigens and low levels of LPS and other microbial products. Those do not represent an inflammatory stimulus in steady state conditions but elicit immunosuppressive responses in order to prevent constant detrimental immune activation (Tacke et al., 2009). Intrahepatic macrophages accommodate for both opposing scenarios: promoting immune tolerance during homeostasis as well as implementing proinflammatory mechanisms in acute and chronic liver injury. KCs traditionally denote hepatic (resident) macrophages and represent up to 80–90% of the total body macrophage pool (Ishibashi et al., 2009). Together with LSEC, hepatic stellate cells (HSC), and local immune cells [in particular atypical T-cells, NK-cells (pit cells), and hepatic dendritic cells (DCs)] KCs constitute the non-parenchymal liver cells. They dwell in the lumen of liver sinusoids in close contact to the sinusoidal endothelial cells and sense the circulating blood for food-borne antigens and microbial constituents stemming from the splanchnic circulation. The sinusoidal site also guarantees intimate contact and communication with immune cells that enter the liver via the portal vein. In the non-inflamed liver one of the key functions of KCs is the removal of insoluble macromolecules through phagocytosis mediated by a wide repertoire of pattern-recognition receptors (PRRs) on their surface including scavenger receptors SR-AI and SR-AII, mannose receptor, and Fc-γ receptors (Gao et al., 2008; **Figure 1**). Thereby, hepatic macrophages eliminate potential harmful threats elicited by degenerated cells, microbes, immune complexes, and toxins (Kolios et al., 2006). KC show functional disparities related to their localization within the liver lobule (Bilzer et al., 2006). Periportal KCs, which are the first macrophages to encounter inflowing portal blood, are more abundant, bigger in size, and exhibit greater phagocytic and lysosomal capacities in addition to an increased production of inflammatory mediators [such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) alpha (TNF-alpha)], in comparison to those hepatic macrophages located in the midzonal area and around the central vein (Sleyster and Knook, 1982; Hoedemakers et al., 1995). Moreover, KCs are not entirely static in the sinusoidal lumen but migrate across the sinusoidal walls and are capable of reducing the sinusoidal blood velocity hence supporting the contact of circulating immune cells with sinusoidal endothelial cells (MacPhee et al., 1992, 1995).

ORIGIN AND PHENOTYPE OF KCs DURING ABSENCE OR PRESENCE OF HEPATIC INJURY

In absence of liver inflammation, the number of intrahepatic macrophages is maintained at constant numbers. Various cytokines comprising IL-1, IL-4, interferon-gamma (IFN-gamma), granulocyte–macrophage colony-stimulating factor (GM-CSF), and other hematopoietic factors promote macrophage apoptosis and survival *in vitro* (Naito et al., 2004). Results of studies covering the life span of KCs are inconsistent and range between 14 days and several months (Naito et al., 2004). Interestingly, even in monocytopenic species KC persistence *in situ* may exceed 6 weeks, suggesting that KCs constitute long-lived resident macrophages (Naito et al., 2004). Nevertheless, constant turnover is present and hepatic macrophages are incessantly repopulated. Previous concepts of resident macrophages

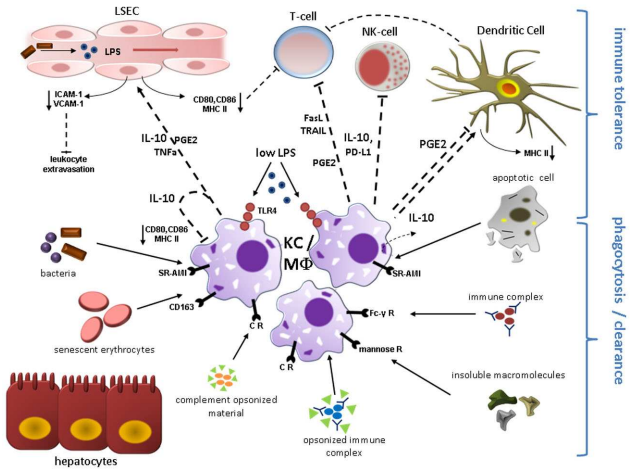


FIGURE 1 | Kupffer cell (KC)/Macrophage (MΦ) function during liver homeostasis. Phagocytosis and induction of immune tolerance as the two main functions of KCs/hepatic MΦ in the steady state are depicted here. KCs reside in the liver sinusoids in close proximity to sinusoidal endothelial cells (LSEC) and immune cells entering the liver microvasculature mainly through the portal vein. KCs express a broad range of surface receptors mediating phagocytosis, which renders these cells as highly effective filters of endogenous and exogenous antigens. Complement receptors mediate removal of complement-opsonized material. Circulating non-opsonized immune globulin complexes are cleared through Fc-γ Receptors. Insoluble macromolecules from multiple sources are effectively cleared after binding to Scavenger Receptors including CD163 for senescent erythrocytes. Molecules with a mannosyl motif are phagocytized following engagement of mannose receptors. Engulfment of apoptotic cell constituents can induce secretion of immunosuppressive IL-10 which likely contributes to the immune modulatory function of quiescent KCs. Constant exposure to gut-derived LPS via TLR4 also results in expression of IL-10 and PGE2 that can directly inhibit T-cell and NK-cell function and mediate down-regulation of co-stimulatory proteins including CD80, CD86, and MHC class II on endothelial cells, dendritic cells, and KCs constituting liver APCs, which further attenuates T-cell activation. KC-secreted PD-L1 and release of apoptosis-inducing mediators (TRAIL, FasL) contribute to suppression of adaptive and innate immune response through inactivation/elimination of T-cells and NK-cells. IL-10, PGE2, and TNF-alpha lead to reduced expression of adhesion molecules (VCAM-1; ICAM-1) on LSEC, thereby limiting leukocyte influx. Abbreviations: APC, antigen presenting cell; CR, complement receptor; FasL, Fas ligand; ICAM-1, intercellular adhesion molecule 1; PD-L, programmed cell death 1 ligand 1; PGE2, prostaglandin E2; SR-AI/II, scavenger receptor AI/AII; TLR4, toll-like receptor 4; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; VCAM-1, vascular adhesion molecular 1.

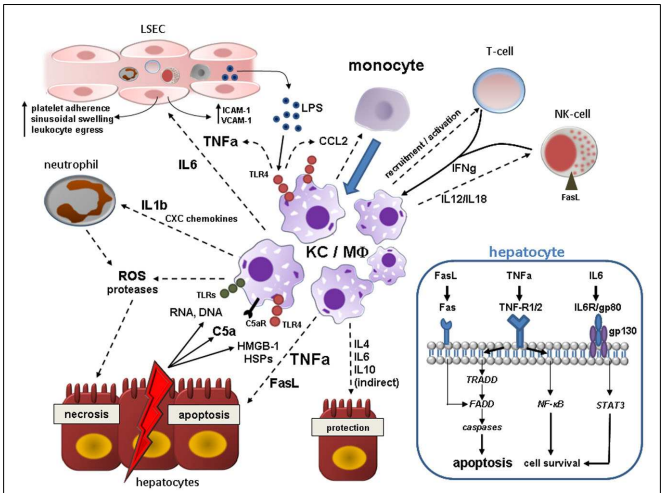


FIGURE 2 | Kupffer cell (KC)/Macrophage (MΦ) contribution to acute liver injury. Acute hepatocyte damage in response to multitude events leads to release of various DAMPs including HSPs and HMGB-1, which bind to TLR4 on KCs, and other cell contents (RNA, DNA) binding to various TLRs. TLR4 engagement activates NF-κB pathways in KC resulting in the synthesis of a myriad of proinflammatory cytokines, chemokines, reactive oxygen, and nitrogen species. KC-secreted TNF-alpha is central in the augmentation of liver injury mainly by inducing hepatocyte apoptosis, but also by deterioration of hepatic microcirculation through swelling and activation of endothelial cells with subsequent sinusoidal platelet aggregation and facilitation of peripheral immune cells entry. Activated KCs secrete IL-1beta and CXC chemokines such as CXCL2 and CXCL8 (IL-8), whereby neutrophils are massively attracted and start releasing ROS and proteases evoking hepatocyte necrosis. In addition, KCs, injured hepatocytes and activated hepatic stellate cells secrete CCL2 and other CC chemokines mediating liver influx of bone-marrow-derived monocytes that expand the local macrophage pool. Hepatic macrophages are also stimulated by IFN-gamma from resident and recruited T-cells and NK-cells and by C5a. KCs actively govern NK cell activation and recruitment by the production of IL-12/IL-18 which in turn induces hepatocyte death via membrane-bound FasL. High levels of LPS arising from Gram-negative bacteria in the context of increased bacterial translocation during acute liver injury magnify the activation the liver macrophages. As a counter-regulatory effect, hepatic macrophages also secrete IL-4, IL-6, and IL-10 amongst others that may dampen hepatic injury by either direct or indirect mechanisms. Engulfment of cell debris may either contain or amplify tissue injury (not depicted here). The blue box in the right lower corner illustrates important proapoptotic and prosurvival pathways in hepatocytes after binding of KC-related effector molecules during acute liver injury. TNF-alpha elicits both apoptosis (mainly via TNF-R1 and subsequent downstream signaling involving TRADD, FADD, and effector caspases) and cell survival through activation of NF-κB downstream cascade. Soluble and membrane-bound FasL derived from activated KCs and NK-cells also leads to caspase-dependent cell death after direct engagement of FADD. IL-6 complexes with gp80 and gp130 resulting in STAT3 activation via Janus kinases (JAKs) that can promote cell protection. Abbreviations: C5a, complement factor 5a; FADD, Fas-associated death domain; HMGB-1, high mobility group box-1; HSP, heat-shock protein; IL, interleukin; NF-κB, nuclear-factor-kappaB; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TNFa, tumor necrosis factor alpha; TRADD, TNF receptor associated death domain.

deriving from precursor cells (Yamamoto et al., 1996; Naito et al., 1997; Duffield et al., 2005) have been refuted by more recent studies that indicate that a significant extent of repopulation of these cells is from bone-marrow-derived myeloid precursors (Klein et al., 2007). In line, in one study only 1.5% of hepatic macrophages incorporated ^{3}H-thymidine during steady state, indicative of a low proliferation index (Crofton et al., 1978). In acute and chronic liver injury the intrahepatic macrophage count is massively expanded following the influx of peripheral monocytes (**Figure 2**) rather than augmentation of tissue-resident macrophages (Duffield et al., 2005; Imamura et al., 2005; Holt et al., 2008; Karlmark et al., 2009; Zimmermann et al., 2010). However, a current paper indicating that IL-4 dependent rapid *in situ* proliferation of local macrophages in Th2-biased inflammation accounts for extension of tissue alternatively activated macrophages (AAM), has challenged our prevailing understanding of a predominant

monocytic contribution to augmented hepatic macrophage pool and sparked intensive debate (Jenkins et al., 2011; Tacke and Kurts, 2011). Indeed, under certain circumstances the intrahepatic macrophage infiltrate might actually be preferentially polarized towards the M2 phenotype (synonymous for AAM) as it has been observed in acetaminophen (APAP) treated mice by Holt et al. (2008). Congruently, these cells elicited a protective role by promoting inflammation resolution and tissue repair. Yet, as far as other experimental models of ALI are concerned, it is tempting to speculate that macrophage actions in these conditions are rather dominated by classically activated M1 macrophages (CAM) emanating from infiltrating monocytes. This hypothesis is supported by observations in acute carbon tetrachloride (CCl_4) mediated liver injury in mice. Upon injury, the fraction of $Ly6c^{hi}$ $CD11b^{+}$ $F4/80^{+}$ monocytes, representing the peripheral inflammatory monocyte subset, is significantly enlarged, whereas $Ly6c^{lo}$ $CD11b^{+}$ $F4/80^{-}$ or $Ly6c^{lo}$ $CD11b^{-}$ $F4/80^{++}$ cells, corresponding to either unconventional or resident macrophages, remain stable (Karlmark et al., 2009). Thus, the major body of evidence indicates that a large proportion of intrahepatic macrophages directly derives from blood-borne monocytes in conditions of experimental ALI. However, a thorough inspection of the nature of infiltrating macrophages is warranted, and it is likely that the paramount macrophage phenotype strongly depends on the respective injury model.

Monocyte migration into the liver is facilitated by a profound secretion of CCL2 (MCP-1) and presumably other chemokines by parenchymal and non-parenchymal liver cells. Liver macrophages represent a vigorous source of CCL2 (Karlmark et al., 2008). CCL2 is secreted and released into the systemic circulation inducing monocyte egress from the bone-marrow. However, available research activity has failed to elucidate a direct role of CCL2 in the transendothelial migration of monocyte into the abluminal liver compartment (Karlmark et al., 2009).

PHYSIOLOGICAL STIMULI TRIGGER IMMUNE MODULATORY RESPONSES IN KCs

Under homeostatic conditions KC response to stimulation with physiologically low levels of LPS is considered to restrict inflammation (Knolle and Gerken, 2000). Although KCs also secrete proinflammatory cytokines like TNF-alpha in the steady state context, this response may be too faint to elicit overt inflammation and is seemingly blunted by the concomitant release of anti-inflammatory signals. In fact, TNF-alpha secreted by KC can even contribute to dampen T-cell response (Knolle and Gerken, 2000). Various mechanisms are believed to render KCs to anti-inflammatory cells during homeostasis. Strikingly, KC activation in response to low LPS concentrations has profound impact on LSEC biology, emphasizing the close reciprocity between these two cell types. After low concentrated LPS challenge, hepatic macrophages secrete immunosuppressive IL-10, which acts in an autocrine manner on KC, but also paracrine on LSEC. IL-10 entails down-regulation of MHC-II and co-stimulatory molecules such as CD80 and CD86 on LSEC, thereby abrogating $CD4^{+}$ T-cell activation (Knolle et al., 1998). Similar effects have been observed for prostanoids [i.e., Prostaglandin 2a (PGE2)] under non-inflammatory conditions (Knolle et al., 1998). Besides, naïve KC themselves are poor allostimulatory T-cell activators due to low expression of MHC-II, B7-1 (CD80), B7-2 (CD86) and CD40, and limit DC-induced antigen specific T-cell activation. This may represent another pathway of T-cell tolerance induction by KCs (You et al., 2008). Moreover, *in vitro* studies elucidated that IL-10 and prostanoids decrease expression of certain leukocyte adhesion molecules (ICAM-1, VCAM-1) on LSEC diminishing leukocyte endothelial transmigration into the liver parenchyma in homeostasis (Knolle and Gerken, 2000). Interestingly, KCs are also capable of disposing activated neutrophils invading the liver, thereby confining inflammatory processes (Bilzer et al., 2006). Phagocytosis of apoptotic neutrophil remnants by macrophages also attenuates production of proinflammatory cytokines like IL-1β and IL-8 in a TGF-β1, PGE2, and platelet-activating-factor (PAF) dependent fashion (Fadok et al., 1998). Accordingly, in a more recent publication it was demonstrated that KC priming with apoptotic splenocytes enhances production of IL-10 and reduces the release of proinflammatory signals (TNF-α and nitric oxide) through the Smad3 pathway after endotoxin challenge. Membrane-bound TGF-beta on apoptotic cells was reported to be the driving force of this phenomenon (Zhang et al., 2011). This is conclusive evidence that engulfment of apoptotic cell remnants by KCs favors tolerogenic immune response, as it has been also confirmed for "steady state" macrophages in general (Lucas et al., 2006; Chung et al., 2007). Efferocytosis of neutrophils has been demonstrated to induce IL-10 and TGF-beta production by macrophages and these cytokines are also closely linked to tissue repair (Ribeiro-Gomes et al., 2004; Filardy et al., 2010) Mechanisms of immune tolerance induction by quiescent KCs are illustrated in **Figure 1**.

KC ACTIVATION AND ITS PROINFLAMMATORY CONSEQUENCES UPON ACUTE LIVER INSULTS

The response of KC to acute hazardous events contributes to hepatocyte killing directly and indirectly. Prelude of KC activation is the release of intracellular constituents from necrotic cells due to whatever cause (e.g., chemicals, physical, viral, hypoxia). The liberation of "danger" signals from inflamed, necrotic, or hypoxic parenchyma cells and the secretion of complement C5a directly contribute to KC activation. These endogenous damage-associated-molecular-pattern-molecules (DAMPs) bind to PRRs. The nuclear transcription factor high mobility group box-1 (HMGB-1) is a ligand of toll-like receptor 4 (TLR4) Its binding engages several downstream transcriptional factors (nuclear factor kappa B, NF-κB, AP-1, IRF-3, STAT-1) and kinases, eventually cumulating in upregulated synthesis of proinflammatory mediators with TNF-alpha being the most intensively studied mediator (Abu-Amara et al., 2010). Congruently, pharmacological inhibition of HMGB-1 by the triterpene glycyrrhizin ameliorated liver injury after ischemia-reperfusion in rats (Ogiku et al., 2011). Exogenous DAMPs (i.e., LPS) accumulating in the anhepatic phase of liver transplantation through translocation of intestinal microbes have been evidenced to play a role in TLR4 signaling in ALI as well and boost the inflammatory cascade (Fiorini et al., 2004). It can be concluded, that KCs are more sensitized to endotoxins in the presence of an additional hepatic insult, which lowers the threshold to recruit proinflammatory signals. Although considered a rather protective mechanism in

homeostasis, engulfment of apoptotic hepatocytes can result in Fas ligand and TNF-alpha release by KC promoting tissue injury (Canbay et al., 2003), yet contradictory results in fulminant hepatitis exist (Zhang et al., 2011). After TNF-alpha binds to TNF-R1/TNF-R2 surface receptor on hepatocytes, downstream cascades via various domains (TRADD, FADD) imply activation of initiator caspases 8/9 and executioner caspases 3/6/7, which orchestrate cell death (Tacke et al., 2009). TNF-alpha and Fas (CD95) binding Fas ligand (FasL) expression are activated in fulminant hepatic failure by $CD8^+$-cells, NK-cells, and KCs (Miyagawa-Hayashino et al., 2007; Malhi and Gores, 2008) and share common molecular pathways of instigation of apoptosis (Tacke et al., 2009). Furthermore, TNF-alpha can mediate caspase-independent launch of cellular death via formation of ROS and prolonged activation of JNK-pathway leading to extensive necrosis (Malhi and Gores, 2008). Concomitantly, TNF-alpha elicits protective antiapoptotic actions via NF-κB activation, resulting in transcription of survival genes (e.g., Bcl-xl, cFLIP). It is largely unknown which factors disarrange the balance of TNF-signaling from the predominate "prosurvival" side to the "proapoptotic" side in ALI (Malhi and Gores, 2008). Counterbalancing cytokines released by KCs include IL-4, IL-6, IL-10, and have shown to play a compensatory role in ALI by abrogating deleterious TNF signals, among others (Abu-Amara et al., 2010).

Another detrimental aspect of KC activation is secretion of various CC- and CXC-motif chemokines by activated KCs, which promote attraction of polymorphonuclear cells (neutrophil granulocytes), $CD4^+$ T-cells, and monocyte-derived macrophages from the circulation to the hepatic microvasculature (Adams et al., 2010). When adhered to their target cell, granulocytes release ROS that trigger hepatocyte death and intensify resulting liver damage. This emphasizes the conception of heterogeneous cell types acting synergistically in ALI. $CD4^+$ T-cell and neutrophil extravasation is facilitated by the induction of sinusoidal ICAM-1 and VCAM-1, interacting with the integrins CD11b/CD18 and CD29/49, through the KC-cytokines TNF-alpha and IL-6 (Sakamoto et al., 2002; Hanschen et al., 2008). **Figure 2** summarizes by which mechanisms hepatic macrophages contribute to ALI.

MONOCYTE SUBPOPULATIONS POSSESS DISTINCT PHENOTYPES AND FUNCTIONS

MONOCYTES ARE MACROPHAGE/DENDRITIC CELL PROGENITORS AND IMMUNE EFFECTOR CELLS

Monocytes link the dramatic processes within the systemic and the hepatic compartment during ALI and failure by dictating systemic responses to local incidents and by providing hepatic macrophages that drive tissue injury. As to general features, monocytes are innate immune cells endowed with a broad panel of chemokine and PRRs ensuring their role as a potent antimicrobial and migratory leukocyte subset. They are able to identify a broad range of antigens and stimuli including dead cells, lipids, and bacterial pathogens (Geissmann et al., 2010). Based on their heterogeneity and versatility, monocytes exert multiple, occasionally opposed functions, and it is equivocal to which extent distinct monocyte subsets reflect diverging maturity stages (Gordon and Taylor, 2005; Geissmann et al., 2010). Lately, comprehensive phenotyping efforts have indicated that probably a continuum of phenotypes exists while the circumscribed profiles attributed to the defined subtypes merely constitute extreme polarizations (Wong et al., 2011). The capacity of rapidly releasing abundant amounts of proinflammatory cytokines (e.g., TNF-alpha, IL-1beta, IL-6, IL-8), reactive oxygen species, complement factors, and proteolytic enzymes upon various stimulatory events may render them as crucial contributors to the early systemic inflammatory response of acute and devastating systemic diseases. Opposite to this, the synthesis of immunosuppressive mediators (e.g., IL-10), which has been highlighted by numerous studies, can drive immune paralysis observed in ALI (Antoniades et al., 2008). Besides secretion of soluble factors, they augment the local macrophage pools following transendothelial migration. Moreover, they give rise to certain DCs (e.g., inflammatory TNF-alpha/iNOS-producing TipDCs) during inflammation (Tacke and Randolph, 2006) and repopulate tissue macrophages under physiological conditions (Klein et al., 2007).

DEVELOPMENT OF BLOOD MONOCYTES FROM HEMATOPOIETIC PRECURSOR CELLS

Monocytes circulate in blood vessels, spleen, and bone-marrow (Geissmann et al., 2010). Their development entails several precursor cells ranging from a pluripotent hematopoietic stem cell over a common myeloid (CMP) to a macrophage/DC progenitor (MDP) along a path of rising lineage restriction and commitment encompassing additional intermediate stages (Robbins and Swirski, 2010). This process is under control of the growth factors Csf-1 (alternatively termed M-CSF; Kawasaki et al., 1985) and IL-34 (Lin et al., 2008) that both bind to Csf-1R (M-CSFR/CD115; Wei et al., 2010) and synergistically govern monocyte differentiation in a non-redundant manner (Geissmann et al., 2008). CMPs are referred to as $Lin^-Sca^-IL\text{-}7Ra^-CD117^{low}CD34^+CD16^+$ cells that exhibit CD115 and CX_3CR1 on their surface and differentiate into macrophages, DCs, and monocytes but not into granulocytes (Akashi et al., 2000; Fogg et al., 2006; Varol et al., 2007). Development from MDP into monocytes occurs in the bone-marrow as the last step of monocyte differentiation before they exit into circulation. This event that is mainly directed by CCR2/CCL2 interactions (especially upon systemic inflammation), but may involve other homeostatic pathways such as CXCR4/CXCL12 (Serbina and Pamer, 2006; Geissmann et al., 2008; **Figure 3**). The prevalent paradigm of the bone-marrow as the sole source of monocytes, however, has been challenged by more recent investigations. According to these findings, a certain fraction of circulating monocytes delineates from a splenic reservoir in the cords in the subcapsular red pulp and does not require CCR2 signals to enter systemic circulation (Swirski et al., 2009). In fact, release from the splenic site relies on factors including angiotensin II (Swirski et al., 2009). Concordantly, angiotensin-II-lowering agents like angiotensin-converting-enzyme inhibitors confined the mobilization of splenic monocytes in a model of myocardial infarction (Leuschner et al., 2010). CCR2-independent non-medullar monocyte resources therefore have to be incorporated in further studies dedicated to intervene at monocyte allocation in pathological settings.

PHENOTYPE AND FUNCTION OF MURINE "CLASSICAL" MONOCYTES

Irrespective of their human or murine origin, peripheral monocytes are defined by expressing the pan-monocytic markers

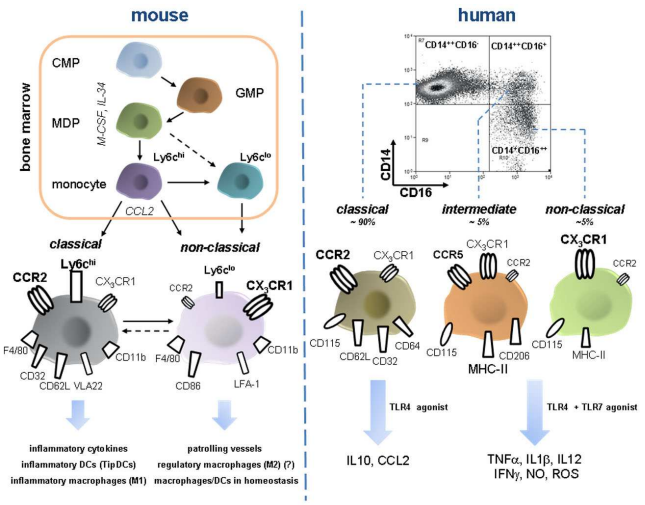


FIGURE 3 | Development and features of murine and human monocyte subsets. Monocyte development from a common myeloid progenitor (CMP) occurs in the bone-marrow under the control of M-CSF and IL-34 and increasing lineage commitment. A macrophage dendritic progenitor (MDP) gives rise to Ly6c^{hi} monocytes and probably also to Ly6c^{lo} monocytes. Bone-marrow egress is directed by CCR2, maybe also by other pathways such as CXCR4/CXCL12 (not depicted). Peripheral Ly6c^{hi} can shuttle back to the bone-marrow and possibly transdifferentiate into Ly6c^{lo} monocytes, a process that might also occur in the periphery (e.g., in the spleen). Murine monocyte subsets constantly express F4/80 and CD11b. Classical Ly6c^{hi} are characterized by high expression of CCR2 but only moderate levels of the fractalkine receptor CX_3CR1. Furthermore, they express several scavenger receptors (e.g., CD32), selectin ligand CD62L, and the integrin VLA-2. Ly6c^{hi} monocytes have a proinflammatory profile since they secrete inflammatory cytokines, migrate into inflamed tissue and give rise to inflammatory macrophages (sharing qualities with classical M1 macrophages), and DC subsets. Ly6c^{lo} are less abundant in the periphery and express high CX_3CR1, CD86, and LFA-1 whereas CCR2 is almost absent. They patrol blood vessels by crawling across endothelium. During inflammation they can rapidly or protracted enter tissue and presumably develop into macrophages with a rather anti-inflammatory phenotype eliciting wound repair (reminiscent of M2 macrophages). In homeostasis they are currently designated as precursor cells of local macrophages and dendritic cells. Importantly, Ly6C^{hi} derived macrophages can very likely also acquire anti-inflammatory phenotypes in peripheral tissues such as the liver. Intermediate Ly6c^{int} are not depicted here. FACS dot plot in the right upper corner illustrates characteristic distribution of human monocyte subsets. CCR2 high expressing CD14^{++}CD16^{-} monocytes are termed classical monocytes, representing the vast majority of circulating monocytes. Despite phenotypic homology to murine inflammatory Ly6c^{hi} monocytes they release immune suppressive IL-10 upon LPS stimulation. In turn, intermediate and non-classical monocytes that are defined by the expression of CD16, display moderate to high CX_3CR1 and varying density of CD14 and are far less abundant, but release various inflammatory cytokines after challenge with TLR4 and TLR7 agonists. These functions sharply contrast the rather anti-inflammatory phenotype of Ly6c^{lo} monocytes as their putative murine counterparts. Intermediate monocytes and classical monocytes have a distinct surface protein profile (e.g., CCR5 is almost exclusively expressed on the intermediate subset) though functional differences are not fully defined. Abbreviations: CMP, common myeloid progenitor; GMP, granulocyte myeloid progenitor; IFNg, interferon gamma; LFA-1, lymphocyte function antigen 1; M-CSF, macrophage colony-stimulating factor; MDP, macrophage dendritic cell progenitor; NO, nitric oxide; TipDCs, TNF–iNOS-producing dendritic cells; VLA-2, very late antigen 2.

CD115/CX_3CR1 and they lack expression of surface molecules such as Nkp-46, CD3, CD19, or CD15 that characterize NK-cells, T-cells, B-cells, and neutrophils, respectively (Auffray et al., 2009). In mice discrimination of CD11b^{+}F4/80^{+} monocyte subtypes relies on the differential expression levels of Ly6C which is recognized by the antibody RB6-8C5 (Gr1; Geissmann et al., 2003; Taylor and Gordon, 2003). Owing to a deficit of functional data, further separation of murine monocyte subsets on the grounds of variable CD43 expression, as it has been proposed in the current nomenclature (Ziegler-Heitbrock et al., 2010), will be neglected here. Ly6c^{hi} (Gr1^{hi}) monocytes constitute comparatively big cells that are distinguished by CCR2^{hi}CX_3CR1^{lo} expression and the presence of VLA-2 and CD62L adhesion molecules (Taylor and Gordon, 2003; Robbins and Swirski, 2010; **Figure 3**). Representing the major subpopulation, they are designated "inflammatory" or "classical" monocytes due to the extensive capacity of secreting proinflammatory mediators (i.e., TNF-alpha, iNOs, IL-12, type 1 interferon) and migrating into inflamed tissues, as it has been demonstrated for inflamed peritoneum, skin, and infarcted myocardium (Gordon and Taylor, 2005; Tacke and Randolph, 2006; Robbins and Swirski, 2010). If activating inflammatory signals hold off, egressed Ly6c^{hi} monocyte shuttle back to the bone-marrow and down-regulate CCR2 expression. Upon inflammatory stimuli including ALI, Ly6c^{hi} monocytes massively translocate from bone marrow into the circulation and decrease again as the inflammation abates (Karlmark et al., 2009; Robbins and Swirski, 2010). Subsequent to tissue entry, "classical" monocytes give rise to macrophages and inflammatory DCs including TipDCs after MyD88-pathway activation and hence endorse microbial killing (Strauss-Ayali et al., 2007; Serbina et al., 2008). Likewise, accumulation of these cells also occurs in non-infectious inflammation settings (Nahrendorf et al., 2007; Robays et al., 2007; Tacke et al., 2007). In the chronically inflamed liver, Ly6c^{hi} monocytes acquire a phenotype reminiscent of CAM thereby perpetuating the intrahepatic inflammatory milieu that promotes fibrogenesis (Karlmark et al., 2009; Tacke and Kurts, 2011). The concept of the progeny of M1 macrophages from Ly6c^{hi} monocytes bases on observations on mucosal TNF-alpha- and iNOs-producing macrophages in toxoplasma gondii infection (Dunay et al., 2008).

CHARACTERISTICS OF "NON-CLASSICAL" MONOCYTES IN MICE

In contrast to "classical" monocytes, the functional spectrum of the less frequent murine Ly6c^{lo} (Gr1^{-}) "non-classical" or "resident" monocytes has not been sufficiently clarified. Controversial reports have hampered the formation of a clear concept for this cell type. Some experimental evidence indicates that Ly6c^{lo} monocytes originate from Ly6c^{hi} cells during maturation (Sunderkotter et al., 2004; Tacke et al., 2006; Varol et al., 2007), some of the Ly6c^{lo} monocytes, however, develop independently in the bone-marrow (Geissmann et al., 2003; Hanna et al., 2011). It is well acknowledged, that "non-classical" monocytes have the potential to home to uninflamed tissue in a G-protein-dependent manner and thus renew local macrophages and DCs, whereas tissue inflammation does not induce Ly6c^{lo} monocyte recruitment in early phases of inflammation (Geissmann et al., 2003). Furthermore, their human counterpart cells (CD16^{+} monocytes) were

capable of reversely transmigrating across unstimulated human umbilical vein endothelial cells (HUVEC), a process that involved the adoption of a DC-like profile (Randolph et al., 2002). Consecutive investigations, however, indicated that Ly6c^{lo} monocytes constantly crawl in the lumen of the blood vessels across the endothelial cell layer, mediated by the integrin LFA-1 and CX_3CL1, and sense the subjacent space for danger signals (Auffray et al., 2007). Yet, when manifest inflammatory processes are absent, Ly6c^{lo} monocytes exhibited very low tendency to extravasate. In turn, during acute *Listeria monocytogenes* infection patrolling "non-classical" monocytes rapidly transmigrate toward the local irritant stimulus and transiently produce inflammatory cytokines (Auffray et al., 2009). As inflammation evolves, Ly6c^{lo} then might differentiate into alternatively activated (M2) macrophages that suppress inflammation and convey repair, angiogenesis, and wound healing (Nahrendorf et al., 2007; Auffray et al., 2009). CCR2 expression of murine Ly6c^{lo} is low to absent in agreement with a rather anti-inflammatory profile. Instead, they express abundant CX_3CR1, which might potentially explain their longer live-span, and express different adhesion molecules (high LFA-1 expression) in comparison to the "classical" counterpart (**Figure 3**).

DIVERGENT PROPERTIES OF HUMAN MONOCYTE SUBTYPES

Despite increasing knowledge about phenotypic and genetic congruency between murine and human monocyte subsets (Ingersoll et al., 2010), there seem to be profound functional disparities between monocytes in different species. In addition, some differences on the phenotype level are present as well that hinder the experimental analysis of monocytes in acute murine liver injury. For instance, differential regulation of monocytic HLA-DR expression in ALI cannot be studied in mice since HLA-DR is not present on circulating monocytes in that species. By now the distinction of three different human monocyte subpopulations has been implemented in the official nomenclature (Ziegler-Heitbrock et al., 2010; Wong et al., 2011; Zawada et al., 2011). Accounting for approximately 90% of circulating monocytes, human "classical" monocytes are $CD14^{++}CD16^{-}$ cells with high levels of CCR2 and low levels of CX_3CR1 thereby closely resembling murine Ly6c^{hi}. They feature relatively high CCR1, CXCR1, CXCR2, CXCR4, CD32, CD62L and CD64 expression but only low levels HLA-DR and have significant phagocytic activity (Wong et al., 2011; Zimmermann et al., 2011). Following endothelial transmigration, these cells tend to persist in the subendothelial space and develop into macrophages whereas CD16 positive monocytes are predisposed to develop into migratory DCs with T-cell-stimulatory capacity (Randolph et al., 2002). Monocytes that display CD16 on their surface are now further divided into an "intermediate" subset (with maintained high expression of CD14, therefore termed $CD14^{++}CD16^{+}$) and "non-classical" $CD14^{lo}$ cells with equal to increased amounts of CD16 ($CD14^{+}CD16^{++}$; **Figure 3**). Some striking discrepancies exist between the latter subsets. "Intermediate" monocytes exhibit highest HLA-DR, mannose receptor CD206 [a M2 marker (Zimmermann and Adams, unpublished data)] and CCR5 levels, whereas the fractalkine receptor is most abundant on the $CD14^{lo}$ subtype (Wong et al., 2011; Zawada et al., 2011). Selective CD206 expression by $CD14^{++}CD16^{+}$ cells might refer to their preferential capacity to give rise to AAM, though this needs functional confirmation. Both subsets show comparable levels of CD32 and only marginal CCR2 expression (Zawada et al., 2011). Functional differences between the $CD16^{+}$ monocytes are still somewhat ill-defined and the majority of available functional data does not distinguish between the various subtypes. "Intermediate" monocytes seem to be specifically involved in HIV infection, possibly because of their CCR5 expression that confers viral cell-entry in CCR5-tropic-HIV-1-(M)-strains (Jaworowski et al., 2007). The prominent feature of $CD16^{+}$ monocytes to release proinflammatory mediators upon *in vitro* stimulation with LPS has been attributed to the $CD14^{hi}$ expression subset (Grage-Griebenow et al., 2001), whereas, according to another study, $CD14^{lo}$ monocytes show only weak phagocytic activity and secrete only low amounts of cytokines and reactive oxygen species after LPS exposure but synthesize TNF-alpha, IL-1beta as well as CCL3 subsequent to viral and nucleic acid stimuli involving activated TLR7–TLR8–MyD88–MEK pathway (Cros et al., 2010). Similar to murine Ly6c^{lo} monocytes, these cells patrol in the blood vessels in an integrin-dependent manner (Cros et al., 2010).

EXPANSION AND FUNCTION OF CD16 EXPRESSING MONOCYTES DURING HUMAN DISEASES

Regardless of the heterogeneity of $CD16^{+}$ monocytes, there are a multitude of pathological conditions leading to an expansion of the minor $CD16^{+}$ subset. Increased proportions of $CD16^{+}$ monocytes in "sterile" inflammation have been reported in rheumatoid arthritis (Kawanaka et al., 2002; Wijngaarden et al., 2003), hemodialysis (Nockher and Scherberich, 1998), and atherosclerosis/coronary artery disease (Rothe et al., 1996; Schlitt et al., 2004), among others, and frequently mirrored disease activity and severity. Infectious diseases such as HIV (Thieblemont et al., 1995), erysipelas (Horelt et al., 2002), and bacterial sepsis (Fingerle et al., 1993) also exhibit a substantial rise in $CD16^{+}$ monocytes. Interestingly, peripheral counts of this subset were pronounced in patients with Gram-negative bacteremia (Herra et al., 1996), which is highly prevalent in patients with ALF. Resolution of inflammation and successful antimicrobial treatment result in decline of peripheral monocytes (Horelt et al., 2002). In the context of liver disease, a shift in distribution of peripheral monocytes and monocyte-derived hepatic macrophage subsets toward the $CD16^{+}$ subtype indicates disease progression and is highest in patients with end-stage Child C cirrhosis (Zimmermann et al., 2010). Furthermore, under constant flow mimicking sinusoidal shear stress $CD16^{+}$ monocytes are more prone to transmigrate across TNF-alpha/IFN-gamma stimulated hepatic sinusoidal endothelium than their $CD16^{-}$ counterpart (Liaskou et al., unpublished data). It is obvious to anticipate that $CD16^{+}$ monocytes also expand in ALF, yet, investigations in this subject are still pending. The total number of monocytes harshly decline in acetaminophen-induced ALF (AALF), but the relative distribution of monocyte subsets was not addressed in this study (Antoniades et al., 2012).

In sharp contrast to their putative murine counterparts, $CD16^{+}$ monocytes are tagged "proinflammatory" since *in vitro* they are potent producers of proinflammatory mediators such as TNF-alpha, IL-12, IFN-gamma, CCL3, CCL4, CXCL9, and nitric oxide after stimulation with either TLR2-/TLR4-agonists, tumors or even spontaneously. They hence might pivotally contribute to

systemic enhancement of the proinflammatory environment in ALF (Belge et al., 2002; Szaflarska et al., 2004; Zimmermann et al., 2010). Vice versa, human $CD16^-$ monocytes secrete immunosuppressant IL-10, which is nearly absent on protein as well as transcriptional level in the $CD16^+$ subset. They thus clearly feature anti-inflammatory traits (Mizuno et al., 2005; Zimmermann et al., 2010; **Figure 3**). Of note, the profound discrepancies between similar phenotypic features and functional disparities between murine and their putative homolog human monocyte subpopulations is beyond clarification. In addition, the biological relevance of proinflammatory qualities attributed to human $CD16^+$ monocytes has not been completely uncovered but future scientific studies will certainly help to elucidate open questions in that controversy.

MONOCYTES/MACROPHAGES IN EXPERIMENTAL MODELS OF ACUTE LIVER INJURY

Given the heterogeneous nature of the various triggers of ALI as a common sequela, it is a rational approach to use different experimental models in order to shed light on the pathogenesis of this disorder. Despite a broad panel of different models of ALI in rodents and other mammals, there are currently no experimental models that appropriately reproduce all aspects of acute human liver injury/failure (Newsome et al., 2000). Importantly, the diagnostic King's college criteria for ALI and ALF in human mainly incorporate biochemical and clinical deteriorations that are scarcely applicable to (small) animals (Tunon et al., 2009). Despite prevailing limitations, animal models have unraveled relevant aspects of macrophage action in this devastating disease condition. Selected models will be summarized in this paragraph. Approaches that aimed at ablating monocyte/macrophage migration and function by targeting chemokine pathways are discussed in subsequent sections.

EXPERIMENTAL MODELS OF ALI INVOLVING CHEMICAL SUBSTANCES

Chemical models of ALI comprise substances such as acetaminophen (APAP), galactosamine and LPS (GalN–LPS), the plant-derived lectin Concanavalin A (ConA), double-stranded RNA (polyI:C), alpha-Galactosylceramide (alpha-GalCer), single endotoxins, carbon tetrachloride (CCl_4), CpG, DMSO, CD40L, amanitin, and thioacetamide, but only very few have been used to substantially unravel role and mechanisms of macrophage activity in this disease complex (Wu et al., 2010).

Concanavalin A

Concanavalin A (ConA) is a plant mitogen with carbohydrate-binding (lectin) properties extracted from *Canavalia ensiformis*, which causes a vigorous $CD4^+$ T-cell stimulation resulting in hepatic TNF-related hepatic necrosis after single administration (Mizuhara et al., 1994). Initial evidence for a causative role of KCs in ALI following ConA challenge stems from a study involving gadolinium-chloride-($GdCl_3$)-treatment (Okamoto et al., 1998). $GdCl_3$ reduces KCs and compromises their function, though a complete depletion is not achieved (Michael et al., 1999). Reduction/alteration of KCs yielded less damage, though intrahepatic TNF-alpha expression was not changed (Okamoto et al., 1998). Based on a similar strategy of KC manipulation, liver injury could be markedly ameliorated, accompanied by reduced intrahepatic cytokine levels (including TNF-alpha) and diminished infiltration of $CD4^+$ T-cells, in another study (Morita et al., 2003). Schumann et al. (2000) could demonstrate that KC elimination by clodronate-loaded liposomes significantly restricted the spreading of focal confluent necroses. This phenomenon was attributed to KC-dominant TNF-alpha secretion in that model. However, systemic levels of TNF-alpha were not affected and injection of soluble rmuTNF could not abolish the protective effect of KC-ablation (Schumann et al., 2000). The latter observation suggests that membrane-bound TNF-alpha is pivotal in driving ConA-induced liver injury. The authors could also observe a harsh decline of systemic IL-6 levels, a cytokine which has hepatoprotective qualities and governs liver regeneration (Streetz et al., 2003; Tacke et al., 2009). This might appear contradictory to the blunted liver damage observed here, since IL-6-deficient mice were more susceptible to ConA-mediated fulminant hepatitis than wild-type animals (Tagawa et al., 2000). Succeeding investigations underscored the relevance of KCs for the contribution to a dominant Th1 immune response in the ConA model (Chen et al., 2011). Of note, several studies demonstrated that repetitive sublethal ConA injections induced immunotolerance, which could be attributed to instigation of IL-10 production by KCs acting synergistically with regulatory T-cells (Erhardt et al., 2007; Erhardt and Tiegs, 2010). Mechanistically, inhibition of NF-κB activation in KCs by intraportally injected decoy oligonucleotide-loaded gelated particles during ConA hepatitis but not ischemia/reperfusion related liver injury led to diminished TNF-alpha production and reduced phosphorylation of the proapoptotic jun N-terminal kinase (JNK), that essentially drives hepatocyte apoptosis (Hoffmann et al., 2009).

D-*galactosamine/lipopolysaccharide*

The coadministration of D-galactosamine and LPS (GalN/LPS) is a model of synchronous liver injury and endotoxin-shock and is governed by monocyte/macrophage-released TNF-alpha that essentially induces hepatocyte apoptosis (Josephs et al., 2000; Stuart et al., 2011). In accordance to the ConA model described precedingly, selective pharmacological inhibition of the NF-κB downstream cascade in KCs abrogated TNF-alpha synthesis and blunted liver damage (Hoffmann et al., 2009). TNF-alpha release by KCs in this model is negatively modulated by the Ron receptor tyrosine kinase and Lys-Cre Ron $TK^{ft/ft}$ mice with a conditional myeloid-specific Ron deletion featured exacerbated damage and increased lethality in response GalN/LPS induced injury (Stuart et al., 2011). Ron deficient mice correspondingly had higher *Tnf-alpha* gene transcription (Stuart et al., 2011).

Acetaminophen (APAP)

In terms of clinical relevance and dose-dependent toxicity, APAP meets the criteria for a suitable model of ALF (Newsome et al., 2000). Following exposure to toxic doses, physiological detoxifying pathways (glucuronidation, sulfation, renal excretion) are depleted and acetaminophen enters Cytochrom-P450 metabolism, which in turn yields abundant production of the toxic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAQI; Corcoran et al., 1980). Centrilobular necrosis and fulminant organ failure emerge when protective glutathione levels are exhausted (Roberts et al., 1990). This is significantly accelerated during concomitant exposure to

cytochrom-P450-inducing drugs and alcohol. Covalent binding and arylation of important cell proteins by NAQI results in loss of protein function and profoundly impairs organelle and cell integrity (Cohen and Khairallah, 1997). Hepatic macrophages contribute to hepatotoxicity via different mechanisms encompassing the formation of nitric oxide and superoxide that react together to produce peroxynitrite, which again exhibits hydroxyl radical-like activity (Michael et al., 1999). Importantly, KCs release a multitude of inflammatory cytokines, in particular TNF-alpha, after toxic APAP exposure (Laskin et al., 1986). In line, earlier studies reported that $GdCl_3$ treatment prior to APAP challenge in mice significantly attenuated liver damage and associated mortality (Blazka et al., 1995; Laskin et al., 1995; Michael et al., 1999). Subsequent series of experiments employing clodronate-loaded liposomes, a strategy that enables more profound KC depletion than $GdCl_3$, showed contradictory results. Pretreatment with clodronate liposomes led to almost complete KC depletion, decrease of intrahepatic immune modulatory cytokines such as IL-6, IL-10, or IL-18, and markedly enhanced susceptibility to acetaminophen (Ju et al., 2002). IL-10 was demonstrated to diminish APAP-associated hepatic necrosis and lethality (Bourdi et al., 2002). In support of this work, Holt et al. (2010) found that KC-dependent maintenance of liver sinusoidal endothelial cell integrity is another contributory factor to hepatoprotection during acetaminophen toxicity. Ambiguously, a protective role of KCs was not reproducible in a successive study also using clodronate liposomes (Campion et al., 2008). Conclusively, the precise nature of the functional dichotomy of hepatic macrophages in APAP toxicity is yet to be elucidated. A more recent study portended that opposing roles of hepatic macrophages might be attributable to cell origin since prolonged infiltration of monocyte-derived macrophages clearly promoted resistance to APAP damage (Holt et al., 2008). The concept of sequential immigration of different macrophage subpopulations was also evidenced in acute thioacetamide-mediated injury (Mori et al., 2009), in which KC-inactivation alleviates the extent of liver deterioration and accelerates liver regeneration (Andres et al., 2003; Bautista et al., 2010). Of note, beneficial properties of recently infiltrated macrophages were note reproducible in the carbon tetrachloride (CCl_4) model of acute injury (Karlmark et al., 2009; Mitchell et al., 2009).

Carbon tetrachloride (CCl_4)

CCl_4 is widely employed in liver research as it reliably induces an acute toxic liver injury and even liver fibrosis within several weeks after repetitive injection. Cessation of CCl_4 administration after single and long-term use allows the study of injury regression (Berres et al., 2010; Karlmark et al., 2010). Acute hepatic toxicity is a consequence of carbon-tetrachloride-induced formation of a highly reactive carbon-entered trichloromethyl radical through cytochrom-P450 isoenzymes that interacts with hepatic proteins as well as lipids and deteriorates cellular membranes (Luckey and Petersen, 2001). Rapid centrilobular damage succeeds already after one injection. CCl_4-exposure entails upregulation of *Tnf-alpha*, *Il-1beta*, *Il-6*, *Tgf-beta*, and *cyclooxygenase-2* mRNA transcripts and synthesis of reactive oxygen species plus eicosanoids in KCs (Alric et al., 2000; Luckey and Petersen, 2001). However, mechanistic evidence for a relevant role of KCs in CCl_4-mediated liver injury is scarce. Transient elimination of blood monocytes and hepatic macrophages cells prior to toxin-injection did not alter the magnitude of resulting liver damage, suggesting that monocytes/macrophages do not endorse acute liver damage in that specific model, but rather modify the subsequent inflammatory response within the injured liver. These findings were corroborated by data from chemokine-directed targeting of infiltrating macrophages in the same work (Karlmark et al., 2009). Basically, the secretory profile of hepatic macrophages might implicate both harmful and beneficial effects for the hepatic outcome of CCl_4-mediated injury, since it has been shown that disruption of TNF-alpha-pathways partially restores liver integrity (Morio et al., 2001), whereas mice lacking the gene for nitric oxidase synthase (NOS II) or IL-6 are more susceptible to liver damage in that model (Morio et al., 2001; Bansal et al., 2005). Noteworthy, the reliability of CCl_4 for studying pathogenesis of ALI is hampered by the considerable inherent variability of this model related to differences in species, age, and development of the metabolizing cytochrome-P-450 system (Newsome et al., 2000).

KC IN THE MOLECULAR CASCADE OF ISCHEMIA/REPERFUSION AS A SURGICAL MODEL OF ALI

Acute liver damage is an important sequel to hepatic ischemia/reperfusion (I/R) injury as it occurs in the clinical settings of trauma, shock of any cause, transient surgical interruption of hepatic blood flow and liver transplantation (Howard et al., 1990). A vast array of I/R animal studies have highlighted the role of inflammatory cytokines, reactive oxygen species, and sequestration/activation of leukocytes in the self-amplified cytotoxic cascade entailing the demise of liver parenchyma (Zhai et al., 2011). During I/R injury, KC swelling as a consequence of ion channel breakdown due to the depletion of adenosine triphosphate (ATP) contributes to devastating hepatic microcirculatory dysfunction. Moreover, complement factors and lymphocyte-released IFN-gamma are strong activating stimuli for KCs (Wanner et al., 1996). NADPH oxidase activation in KCs has been shown to be an integral component in the postischemic injury phase (Jaeschke, 2003). They secrete critical amounts of TNF-alpha, IL-1beta, promigratory chemokines, and free radicals. These humoral factors and reactive species directly promote hepatocyte death, sinusoidal endothelial damage and favor leukocyte adhesion, and infiltration, all of which processes propagate I/R injury (Abu-Amara et al., 2010). Monocyte-derived macrophages enter the liver in the rather late postischemic phase and sustain the inflammatory milieu responsible for continuous destruction of local tissue (Zhai et al., 2011). In the scope of ongoing liver inflammation due to I/R injury, KCs are able to change from a proinflammatory to a more immune modulatory phenotype by releasing IL-10 (Bamboat et al., 2010; Ellett et al., 2010).

In agreement with the well-described inflammatory properties of hepatic macrophages in that model, many investigators observed that KC blockade improves ischemic liver injury (Hardonk et al., 1992; Giakoustidis et al., 2003, 2006; Tomiyama et al., 2008). Ellett et al. (2010) demonstrated an imbalanced cytokine milieu in the absence of KC in I/R combined with bowel congestion, presumably owing to a lack of protective IL-10. This was associated with largely enhanced liver injury and

increased mortality. Beneficial effects of hepatic macrophages during ischemic insults could be attributed to liver resident but not infiltrating macrophages in a previous paper, that elegantly delineated the differential contribution of resident and infiltrating macrophages by using clodronate liposomes in CD11b diphtheria toxin receptor (DTR) mice. The KC-specific antioxidant heme-oxygenase was described to be protective in this model (Richards et al., 2010). Conflicting results regarding KC function in I/R injury may be explained by substantial methodic differences in KC depletion in the pertinent studies (e.g., $GdCl_3$ vs. clodronate liposomes) or by the fact that the different observations merely depict one extreme of the diametrically opposed biphasic functions of hepatic macrophages in ALI. In conclusion, the net effect of KC activity in I/R injury remains to be clarified.

MONOCYTE AND MACROPHAGE RELATED CHEMOKINE PATHWAYS IN ACUTE LIVER INJURY

CLASSIFICATION OF THE CHEMOKINE SYSTEM

Chemokines belong to a family of heparin-binding small promigratory cytokines that orchestrate the trafficking of immune cells, which is indispensable for immune cell functions in homeostasis (recirculation of leukocytes to secondary lymphoid tissues) and inflammation. They have received tremendous attention throughout the last years in terms of their function in liver inflammation and resulting fibrosis and many studies have investigated whether modifications of chemokine pathways could imply therapeutic benefits (comprehensively reviewed in Zimmermann and Tacke, 2011). Apart from CX_3CR1 and CCR2 monocyte differentially express CCR1, CCR5, CCR8, CXCR1, CXCR2, and CXCR4 that may all influence their migratory fate to varying degrees (Gordon and Taylor, 2005; Zawada et al., 2011; Zimmermann and Tacke, 2011).

CCL2 (MONOCYTE-CHEMOATTRACTANT-PROTEIN-1) IN ACUTE LIVER INJURY

CCL2 (monocyte-chemoattractant-protein-1, MCP-1) is the pivotal ligand of CCR2 and has been intensively studied in the context of liver injury. Many intrahepatic cell subsets release CCL2 upon a deleterious stimulus encompassing hepatocytes (Dambach et al., 2002) and non-parenchymal cells such as KCs (Jaeschke and Smith, 1997; Marra et al., 1998; Dambach et al., 2002; Seki et al., 2007), biliary epithelial cells (Marra et al., 1998; Tsuneyama et al., 2001; Kruglov et al., 2006; Harada et al., 2011), and quiescent or activated HSC (Marra et al., 1993, 1995, 1998) supporting the notion that CCL2-release represents an ubiquitous inflammatory pathway that is highly conserved in cells from entirely distinct ontogenic backgrounds. In the context of ALI, CCL2 as well as other proinflammatory chemokines such as CCL3 (MIP-1alpha), CCL4 (MIP-1beta), CXCL9 (MIG), and CXCL10 (IP-10) are secreted in response to TNF-alpha, IL-1alpha, IL-1beta, LPS, and reactive oxygen species stimuli originating from damaged hepatocytes, KCs, intestinal bacteria, and other sources (Czaja et al., 1994; Heymann et al., 2009). Interestingly, hepatic upregulation of CCL2 marks a very early event in the course of acute hepatic damage, since it is detectable in the first 1–4 h after the onset of CCl_4-induced liver injury (Czaja et al., 1994; Marra et al., 1999; Leifeld et al., 2003; Karlmark et al., 2009). First evidence of the relevance of CCL2 in ALI delineate from a study involving CCl_4- and galactosamine exposed rats, that displayed marked increase of CCL2 (Czaja et al., 1994). Apart from CCl_4-mediated ALI, induction of CCL2 expression has been observed in GalN/LPS (Leifeld et al., 2003), thioacetamide (Mori et al., 2009), ConA (Ajuebor et al., 2003; Leifeld et al., 2003), and drug-(APAP)-induced liver toxicity (Dambach et al., 2002). In human ALF increased CCL2 levels have been detected in various studies (Leifeld et al., 2003; James et al., 2005; Roth et al., 2009; Antoniades et al., 2012). Of note, CCL2 serum concentrations in a pediatric study cohort correlated with clinical disease severity, transaminase levels, and coagulopathy (James et al., 2005) and highest CCL2 levels were associated with fatal outcome in patients with acetaminophen-induced liver failure (Antoniades et al., 2012). Functional cues for the importance of increased intrahepatic chemokines for the hepatopetal trafficking of human monocytes/macrophages in ALF arise from one study that used homogenized liver samples from ALF patients livers containing elevated amounts of CCL2. Therein, a chemotactic stimulus on monocytes could be observed (Leifeld et al., 2003). Chemotaxis was significantly decreased by simultaneous treatment with CCL2, CCL3, CCL4, and CCL5 neutralizing antibodies. The latter chemokines were comparably elevated in acute human liver failure specimen suggesting that they also might contribute to the monocyte/macrophage attraction in this disease (Leifeld et al., 2003).

CCR2 IN ACUTE LIVER INJURY

Experimental studies involving rodents with genetic CCR2 deficiency have shed light onto the functional relevance of the CCR2/CCL2 axis for the influx of monocytes/macrophages in ALI. In a study performed by our group, wild-type mice exhibited a vigorous influx of $CD11b^+$ $F4/80^+$ macrophages detectable 4 h and peaking at 48 h post-CCl_4-administration. Macrophage infiltration paralleled tissue and hepatic CCL2 upregulation suggesting that CCL2/CCR2 instructed macrophage-tissue-ingress. This was corroborated by the mitigation of $CD45^+$ cell and macrophage accumulation in the liver in *Ccr2*$^{-/-}$ knockout mice occurring 4 h after toxin-challenge (Karlmark et al., 2009). Since CCL2 mainly controls monocyte bone-marrow egress, levels of circulating monocytes declined as well, yet to a lesser extent. Mitchell et al. (2009) also detected diminished proportions of $CD11b^+$ $F4/80^+$ macrophages as well as their respective blood precursors but not $CD11c^+$ cells post-CCl_4-challenge in *Ccr2* deficient mice at an early time-point. In contrast to the previous work, hepatic damage was reduced in the latter study, as expressed by lower aminotransferases. This suggests a detrimental role of $CCR2^+$ infiltrating macrophages in the setting of acute liver damage. Furthermore, the critical upregulation of the proinflammatory cytokines and chemokines TNF-alpha, IL-1beta, IL-6, CCL2, CCL3, CXCL9, and CXCL10 was markedly attenuated, which might provide a possible mechanism for the observed decrease in liver injury. In addition, this observation implies that invading macrophages are an important source of inflammatory mediators and thereby directly exacerbate tissue harm. Despite this, the putative disadvantageous effects of CCR2-controled macrophage influx observed here cannot be readily extrapolated from the carbon tetrachloride model to other injury settings. Although

data obtained from various studies involving acetaminophen-challenged CCR2$^{-/-}$ mice confirmed that genetic disruption of CCR2 yields less infiltration of macrophages (Dambach et al., 2002; Holt et al., 2008), CCR2 deletion did not limit hepatic necrosis (Dambach et al., 2002) but effectuated increased liver damage with amplified apoptosis (Hogaboam et al., 2000). Noteworthy, Dambach et al. (2002) reported augmented serum CCL2 levels and induction of TNF-alpha in knockout animals in contrast to the CCl_4 model. These findings support the assumption that immigrating CCR2^{+} macrophages in APAP-induced ALI convey their hepatoprotective effect via resolution of inflammation (e.g., through induction of neutrophil apoptosis) and direct necrosis regression due to phagocytosis of cell debris. In line, the induction of several hallmark genes of a M2 macrophage phenotype (*Ym1, Fizz1, Arg-1*) associated with tissue repair and wound healing in infiltrating macrophages has been reported (Holt et al., 2008). Concordantly, clearance of necrotic areas in *Ccr2*$^{-/-}$ animals was substantially delayed in the reparation phase. The controversy regarding the consequence of disruption of the CCR2/CCL2 axis in acute liver toxicity has been further substantiated by a study in which CCL2-deficient mice were characterized by delayed necrosis formation, ameliorated liver enzyme profile, extenuated elevation of TNF-alpha, lymphotoxin-beta, and reduced oxidative stress after single gastric CCl_4 administration (Zamara et al., 2007). These antithetic results suggest CCR2-independent effects of CCL2 which have been described in *in-vitro*-assays before (Schecter et al., 2004; Kruglov et al., 2006). The proposed protective role of CCL2 in acute injury settings recapitulates earlier findings indicating that CCL2 neutralization caused enhanced mortality in a murine model of lethal endotoxemia (Zisman et al., 1997). A monocyte-independent mechanism of CCL2-related hepatoprotection in acute T-cell mediated hepatitis has been provided by Ajuebor et al. (2003) demonstrating that CCL2 also directly hinders IL-4 production by NKT cells.

Importantly, biological effects of CCL2 deficiency in liver injury may be reverted by the compensatory upregulation of CCL8 (MCP-2) and CCL7 (MCP-3), which also bind to CCR2 and mediate monocyte recruitment to the liver in *L. monocytogenes* infection (Jia et al., 2008). Furthermore, monocyte/macrophage trafficking to the site of hepatic injury might also occur without the involvement of chemokines (Shi et al., 2010). Despite these possible confinements, intriguing data from murine steatohepatitis have given proof of the generic feasibility and efficacy of CCL2 antagonism by small molecules in combating liver diseases (Baeck et al., 2012), Clinical trials involving CCR2 antagonists in non-liver disease entities are already at hand. Pharmacological inhibition of CCL2 by *Spiegelmer* technology resulted in a pronounced decrease of monocyte infiltration in acute CCl_4-induced toxicity (Baeck et al., 2012). Further insight into the precise mechanisms of CCR2/CCL2 in ALI and identification of disease settings in which modification of this axis might be of avail are required to benefit from the recent advances in the development of therapeutic strategies.

CCL25/CCR9 PATHWAY

Apart from the CCR2/CCL2 pathway, other chemokine networks are likely to promote monocytes/macrophages to enter the acutely inflamed liver as well. CCR9, a chemokine receptor traditionally associated with gut-homing properties of CD4^{+} T-lymphocytes in response to a CCL25 gradient, has been shown to be expressed on liver-infiltrating macrophages (Nakamoto et al., 2012). Following ConA-exposure TNF-alpha expressing CCR9^{+}CD11b^{+}CD11c^{-} monocytes abundantly accumulated in the liver of wild-type mice in accordance to increased hepatic *ccl25* mRNA expression. In turn, acute hepatitis was largely prevented in CCR9$^{-/-}$ animals and severe inflammation could be restored by adoptive transfer of macrophages from *CCR9*$^{+/+}$ mice. Concordantly, antagonizing CCL25 was able to confine ConA-induced liver damage and reduced the number of CCR9^{+} macrophages (Nakamoto et al., 2012).

CX3CL1 (FRACTALKINE)/CX3CR1 PATHWAY

The CX_3CL1 (fractalkine) receptor CX_3CR1 is constitutively expressed on monocytes and represents the predominant chemokine receptor on the non-classical monocyte subset (Tacke and Randolph, 2006). It is not only implicated in cell migration but also controls several pleiotropic effects which are of vital importance for monocyte biology. In chronic liver injury CX_3CL1 hepatic and serum levels are strongly regulated; CX_3CR1 elicits liver protective functions by promoting hepatic macrophage survival and restricting adoption of a proinflammatory phenotype (Aoyama et al., 2010; Karlmark et al., 2010). Of note, CX_3CR1-mediated macrophage survival suppresses the monocyte influx and thus virtually counteracts CCR2/CCL2 transduced promigratory effects in murine experimental injury models (Karlmark et al., 2010). Besides, CX_3CR1 enables human CD16^{+} monocytes to migrate across inflamed sinusoidal endothelial cell layer in conjunction with vascular-adhesion-protein-1 (VAP-1; Aspinall et al., 2010). Data concerning the relevance of CX_3CR1/CX_3CL1 in ALI is scarce. *In situ* hybridization of *Cx*$_3$*cl1* and *Cx3cr1* revealed increased expression of both gene transcripts in ALI (Efsen et al., 2002), although experimental liver damage was not altered in $CX_3CR1^{-/-}$ mice in early phases after CCl_4-exposure (Karlmark et al., 2010). In the later time-course 72–120 h post-injury when resolution had occurred, intrahepatic CD11b^{+}F4/80^{+} remained fairly stable in knockout animals instead of declining as observed in wild-type controls. In parallel, liver damage was slightly prolonged, even though differences were rather humble (Karlmark et al., 2010). Conclusively, CX_3CL1/CX_3CR1 interactions might spur on wound repair, though further investigations are required to underpin this theory.

IMPLICATIONS OF HUMAN MONOCYTE AND MACROPHAGE ACTIVITY FOR ACUTE LIVER FAILURE

Acute human liver failure is rare clinical syndrome with only 200–500 annual cases in Germany (Canbay et al., 2011). The resulting paucity of data concerning the role of human monocytes and macrophages in this syndrome significantly hampers the translation of findings from animal experimental studies into man. ALF exhibits striking parallels to the systemic inflammatory response syndrome (SIRS) and concomitant compensatory anti-inflammatory responses (CARS) observed in the evolution to septic shock, for instance (Antoniades et al., 2008; Possamai et al., 2010). Multiple organ dysfunction syndrome (MODS) represents

a frequent common final path of SIRS/CARS and ALF, eventually leading to death. Therefore, the dysfunction of human monocytes/macrophages in SIRS/CARS may be regarded as a prototype for the respective processes that govern fatal outcome in ALF. The concordant acquired disturbances in the innate immune system of critically ill patients from a liver and a non-liver background have been extensively presented in a review by Antoniades et al. (2008).

REDUCED MONOCYTIC HLA-DR EXPRESSION AS PROGNOSTIC MARKER IN ALF

Expression of the MHC-class-II-molecule HLA-DR and *ex vivo* cytokine releasing-capacity are the most widely employed tools to study monocyte activation and function. In seminal work involving 50 patients with AALF and 20 patients with ALF due to other causes (NAALF), a dramatic reduced monocytic HLA-DR expression and declined number of total HLA-DR positive monocytes as a sign of immune paralysis could be observed in comparison to patients with stable advance chronic liver disease or healthy controls. HLA-DR levels were even more decreased on monocytes isolated from AALF patients who died or were subjected to liver transplantation in comparison AALF-subjects with transplant-free survival. The authors consecutively proposed HLA-DR expression as a marker to predict an unfavorable outcome in ALF. Simultaneous measurements of proinflammatory (IL-6, TNF-alpha, IFN-gamma) and anti-inflammatory cytokines (IL-4, IL-10) revealed highest concentrations in patients with low HLA-DR expression (Antoniades et al., 2006). The observation of markedly decreased MHC-class-II-molecule expression is highly reminiscent of the monocyte phenotype reported in numerous studies with sepsis and trauma patients and underscores the vicinity of these clinical conditions (Antoniades et al., 2008). Indeed, reduction of monocytic HLA-DR expression as marker of poor prognosis could be recapitulated in patients with acute on chronic liver injury due to superimposed bleeding and infectious complications (Wasmuth et al., 2005). In a recent study, Antoniades et al. (2012) detected depletion of circulating monocytes in AALF patients which was pronounced in individuals who died or required transplantation.

MONOCYTE-RELEASED TNF-ALPHA AND IL-10

Interestingly, *ex vivo* analysis showed a reduced capacity of monocytes to secrete TNF-alpha in response to endotoxin stimulation which was associated with poor outcome (de la Mata et al., 1990; Wigmore et al., 1998). In conjunction with reduced monocytic HLA-DR levels this suggests profound monocyte deactivation, which might contribute to immune paralysis. Immune stunning in the context of a CARS-like state is a predisposing factor to bacterial infections frequently aggravating clinical course of ALF (Antoniades et al., 2008). IL-10 is an inflammation counterbalancing cytokine that dominates the immune paralysis during CARS. Unfavorable elevated systemic IL-10 concentrations observed in ALF might thus be responsible for monocytic dysfunction since IL-10 dampens TNF-alpha release and reduces HLA-DR expression in monocytes (Koppelman et al., 1997; Possamai et al., 2010). Considering the strong capacity of $CD16^-$ monocytes to secrete IL-10, it is conceivable that monocytes are simultaneously a source of and a target for IL-10. In contrast, $CD16^+$ monocytes likely release TNF-alpha in conditions of ALF as well, and boost the SIRS. These oppositional roles, however, have to be scrutinized in this scenario.

ACTIVITY OF INTRAHEPATIC MACROPHAGES IN HUMAN ALF

There is very sparse data aiming at the hepatic compartment in human ALF with respect to macrophages. In acetaminophen-induced liver failure locally proliferating and monocyte-derived macrophages accumulate in necrotic areas in an anti-inflammatory/regenerative niche of high IL-6, IL-10, and TGF-beta1 concentration (Antoniades et al., 2012). Increased circulating soluble CD163 (a hemoglobin scavenger receptor) levels in fulminant liver failure have been suggested to mirror intrahepatic macrophage activity and were associated with fatality (Hiraoka et al., 2005; Moller et al., 2007). Immunohistochemical studies of explanted ALF livers revealed marked expansion of $CD68^+$ macrophages and augmented Fas Ligand expression by KCs, hinting at the induction of macrophage-mediated hepatocyte apoptosis through Fas–FasL interactions as a pathogenic pathway in human liver failure (Mita et al., 2005). A Japanese group proposed massively elevated serum ferritin (>5000 ng/mL) levels as surrogate marker for excessive macrophage hyperactivity, comparable to the macrophage activating syndrome (Kotoh and Takayanagi, 2010). Transarterial injection of 1000 mg methylprednisone via the *arteria hepatica propria* (a procedure named TASIT by the authors) at three consecutive days was performed in 17 patients in a single-center study. In some cases liver biopsies were obtained prior to and 1 week after the intervention showing a decline of hepatic macrophages post-treatment. In addition, survival of treated patients exceeded 70%, whereas only 24% of patients from a conservatively treated control group survived (Kotoh and Takayanagi, 2010). The authors claim a clinical benefit of this tool for selected ALI/ALF patients featuring macrophage hyperactivation as indicated by serum ferritin concentrations. However, severe limitations in the study design hamper to judge the true clinical relevance.

CONCLUSION

Acute liver failure is a rare but fatal clinical condition. Despite its low prevalence it is a significant burden to public health systems. Some of the hallmark molecular events in ALI have been unraveled throughout the last decades but a considerable therapeutic breakthrough has not been accomplished so far. Indeed, new medical tools in counterbalancing the devastating inflammatory mechanisms in the acutely inflamed liver are required to prevent liver transplantation and consecutive live-long immunosuppressive therapy. Tremendous research efforts have corroborated the concept that hepatic macrophages are central in the pathogenesis of acute hepatic injury. Liver resident macrophages do not only contribute to but frequently initiate the inflammatory cascade after an acute hepatic insult. They are an important source of the prototypical proinflammatory cytokine TNF-alpha and other key mediators propagating liver injury. Liver necrosis as the macroscopic sequel to extensive hepatocyte cell death can be reverted by depletion of macrophages in many experimental models. Due to the complexity of the underlying inflammatory network, hepatic macrophages do not only compromise

the integrity of liver parenchyma but also integrate sinusoidal endothelial cells and sessile as well as migrating immune cells into the detriment cascade. However, as summarized in the present review, hepatic macrophages are not exclusively deleterious. They also secrete messengers such as IL-6 or IL-10 that display local hepatoprotective properties. The ambiguous consequences of KC activation in the process of liver injury therefore might arise from a sequential release of pro- and anti-inflammatory cytokines possibly related to a classically or alternatively activated phenotype but might also be attributable to the origin of hepatic macrophages. In this light, KC-ablation even impaired liver damage in some sets of experiments. Furthermore, experimental interventions inhibiting monocyte ingress like disruption of CCR2/CCL2 and other chemokine pathways have partially deciphered the differential role of monocyte-derived macrophages in the context of ALI. Newly recruited macrophages may account for some of the dichotomous functions of hepatic macrophages that have been reported. Moreover, the nature of the underlying injury seemingly impacts macrophage function. In conclusion, although the overall contribution of monocytes and hepatic macrophages to acute hepatic injury may remain elusive, targeting macrophages exhibits a high potential for improving management of patients with ALF in the future.

ACKNOWLEDGMENTS

We thank all members of the Tacke lab for inspiring discussions. This work was supported by the German Research Foundation (DFG-Ta434/2-1, SFB-TRR57), the Start-Program for Young Investigators, and the Interdisciplinary Centre for Clinical Research (IZKF) at the RWTH Aachen.

REFERENCES

Abu-Amara, M., Yang, S. Y., Tapuria, N., Fuller, B., Davidson B., and Seifalian, A. (2010). Liver ischemia/reperfusion injury: processes in inflammatory networks–a review. *Liver Transpl.* 16, 1016–1032.

Adams, D. H., Ju, C., Ramaiah, S. K., Uetrecht, J., and Jaeschke, H. (2010). Mechanisms of immune-mediated liver injury. *Toxicol. Sci.* 115, 307–321.

Ajuebor, M. N., Hogaboam, C. M., Le, T., and Swain, M. G. (2003). C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. *J. Immunol.* 170, 5252–5259.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–197.

Alric, L., Orfila, C., Carrere, N., Beraud, M., Carrera, G., Lepert, J. C., Duffaut, M., Pipy, B., and Vinel, J. P. (2000). Reactive oxygen intermediates and eicosanoid production by Kupffer cells and infiltrated macrophages in acute and chronic liver injury induced in rats by CCl4. *Inflamm. Res.* 49, 700–707.

Andres, D., Sanchez-Reus, I., Bautista, M., and Cascales, M. (2003). Depletion of Kupffer cell function by gadolinium chloride attenuates thioacetamide-induced hepatotoxicity. Expression of metallothionein and HSP70. *Biochem. Pharmacol.* 66, 917–926.

Antoniades, C. G., Berry, P. A., Davies, E. T., Hussain, M., Bernal, W., Vergani, D., and Wendon, J. (2006). Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. *Hepatology* 44, 34–43.

Antoniades, C. G., Berry, P. A., Wendon, J. A., and Vergani, D. (2008). The importance of immune dysfunction in determining outcome in acute liver failure. *J. Hepatol.* 49, 845–861.

Antoniades, C. G., Quaglia, A., Taams, L. S., Mitry, R. R., Hussain, M., Abeles, R., Possamai, L. A., Bruce, M., McPhail, M., Starling, C., Wagner, B., Barnardo, A., Pomplun, S., Auzinger, G., Bernal, W., Heaton, N., Vergani, D., Thursz, M. R., and Wendon, J. (2012). Source and characterisation of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology* 56, 735–746.

Aoyama, T., Inokuchi, S., Brenner, D. A., and Seki, E. (2010). CX3CL1-CX3CR1 interaction prevents carbon tetrachloride-induced liver inflammation and fibrosis in mice. *Hepatology* 52, 1390–1400.

Aspinall, A. I., Curbishley, S. M., Lalor, P. F., Weston, C. J., Liaskou, E., Adams, R. M., Holt, A. P., and Adams, D. H. (2010). CX(3)CR1 and vascular adhesion protein-1-dependent recruitment of CD16(+) monocytes across human liver sinusoidal endothelium. *Hepatology* 51, 2030–2039.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317, 666–670.

Auffray, C., Sieweke, M. H., and Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu. Rev. Immunol.* 27, 669–692.

Baeck, C., Wehr, A., Karlmark, K. R., Heymann, F., Vucur, M., Gassler, N., Huss, S., Klussmann, S., Eulberg, D., Luedde, T., Trautwein, C., and Tacke, F. (2012). Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. *Gut* 61, 416–426.

Bamboat, Z. M., Ocuin, L. M., Balachandran, V. P., Obaid, H., Plitas, G., and DeMatteo, R. P. (2010). Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion. *J. Clin. Invest.* 120, 559–569.

Bansal, M. B., Kovalovich, K., Gupta, R., Li, W., Agarwal, A., Radbill, B., Alvarez, C. E., Safadi, R., Fiel, M. I., Friedman, S. L., and Taub, R. A. (2005). Interleukin-6 protects hepatocytes from CCl4-mediated necrosis and apoptosis in mice by reducing MMP-2 expression. *J. Hepatol.* 42, 548–556.

Bautista, M., Andres, D., Cascales, M., Morales-Gonzalez, J. A., and Sanchez-Reus, M. I. (2010). Effect of gadolinium chloride on liver regeneration following thioacetamide-induced necrosis in rats. *Int. J. Mol. Sci.* 11, 4426–4440.

Belge, K. U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T., and Ziegler-Heitbrock, L. (2002). The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J. Immunol.* 168, 3536–3542.

Bell, L. N., and Chalasani, N. (2009). Epidemiology of idiosyncratic drug-induced liver injury. *Semin. Liver Dis.* 29, 337–347.

Bernal, W., Auzinger, G., Dhawan, A., and Wendon, J. (2010). Acute liver failure. *Lancet* 376, 190–201.

Berres, M. L., Koenen, R. R., Rueland, A., Zaldivar, M. M., Heinrichs, D., Sahin, H., Schmitz, P., Streetz, K. L., Berg, T., Gassler, N., Weiskirchen, R., Proudfoot, A., Weber, C., Trautwein, C., and Wasmuth, H. E. (2010). Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. *J. Clin. Invest.* 120, 4129–4140.

Bilzer, M., Roggel, F., and Gerbes, A. L. (2006). Role of Kupffer cells in host defense and liver disease. *Liver Int.* 26, 1175–1186.

Blazka, M. E., Germolec, D. R., Simeonova, P., Bruccoleri, A., Pennypacker, K. R., and Luster, M. I. (1995). Acetaminophen-induced hepatotoxicity is associated with early changes in NF-kB and NF-IL6 DNA binding activity. *J. Inflamm.* 47, 138–150.

Bourdi, M., Masubuchi, Y., Reilly, T. P., Amouzadeh, H. R., Martin, J. L., George, J. W., Shah, A. G., and Pohl, L. R. (2002). Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. *Hepatology* 35, 289–298.

Campion, S. N., Johnson, R., Aleksunes, L. M., Goedken, M. J., van Rooijen, N., Scheffer, G. L., Cherrington, N. J., and Manautou, J. E. (2008). Hepatic Mrp4 induction following acetaminophen exposure is dependent on Kupffer cell function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G294–G304.

Canbay, A., Feldstein, A. E., Higuchi, H., Werneburg, N., Grambihler, A., Bronk, S. F., and Gores, G. J. (2003). Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. *Hepatology* 38, 1188–1198.

Canbay, A., Tacke, F., Hadem, J., Trautwein, C., Gerken, G., and Manns, M. P. (2011). Acute liver failure: a life-threatening disease. *Dtsch Arztebl Int* 108, 714–720.

Chen, L., Xie, X. J., Ye, Y. F., Zhou, L., Xie, H. Y., Xie, Q. F., Tian, J., and Zheng, S. S. (2011). Kupffer cells contribute to concanavalin A-induced hepatic injury through a Th1 but not Th17 type response-dependent pathway in mice. *HBPD INT* 10, 171–178.

Chung, E. Y., Liu, J., Homma, Y., Zhang, Y., Brendolan, A., Saggese, M., Han, J., Silverstein, R., Selleri, L., and Ma, X. (2007). Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity* 27, 952–964.

Cohen, S. D., and Khairallah, E. A. (1997). Selective protein arylation and acetaminophen-induced hepatotoxicity. *Drug Metab. Rev.* 29, 59–77.

Corcoran, G. B., Mitchell, J. R., Vaishnav, Y. N., and Horning, E. C. (1980). Evidence that acetaminophen and *N*-hydroxyacetaminophen form a common arylating intermediate, *N*-acetyl-*p*-benzoquinoneimine. *Mol. Pharmacol.* 18, 536–542.

Crofton, R. W., Diesselhoff-den Dulk, M. M., and van Furth, R. (1978). The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. *J. Exp. Med.* 148, 1–17.

Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S. Y., Senechal, B., Puel, A., Biswas, S. K., Moshous, D., Picard, C., Jais, J. P., D'Cruz, D., Casanova, J. L., Trouillet, C., and Geissmann, F. (2010). Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 33, 375–386.

Czaja, M. J., Geerts, A., Xu, J., Schmiedeberg, P., and Ju, Y. (1994). Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. *J. Leukoc. Biol.* 55, 120–126.

Dambach, D. M., Watson, L. M., Gray, K. R., Durham, S. K., and Laskin, D. L. (2002). Role of CCR2 in macrophage migration into the liver during acetaminophen-induced hepatotoxicity in the mouse. *Hepatology* 35, 1093–1103.

de la Mata, M., Meager, A., Rolando, N., Daniels, H. M., Nouri-Aria, K. T., Goka, A. K., Eddleston, A. L., Alexander, G. J., and Williams, R. (1990). Tumour necrosis factor production in fulminant hepatic failure: relation to aetiology and superimposed microbial infection. *Clin. Exp. Immunol.* 82, 479–484.

Duffield, J. S., Forbes, S. J., Constandinou, C. M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R., and Iredale, J. P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J. Clin. Invest.* 115, 56–65.

Dunay, I. R., Damatta, R. A., Fux, B., Presti, R., Greco, S., Colonna, M., and Sibley, L. D. (2008). Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen Toxoplasma gondii. *Immunity* 29, 306–317.

Efsen, E., Grappone, C., DeFranco, R. M., Milani, S., Romanelli, R. G., Bonacchi, A., Caligiuri, A., Failli, P., Annunziato, F., Pagliai, G., Pinzani, M., Laffi, G., Gentilini, P., and Marra, F. (2002). Up-regulated expression of fractalkine and its receptor CX3CR1 during liver injury in humans. *J. Hepatol.* 37, 39–47.

Ellett, J. D., Atkinson, C., Evans, Z. P., Amani, Z., Balish, E., Schmidt, M. G., van Rooijen, N., Schnellmann, R. G., and Chavin, K. D. (2010). Murine Kupffer cells are protective in total hepatic ischemia/reperfusion injury with bowel congestion through IL-10. *J. Immunol.* 184, 5849–5858.

Erhardt, A., Biburger, M., Papadopoulos, T., and Tiegs, G. (2007). IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice. *Hepatology* 45, 475–485.

Erhardt, A., and Tiegs, G. (2010). Tolerance induction in response to liver inflammation. *Dig. Dis.* 28, 86–92.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* 101, 890–898.

Filardy, A. A., Pires, D. R., Nunes, M. P., Takiya, C. M., Freire-de-Lima, C. G., Ribeiro-Gomes, F. L., and DosReis, G. A. (2010). Proinflammatory clearance of apoptotic neutrophils induces an IL-12(low)IL-10(high) regulatory phenotype in macrophages. *J. Immunol.* 185, 2044–2050.

Fingerle, G., Pforte, A., Passlick, B., Blumenstein, M., Strobel, M., and Ziegler-Heitbrock, H. W. (1993). The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood* 82, 3170–3176.

Fiorini, R. N., Shafizadeh, S. F., Polito, C., Rodwell, D. W., Cheng, G., Evans, Z., Wan, C., Belden, S., Haines, J. K., Birsner, J., Lewin, D., Wasiluk, K. R., Dunn, D. L., Schmidt, M. G., and Chavin, K. D. (2004). Anti-endotoxin monoclonal antibodies are protective against hepatic ischemia/reperfusion injury in steatotic mice. *Am. J. Transplant.* 4, 1567–1573.

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311, 83–87.

Gao, B., Jeong, W. I., and Tian, Z. (2008). Liver: an organ with predominant innate immunity. *Hepatology* 47, 729–736.

Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A., Campisi, L., Narni-Mancinelli, E., and Lauvau, G. (2008). Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. *Immunol. Cell Biol.* 86, 398–408.

Geissmann, F., Jung, S., and Littman, D. R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19, 71–82.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., and Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science* 327, 656–661.

Giakoustidis, D., Papageorgiou, G., Iliadis, S., Giakoustidis, A., Kostopoulou, E., Kontos, N., Botsoglou, E., Tsantilas, D., Papanikolaou, V., and Takoudas, D. (2006). The protective effect of alpha-tocopherol and GdCl3 against hepatic ischemia/reperfusion injury. *Surg. Today* 36, 450–456.

Giakoustidis, D. E., Iliadis, S., Tsantilas, D., Papageorgiou, G., Kontos, N., Kostopoulou, E., Botsoglou, N. A., Gerasimidis, T., and Dimitriadou, A. (2003). Blockade of Kupffer cells by gadolinium chloride reduces lipid peroxidation and protects liver from ischemia/reperfusion injury. *Hepatogastroenterology* 50, 1587–1592.

Gordon, S., and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5, 953–964.

Grage-Griebenow, E., Zawatzky, R., Kahlert, H., Brade, L., Flad, H., and Ernst, M. (2001). Identification of a novel dendritic cell-like subset of CD64(+)/CD16(+) blood monocytes. *Eur. J. Immunol.* 31, 48–56.

Hanna, R. N., Carlin, L. M., Hubbeling, H. G., Nackiewicz, D., Green, A. M., Punt, J. A., Geissmann, F., and Hedrick, C. C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat. Immunol.* 12, 778–785.

Hanschen, M., Zahler, S., Krombach, F., and Khandoga, A. (2008). Reciprocal activation between CD4+ T cells and Kupffer cells during hepatic ischemia-reperfusion. *Transplantation* 86, 710–718.

Harada, K., Chiba, M., Okamura, A., Hsu, M., Sato, Y., Igarashi, S., Ren, X. S., Ikeda, H., Ohta, H., Kasashima, S., Kawashima, A., and Nakanuma, Y. (2011). Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to hepatic fibrogenesis. *J. Clin. Pathol.* 64, 660–665.

Hardonk, M. J., Dijkhuis, F. W., Hulstaert, C. E., and Koudstaal, J. (1992). Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J. Leukoc. Biol.* 52, 296–302.

Herra, C. M., Keane, C. T., and Whelan, A. (1996). Increased expression of Fc gamma receptors on neutrophils and monocytes may reflect ongoing bacterial infection. *J. Med. Microbiol.* 44, 135–140.

Heymann, F., Trautwein, C., and Tacke, F. (2009). Monocytes and macrophages as cellular targets in liver fibrosis. *Inflamm Allergy Drug Targets* 8, 307–318.

Hiraoka, A., Horiike, N., Akbar, S. M., Michitaka, K., Matsuyama, T., and Onji, M. (2005). Soluble CD163 in patients with liver diseases: very high levels of soluble CD163 in patients with fulminant hepatic failure. *J. Gastroenterol.* 40, 52–56.

Hoedemakers, R. M., Morselt, H. W., Scherphof, G. L., and Daemen, T. (1995). Heterogeneity in secretory responses of rat liver macrophages of different size. *Liver* 15, 313–319.

Hoffmann, F., Sass, G., Zillies, J., Zahler, S., Tiegs, G., Hartkorn, A., Fuchs, S., Wagner, J., Winter, G., Coester, C., Gerbes, A. L., and Vollmar, A. M. (2009). A novel technique for selective NF-kappaB inhibition in Kupffer cells: contrary effects in fulminant hepatitis and ischaemia-reperfusion. *Gut* 58, 1670–1678.

Hogaboam, C. M., Bone-Larson, C. L., Steinhauser, M. L., Matsukawa, A., Gosling, J., Boring, L., Charo, I. F., Simpson, K. J., Lukacs, N. W., and Kunkel, S. L. (2000). Exaggerated hepatic injury due to acetaminophen challenge in mice lacking C-C chemokine receptor 2. *Am. J. Pathol.* 156, 1245–1252.

Holt, M. P., Cheng, L., and Ju, C. (2008). Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J. Leukoc. Biol.* 84, 1410–1421.

Holt, M. P., Yin, H., and Ju, C. (2010). Exacerbation of acetaminophen-induced disturbances of liver sinusoidal endothelial cells in the absence of Kupffer cells in mice. *Toxicol. Lett.* 194, 34–41.

Horelt, A., Belge, K. U., Steppich, B., Prinz, J., and Ziegler-Heitbrock, L. (2002). The CD14+CD16+ monocytes in erysipelas are expanded and show reduced cytokine production. *Eur. J. Immunol.* 32, 1319–1327.

Howard, T. K., Klintmalm, G. B., Cofer, J. B., Husberg, B. S., Goldstein, R. M., and Gonwa, T. A. (1990). The influence of preservation injury on rejection in the hepatic transplant recipient. *Transplantation* 49, 103–107.

Imamura, M., Ogawa, T., Sasaguri, Y., Chayama, K., and Ueno, H. (2005). Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. *Gastroenterology* 128, 138–146.

Ingersoll, M. A., Spanbroek, R., Lottaz, C., Gautier, E. L., Frankenberger, M., Hoffmann, R., Lang, R., Haniffa, M., Collin, M., Tacke, F., Habenicht, A. J., Ziegler-Heitbrock, L., and Randolph, G. J. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 115, e10–e19.

Ishibashi, H., Nakamura, M., Komori, A., Migita, K., and Shimoda, S. (2009). Liver architecture, cell function, and disease. *Semin. Immunopathol.* 31, 399–409.

Jaeschke, H. (2003). Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning. *J. Invest. Surg.* 16, 127–140.

Jaeschke, H., and Smith, C. W. (1997). Mechanisms of neutrophil-induced parenchymal cell injury. *J. Leukoc. Biol.* 61, 647–653.

James, L. P., Simpson, P. M., Farrar, H. C., Kearns, G. L., Wasserman, G. S., Blumer, J. L., Reed, M. D., Sullivan, J. E., and Hinson, J. A. (2005). Cytokines and toxicity in acetaminophen overdose. *J. Clin. Pharmacol.* 45, 1165–1171.

Jaworowski, A., Kamwendo, D. D., Ellery, P., Sonza, S., Mwapasa, V., Tadesse, E., Molyneux, M. E., Rogerson, S. J., Meshnick, S. R., and Crowe, S. M. (2007). CD16+ monocyte subset preferentially harbors HIV-1 and is expanded in pregnant Malawian women with *Plasmodium falciparum* malaria and HIV-1 infection. *J. Infect. Dis.* 196, 38–42.

Jenkins, S. J., Ruckerl, D., Cook, P. C., Jones, L. H., Finkelman, F. D., van Rooijen, N., MacDonald, A. S., and Allen, J. E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332, 1284–1288.

Jia, T., Serbina, N. V., Brandl, K., Zhong, M. X., Leiner, I. M., Charo, I. F., and Pamer, E. G. (2008). Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during *Listeria monocytogenes* infection. *J. Immunol.* 180, 6846–6853.

Josephs, M. D., Bahjat, F. R., Fukuzuka, K., Ksontini, R., Solorzano, C. C., Edwards, C. K. III, Tannahill, C. L., MacKay, S. L., Copeland, E. M. III, and Moldawer, L. L. (2000). Lipopolysaccharide and D-galactosamine-induced hepatic injury is mediated by TNF-alpha and not by Fas ligand. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278, R1196–R1201.

Ju, C., Reilly, T. P., Bourdi, M., Radonovich, M. F., Brady, J. N., George, J. W., and Pohl, L. R. (2002). Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem. Res. Toxicol.* 15, 1504–1513.

Karlmark, K. R., Wasmuth, H. E., Trautwein, C., and Tacke, F. (2008). Chemokine-directed immune cell infiltration in acute and chronic liver disease. *Expert Rev. Gastroenterol. Hepatol.* 2, 233–242.

Karlmark, K. R., Weiskirchen, R., Zimmermann, H. W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C., and Tacke, F. (2009). Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 50, 261–274.

Karlmark, K. R., Zimmermann, H. W., Roderburg, C., Gassler, N., Wasmuth, H. E., Luedde, T., Trautwein, C., and Tacke, F. (2010). The fractalkine receptor CXCR1 protects against liver fibrosis by controlling differentiation and survival of infiltrating hepatic monocytes. *Hepatology* 52, 1769–1782.

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., and Makino, H. (2002). CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum.* 46, 2578–2786.

Kawasaki, E. S., Ladner, M. B., Wang, A. M., Van Arsdell, J., Warren, M. K., Coyne, M. Y., Schweickart, V. L., Lee, M.-T., Wilson, K. J., Boosman, A., Stanley, E. R., Ralph, P., and Mark, D. F. (1985). Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). *Science* 230, 291–296.

Klein, I., Cornejo, J. C., Polakos, N. K., John, B., Wuensch, S. A., Topham, D. J., Pierce, R. H., and Crispe, I. N. (2007). Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood* 110, 4077–4085.

Knolle, P. A., and Gerken, G. (2000). Local control of the immune response in the liver. *Immunol. Rev.* 174, 21–34.

Knolle, P. A., Uhrig, A., Hegenbarth, S., Loser, E., Schmitt, E., Gerken, G., and Lohse, A. W. (1998). IL-10 down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells through decreased antigen uptake via the mannose receptor and lowered surface expression of accessory molecules. *Clin. Exp. Immunol.* 114, 427–433.

Kolios, G., Valatas, V., and Kouroumalis, E. (2006). Role of Kupffer cells in the pathogenesis of liver disease. *World J. Gastroenterol.* 12, 7413–7420.

Koppelman, B., Neefjes, J. J., de Vries, J. E., and de Waal Malefyt, R. (1997). Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity* 7, 861–871.

Kotoh, K., and Takayanagi, R. (2010). A new treatment strategy for fulminant hepatic failure with transcatheter arterial steroid injection therapy. *Fukuoka Igaku Zasshi* 101, 109–118.

Kruglov, E. A., Nathanson, R. A., Nguyen, T., and Dranoff, J. A. (2006). Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290, G765–G771.

Lalor, P. F., Lai, W. K., Curbishley, S. M., Shetty, S., and Adams, D. H. (2006). Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. *World J. Gastroenterol.* 12, 5429–5439.

Laskin, D. L., Gardner, C. R., Price, V. F., and Jollow, D. J. (1995). Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology* 21, 1045–1050.

Laskin, D. L., Pilaro, A. M., and Ji, S. (1986). Potential role of activated macrophages in acetaminophen hepatotoxicity. IMechanism, I., of macrophage accumulation and activation. *Toxicol. Appl. Pharmacol.* 86, 216–226.

Leifeld, L., Dumoulin, F. L., Purr, I., Janberg, K., Trautwein, C., Wolff, M., Manns, M. P., Sauerbruch, T., and Spengler, U. (2003). Early up-regulation of chemokine expression in fulminant hepatic failure. *J. Pathol.* 199, 335–344.

Leuschner, F., Panizzi, P., Chico-Calero, I., Lee, W. W., Ueno, T., Cortez-Retamozo, V., Waterman, P., Gorbatov, R., Marinelli, B., Iwamoto, Y., Chudnovskiy, A., Figueiredo, J. L., Sosnovik, D. E., Pittet, M. J., Swirski, F. K., Weissleder, R., and Nahrendorf, M. (2010). Angiotensin-converting enzyme inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. *Circ. Res.* 107, 1364–1373.

Lin, H., Lee, E., Hestir, K., Leo, C., Huang, M., Bosch, E., Halenbeck, R., Wu, G., Zhou, A., Behrens, D., Hollenbaugh, D., Linnemann, T., Qin, M., Wong, J., Chu, K., Doberstein, S. K., and Williams, L. T. (2008). Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science* 320, 807–811.

Lucas, M., Stuart, L. M., Zhang, A., Hodivala-Dilke, K., Febbraio, M., Silverstein, R., Savill, J., and Lacy-Hulbert, A. (2006). Requirements for apoptotic cell contact in regulation of macrophage responses. *J. Immunol.* 177, 4047–4054.

Luckey, S. W., and Petersen, D. R. (2001). Activation of Kupffer cells during the course of carbon tetrachloride-induced liver injury and fibrosis in rats. *Exp. Mol. Pathol.* 71, 226–240.

MacPhee, P. J., Schmidt, E. E., and Groom, A. C. (1992). Evidence for Kupffer cell migration along liver sinusoids, from high-resolution in vivo microscopy. *Am. J. Physiol.* 263(1 Pt 1), G17–G23.

MacPhee, P. J., Schmidt, E. E., and Groom, A. C. (1995). Intermittence of blood flow in liver sinusoids, studied by high-resolution in vivo microscopy. *Am. J. Physiol.* 269(5 Pt 1), G692–G698.

Malhi, H., and Gores, G. J. (2008). Cellular and molecular mechanisms

of liver injury. *Gastroenterology* 134, 1641–1654.

Marra, F., Grandaliano, G., Valente, A. J., and Abboud, H. E. (1995). Thrombin stimulates proliferation of liver fat-storing cells and expression of monocyte chemotactic protein-1: potential role in liver injury. *Hepatology* 22, 780–787.

Marra, F., DeFranco, R., Grappone, C., Milani, S., Pastacaldi, S., Pinzani, M., Romanelli, R. G., Laffi, G., and Gentilini, P. (1998). Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am. J. Pathol.* 152, 423–430.

Marra, F., DeFranco, R., Grappone, C., Parola, M., Milani, S., Leonarduzzi, G., Pastacaldi, S., Wenzel, U. O., Pinzani, M., Dianzani, M. U., Laffi, G., and Gentilini, P. (1999). Expression of monocyte chemotactic protein-1 precedes monocyte recruitment in a rat model of acute liver injury, and is modulated by vitamin E. *J. Investig. Med.* 47, 66–75.

Marra, F., Valente, A. J., Pinzani, M., and Abboud, H. E. (1993). Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. *J. Clin. Invest.* 92, 1674–1680.

Michael, S. L., Pumford, N. R., Mayeux, P. R., Niesman, M. R., and Hinson, J. A. (1999). Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. *Hepatology* 30, 186–95.

Mita, A., Hashikura, Y., Tagawa, Y., Nakayama, J., Kawakubo, M., and Miyagawa, S. (2005). Expression of Fas ligand by hepatic macrophages in patients with fulminant hepatic failure. *Am. J. Gastroenterol.* 100, 2551–2559.

Mitchell, C., Couton, D., Couty, J. P., Anson, M., Crain, A. M., Bizet, V., Renia, L., Pol, S., Mallet, V., and Gilgenkrantz, H. (2009). Dual role of CCR2 in the constitution and the resolution of liver fibrosis in mice. *Am. J. Pathol.* 174, 1766–1775.

Miyagawa-Hayashino, A., Tsuruyama, T., Egawa, H., Haga, H., Sakashita, H., Okuno, T., Toyokuni, S., Tamaki, K., Yamabe, H., Manabe, T., and Uemoto, S. (2007). FasL expression in hepatic antigen-presenting cells and phagocytosis of apoptotic T cells by FasL+ Kupffer cells are indicators of rejection activity in human liver allografts. *Am. J. Pathol.* 171, 1499–1508.

Mizuhara, H., O'Neill, E., Seki, N., Ogawa, T., Kusunoki, C., Otsuka, K., Satoh, S., Niwa, M., Senoh, H., and Fujiwara, H. (1994). T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J. Exp. Med.* 179, 1529–1537.

Mizuno, K., Toma, T., Tsukiji, H., Okamoto, H., Yamazaki, H., Ohta, K., Kasahara, Y., Koizumi, S., and Yachie, A. (2005). Selective expansion of CD16highCCR2- subpopulation of circulating monocytes with preferential production of haem oxygenase (HO)-1 in response to acute inflammation. *Clin. Exp. Immunol.* 142, 461–470.

Moller, H. J., Gronbaek, H., Schiodt, F. V., Holland-Fischer, P., Schilsky, M., Munoz, S., Hassanein, T., and Lee, W. M. (2007). Soluble CD163 from activated macrophages predicts mortality in acute liver failure. *J. Hepatol.* 47, 671–676.

Mori, Y., Izawa, T., Takenaka, S., Kuwamura, M., and Yamate, J. (2009). Participation of functionally different macrophage populations and monocyte chemoattractant protein-1 in early stages of thioacetamide-induced rat hepatic injury. *Toxicol Pathol* 37, 463–473.

Morio, L. A., Chiu, H., Sprowles, K. A., Zhou, P., Heck, D. E., Gordon, M. K., and Laskin, D. L. (2001). Distinct roles of tumor necrosis factor-alpha and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. *Toxicol. Appl. Pharmacol.* 172, 44–51.

Morita, A., Itoh, Y., Toyama, T., Fujii, H., Nishioji, K., Kirishima, T., Makiyama, A., Yamauchi, N., and Okanoue, T. (2003). Activated Kupffer cells play an important role in intra-hepatic Th1-associated necroinflammation in Concanavalin A-induced hepatic injury in mice. *Hepatol. Res.* 27, 143–150.

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J. L., Libby, P., Weissleder, R., and Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J. Exp. Med.* 204, 3037–3047.

Naito, M., Hasegawa, G., Ebe, Y., and Yamamoto, T. (2004). Differentiation and function of Kupffer cells. *Med. Electron. Microsc.* 37, 16–28.

Naito, M., Hasegawa, G., and Takahashi, K. (1997). Development, differentiation, and maturation of Kupffer cells. *Microsc. Res. Tech.* 39, 350–364.

Nakamoto, N., Ebinuma, H., Kanai, T., Chu, P. S., Ono, Y., Mikami, Y., Ojiro, K., Lipp, M., Love, P. E., Saito, H., and Hibi, T. (2012). CCR9(+) macrophages are required for acute liver inflammation in mouse models of hepatitis. *Gastroenterology* 142, 366–376.

Newsome, P. N., Plevris, J. N., Nelson, L. J., and Hayes, P. C. (2000). Animal models of fulminant hepatic failure: a critical evaluation. *Liver Transpl.* 6, 21–31.

Nockher, W. A., and Scherberich, J. E. (1998). Expanded CD14+ CD16+ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infect. Immun.* 66, 2782–2790.

Ogiku, M., Kono, H., Hara, M., Tsuchiya, M., and Fujii, H. (2011). Glycyrrhizin prevents liver injury by inhibition of high-mobility group box 1 production by Kupffer cells after ischemia-reperfusion in rats. *J. Pharmacol. Exp. Ther.* 339, 93–98.

Okamoto, T., Maeda, O., Tsuzuike, N., and Hara, K. (1998). Effect of gadolinium chloride treatment on concanavalin A-induced cytokine mRNA expression in mouse liver. *Jpn. J. Pharmacol.* 78, 101–103.

Ostapowicz, G., Fontana, R. J., Schiodt, F. V., Larson, A., Davern, T. J., Han, S. H., McCashland, T. M., Shakil, A. O., Hay, J. E., Hynan, L., Crippin, J. S., Blei, A. T., Samuel, G., Reisch, J., and Lee, W. M. (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann. Intern. Med.* 137, 947–954.

Possamai, L. A., Antoniades, C. G., Anstee, Q. M., Quaglia, A., Vergani, D., Thursz, M., and Wendon, J. (2010). Role of monocytes and macrophages in experimental and human acute liver failure. *World J. Gastroenterol.* 16, 1811–1819.

Racanelli, V., and Rehermann, B. (2006). The liver as an immunological organ. *Hepatology* 43(2 Suppl. 1), S54–S62.

Randolph, G. J., Sanchez-Schmitz, G., Liebman, R. M., and Schakel, K. (2002). The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J. Exp. Med.* 196, 517–527.

Ribeiro-Gomes, F. L., Otero, A. C., Gomes, N. A., Moniz-De-Souza, M. C., Cysne-Finkelstein, L., Arnholdt, A. C., Calich, V. L., Coutinho, S. G., Lopes, M. F., and DosReis, G. A. (2004). Macrophage interactions with neutrophils regulate Leishmania major infection. *J. Immunol.* 172, 4454–4462.

Richards, J. A., Wigmore, S. J., and Devey, L. R. (2010). Heme oxygenase system in hepatic ischemia-reperfusion injury. *World J. Gastroenterol.* 16, 6068–6078.

Robays, L. J., Maes, T., Lebecque, S., Lira, S. A., Kuziel, W. A., Brusselle, G. G., Joos, G. F., and Vermaelen, K. V. (2007). Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J. Immunol.* 178, 5305–5311.

Robbins, C. S., and Swirski, F. K. (2010). The multiple roles of monocyte subsets in steady state and inflammation. *Cell. Mol. Life Sci.* 67, 2685–2693.

Roberts, S. A., Price, V. F., and Jollow, D. J. (1990). Acetaminophen structure-toxicity studies: in vivo covalent binding of a nonhepatotoxic analog, 3-hydroxyacetanilide. *Toxicol. Appl. Pharmacol.* 105, 195–208.

Roth, G. A., Faybik, P., Hetz, H., Ankersmit, H. J., Hoetzenecker, K., Bacher, A., Thalhammer, T., and Krenn, C. G. (2009). MCP-1 and MIP3-alpha serum levels in acute liver failure and molecular adsorbent recirculating system (MARS) treatment: a pilot study. *Scand. J. Gastroenterol.* 44, 745–751.

Rothe, G., Gabriel, H., Kovacs, E., Klucken, J., Stohr, J., Kindermann, W., and Schmitz, G. (1996). Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arterioscler. Thromb. Vasc. Biol.* 16, 1437–147.

Sakamoto, S., Okanoue, T., Itoh, Y., Nakagawa, Y., Nakamura, H., Morita, A., Daimon, Y., Sakamoto, K., Yoshida, N., Yoshikawa, T., and Kashima, K. (2002). Involvement of Kupffer cells in the interaction between neutrophils and sinusoidal endothelial cells in rats. *Shock* 18, 152–157.

Schecter, A. D., Berman, A. B., Yi, L., Ma, H., Daly, C. M., Soejima, K., Rollins, B. J., Charo, I. F., and Taubman, M. B. (2004). MCP-1-dependent signaling in CCR2(-/-) aortic smooth muscle cells. *J. Leukoc. Biol.* 75, 1079–1085.

Schlitt, A., Heine, G. H., Blankenberg, S., Espinola-Klein, C., Dopheide, J. F., Bickel, C., Lackner, K. J., Iz, M., Meyer, J., Darius, H., and Rupprecht, H. J. (2004). CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thromb. Haemost.* 92, 419–424.

Schumann, J., Wolf, D., Pahl, A., Brune, K., Papadopoulos, T., van Rooijen, N., and Tiegs, G. (2000). Importance of Kupffer cells for T-cell-dependent liver injury in mice. *Am. J. Pathol.* 157, 1671–1683.

Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., and Schwabe, R. F. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332.

Serbina, N. V., Jia, T., Hohl, T. M., and Pamer, E. G. (2008). Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* 26, 421–452.

Serbina, N. V., and Pamer, E. G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7, 311–317.

Shi, C., Velazquez, P., Hohl, T. M., Leiner, I., Dustin, M. L., and Pamer, E. G. (2010). Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. *J. Immunol.* 184, 6266–6274.

Sleyster, E. C., and Knook, D. L. (1982). Relation between localization and function of rat liver Kupffer cells. *Lab. Invest.* 47, 484–490.

Strauss-Ayali, D., Conrad, S. M., and Mosser, D. M. (2007). Monocyte subpopulations and their differentiation patterns during infection. *J. Leukoc. Biol.* 82, 244–252.

Streetz, K. L., Tacke, F., Leifeld, L., Wustefeld, T., Graw, A., Klein, C., Kamino, K., Spengler, U., Kreipe, H., Kubicka, S., Muller, W., Manns, M. P., and Trautwein, C. (2003). Interleukin 6/gp130-dependent pathways are protective during chronic liver diseases. *Hepatology* 38, 218–229.

Stuart, W. D., Kulkarni, R. M., Gray, J. K., Vasiliauskas, J., Leonis, M. A., and Waltz, S. E. (2011). Ron receptor regulates Kupffer cell-dependent cytokine production and hepatocyte survival following endotoxin exposure in mice. *Hepatology* 53, 1618–1628.

Sunderkotter, C., Nikolic, T., Dillon, M. J., Van Rooijen, N., Stehling, M., Drevets, D. A., and Leenen, P. J. (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* 172, 4410–4417.

Swirski, F. K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J. L., Kohler, R. H., Chudnovskiy, A., Waterman, P., Aikawa, E., Mempel, T. R., Libby, P., Weissleder, R., and Pittet, M. J. (2009). Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325, 612–616.

Szaflarska, A., Baj-Krzyworzeka, M., Siedlar, M., Weglarczyk, K., Ruggiero, I., Hajto, B., and Zembala, M. (2004). Antitumor response of CD14+/CD16+ monocyte subpopulation. *Exp. Hematol.* 32, 748–755.

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., Lira, S. A., Habenicht, A. J., and Randolph, G. J. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 117, 185–194.

Tacke, F., Ginhoux, F., Jakubzick, C., van Rooijen, N., Merad, M., and Randolph, G. J. (2006). Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. *J. Exp. Med.* 203, 583–597.

Tacke, F., and Kurts, C. (2011). Infiltrating monocytes versus resident Kupffer cells: do alternatively activated macrophages need to be targeted alternatively? *Hepatology* 54, 2269–2271.

Tacke, F., Luedde, T., and Trautwein, C. (2009). Inflammatory pathways in liver homeostasis and liver injury. *Clin. Rev. Allergy Immunol.* 36, 4–12.

Tacke, F., and Randolph, G. J. (2006). Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* 211, 609–618.

Tagawa, Y., Matthys, P., Heremans, H., Dillen, C., Zaman, Z., Iwakura, Y., and Billiau, A. (2000). Bimodal role of endogenous interleukin-6 in concanavalin A-induced hepatitis in mice. *J. Leukoc. Biol.* 67, 90–96.

Taylor, P. R., and Gordon, S. (2003). Monocyte heterogeneity and innate immunity. *Immunity* 19, 2–4.

Thieblemont, N., Weiss, L., Sadeghi, H. M., Estcourt, C., and Haeffner-Cavaillon, N. (1995). CD14lowCD16high: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. *Eur. J. Immunol.* 25, 3418–3424.

Tomiyama, K., Ikeda, A., Ueki, S., Nakao, A., Stolz, D. B., Koike, Y., Afrazi, A., Gandhi, C., Tokita, D., Geller, D. A., and Murase, N. (2008). Inhibition of Kupffer cell-mediated early proinflammatory response with carbon monoxide in transplant-induced hepatic ischemia/reperfusion injury in rats. *Hepatology* 48, 1608–1620.

Tsuneyama, K., Harada, K., Yasoshima, M., Hiramatsu, K., Mackay, C. R., Mackay, I. R., Gershwin, M. E., and Nakanuma, Y. (2001). Monocyte chemotactic protein-1, -2, and -3 are distinctively expressed in portal tracts and granulomata in primary biliary cirrhosis: implications for pathogenesis. *J. Pathol.* 193, 102–109.

Tunon, M. J., Alvarez, M., Culebras, J. M., and Gonzalez-Gallego, J. (2009). An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure. *World J. Gastroenterol.* 15, 3086–3098.

Varol, C., Landsman, L., Fogg, D. K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F., and Jung, S. (2007). Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J. Exp. Med.* 204, 171–180.

Wanner, G. A., Ertel, W., Muller, P., Hofer, Y., Leiderer, R., Menger, M. D., and Messmer, K. (1996). Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock* 5, 34–40.

Wasmuth, H. E., Kunz, D., Yagmur, E., Timmer-Stranghoner, A., Vidacek, D., Siewert, E., Bach, J., Geier, A., Purucker, E. A., Gressner, A. M., Matern, S., and Lammert, F. (2005). Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. *J. Hepatol.* 42, 195–201.

Wei, S., Nandi, S., Chitu, V., Yeung, Y. G., Yu, W., Huang, M., Williams, L. T., Lin, H., and Stanley, E. R. (2010). Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J. Leukoc. Biol.* 88, 495–505.

Wigmore, S. J., Walsh, T. S., Lee, A., and Ross, J. A. (1998). Pro-inflammatory cytokine release and mediation of the acute phase protein response in fulminant hepatic failure. *Intensive Care Med.* 24, 224–229.

Wijngaarden, S., van Roon, J. A., Bijlsma, J. W., van de Winkel, J. G., and Lafeber, F. P. (2003). Fcgamma receptor expression levels on monocytes are elevated in rheumatoid arthritis patients with high erythrocyte sedimentation rate who do not use anti-rheumatic drugs. *Rheumatology (Oxford)* 42, 681–688.

Wong, K. L., Tai, J. J., Wong, W. C., Han, H., Sem, X., Yeap, W. H., Kourilsky, P., and Wong, S. C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* 118, e16–e31.

Wu, Z., Han, M., Chen, T., Yan, W., and Ning, Q. (2010). Acute liver failure: mechanisms of immune-mediated liver injury. *Liver Int.* 30, 782–794.

Yamamoto, T., Naito, M., Moriyama, H., Umezu, H., Matsuo, H., Kiwada, H., and Arakawa, M. (1996). Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. *Am. J. Pathol.* 149, 1271–1286.

You, Q., Cheng, L., Kedl, R. M., and Ju, C. (2008). Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology* 48, 978–990.

Zamara, E., Galastri, S., Aleffi, S., Petrai, I., Aragno, M., Mastrocola, R., Novo, E., Bertolani, C., Milani, S., Vizzutti, F., Vercelli, A., Pinzani, M., Laffi, G., LaVilla, G., Parola, M., and Marra, F. (2007). Prevention of severe toxic liver injury and oxidative stress in MCP-1-deficient mice. *J. Hepatol.* 46, 230–238.

Zawada, A. M., Rogacev, K. S., Rotter, B., Winter, P., Marell, R. R., Fliser, D., and Heine, G. H. (2011). SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood* 118, e50–e61.

Zhai, Y., Busuttil, R. W., and Kupiec-Weglinski, J. W. (2011). Liver ischemia and reperfusion injury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation. *Am. J. Transplant.* 11, 1563–1569.

Zhang, M., Xu, S., Han, Y., and Cao, X. (2011). Apoptotic cells attenuate fulminant hepatitis by priming Kupffer cells to produce interleukin-10 through membrane-bound TGF-beta. *Hepatology* 53, 306–316.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J., Liu, Y. J., MacPherson, G., Randolph, G. J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J. M., and Lutz, M. B. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood* 116, e74–e80.

Zimmermann, H. W., Seidler, S., Gassler, N., Nattermann, J., Luedde, T., Trautwein, C., and Tacke, F. (2011). Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. *PLoS ONE* 6, e21381. doi:10.1371/journal.pone.0021381

Zimmermann, H. W., Seidler, S., Nattermann, J., Gassler, N., Hellerbrand, C., Zernecke, A., Tischendorf, J. J., Luedde, T., Weiskirchen, R., Trautwein, C., and Tacke, F. (2010). Functional contribution of elevated circulating and hepatic non-classical CD14CD16 monocytes to inflammation and human liver fibrosis. *PLoS ONE* 5, e11049. doi:10.1371/journal.pone.0011049

Zimmermann, H. W., and Tacke, F. (2011). Modification of chemokine pathways and immune cell infiltration as a novel therapeutic approach in liver inflammation and fibrosis. *Inflamm Allergy Drug Targets* 10, 509–536.

Zisman, D. A., Kunkel, S. L., Strieter, R. M., Tsai, W. C., Bucknell, K., Wilkowski, J., and Standiford, T. J. (1997). MCP-1 protects mice in lethal endotoxemia. *J. Clin. Invest.* 99, 2832–2836.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 January 2012; accepted: 27 February 2012; published online: 19 October 2012.

Citation: Zimmermann HW, Trautwein C and Tacke F (2012) Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. Front. Physio. ***3****:56. doi: 10.3389/fphys.2012.00056*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 26 September 2013
doi: 10.3389/fphys.2013.00258

Role of liver progenitors in acute liver injury

Jan Best[1,2], Laurent Dollé[2], Paul Manka[1], Jason Coombes[3], Leo A. van Grunsven[2] and Wing-Kin Syn[3]*

[1] *Department of Gastroenterology and Hepatology, University Hospital Essen, Essen, Germany*
[2] *Liver Cell Biology Lab (LIVR), Department of Cell Biology (CYTO), Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium*
[3] *Regeneration and Repair Group, Foundation for Liver Research, The Institute of Hepatology, London, UK*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Frank Tacke, University Hospital Aachen, Germany
Bruno Christ, Department of Surgery, Germany

****Correspondence:***
Wing-Kin Syn, Regeneration and Repair Group, The Institute of Hepatology, Harold Samuel House, 69-75 Chenies Mews, London WC1E 6HX, UK
e-mail: wsyn@doctors.org.uk

Acute liver failure (ALF) results from the acute and rapid loss of hepatocyte function and frequently exhibits a fulminant course, characterized by high mortality in the absence of immediate state-of-the-art intensive care and/or emergency liver transplantation (ELT). The role of hepatocyte-mediated liver regeneration during acute and chronic liver injury has been extensively investigated, and recent studies suggest that hepatocytes are not exclusively responsible for the regeneration of the injured liver during fulminant liver injury. Liver progenitor cells (LPC) (or resident liver stem cells) are quiescent in the healthy liver, but may be activated under conditions where the regenerative capacity of mature hepatocytes is severely impaired. This review aims to provide an overview of the role of the LPC population during ALF, and the role of putative cytokines, growth factors, mitogens, and hormones in the LPC response. We will highlight the potential interaction among cellular compartments during ALF, and discuss the possible prognostic value of the LPC response on ALF outcomes.

Keywords: hepatic failure, acute liver failure, severe acute liver injury, liver progenitor cells, oval cells, stem cells, liver regeneration

INTRODUCTION

Acute liver failure (ALF) is a rare clinical syndrome that affects around 2000 individuals in the USA annually (Polson and Lee, 2005) and frequently exhibits a fulminant course, characterized by high mortality in the absence of immediate state-of-the-art intensive care and/or emergency liver transplantation (ELT).

ALF results from the acute and rapid loss of hepatocyte function, and is associated with coagulopathy [International Normalized Ratio (INR) >1.5] and hepatic encephalopathy (HE) in a patient without pre-existing liver disease. Typically, the time from onset of symptoms to development of HE is up to 8 weeks, but may take up to 26 weeks (Polson and Lee, 2005). Individuals who develop hepatic dysfunction (i.e., coagulopathy), in the absence of HE are defined as having severe acute liver injury (sALI). ALF can occur as a result of various etiologies. In a German study, triggers such as non-acetaminophen drug-induction (including idiosyncratic toxic reactions, e.g., Phenprocoumon) (32%), indeterminate or sero-negative hepatitis (24%), viral hepatitis (such as hepatitis A, B, E) (21%) (Jochum et al., 2009), and acetaminophen overdose (9%) (Hadem et al., 2012) appear to be most frequent causes of ALF. Other etiologies include autoimmune disease (or hepatitis) (Czaja, 2013), ischemia (Henrion, 2012) pregnancy (Ichai and Saliba, 2009), Wilsons disease (Okada et al., 2010), and congestive heart failure (Saner et al., 2009).

The prognosis of ALF is primarily dependent upon the underlying etiology. During ALF, viral-mediated (i.e., direct cytopathic effects), cytokine and/or immune-mediated (i.e., indirect cytopathic effects) hepatocyte necrosis, and apoptosis occur. A regenerative process is triggered, and replication of the remaining healthy hepatocytes ensues, in an attempt to restore hepatic architecture and function. This process is initiated or regulated, at least in part, by three major factors which include cytokines, growth factors, and metabolic signaling pathways. During the early stages of liver damage, inflammatory cytokines trigger healthy hepatocytes to enter the cell cycle. If hepatocyte replication is hampered by excessive parenchymal damage (as generally observed in ALF), or hepatocyte senescence (as occurring in steatotic livers or livers with concomitant chronic injury), resident liver progenitor cells (LPCs) are activated to support, or take over the role of regeneration. However, for many with ALF, this regenerative process is inadequate to match the rapid, confluent loss of hepatocyte mass and function, and liver transplantation offers the only potential hope for survival. Further studies will be needed to ascertain if an enhanced liver progenitor response could lead to better patient outcomes.

A proportion of individuals will recover spontaneously from ALF, and they exemplify the unique capacity of the liver to regenerate completely after injury. Currently used ALF scores/criteria such as the King's College criteria (KCC), Model of end stage liver disease score (MELD), and Bilirubin-lactate-etiology score (BiLE) (Hadem et al., 2008) utilize clinical parameters at the time of admission and/or during the course of ALF, and reliably predict death, but are poor at predicting survival. Recent studies suggest that cell death markers (M65)-based MELD may improve prediction of spontaneous survival (Bechmann et al., 2008, 2010). Hepatocyte cell death is intricately linked to LPC response in chronic liver disease (Jung et al., 2012; Sancho-Bru et al., 2012), hence, raising the possibility that the amount or type of LPC activation (i.e., progenitor cell response) during ALF could also predict ALF outcomes.

The aims of the review are: (A) To provide an overview of the mechanisms of LPC activation and to highlight potential therapeutic targets/strategies in context of ALF, and (B) To discuss the clinical relationship between LPC activation and acute liver damage, and the possible role of LPC activation in predicting ALF outcomes.

DIFFERENT CELL POPULATIONS ARE ACTIVATED DURING ACUTE AND CHRONIC LIVER DAMAGE

ALF (fulminant hepatic failure) occurs when there is rapid, massive hepatocyte cell death, which leads to significant impairment of liver function. On the other hand, chronic liver injury is driven by progressive hepatocyte injury and death that spans months, years and even decades.

The cellular response that occurs during hepatic injury essentially mirrors the clinical scenario, and as such, is strongly associated with the etiology and severity of injury. Mature hepatocytes constitute the majority cell type in the liver, and are unipotent cells that contribute to normal cell turnover and are able to respond rapidly to injurious stimuli (such as liver resection in man, or partial hepatectomy in mice). In contrast, the LPC compartment is triggered to expand when hepatocyte loss occurs in the presence of residual hepatocyte senescence (i.e., replicative senescence), a feature common to chronic liver disease (Santoni-Rugiu et al., 2005; Dollé et al., 2010). Upon transit amplification, the LPCs infiltrate along the liver plate toward the central vein, and differentiate into hepatocytes to restore liver function and cell mass (Espanol-Suner et al., 2012).

The role of LPC in acute injury or ALF remains poorly defined (Theise et al., 2013). Nevertheless, we and others have recently reported that LPC expansion occurs in mice and humans during acute hepatic injury (or hepatic failure), and propose that the LPC compartment is an important contributor to the restoration of liver parenchyma or function in mice (Ochoa et al., 2010; Khuu et al., 2013). Inhibiting the LPC response in mice after 70% partial hepatectomy led to impaired liver regeneration, as assessed by the liver to body weight ratios, and reduced overall survival. It is likely, however, that the LPC compartment is only activated when there is an insufficient number of healthy residual hepatocytes to undertake the regenerative process. Indeed, Katoonizadeh et al. suggest, that a minimum 50% hepatocyte loss and presence of hepatocyte replicative senescence are necessary triggers for LPC activation (Katoonizadeh et al., 2006).

The activation and expansion of the LPC compartment occurs roughly over 7 days, while process of LPC differentiation into intermediate hepatocytes requires an additional 7 days (Fausto, 2004; Fausto et al., 2006). Thus, the LPC response is a much slower regenerative process (compared with hepatocyte replication), and can be more easily detected in the livers of patients with a sub-acute form of ALF (i.e., such as in those with sero-negative hepatitis). Although there is no direct evidence on the role of LPC in human liver regeneration during ALF, the presence of a ductular response after acute alcoholic hepatitis or ALF, and aggregate data from small animal studies support the hypothesis that activation and differentiation of LPCs might play a pivotal role in regeneration following fulminant hepatocyte loss (Katoonizadeh et al., 2006; Sancho-Bru et al., 2012).

SIGNALING PATHWAYS AND MARKERS OF LPC

Under normal circumstances (i.e., in a healthy adult liver), the responsibility of regenerating a liver after an acute insult falls upon the residual hepatocytes. Ordinarily, hepatocytes turn over once or twice a year (Fausto et al., 2006). Upon acute injury, complete hepatocyte regeneration can occur after 2–3 cycles of hepatocyte replication (Michalopoulos, 2010). This regenerative process is orchestrated by cross talk between different liver cell compartments, and mediated by multiple cytokines, growth factors, and mitogens.

The regulation of the LPC response is best characterized in humans and animal models of chronic liver disease. Chronic liver disease is characterized by hepatocyte apoptosis, necrosis, and senescence (Ghavami et al., 2005), and is concomitantly associated with a robust expansion of the LPC compartment (Duncan et al., 2009; Fellous et al., 2009). Some of the putative factors that promote LPC expansion include cytokines IL6, TNFα, TGFβ, as well as cytokine regulated transcription factors nuclear factor kappa B, CCAAT enhancer binding protein beta, and growth factors HGF, EGF (Campbell et al., 2001). Hormones (such as insulin, somatostatin) (Jung et al., 2012), adipokines (cytokines released by adipocytes, such as leptin) (Diehl, 2005; Nobili et al., 2012), and neurotransmitters (such as serotonin, epinephrine or norepinephrine) have also been reported to regulate LPC response or growth. The interactions between these factors and signaling pathways are complex, and remain poorly understood. In aggregate, they act to stimulate the proliferation of LPC, and promote their differentiation into new hepatocytic cells and cholangiocytes. Recent studies show that morphogens (factors important during embryonic development) such as Wnt, Notch, and Hedgehog (Hh) are also important drivers of LPC response. For example, Hh ligands released by apoptotic hepatocytes can act on surrounding LPC and hepatic stellate cells (the key cell involved in scar tissue accumulation) to promote liver repair (Jung et al., 2010), while Wnt and Notch signals within the microenvironment could modulate LPC differentiation into either hepatocytes or cholangiocytes, respectively (Boulter et al., 2012).

The regenerative process that follows ALF is not well described or understood, but is likely to resemble the liver repair process occurring during chronic liver disease. During ALF, the liver would have been subject to a significant insult that results in widespread hepatocyte necrosis and apoptosis, which far exceeds the capacity of the remaining healthy hepatocytes to replicate and to restore homeostatic function.

For individuals who have other co-morbidities (such as hepatic steatosis associated with obesity or type 2 diabetes mellitus), hepatocytes may already exhibit replicative senescence which would further limit the regenerative capability of residual hepatocytes. The LPC compartment located within the canals of Herring (Petersen and Shupe, 2008) is therefore tasked to restore hepatocytic function in the failing liver. Indeed, in a recent study (Dechene et al., 2010), we observed a robust ductular reaction among survivors of ALF. Consistently, LPC markers appeared to correlate with severity and short-term mortality among individuals with alcoholic hepatitis (Sancho-Bru et al., 2012). Following

fulminant liver injury, several different cell signaling axes have also been postulated to regulate LPC-mediated liver regeneration.

The TNF-like weak apoptosis inducing factor (TWEAK)/Fibroblast growth factor inducible 14 (Fn14) pathway plays a crucial role in activation of LPCs. TWEAK/Fn 14 activation has been reported to selectively expand LPCs, without affecting growth and viability of mature resident hepatocytes (Jakubowski et al., 2005). Recent studies in humans and mice with chronic liver disease confirmed that Fn14, the receptor of TWEAK, is dramatically upregulated during chronic injury, and directly modulates the LPC response (Tirnitz-Parker et al., 2010). Liver expression of TWEAK/Fn14 is also upregulated significantly, early after partial hepatectomy (Ochoa et al., 2010). Using the Fn14-deficient mice, Karaca and colleagues further propose that TWEAK/Fn14 axis could directly stimulate LPC expansion after acute liver injury (Karaca et al., 2010).

Following activation of progenitor cell niche, it has been postulated, that migration of LPCs is mediated by SCF/c-Kit (Hu and Colletti, 2008) and SDF1/CXCR4 (Hatch et al., 2002):

Stem cell factor (SCF) and its receptor c-kit play a key role in hematopoiesis and cellular proliferation. It is well accepted, that c-kit is a cell surface marker for progenitor cells (Heinemann et al., 2005). The biologic effects of the SCF/c-kit system are believed to involve survival, proliferation, and migration of early stem cell progeny (Fujio et al., 1994). There is a large reservoir of hepatic SCF, and this molecule has proven to play a pivotal role in liver reconstitution following 70% partial hepatectomy in mice. In another mouse model of ALF, Hu and co-workers found SCF-deficient mice administered APAP exhibited significantly higher mortality compared with litter-mate controls. Furthermore, administration of exogenous SCF significantly reduced mortality of APAP-treated wild-type mice (Hu and Colletti, 2008).

Stromal derived factor-1 alpha (SDF-1alpha) and its cognate receptor CXCR4 have similarly been shown to regulate migration of hematopoietic stem cells (HSC) in the fetal and adult stages of hematopoiesis. Previously, others have shown that bone marrow-derived mesenchymal stem cells promote hepatic regeneration *after CCl 4 treatment in rats* (Gruttadauria et al., 2013; Li et al., 2013). Hatch and colleagues recently proposed that the SDF-1alpha-CXCR4 axis is important for oval (LPC) cell activation during liver regeneration. They show that up-regulation of hepatocyte-derived SDF-1alpha expression during fulminant liver injury could not only promote recruitment of HSC from the bone-marrow (Hatch et al., 2002), but also enhance LPC accumulation, as both of these progenitor populations express CXCR4, the known receptor for SDF-1alpha.

The Hh pathway normally orchestrates fetal tissue and organ development, but has been shown to play an important role during adult tissue repair (Omenetti and Diehl, 2008). Hh ligands stimulate the expansion and viability of various stem cells (Yang et al., 2008b) and have been shown to function as viability factors for human and rodent liver progenitors. Recent studies have shown that Hh pathway activation occurs during liver regeneration after partial hepatectomy (Ochoa et al., 2010; Cai et al., 2011; Hanaoka et al., 2013). Importantly, inhibiting the Hh signaling (with cyclopamine, an antagonist of Smoothened), led to an attenuated LPC response, a lower expression of liver progenitor markers, AFP, Fn14, and K19, and reduced overall survival (Ochoa et al., 2010). The aggregate observations confirm the importance of Hh signaling in the LPC response after acute liver injury, and suggest that the degree of Hh pathway activation may dictate the extent of liver regeneration and clinical outcome in ALF. Although consistent with its recognized role during development, further studies will be needed to ascertain the importance of Hh-mediated LPC response in other models of adult ALF. A better understanding of the role of LPC in ALF, and more detailed study of some of these pathways may help identify potential treatment strategies for the treatment of ALF.

The role of Tri-iodothyronine (T3) in tissue regeneration is well recognized (Leffert and Alexander, 1976; Short and Ove, 1983). Experimental models of liver regeneration have, in most cases, focused on characterizing hepatocyte replication, but not of LPC-mediated parenchymal reconstitution. T3 affects cell growth, differentiation, and regulates metabolic functions via its interaction with the thyroid hormone nuclear receptors (TRs). Cumulative studies suggest that T3 is a potent stimulator of liver regeneration. Bockhorn et al. found out that the exogenous administration of T3 enhanced liver regeneration after 70 and 90% hepatectomy in terms of increased liver to body weight ratio and Ki-67 index (Bockhorn et al., 2007). Several molecular mechanisms have been postulated to mediate T3 effects on liver regeneration. T3 stimulated rats that were subjected to partial hepatectomy expressed the cell cycle protein, cyclin D1 at earlier time points compared with control rats that did not receive T3, suggesting that T3-TR signaling is an important regulator of the cell cycle in an experimental model of liver resection (Leffert and Alexander, 1976; Short and Ove, 1983). Recent reports suggest that T3 not only stimulates hepatocyte proliferation, but may induce LPC activation during fulminant liver injury. In a rodent model of combined AAF/PH, László et al. showed that administration of T3 led to an accelerated differentiation of LPCs into hepatocytes (Laszlo et al., 2008). Nevertheless, the molecular mechanisms underlying the LPC differentiation response have yet to be fully understood.

IS THERE A CELLULAR MICRO-ENVIRONMENT CHAPERONING LPC MIGRATION?

LPCs are believed to originate from the canals of Hering (CoH), which is lined proportionately by cholangiocytes and hepatocytes (see **Figure 1**). It serves to conduct bile from the bile canaliculi to the terminal bile ducts located in the portal tracts (Saxena and Theise, 2004). Because the CoH constitutes the biliary-hepatocytic interface, it makes biological sense that LPCs, being bipotential cells, are located in this niche. The LPC neighborhood includes epithelial (hepatocytes and cholangiocytes) cells, hepatic stellate cells, immune cells (i.e., Kupffer cells), and the extracellular matrix (ECM). The proximity of these cells suggests that crosstalk is important, and occurs not only under basal, homeostatic conditions, but also during injury and repair. Indeed, during liver injury, soluble factors released by one cell type act in a autocrine and paracrine manner to regulate

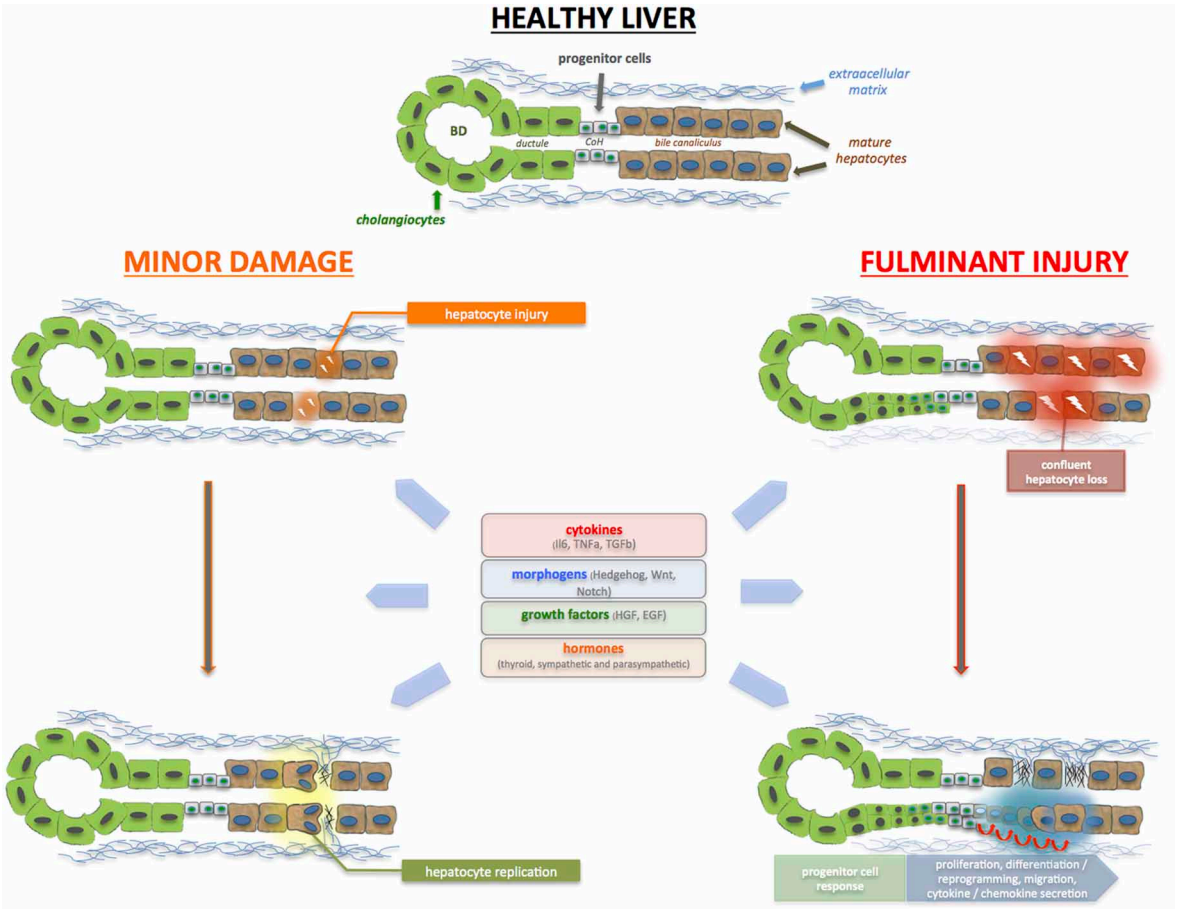


FIGURE 1 | During acute liver injury, hepatocyte (and cholangiocyte) apoptosis, and necrosis occur. With minor injury, restoration of hepatocyte mass and function is mediated by the replication of remaining healthy hepatocytes (and cholangiocytes). During a major insult, massive, and confluent hepatocyte loss occurs. There are insufficient healthy remaining hepatocytes mass to restore hepatic function; as such, the liver progenitor (LPC) or liver stem cell compartment is activated in an attempt to restore epithelial cell mass, architecture, and function. The bipotential LPCs reside in the Canals of Herring (CoH), located in the niche of the biliary-hepatocytic interface, and are able to infiltrate along the liver plate and differentiate into hepatocytes and cholangiocytes. LPCs are surrounded by epithelial cells, non-parenchymal cells such as hepatic stellate cells, as well as immune cells and extracellular matrix. These regenerative processes are triggered and regulated by the plethora of cytokines, growth factors and metabolic signals. Resurrection of morphogenic signals (i.e., Hedgehog, Wnt, Notch) also occurs, particularly during massive injury, to invoke the liver progenitor cell compartment. In brief, these molecules act in concert to ensure that sufficient regeneration occurs, and yet, not exceed normal homeostatic requirements.

the growth and differentiation of a neighboring cell compartment (Parola and Pinzani, 2009). We reported that Hh ligands which are over-expressed during acute and chronic liver injury could directly stimulate LPCs to secrete chemokines that lead to the additional recruitment of inflammatory cells which participate in the regeneration or repair process (Omenetti et al., 2009). Inflammatory cells produce a range of cytokines and chemokines. SDF-1 attracts $CXCR4^{+}$ T cells, which express TNF-like weak inducer of apoptosis (TWEAK), that in turn stimulates LPC response by engaging its receptor Fn14 (Alison et al., 2009).

Recent studies further show that the expansion of the LPC compartment occurs in association with ECM remodeling (Van Hul et al., 2009; Lorenzini et al., 2010; Lozoya et al., 2011), while failure of ECM remodeling lead to impaired ability of the liver to activate LPCs (Kallis et al., 2011). In this study, laminin-LPC interactions were shown to be critical for LPC-mediated repair. Separately, Van Hul and co-workers observed that ECM deposition and activation of matrix-secreting cells occurred, not only before the increase in number of LPCs, but also in front of LPCs along the porto-venous gradient of lobular invasion (Van Hul et al., 2009). During migration, LPCs are embedded within ECM and are chaperoned by alpha-smooth muscle actin (alpha-SMA)-positive cells (Van Hul et al., 2009). In addition to a direct effect of Hh on LPC, Hh pathway activation could also enhance LPC proliferation, indirectly, through the activation of hepatic stellate cells into matrix-producing myofibroblasts (Choi et al., 2009). These findings in mice support our study in man, where we showed that LPC expansion (ductular reaction) occurred in context of the fibrogenic response (Dechene et al., 2010). We

propose that short term accumulation of collagen matrix might be a physiological repair response that precedes parenchymal cell reconstitution. Similar to findings in mice, the fibrous tissue scaffold is the LPC niche that facilitates LPC activation and differentiation.

Further studies will be needed to understand if, and how ECM composition could modulate LPC responses after ALF, and whether modifying individual components of ECM could regulate LPC proliferation and differentiation.

IS THERE A CORRELATION BETWEEN LPC ACTIVATION AND CLINICAL PARAMETERS IN ALF?

Currently used ALF scoring systems such as the KCC, MELD, and BiLE reliably predict death, but are poor at predicting survival (Polson and Lee, 2005). None of these systems take into account the histological changes that occur during ALF. The role of histology and presence or absence of hepatocyte cell death has only recently come to fore. We recently reported that a CK18 M65 (marker of cell death)-based MELD score could predict survival from ALF with greater sensitivity and specificity (Bechmann et al., 2010). Among individuals with acute alcoholic hepatitis, a condition with high mortality, expression of LPC markers correlated positively with clinical severity and short-term mortality (Sancho-Bru et al., 2012).

A similar study of 74 patients with ALF or sub-acute liver failure demonstrated a positive correlation between histopathological findings (of hepatocyte loss, the number of proliferating hepatocytes and the number of LPCs) and clinical severity by the MELD score. The fact that the number of LPCs was not relevantly different between mild (30%) and moderate (30–50%) hepatocyte loss, but significantly increased upon severe (50–75%) or very severe (>75%) injury, clearly indicates that LPC compartment activation requires a high parenchymal injury threshold prior to its recruitment (Katoonizadeh et al., 2006). Importantly, surviving patients exhibited significantly fewer hepatocyte losses, less LPC activation and more mature hepatocyte proliferative activity, compared with those who either died or needed a liver transplant.

The cumulative data show that the degree of LPC activation and expansion of LPC compartment correlates strongly with the extent of hepatic injury and severity of ALF. Further studies will certainly be needed to evaluate if liver histology would improve prognostication (or predicting survival) for patients with ALF.

COULD LPCs REPRESENT A FUTURE CELL THERAPY OPTION IN ALF?

Whether the LPC response is simply a bystander effect as a result of the rich cytokine milieu, or whether it is an incomplete or unsuccessful attempt at liver regeneration remains to be seen. However, the significance of LPC activation during recovery from acute liver injury remains subject of controversy. Lineage tracing models utilizing reporter mouse models might represent a feasible tool to quantify the contribution of LPC during regeneration (Malato et al., 2011; Espanol-Suner et al., 2012; Diehl and Chute, 2013).

At present, the only curative treatment for patients with fulminant hepatic failure is an ELT. However, this is significantly limited by the shortage of suitable donor organs. As such, hepatocyte (cell) transplantation has been evaluated as an alternative for those ineligible for liver transplantation, or as a bridge to liver transplant. This is particularly attractive because cryopreserved cells are readily available. However, the number of cells that can be delivered (via the portal vein) is limited by the risks of portal hypertension (Weber et al., 2009a,b), and their large size lead to reduced cellular engraftment (Fox et al., 1998). LPC, on the other hand, are small in size, and are capable of differentiating into both hepatocytes and cholangiocytes (Sandhu et al., 2001). It would be important to study if enhanced expansion of the LPC compartment, with or without changes in the LPC niche, could lead to amelioration of ALF.

Previously, the limiting factor in the study of LPC has been the inability to identify, isolate or purify these cells in a reliable fashion. Recently, Cardinale and colleagues successfully isolated multipotent stem/progenitor cells from the human biliary tree by extended cell culture techniques (Cardinale et al., 2011) and demonstrated that these progenitor cells are capable of giving rise to hepatocytes, cholangiocytes, and pancreatic islets. We have similarly developed a LPC isolation protocol for mouse and human liver tissue, but using fluorescence-activated cell sorting (FACS). Our technique was based on the observation that progenitor cells express high levels of aldehyde dehydrogenase (ALDH) activity. FACS-ALDH positive LPC in culture could give rise to functional hepatocyte-like cells as illustrated by albumin and urea secretion and cytochrome P450 activity. These novel methods of LPC isolation could well pave the way for the development of future ALF therapies (Dollé et al., 2012).

A non-parenchymal cell population that might have an implication in liver regeneration are hepatic stellate cells. Their contribution to liver regeneration was recently confirmed with pancreatic stellate cells that were transplanted via tail vein injection into rats that were previously subjected to 70% PH and substantially contributed to organ reconstitution by differentiating into epithelial cells. The contribution of stem cells in tissue repair remains controversial, but prevailing evidence suggest that bone marrow or adipose tissue derived MSCs might contribute to liver regeneration through differentiation (Sato et al., 2005; Aurich et al., 2007; Chamberlain et al., 2007). Hepatic stellate cells could possibly fulfill a dual role as supportive cells producing a connective tissue scaffold facilitating LPC expansion and migration on the one hand and as progenitor cells on the other (Yang et al., 2008a; Kordes and Haussinger, 2013).

The inter-relationship between liver and non-liver progenitor or stem cells (i.e., bone marrow derived), and their roles in liver regeneration after ALF remains complex and poorly understood. Further studies will be necessary to understand and tap this potential source of new liver cells. Administration of granulocyte colony stimulating factor (G-CSF) during myocardial infarction for example, leads to the mobilization and differentiation of HSC to a committed lineage (Theiss et al., 2013). Therefore, a potential attempt to enhance liver regeneration during ALF might be a mobilization of bone marrow progenitor cells by an administration of G-CSF.

CONCLUSIONS

Further studies will be needed to understand the role of the LPC response during ALF. Cumulative data to date suggest that the LPC compartment is activated when there is confluent loss of hepatocyte mass, that lead to insufficient regenerative capacity of residual hepatocytes. The cytokine storm that ensues during acute liver injury, in combination with growth factors, morphogens, hormones, and neurotransmitters, all act in concert to dictate the LPC response. LPC activation and differentiation appears to require ECM, and forms the LPC niche. Hence, modulating the ECM composition and/or enhancing the LPC response could be useful strategies to promote liver regeneration.

Observational studies from cohorts of patients with ALF show that the amount and type of LPC activation/expansion correlate with severity of liver injury, and clinical outcomes. This is unsurprising as hepatocyte cell death is intricately associated with liver repair (i.e., the greater the injury, the greater the attempt at repair). Currently used scoring systems have not been able to reliably predict those who may survive from ALF. Future studies will be needed to evaluate if the degree of LPC response and/or ECM accumulation could be useful biomarkers of regenerative capability, thus improving clinical decision making in ALF.

FUNDING

Foundation for Liver Research (Wing-Kin Syn, Jason Coombes), CORE (Wing-Kin Syn), BRET (Wing-Kin Syn), Belgian Federal Science Policy Office (Interuniversity Attraction Poles program - P6/20 and P7/83-HEPRO) (Jan Best, Laurent Dollé, and Leo A. van Grunsven), the Brussels Capital Region (INNOVIRIS Impulse programme-Life Sciences 2007 and 2011; BruStem) (Jan Best, Laurent Dollé, and Leo A. van Grunsven) and the Institute for the Promotion of Innovation through Science and Technology in Flanders (SBO-IWT-090066 HEPSTEM) (Jan Best, Laurent Dollé, and Leo A. van Grunsven).

REFERENCES

Alison, M. R., Islam, S., and Lim, S. (2009). Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly. *J. Pathol.* 217, 282–298. doi: 10.1002/path.2453

Aurich, I., Mueller, L. P., Aurich, H., Luetzkendorf, J., Tisljar, K., Dollinger, M. M., et al. (2007). Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. *Gut* 56, 405–415. doi: 10.1136/gut.2005.090050

Bechmann, L. P., Jochum, C., Kocabayoglu, P., Sowa, J. P., Kassalik, M., Gieseler, R. K., et al. (2010). Cytokeratin 18-based modification of the MELD score improves prediction of spontaneous survival after acute liver injury. *J. Hepatol.* 53, 639–647. doi: 10.1016/j.jhep.2010.04.029

Bechmann, L. P., Marquitan, G., Jochum, C., Saner, F., Gerken, G., and Canbay, A. (2008). Apoptosis versus necrosis rate as a predictor in acute liver failure following acetaminophen intoxication compared with acute-on-chronic liver failure. *Liver Int.* 28, 713–716. doi: 10.1111/j.1478-3231.2007.01566.x

Bockhorn, M., Frilling, A., Benko, T., Best, J., Sheu, S. Y., Trippler, M., et al. (2007). Tri-iodothyronine as a stimulator of liver regeneration after partial and subtotal hepatectomy. *Eur. Surg. Res.* 39, 58–63. doi: 10.1159/000098443

Boulter, L., Govaere, O., Bird, T. G., Radulescu, S., Ramachandran, P., Pellicoro, A., et al. (2012). Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat. Med.* 18, 572–579. doi: 10.1038/nm.2667

Cai, Y., Zheng, H., Gong, W., Che, Y., and Jiang, B. (2011). The role of hedgehog signaling pathway in liver regeneration. *Hepatogastroenterology* 58, 2071–2076.

Campbell, J. S., Prichard, L., Schaper, F., Schmitz, J., Stephenson-Famy, A., Rosenfeld, M. E., et al. (2001). Expression of suppressors of cytokine signaling during liver regeneration. *J. Clin. Invest.* 107, 1285–1292. doi: 10.1172/JCI11867

Cardinale, V., Wang, Y., Carpino, G., Cui, C. B., Gatto, M., Rossi, M., et al. (2011). Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. *Hepatology* 54, 2159–2172. doi: 10.1002/hep.24590

Chamberlain, J., Yamagami, T., Colletti, E., Theise, N. D., Desai, J., Frias, A., et al. (2007). Efficient generation of human hepatocytes by the intrahepatic delivery of clonal human mesenchymal stem cells in fetal sheep. *Hepatology* 46, 1935–1945. doi: 10.1002/hep.21899

Choi, S. S., Omenetti, A., Witek, R. P., Moylan, C. A., Syn, W. K., Jung, Y., et al. (2009). Hedgehog pathway activation and epithelial-to-mesenchymal transitions during myofibroblastic transformation of rat hepatic cells in culture and cirrhosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G1093–G1106. doi: 10.1152/ajpgi.00292.2009

Czaja, A. J. (2013). Acute and acute severe (fulminant) autoimmune hepatitis. *Dig. Dis. Sci.* 58, 897–914. doi: 10.1007/s10620-012-2445-4

Dechene, A., Sowa, J. P., Gieseler, R. K., Jochum, C., Bechmann, L. P., El Fouly, A., et al. (2010). Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 52, 1008–1016. doi: 10.1002/hep.23754

Diehl, A. M. (2005). Lessons from animal models of NASH. *Hepatol. Res.* 33, 138–144. doi: 10.1016/j.hepres.2005.09.022

Diehl, A. M., and Chute, J. (2013). Underlying potential: cellular and molecular determinants of adult liver repair. *J. Clin. Invest.* 123, 1858–1860. doi: 10.1172/JCI69966

Dollé, L., Best, J., Empsen, C., Mei, J., Van Rossen, E., Roelandt, P., et al. (2012). Successful isolation of liver progenitor cells by aldehyde dehydrogenase activity in naive mice. *Hepatology* 55, 540–552. doi: 10.1002/hep.24693

Dollé, L., Best, J., Mei, J., Al Battah, F., Reynaert, H., Van Grunsven, L. A., et al. (2010). The quest for liver progenitor cells: a practical point of view. *J. Hepatol.* 52, 117–129. doi: 10.1016/j.jhep.2009.10.009

Duncan, A. W., Dorrell, C., and Grompe, M. (2009). Stem cells and liver regeneration. *Gastroenterology* 137, 466–481. doi: 10.1053/j.gastro.2009.05.044

Espanol-Suner, R., Carpentier, R., Van Hul, N., Legry, V., Achouri, Y., Cordi, S., et al. (2012). Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. *Gastroenterology* 143, 1564–1575.e7. doi: 10.1053/j.gastro.2012.08.024

Fausto, N. (2004). Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 39, 1477–1487. doi: 10.1002/hep.20214

Fausto, N., Campbell, J. S., and Riehle, K. J. (2006). Liver regeneration. *Hepatology* 43, S45–S53. doi: 10.1002/hep.20969

Fellous, T. G., Islam, S., Tadrous, P. J., Elia, G., Kocher, H. M., Bhattacharya, S., et al. (2009). Locating the stem cell niche and tracing hepatocyte lineages in human liver. *Hepatology* 49, 1655–1663. doi: 10.1002/hep.22791

Fox, I. J., Chowdhury, J. R., Kaufman, S. S., Goertzen, T. C., Chowdhury, N. R., Warkentin, P. I., et al. (1998). Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N. Engl. J. Med.* 338, 1422–1426. doi: 10.1056/NEJM199805143382004

Fujio, K., Evarts, R. P., Hu, Z., Marsden, E. R., and Thorgeirsson, S. S. (1994). Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. *Lab. Invest.* 70, 511–516.

Ghavami, S., Hashemi, M., Kadkhoda, K., Alavian, S. M., Bay, G. H., and Los, M. (2005). Apoptosis in liver diseases–detection and therapeutic applications. *Med. Sci. Monit.* 11, RA337–RA345.

Gruttadauria, S., Grosso, G., Pagano, D., Biondi, A., Echeverri, G. J., Seria, E., et al. (2013). Marrow-derived mesenchymal stem cells restore biochemical markers of acute liver injury in experimental model. *Transplant. Proc.* 45, 480–486. doi: 10.1016/j.transproceed.2012.06.087

Hadem, J., Stiefel, P., Bahr, M. J., Tillmann, H. L., Rifai, K., Klempnauer, J., et al. (2008). Prognostic implications of lactate, bilirubin, and etiology in German patients with acute liver failure. *Clin. Gastroenterol. Hepatol.* 6, 339–345. doi: 10.1016/j.cgh.2007.12.039

Hadem, J., Tacke, F., Bruns, T., Langgartner, J., Strnad, P., Denk, G. U., et al. (2012). Etiologies and outcomes of acute liver failure in Germany. *Clin. Gastroenterol. Hepatol.* 10, 664.e2–669.e2. doi: 10.1016/j.cgh.2012.02.016

Hanaoka, J., Shimada, M., Utsunomiya, T., Morine, Y., Imura, S., Ikemoto, T., et al. (2013). Significance of sonic hedgehog signaling after massive hepatectomy in a rat. *Surg. Today* 43, 300–307. doi: 10.1007/s00595-012-0248-z

Hatch, H. M., Zheng, D., Jorgensen, M. L., and Petersen, B. E. (2002). SDF-1alpha/CXCR4: a mechanism for hepatic oval cell activation and bone marrow stem cell recruitment to the injured liver of rats. *Cloning Stem Cells* 4, 339–351. doi: 10.1089/153623002321025014

Heinemann, A., Sturm, G. J., Ofner, M., Sturm, E. M., Weller, C., Peskar, B. A., et al. (2005). Stem cell factor stimulates the chemotaxis, integrin upregulation, and survival of human basophils. *J. Allergy Clin. Immunol.* 116, 820–826. doi: 10.1016/j.jaci.2005.06.008

Henrion, J. (2012). Hypoxic hepatitis. *Liver Int.* 32, 1039–1052. doi: 10.1111/j.1478-3231.2011.02655.x

Hu, B., and Colletti, L. M. (2008). Stem cell factor and c-kit are involved in hepatic recovery after acetaminophen-induced liver injury in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G45–G53. doi: 10.1152/ajpgi.00024.2008

Ichai, P., and Saliba, F. (2009). Fulminant and subfulminant hepatitis: causes and treatment. *Presse Med* 38, 1290–1298.

Jakubowski, A., Ambrose, C., Parr, M., Lincecum, J. M., Wang, M. Z., Zheng, T. S., et al. (2005). TWEAK induces liver progenitor cell proliferation. *J. Clin. Invest.* 115, 2330–2340. doi: 10.1172/JCI23486

Jochum, C., Gieseler, R. K., Gawlista, I., Fiedler, A., Manka, P., Saner, F. H., et al. (2009). Hepatitis B-associated acute liver failure: immediate treatment with entecavir inhibits hepatitis B virus replication and potentially its sequelae. *Digestion* 80, 235–240. doi: 10.1159/000236009

Jung, Y., Oh, S. H., Witek, R. P., and Petersen, B. E. (2012). Somatostatin stimulates the migration of hepatic oval cells in the injured rat liver. *Liver Int.* 32, 312–320. doi: 10.1111/j.1478-3231.2011.02642.x

Jung, Y., Witek, R. P., Syn, W. K., Choi, S. S., Omenetti, A., Premont, R., et al. (2010). Signals from dying hepatocytes trigger growth of liver progenitors. *Gut* 59, 655–665. doi: 10.1136/gut.2009.204354

Kallis, Y. N., Robson, A. J., Fallowfield, J. A., Thomas, H. C., Alison, M. R., Wright, N. A., et al. (2011). Remodelling of extracellular matrix is a requirement for the hepatic progenitor cell response. *Gut* 60, 525–533. doi: 10.1136/gut.2010.224436

Karaca, G. F., Chen, Y. P., Syn, W. K., Ochoa, B., Choi, S. S., Jakubowski, A., et al. (2010). Defective liver regeneration after partial hepatectomy in mice lacking fibroblast growth factor inducible 14. *Hepatology* 52(Suppl.), 969A–970A.

Katoonizadeh, A., Nevens, F., Verslype, C., Pirenne, J., and Roskams, T. (2006). Liver regeneration in acute severe liver impairment: a clinicopathological correlation study. *Liver Int.* 26, 1225–1233. doi: 10.1111/j.1478-3231.2006.01377.x

Khuu, D. N., Nyabi, O., Maerckx, C., Sokal, E., and Najimi, M. (2013). Adult human liver mesenchymal stem/progenitor cells participate to mouse liver regeneration after hepatectomy. *Cell Transplant.* 22, 1369–1380. doi: 10.3727/096368912X659853

Kordes, C., and Haussinger, D. (2013). Hepatic stem cell niches. *J. Clin. Invest.* 123, 1874–1880. doi: 10.1172/JCI66027

Laszlo, V., Dezso, K., Baghy, K., Papp, V., Kovalszky, I., Safrany, G., et al. (2008). Triiodothyronine accelerates differentiation of rat liver progenitor cells into hepatocytes. *Histochem. Cell Biol.* 130, 1005–1014. doi: 10.1007/s00418-008-0482-z

Leffert, H. L., and Alexander, N. M. (1976). Thyroid hormone metabolism during liver regeneration in rats. *Endocrinology* 98, 1241–1247. doi: 10.1210/endo-98-5-1241

Li, D. L., He, X. H., Zhang, S. A., Fang, J., Chen, F. S., and Fan, J. J. (2013). Bone marrow-derived mesenchymal stem cells promote hepatic regeneration after partial hepatectomy in rats. *Pathobiology* 80, 228–234. doi: 10.1159/000346796

Lorenzini, S., Bird, T. G., Boulter, L., Bellamy, C., Samuel, K., Aucott, R., et al. (2010). Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. *Gut* 59, 645–654. doi: 10.1136/gut.2009.182345

Lozoya, O. A., Wauthier, E., Turner, R. A., Barbier, C., Prestwich, G. D., Guilak, F., et al. (2011). Regulation of hepatic stem/progenitor phenotype by microenvironment stiffness in hydrogel models of the human liver stem cell niche. *Biomaterials* 32, 7389–7402. doi: 10.1016/j.biomaterials.2011.06.042

Malato, Y., Naqvi, S., Schurmann, N., Ng, R., Wang, B., Zape, J., et al. (2011). Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. *J. Clin. Invest.* 121, 4850–4860. doi: 10.1172/JCI59261

Michalopoulos, G. K. (2010). Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am. J. Pathol.* 176, 2–13. doi: 10.2353/ajpath.2010.090675

Nobili, V., Carpino, G., Alisi, A., Franchitto, A., Alpini, G., De Vito, R., et al. (2012). Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 56, 2142–2153. doi: 10.1002/hep.25742

Ochoa, B., Syn, W. K., Delgado, I., Karaca, G. F., Jung, Y., Wang, J., et al. (2010). Hedgehog signaling is critical for normal liver regeneration after partial hepatectomy in mice. *Hepatology* 51, 1712–1723. doi: 10.1002/hep.23525

Okada, T., Shiono, Y., Kaneko, Y., Miwa, K., Hasatani, K., Hayashi, Y., et al. (2010). High prevalence of fulminant hepatic failure among patients with mutant alleles for truncation of ATP7B in Wilson's disease. *Scand. J. Gastroenterol.* 45, 1232–1237. doi: 10.3109/00365521.2010.492527

Omenetti, A., and Diehl, A. M. (2008). The adventures of sonic hedgehog in development and repair. II. Sonic hedgehog and liver development, inflammation, and cancer. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G595–G598. doi: 10.1152/ajpgi.00543.2007

Omenetti, A., Syn, W. K., Jung, Y., Francis, H., Porrello, A., Witek, R. P., et al. (2009). Repair-related activation of hedgehog signaling promotes cholangiocyte chemokine production. *Hepatology* 50, 518–527. doi: 10.1002/hep.23019

Parola, M., and Pinzani, M. (2009). Hepatic wound repair. *Fibrogenesis Tissue Repair* 2, 4. doi: 10.1186/1755-1536-2-4

Petersen, B., and Shupe, T. (2008). Location is everything: the liver stem cell niche. *Hepatology* 47, 1810–1812. doi: 10.1002/hep.22333

Polson, J., and Lee, W. M. (2005). AASLD position paper: the management of acute liver failure. *Hepatology* 41, 1179–1197. doi: 10.1002/hep.20703

Sancho-Bru, P., Altamirano, J., Rodrigo-Torres, D., Coll, M., Millan, C., Jose Lozano, J., et al. (2012). Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis. *Hepatology* 55, 1931–1941. doi: 10.1002/hep.25614

Sandhu, J. S., Petkov, P. M., Dabeva, M. D., and Shafritz, D. A. (2001). Stem cell properties and repopulation of the rat liver by fetal liver epithelial progenitor cells. *Am. J. Pathol.* 159, 1323–1334. doi: 10.1016/S0002-9440(10)62519-9

Saner, F. H., Heuer, M., Meyer, M., Canbay, A., Sotiropoulos, G. C., Radtke, A., et al. (2009). When the heart kills the liver: acute liver failure in congestive heart failure. *Eur. J. Med. Res.* 14, 541–546.

Santoni-Rugiu, E., Jelnes, P., Thorgeirsson, S. S., and Bisgaard, H. C. (2005). Progenitor cells in liver regeneration: molecular responses controlling their activation and expansion. *APMIS* 113, 876–902. doi: 10.1111/j.1600-0463.2005.apm_386.x

Sato, Y., Araki, H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., et al. (2005). Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood* 106, 756–763. doi: 10.1182/blood-2005-02-0572

Saxena, R., and Theise, N. (2004). Canals of Hering: recent insights and current knowledge. *Semin. Liver Dis.* 24, 43–48. doi: 10.1055/s-2004-823100

Short, J., and Ove, P. (1983). Synthesis of an hypothesis advocating a prominent role for the thyroid hormones in mammalian liver cell proliferation *in vivo*. *Cytobios* 38, 39–49.

Theise, N. D., Dolle, L., and Kuwahara, R. (2013). Low hepatocyte repopulation from stem cells: a matter of hepatobiliary linkage not massive production. *Gastroenterology* 145, 253–254. doi: 10.1053/j.gastro.2013.02.052

Theiss, H. D., Gross, L., Vallaster, M., David, R., Brunner, S., Brenner, C., et al. (2013). Antidiabetic gliptins in combination with G-CSF enhances myocardial function and survival after acute myocardial infarction. *Int. J. Cardiol.* doi: 10.1016/j.ijcard.2013.04.121. [Epub ahead of print].

Tirnitz-Parker, J. E., Viebahn, C. S., Jakubowski, A., Klopcic, B. R.,

Olynyk, J. K., Yeoh, G. C., et al. (2010). Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. *Hepatology* 52, 291–302. doi: 10.1002/hep.23663

Van Hul, N. K., Abarca-Quinones, J., Sempoux, C., Horsmans, Y., and Leclercq, I. A. (2009). Relation between liver progenitor cell expansion and extracellular matrix deposition in a CDE-induced murine model of chronic liver injury. *Hepatology* 49, 1625–1635. doi: 10.1002/hep.22820

Weber, A., Groyer-Picard, M. T., and Dagher, I. (2009a). Hepatocyte transplantation techniques: large animal models. *Methods Mol. Biol.* 481, 83–96. doi: 10.1007/978-1-59745-201-4_8

Weber, A., Groyer-Picard, M. T., Franco, D., and Dagher, I. (2009b). Hepatocyte transplantation in animal models. *Liver Transpl.* 15, 7–14. doi: 10.1002/lt.21670

Yang, L., Jung, Y., Omenetti, A., Witek, R. P., Choi, S., Vandongen, H. M., et al. (2008a). Fate-mapping evidence that hepatic stellate cells are epithelial progenitors in adult mouse livers. *Stem Cells* 26, 2104–2113. doi: 10.1634/stemcells.2008-0115

Yang, L., Wang, Y., Mao, H., Fleig, S., Omenetti, A., Brown, K. D., et al. (2008b). Sonic hedgehog is an autocrine viability factor for myofibroblastic hepatic stellate cells. *J. Hepatol.* 48, 98–106. doi: 10.1016/j.jhep.2007.07.032

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2013; accepted: 03 September 2013; published online: 26 September 2013.

Citation: Best J, Dollé L, Manka P, Coombes J, van Grunsven LA and Syn W-K (2013) Role of liver progenitors in acute liver injury. Front. Physiol. ***4****:258. doi: 10.3389/fphys.2013.00258*

This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 02 April 2012
doi: 10.3389/fphys.2012.00078

Rodent animal models for surrogate analysis of cell therapy in acute liver failure

Bruno Christ [1,2] **** and Sandra Brückner*** [1]

[1] *Applied Molecular Hepatology Laboratory, Department of Visceral, Transplantation, Thoracic and Vascular Surgery, University Hospital Leipzig, Leipzig, Germany*
[2] *Translational Centre for Regenerative Medicine, Leipzig, Germany*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Isabel Fabregat, University of Barcelona, Spain
Martina Mueller-Schilling, University Hospital Regensburg, Germany

****Correspondence:***
Bruno Christ, Department of Visceral, Transplantation, Thoracic and Vascular Surgery, University Hospital of Leipzig, Liebigstraße 21, D-04103 Leipzig, Germany.
e-mail: bruno.christ@medizin.uni-leipzig.de

Without therapeutic intervention acute liver failure (ALF) is the consequence of a progredient destruction of the liver parenchyma due to metabolic exhaustion of the hepatocytes. Perivenous hepatocytes are responsible for the detoxification of noxious compounds via the cytochrome P450 enzyme system. Liver transplantation is the only remaining therapeutic option in the end-stage of the disease. Assuming that metabolic capacity could be provided by healthy hepatocytes and thus substitute for the genuine parenchymal cells hepatocyte transplantation since quite some time is considered to be an alternative to whole liver transplantation. While this hypothesis achieved proof-of-concept in animal trials clinical breakthrough is still awaiting success, the reasons of which are ongoing matter of debate. In recent times mesenchymal stem cells (MSC) came into focus as a transplantable cell source to treat ALF. Interestingly, as demonstrated in various rodent animal models their mode of action is rather based on trophic support of hepatocytes remaining in the damaged host parenchyma rather than substitution of tissue loss. Mechanistically, either direct or indirect paracrine effects from the transplanted cells acting pro-proliferative, anti-apoptotic, and anti-inflammatory seem to trigger the regenerative response of the residual healthy hepatocytes in the otherwise lethally injured liver parenchyma. Thus, allogeneic MSC may be the best choice for the treatment of ALF taking advantage of their short-term benefit to sustain the critical phase of the acute insult avoiding long-term immunosuppression.

Keywords: cell transplantation, liver stem cells, acute liver injury, stem cell-derived hepatocytes

MOLECULAR PRINCIPLES OF TISSUE TOXICITY IN ALF INDUCED BY PARACETAMOL

Acute liver failure (ALF) is characterized by an initial devastating hepatic insult followed by gross parenchymal dysfunction, which leads to a multitude of systemic organ failures due to the missing metabolic homeostasis normally provided by the healthy liver. The most common causes of ALF are viral hepatitis, idiosyncratic side reactions, chronic liver diseases, autoimmune hepatitis, and dose-dependent drug-induced ALF. The disease occurs rapidly and in general requires intensive care with the known high risk of mortality. Whole liver transplantation very often is the only therapy option of choice (Ostapowicz and Lee, 2000; Gill and Sterling, 2001; Rahman and Hodgson, 2001; O'Grady, 2005). The incidence of acetaminophen (paracetamol)-induced ALF is rather high in the US and in the UK related both to therapy-associated and suicide-driven overdosage of the drug (Reuben et al., 2010; Lee et al., 2011). In the liver acetaminophen is metabolized by the cytochrome P450 enzyme system located predominantly in the hepatocytes surrounding the distal branches of the liver sinusoids, the so-called perivenous hepatocytes (Jungermann and Kietzmann, 2000; Benhamouche et al., 2006; Burke and Tosh, 2006; Hailfinger et al., 2006; Gebhardt and Hovhannisyan, 2010). There are two principle ways of detoxification: (1) conjugation by sulfation and/or glucuronidation followed by elimination and (2) cytochrome P450-dependent oxidation and formation of *N*-acetyl-*p*-benzoquinonimine (NAPQI), which is then conjugated to glutathione and finally eliminated with the bile. Yet, sustained NAPQI formation eventually causes depletion of glutathione, which then in turn leads to formation of protein adducts as well as reactive nitrogen and oxygen species (**Figure 1**). Very likely mitochondrial dysfunction and increased permeability of the mitochondrial membranes contribute to the formation of reactive nitrogen and oxygen metabolites such as peroxynitrate and hydrogen peroxide besides others, which in turn mediate protein nitration and oxidative stress (Jaeschke et al., 2002; James et al., 2003; Jaeschke and Bajt, 2006; Doi and Ishida, 2009). Obviously, besides the hepatocytes non-parenchymal cells such as Kupffer cells and sinusoidal endothelial cells seem to be involved in the generation of reactive nitrogen and oxygen species thus augmenting protein and lipid peroxidation. Since these reactions are ultimately mediated by the perivenous cytochrome P450 enzyme system, apoptotic cell death followed by centrilobular necrosis is a hallmark of acetaminophen-induced hepatotoxicity (**Figure 2**). The inflammatory environment produced during ALF is also responsible for the activation of hepatic stellate cells probably mediated by IL1, which respond with an increase in expression of α-smooth muscle actin and matrix metalloproteinases, mainly MMP9. This seems to favor the remodeling of the extracellular matrix, thus augmenting hepatocyte cell death (Yan et al., 2008; Dechene et al., 2010).

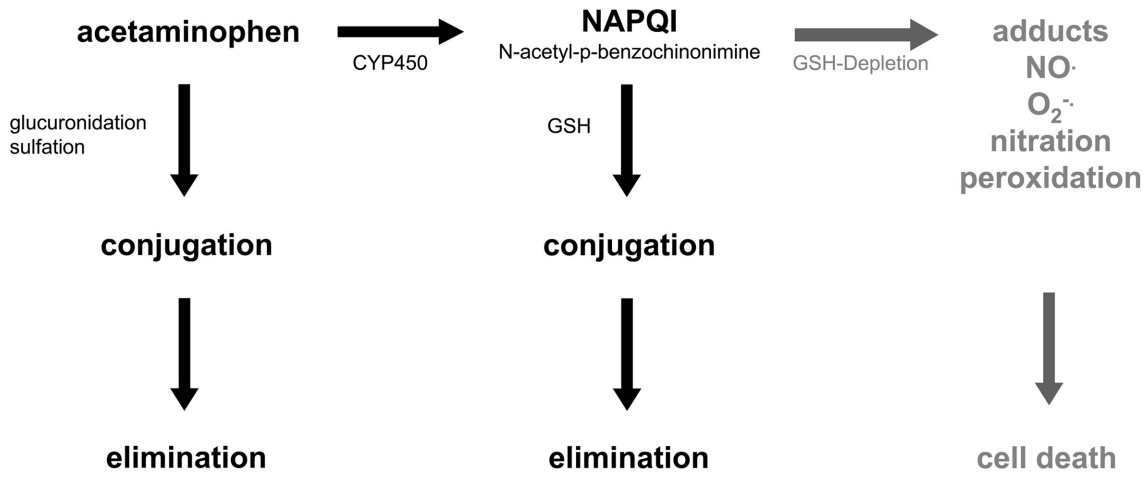


FIGURE 1 | Acetaminophen and hepatotoxicity. Acetaminophen (paracetamol) is detoxified in the liver by conjugation or cytochrome P450-dependent oxidation followed by conjugation to glutathione (GSH). Depletion of GSH leads to formation of reactive nitrogen and oxygen species, which in turn causes cell death. For further details see text.

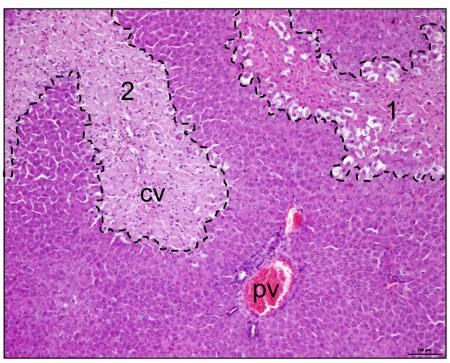


FIGURE 2 | Pericentral necrosis after acetaminophen intoxication. Rats were treated with a repeated oral dose of 4 g/kg body weight of acetaminophen. Eighteen hours later livers were explanted and slices prepared for hemalaun–eosin staining. Dashed lines exemplify initial (area 1) and final necrotic perivenous areas (area 2, cv, central vein) of the liver tissue. Please note that areas around the portal vein (pv) are void of tissue damage.

REGENERATIVE RESPONSE TO ACUTE LIVER INJURY

In the normal healthy liver tissue turnover is in the range of 0.01%. Without any challenge this rather low regenerative rate would reconstitute the whole liver parenchyma within about 1 year (Steiner et al., 1966; Koniaris et al., 2003). One might suspect then that the liver had a poor regenerative potential, which is also corroborated by the fact that after partial hepatectomy the liver is rebuilt to the original organ size, only. After 2/3 partial hepatectomy this would be accomplished by the 1.5-times cell division of the remaining hepatocytes. However, this situation does not reflect the real regenerative potential of hepatocytes. It has been shown in serial transplantation experiments in the albumin promoter-urokinase plasminogen activator (uPA) transgenic mouse that hepatocytes feature a nearly unlimited regenerative capacity. In this model, the intracellular activation of the protease plasmin causes hepatocyte damage and perinatal lethality (Heckel et al., 1990). Eventually, mice survived due to the substitution of hepatocytes bearing the transgene by healthy hepatocytes, which obviously had a survival advantage. Transplantation of these hepatocytes having escaped the lethal phenotype into the livers of transgenic mice revealed the efficient repopulation of the diseased host liver by the donor hepatocytes, thus rescuing the lethal phenotype. This indicates an enormous mitotic potential of hepatocytes (Sandgren et al., 1991; Rhim et al., 1994). In another mouse model, the knockout of fumarylacetoacetate hydrolase (FAH) leads to the accumulation of tyrosine intermediates, which cause toxic insult of hepatocytes. Transplanted healthy hepatocytes display a proliferative advantage over the diseased host hepatocytes, thus achieving nearly complete replacement of the original transgenic hepatocytes by the transplanted cells. In this model, serial transplantation of hepatocytes derived from mutant livers colonized with transplanted wildtype cells revealed that 6 rounds of liver repopulation required a minimum of 69 cell divisions (Overturf et al., 1997, 1999; Wang et al., 2001). Thus, obviously adult hepatocytes have a high replicative and repopulation capacity. This in turn means, that they have the potential of self-renewal and of functional tissue formation *in vivo*, which are ultimate stem cell characteristics.

Experimentally, ALF might be triggered by the use of chemical noxious compounds such as carbon tetrachloride or acetaminophen as mentioned above. As long as the hepatocytes dispose of sufficient metabolic capacity to detoxify the drugs no obvious tissue lesions emerge. Yet, the production of reactive metabolites followed by covalent protein and lipid modification due to metabolic overload as mentioned above finally results in cellular dysfunction, initial cell damage and tissue injury. Depending on the dose applied tissue damage proceeds. The initial insult resulting in injury progression is the mitotic challenge for the hepatocytes to restore the tissue loss by functional hepatocyte progeny. Again, dependent on the dose of the noxious compounds the regenerative potential of the liver is either sufficient for injury regression or overwhelmed resulting in injury progression followed by ALF

(Mehendale, 2005; Palmes et al., 2005; **Figure 3**). Tissue regeneration is accomplished by the hepatocytes themselves as long as a minimal liver tissue mass is compliant with a certain threshold of functional tissue loss. Yet, if this threshold is surpassed the regenerative capacity of the hepatocytes does not suffice for functional tissue restoration. In this case a progenitor cell compartment is activated giving rise to so-called oval cells in rodents, which are agreed upon to be the progeny of adult hepatic stem cells in the liver (Sell, 2001; Kofman et al., 2005). Oval cells appear in the periportal areas after massive liver injury adjacent to the canals of Hering, structural links between the terminal biliary branches and the periportal hepatocytes (Fausto, 2004; Santoni-Rugiu et al., 2005; Oertel and Shafritz, 2008). It is noteworthy that both hepatocytes and hepatic progenitor cells may differentiate into hepatocytes and biliary cells as well indicating their bipotent differentiation capacity. Hence, both cell types meet the minimal definition criteria of a stem cell, i.e., the potential of self-renewal to maintain the stem cell reserve, and a multiple differentiation potential giving rise to progeny of at least two different lineages. In the latter case it is self-evident that proliferation and differentiation of the offspring cells provide the functional backup for tissue repair after injury.

HEPATOCYTE TRANSPLANTATION IN ALF

In ALF, liver transplantation is the gold standard of treatment. However, about one third of patients on the waiting list for liver transplantation in Europe do not profit because of the unavailability of suitable donor organs[1]. The hepatocyte is the smallest functional unit of the liver executing the complete metabolic orchestra, which is provided by the liver as a whole. Therefore, transplantation of hepatocytes might be possible to substitute for the functional tissue loss in ALF provided the donor cells take over hepatocyte functions in the deteriorated host parenchyma for at least the critical period in time either required to bridge to organ transplantation or to allow for tissue recovery from the toxic insult (Najimi and Sokal, 2008; Oertel and Shafritz, 2008; Smets et al., 2008; Ito et al., 2009; Puppi and Dhawan, 2009). Technically, in rodent small animal models the cells are delivered to the liver either after intraportal or intrasplenic injection. It is assumed that the cells distribute homogeneously in the liver by passage with the blood stream where they are entrapped in the sinusoids and eventually penetrate the endothelia, integrate, proliferate, and spread into the host parenchyma. This concept has been verified in various rodent animal models of ALF (for recent reviews, see Fox and Roy-Chowdhury, 2004; Shafritz et al., 2006; Seppen et al., 2009; Weber et al., 2009). There is one major constraint, which probably seriously hampers the clinical translation of hepatocyte transplantation in ALF. Under non-stimulating conditions the repopulation of an acutely injured liver by transplanted hepatocytes is rather low, i.e., in the range of 1–5% of the total liver mass (Ponder et al., 1991; Rajvanshi et al., 1996; Gupta et al., 1999; Fox and Roy-Chowdhury, 2004; Fisher and Strom, 2006). However, if the recipient liver is challenged by a growth stimulus and the proliferation of host hepatocytes is impaired then a significant repopulation by transplanted hepatocytes is achieved. There is an elegant animal model available allowing for the identification of the transplanted cells in the host parenchyma. In this rat model the natural mutation in the CD26 gene leads to the expression of a non-functional protein, however, without obvious pathophysiological consequences. Transplanted wildtype donor cells may then be identified histologically in the host parenchyma by the detection of CD26. Providing selective pressure conditions by partial hepatectomy as a mitotic stimulus and pre-treatment with alkaloids such as retrorsine to inhibit host hepatocyte proliferation a repopulation rate for up to nearly 100% may be achieved in this rat model (Laconi et al., 1998, 1999). Similarly, high rates were obtained using rat fetal liver epithelial cells but without applying selective growth conditions for the transplanted cells (Sandhu et al., 2001; Oertel et al., 2006).

Acute liver failure in mice and rats may be induced under various experimental settings, the most common in use are those acutely applying paracetamol or carbon tetrachloride. In general, when adult hepatocytes or oval cells isolated from donor livers under various inducing conditions are used for transplantation without further selective pressure repopulation of the host liver by the transplanted cells is poor, i.e., in the range of less than 5%. However, cells are functional and survive long-term in the recipient liver indicating support of liver regeneration after acute hepatotoxic injury. If in addition to the acute injury regeneration by host hepatocytes is abrogated by the beforehand treatment with mitotoxins such as the pyrrolizidine alkaloid retrorsine much higher repopulation rates may be achieved, which clearly suffice to substitute for the loss of metabolic capacity due to the toxic parenchymal damage. Similar results were obtained using fetal (ED12.5) rat hepatoblasts. Yet, using ED14 mouse hepatoblasts 10- to 20-fold higher repopulation rates were achieved without applying selective repopulation conditions. A comprehensive summary of models and conditions used to study liver repopulation by transplanted hepatocytes or hepatocyte progenitor cells under normal and injury conditions is available (Sancho-Bru et al., 2009; Shafritz and Oertel, 2011). To summarize, transplanted cells integrate into the host parenchyma and even at low repopulation rates

[1] http://www.eurotransplant.nl/

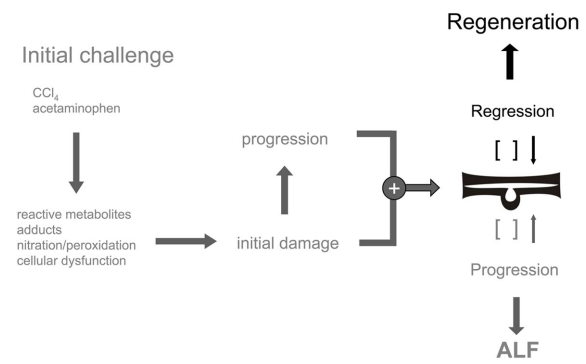


FIGURE 3 | Balance of tissue homeostasis after acute liver injury. The regenerative response of the liver after acute intoxication is triggered by the emergence of initial tissue damage and progression. Dependding on the dose of the noxa and the regenerative capacity of the hepatocytes injury regression and regeneration or progression and ALF develop.

display hepatocyte functions. Thus, hepatocyte transplantation in ALF aims at tissue substitution of the recipient liver in order to functionally reconstitute the injured parenchyma by healthy donor cells.

STEM CELL-DERIVED HEPATOCYTE TRANSPLANTATION IN ALF

In respect to the clinical application of hepatocyte transplantation in ALF the major hurdle is probably the scarcity of donor organs to isolate human hepatocytes in sufficient quality and quantity. Therefore, one feasible alternative to human adult hepatocytes is the use of stem cell-derived hepatocytes. The bone marrow harbors adult stem cells, both hematopoietic and non-hematopoietic, which are clearly superior in choice over embryonic stem cells for clinical application because of their less ethical constraints and the lack of teratoma formation after tissue implantation. Adult stem cells may differentiate into hepatocyte-like cells. In the mouse model of FAH deficiency, hematopoietic bone marrow derived cells rescued the diseased phenotype by complementation of the defective FAH gene with the wildtype gene in the transplanted cells (Lagasse et al., 2000; Wang et al., 2002). It is an open question as to whether hepatocytes derived from the bone marrow are the product of differentiation from hematopoietic stem cells or of the fusion with host hepatocytes (Alvarez-Dolado et al., 2003; Newsome et al., 2003; Vassilopoulos et al., 2003; Wang et al., 2003; Camargo et al., 2004; Jang et al., 2004). In recent years studies in rats (Wang et al., 2004; Lange et al., 2005), mice (Jiang et al., 2002), and humans (Schwartz et al., 2002; Lee et al., 2004; Hong et al., 2005; Seo et al., 2005; Taléns-Visconti et al., 2006; Aurich et al., 2007; Banas et al., 2007) verified that mesenchymal stem cells (MSC) from various tissues like bone marrow, umbilical cord blood, or adipose tissue may differentiate into hepatocyte-like cells following specified growth and differentiation regimens *in vitro*. Yet, under acute injury conditions causing either periportal liver damage induced by allyl alcohol (Sato et al., 2005) or perivenous damage by the use of carbon tetrachloride (Seo et al., 2005; Banas et al., 2007; Yukawa et al., 2009) or acetaminophen (Stock et al., 2009), MSC-derived hepatocyte-like cells integrated into the diseased host liver, though repopulation rates were rather low, i.e., in the range of 1% of the total liver mass.

Reasoning that MSC feature immunomodulatory functions in that they are able to suppress the immune response mediated through T and B cells, dendritic cells and other immune cells (Chamberlain et al., 2007; Götherström, 2007; Krampera et al., 2007; Le Blanc and Ringden, 2007) it might not be surprising that the action of MSC in ALF is rather paracrine than direct tissue support by the transplanted cells. D-galactosamine-induced fulminant hepatic failure in rats was attenuated by MSC-derived molecules through inhibition of apoptosis, stimulation of hepatocyte proliferation, and minimization of the inflammatory response (Parekkadan et al., 2007a; van Poll et al., 2008). The paracrine mode of action of MSC was also corroborated by the amelioration of systemic inflammation induced by LPS or burn indicating in addition pleiotropic effects of the MSC (Yagi et al., 2010). Ectopic recruitment of MSC from the bone marrow to the liver has been shown in mice challenged by acute intoxication with carbon tetrachloride or 2-acetylaminofluorene indicating chemotactic activation of the MSC very likely mediated by stromal cell-derived factor-1 (Jin et al., 2009; Chen et al., 2010). Our own data substantiated that MSC are able to home to and integrate into an acutely injured liver. We treated rats with acetaminophen to induce acute liver damage. MSC derived from rat peritoneal adipose tissue were pre-differentiated into hepatocyte-like cells according to our standard protocol (Stock et al., 2010) and then the cells were administered to the diseased animals via tail vein injection. Eighteen hours after cell delivery donor-derived cells were detected in the liver (unpublished) where they significantly decreased acetaminophen-induced apoptosis as shown immunohistochemically by the TUNEL assay and stimulated proliferation of host hepatocytes as shown by Ki67 staining (**Figure 4**) to regenerate the liver tissue after acute injury (unpublished). Besides their anti-inflammatory and immunomodulatory impact MSC seem also to communicate with target cells by the exchange of mRNA or miRNA molecules (Collino et al., 2010; Deregibus et al., 2010). Thus, genetic material is exchanged, which then might affect the regenerative response of the host tissue cells on the one and the differentiation of donor MSC at the site of their engraftment into the host tissue on the other hand. This, however, means that transplanted cells might be imprinted by their target tissue and the molecular microenvironment induced by a specific type of injury. Acute liver injury may trigger paracrine effects due to the inflammatory environment of the diseased liver, whereas liver regeneration after, e.g., partial hepatectomy is achieved by the engraftment and functional tissue replacement by the MSC differentiated into hepatocytes at the site of their engraftment. This

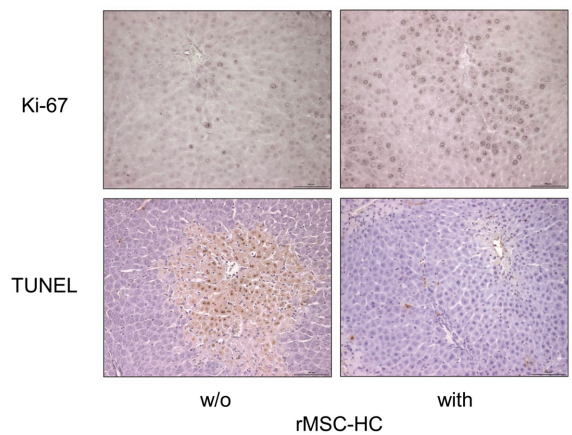


FIGURE 4 | Anti-apoptotic and pro-proliferative action of MSC after acetaminophen intoxication of the rat liver. Rats were treated with a repeated dose of 4 g/kg body weight of acetaminophen. Eighteen hours after the last dose the animals were sacrificed and the livers prepared for the detection of apoptotic cells (dark nuclei) by the TUNEL assay (lower panels) or proliferating cells (dark nuclei) by the Ki67 stain (upper panels). Where indicated animals received adipose tissue-derived rat MSC pre-differentiated into hepatocyte-like cells (rMSC-HC) 6 h after the last dose of acetaminophen. It is obvious that the number of apoptotic cells was significantly lower but of proliferating cells was higher in the livers with MSC (right panels) indicating the anti-apoptotic and pro-proliferative action of the MSC.

Table 1 | Summary of clinical trials involving mesenchymal stem cells of different tissue sources for the treatment of chronic liver diseases.

Study Title	MSC source	Sponsor	Patients	Study phase	Status
Safety and efficacy of human mesenchymal stem cells for treatment of liver failure	Umbilical cord	Beijing 302 Hospital, China	70	Phase I/II	recruiting
Autologous mesenchymal stem cell transplantation in liver cirrhosis	No details	Gulhane Military Medical Academy, Turkey	25	No details	recruiting
Umbilical cord mesenchymal stem cells infusion via hepatic artery in cirrhosis patients	Umbilical cord	Qingdao University, China	50	Phase I/II	Not yet recruiting
Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial	No details	Shaheed Beheshti Medical University, Islamic Republic of Iran	30	Phase I/II	Completed
Allogeneic bone marrow mesenchymal stem cells transplantation in patients with liver failure caused by hepatitis B virus	Bone marrow	Sun Yat-sen University, China	120	Phase II	Active, not recruiting
Human umbilical cord mesenchymal stem cells transplantation for patients with decompensated liver cirrhosis	Umbilical cord	Shenzhen Beike Bio-Technology Co., Ltd., China	20	Phase I/II	Completed
Human menstrual blood-derived mesenchymal stem cells for patients with liver cirrhosis	Menstrual blood	S-Evans Biosciences Co., Ltd., China	50	Phase I/II	Recruiting
Umbilical cord mesenchymal stem cell transfusion in patients with severe liver cirrhosis	Umbilical cord	Chinese Academy of Sciences, China	200	Phase I/II	Recruiting
Mesenchymal stem cells after renal or liver transplantation	No details	University Hospital of Liege, Belgium	40	Phase I/II	Recruiting
Therapeutic effects of liver failure patients caused by chronic hepatitis B after autologous MSCs transplantation	Bone marrow	Sun Yat-sen University, China	158	No details	Completed
Umbilical cord mesenchymal stem cells for patients with liver cirrhosis	Umbilical cord	Beijing 302 Hospital, China	45	Phase I/II	Recruiting
Efficacy of *in vitro* expanded bone marrow derived allogeneic mesenchymal stem cell transplantation via portal vein or hepatic artery or peripheral vein in patients with Wilson cirrhosis	Bone marrow	Murat Kantarcioglu, Gulhane Military Medical Academy, Turkey	10	Phase II	Recruiting
Transplantation of autologous mesenchymal stem cell in decompensate cirrhotic patients with pioglitazone	Bone marrow	Royan Institute, Islamic Republic of Iran	3	Phase I	Recruiting
Efficacy and safety study of allogenic mesenchymal stem cells for patients with chronic graft versus host disease	No comment	Chinese Academy of Medical Sciences, China	100	Phase II, phase III	Not yet recruiting
Efficacy and safety study of allogenic mesenchymal stem cells for patients with refractory primary biliary cirrhosis	Bone marrow	Robert Chunhua Zhao, Chinese Academy of Medical Sciences, China	20	Phase I	Not yet recruiting
Allogenic bone marrow stem cells transplantation in patients with liver cirrhosis	Bone marrow	Sun Yat-sen University, China	60	Phase II	Active, not recruiting
Allogenic bone marrow stem cell transplantation in liver failure	Bone marrow	Sun Yat-sen University, China	60	Phase II	Active, not recruiting

Data are taken from reference (http://clinicaltrials.gov).

potential pleiotropic mode of action makes MSC ideal candidates for stem cell therapy of different liver diseases (Enns and Millan, 2008; Haridass et al., 2008; Alison et al., 2009; Flohr et al., 2009; Soto-Gutierrez et al., 2009).

CLINICAL IMPLICATIONS

It is obvious that experimental settings in animal models aimed to enhance liver repopulation by transplanted hepatocytes are not suited for clinical translation. Thus, the lack of a survival and/or a proliferative advantage of donor vs. host hepatocytes is probably the mechanistical reason for the poor clinical progress of hepatocyte transplantation. The low success rate is augmented by the fact that human hepatocytes are isolated from marginal donor livers not allocated for transplantation. Yet, as outlined above MSC might be an alternate cell resource to generate hepatocyte-like cells. MSC display hepatocyte differentiation potential, which was substantiated both *in vitro* and *in vivo*. Even if biological and biochemical differences might exist between MSC from various tissues they share typical MSC characteristics like marker expression, multiple differentiation capacity, and growth on plastic surfaces, which finally determine quantitative, not qualitative, variability in their hepatocyte differentiation potential. It is feasible to suppose that in respect to ethical, technical and biological aspects the transplantation of stem cell-derived hepatocytes follows the principles of hepatocyte transplantation (Fisher and Strom, 2006). MSC might even open a broader spectrum of activity compared with primary hepatocytes because of their versatile properties such as low immunogenicity as well as their anti-inflammatory, anti-apoptotic, and pro-proliferative activities, which not only substitute the tissue damaged but also actively might temper the inflammatory response, e.g., after toxic or chronic injury. Recently, a couple of clinical trials – most in China – has been initiated or even completed to demonstrate safety and efficacy of the site of application of MSCs concentrating on autologous stem cell transplantation in patients suffering from chronic liver failure (**Table 1**) or acute decompensation after ample liver resection[2]. Yet, so far no published results are available. In these studies, undifferentiated cells have been used bearing a potential tumor-promoting risk (Karnoub et al., 2007), which, however, has not been verified.

Taking ethical considerations into account these clinical conditions may be adequate to assess safety of hepatic MSC transplantation. However, to take advantage of the cells' immunomodulatory, chemotactic, and anti-inflammatory properties, ALF offering a highly inflammatory environment in the liver may be the disease situation of choice for the use of MSC. In this case even the use of allogeneic cell sources may not be a serious problem since only the short-term beneficial actions of the MSC might warrant support of liver regeneration in the critical phase of acute poisoning. Immunosuppression may be applied from the beginning of treatment on or even continued as long as the recovering of the liver is ongoing but then may be ceased, thus avoiding the theoretical risks of potential sensitization of the host for future organ grafts or promoting life-threatening septic episodes during long-term stay in the intensive care units.

CONCLUSION

To overcome the shortage of donor organs for liver transplantation in ALF cell therapy approaches seem to be feasible, which must achieve two principle goals. (1) The loss of metabolic capacity must be substituted by the healthy donor cells, and (2) the emergence of the inflammatory environment in ALF must be decelerated in order to protect hepatocytes from progression into cell death. It is obvious that the first goal might best be reached using primary hepatocytes, which, however, do not have a survival advantage in the deteriorated ALF liver. The second goal might best be met by the use of MSC taking advantage of their anti-inflammatory and – apoptotic as well as pro-proliferative features, which, however, promises no therapeutic benefit in the case that tissue damage has surpassed the lower threshold needed to maintain body metabolic homeostasis. Thus, it might be worthwhile thinking whether a combination of hepatocytes and MSC might be the cell therapeutic of best choice. Indeed, there is evidence that the performance of hepatocytes is improved in co-culture with MSC (Ijima et al., 2008; Shi et al., 2009; Chen et al., 2012), and vice versa MSC differentiation into hepatocyte-like cells is promoted by inflammatory liver injury conditions (Dong et al., 2010; Li et al., 2010). Recent data even demonstrated that not MSC themselves but as yet unequivocally unidentified soluble factors secreted by MSC exert the beneficial effects on hepatocytes under ALF conditions in mice and rats (Parekkadan et al., 2007b; van Poll et al., 2008; Zagoura et al., 2011). The anti-inflammatory cytokine IL10 secreted by MSC seemed to play a major role in alleviating liver damage after acute injury induced by carbon tetrachloride in the NOD/SCID mouse model (Zagoura et al., 2011). Thus, the identification of these factors might open even cell-free therapeutical options for the treatment of ALF with MSC-derived molecules.

Animal models for cell therapy approaches to treat ALF as described above enable us to earn knowledge on the mechanisms of interactions between donor and host cells both on the molecular and cellular level, to identify the hepatotropic effects esp. mediated by MSC and their impact on the noxious challenge in order to optimize integration of transplanted cells into the recipient tissue thereby to support efficacy of cell transplantation and thus optimize the therapeutical outcome.

ACKNOWLEDGMENTS

Original work presented in this paper (**Figures 2** and **4**) was made possible by funding from the German Research Foundation (Ch 109/15-2) as well as by the German Federal Ministry of Education and Research (BMBF, PtJ-Bio, 0315883). The careful assistance of Madlen Hempel is greatly acknowledged.

[2]http://clinicaltrials.gov

REFERENCES

Alison, M. R., Islam, S., and Lim, S. M. (2009). Cell therapy for liver disease. *Curr. Opin. Mol. Ther.* 11, 364–374.

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J. M., Fike, J. R., Lee, H. O., Pfeffer, K., Lois, C., Morrison, S. J., and Alvarez-Buylla, A. (2003). Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425, 968–973.

Aurich, I., Mueller, L. P., Aurich, H., Luetzkendorf, J., Tisljar, K., Dollinger, M. M., Schormann, W., Walldorf, J., Hengstler, J. G., Fleig, W. E., and Christ, B. (2007). Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. *Gut* 56, 405–415.

Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Quinn, G., Okochi, H., and Ochiya, T.

(2007). Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* 46, 219–228.

Benhamouche, S., Decaens, T., Godard, C., Chambrey, R., Rickman, D. S., Moinard, C., Vasseur-Cognet, M., Kuo, C. J., Kahn, A., Perret, C., and Colnot, S. (2006). Apc tumor suppressor gene is the zonation-keeper of mouse liver. *Dev. Cell* 10, 759–770.

Burke, Z. D., and Tosh, D. (2006). The Wnt/beta-catenin pathway: master regulator of liver zonation? *Bioessays* 28, 1072–1077.

Camargo, F. D., Finegold, M., and Goodell, M. A. (2004). Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. *J. Clin. Invest.* 113, 1266–1270.

Chamberlain, G., Fox, J., Ashton, B., and Middleton, J. (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 25, 2739–2749.

Chen, X., Zhang, S., Liu, T., Liu, Y. and Wang, Y. (2012). Maintenance of rat hepatocytes under inflammation by coculture with human orbital fat-derived stem cells. *Cell. Mol. Biol. Lett.* 1–14. doi: 10.2478/s11658-012-0004-9. [Epub ahead of print].

Chen, Y., Xiang, L.-X., Shao, J.-Z., Pan, R.-L., Wang, Y.-X., Dong, X.-J., and Zhang, G.-R. (2010). Recruitment of endogenous bone marrow mesenchymal stem cells towards injured liver. *J. Cell. Mol. Med.* 14, 1494–1508.

Collino, F., Deregibus, M. C., Bruno, S., Sterpone, L., Aghemo, G., Viltono, L., Tetta, C., and Camussi, G. (2010). Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PLoS ONE* 5, e11803. doi:10.1371/journal.pone.0011803

Dechene, A., Sowa, J.-P., Gieseler, R. K., Jochum, C., Bechmann, L. P., El Fouly, A., Schlattjan, M., Saner, F., Baba, H. A., Paul, A., Dries, V., Odenthal, M., Gerken, G., Friedman, S. L., and Canbay, A. (2010). Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 52, 1008–1016.

Deregibus, M. C., Tetta, C., and Camussi, G. (2010). The dynamic stem cell microenvironment is orchestrated by microvesicle-mediated transfer of genetic information. *Histol. Histopathol.* 25, 397–404.

Doi, K., and Ishida, K. (2009). Diabetes and hypertriglyceridemia modify the mode of acetaminophen-induced hepatotoxicity and nephrotoxicity in rats and mice. *J. Toxicol. Sci.* 34, 1–11.

Dong, X. J., Zhang, H., Pan, R. L., Xiang, L. X., and Shao, J. Z. (2010). Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions. *World J. Gastroenterol.* 16, 3267–3278.

Enns, G. M., and Millan, M. T. (2008). Cell-based therapies for metabolic liver disease. *Mol. Genet. Metab.* 95, 3–10.

Fausto, N. (2004). Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 39, 1477–1487.

Fisher, R. A., and Strom, S. C. (2006). Human hepatocyte transplantation. *Transplantation* 82, 441–449.

Flohr, T. R., Bonatti, H. J., Brayman, K. L., and Pruett, T. L. (2009). The use of stem cells in liver disease. *Curr. Opin. Organ Transplant.* 14, 64–71.

Fox, I. J., and Roy-Chowdhury, J. (2004). Hepatocyte transplantation. *J. Hepatol.* 40, 878–886.

Gebhardt, R., and Hovhannisyan, A. (2010). Organ patterning in the adult stage: the role of Wnt/ß-catenin signaling in liver zonation and beyond. *Dev. Dyn.* 239, 45–55.

Gill, R. Q., and Sterling, R. K. (2001). Acute liver failure. *J. Clin. Gastroenterol.* 33, 191–198.

Götherström, C. (2007). Immunomodulation by multipotent mesenchymal stromal cells. *Transplantation* 84, S35–S37.

Gupta, S., Rajvanshi, P., Aragona, E., Lee, C.-D., Yerneni, P. R., and Burk, R. D. (1999). Transplanted hepatocytes proliferate differently after CCl4 treatment and hepatocyte growth factor infusion. *Am. J. Physiol.* 276, G629–G638.

Hailfinger, S., Jaworski, M., Braeuning, A., Buchmann, A., and Schwarz, M. (2006). Zonal gene expression in murine liver: lessons from tumors. *Hepatology* 43, 407–414.

Haridass, D., Narain, N., and Ott, M. (2008). Hepatocyte transplantation: waiting for stem cells. *Curr. Opin. Organ Transplant.* 13, 627–632.

Heckel, J. L., Sandgren, E. P., Degen, J. L., Palmiter, R. D., and Brinster, R. L. (1990). Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 62, 447–456.

Hong, S. H., Gang, E. J., Jeong, J. A., Ahn, C., Hwang, S. H., Yang, I. H., Park, H. K., Han, H. and Kim, H. (2005). In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. *Biochem. Biophys. Res. Commun.* 330, 1153–1161.

Ijima, H., Matsuo, T., and Kawakami, K. (2008). The mixed coculture effect of primary rat hepatocytes and bone marrow cells is caused by soluble factors derived from bone marrow cells. *J. Biosci. Bioeng.* 105, 226–231.

Ito, M., Nagata, H., Miyakawa, S., and Fox, I. J. (2009). Review of hepatocyte transplantation. *J. Hepatobiliary Pancreat. Surg.* 16, 97–100.

Jaeschke, H., and Bajt, M. L. (2006). Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol. Sci.* 89, 31–41.

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., and Lemasters, J. J. (2002). Mechanisms of hepatotoxicity. *Toxicol. Sci.* 65, 166–176.

James, L. P., Mayeux, P. R., and Hinson, J. A. (2003). Acetaminophen-induced hepatotoxicity. *Drug Metab. Dispos.* 31, 1499–1506.

Jang, Y.-Y., Collector, M. I., Baylin, S. B., Diehl, A. M., and Sharkis, S. J. (2004). Hematopoietic stem cells convert into liver cells within days without fusion. 6, 532–539.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M. D. J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., and Verfaillie, C. M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418, 41–49.

Jin, S. Z., Meng, X. W., Han, M. Z., Sun, X., Sun, L. Y., and Liu, B. R. (2009). Stromal cell derived factor-1 enhances bone marrow mononuclear cell migration in mice with acute liver failure. *World J. Gastroenterol.* 15, 2657–2664.

Jungermann, K., and Kietzmann, T. (2000). Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology* 31, 255–260.

Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 449, 557–563.

Kofman, A. V., Morgan, G., Kirschenbaum, A., Osbeck, J., Hussain, M., Swenson, S., and Theise, N. D. (2005). Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury. *Hepatology* 41, 1252–1261.

Koniaris, L. G., McKillop, I. H., Schwartz, S. I., and Zimmers, T. A. (2003). Liver regeneration. *J. Am. Coll. Surg.* 197, 634–659.

Krampera, M., Sartoris, S., Liotta, F., Pasini, A., Angeli, R., Cosmi, L., Andreini, A., Mosna, F., Bonetti, B., Rebellato, E., Testi, M. G., Frosali, F., Pizzolo, G., Tridente, G., Maggi, E., Romagnani, S., and Annunziato, F. (2007). Immune regulation by mesenchymal stem cells derived from adult spleen and thymus. *Stem Cells Dev.* 16, 797–810.

Laconi, E., Oren, R., Mukhopadhyay, D. K., Hurston, E., Laconi, S., Pani, P., Dabeva, M. D., and Shafritz, D. A. (1998). Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. *Am. J. Pathol.* 153, 319–329.

Laconi, S., Curreli, F., Diana, S., Pasciu, D., Filippo, G. D., Sarma, D. S. R., Pani, P., and Laconi, E. (1999). Liver regeneration in response to partial hepatectomy in rats treated with retrorsine: a kinetic study. *J. Hepatol.* 31, 1069–1074.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I. L., and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat. Med.* 6, 1229–1234.

Lange, C., Bassler, P., Lioznov, M. V., Bruns, H., Kluth, D., Zander, A. R., and Fiegel, H. C. (2005). Hepatocytic gene expression in cultured rat mesenchymal stem cells. *Transplant. Proc.* 37, 276–279.

Le Blanc, K., and Ringden, O. (2007). Immunomodulation by mesenchymal stem cells and clinical experience. *J. Intern. Med.* 262, 509–525.

Lee, K. D., Kuo, T. K., Whang-Peng, J., Chung, Y. F., Lin, C. T., Chou, S. H., Chen, J. R., Chen, Y. P. and Lee, O. K. (2004). In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* 40, 1275–1284.

Lee, W. M., Stravitz, R. T., and Larson, A. (2011). Introduction to the revised AASLD position paper on acute liver failure 2011. *Hepatology* 55, 965–967.

Li, J., Tao, R., Wu, W., Cao, H., Xin, J., Guo, J., Jiang, L., Hong, X., Demetriou, A. A., Farkas, D., and Li, L. (2010). Transcriptional profiling and hepatogenic potential of acute hepatic failure-derived bone marrow mesenchymal stem cells. *Differentiation* 80, 166–174.

Mehendale, H. M. (2005). Tissue repair: an important determinant of final

outcome of toxicant-induced injury. *Toxicol. Pathol.* 33, 41–51.

Najimi, M., and Sokal, E. M. (2008). Cell transplantation in the treatment of liver diseases. 12, 6–13.

Newsome, P. N., Johannessen, I., Boyle, S., Dalakas, E., McAulay, K. A., Samuel, K., Rae, F., Forrester, L., Turner, M. L., Hayes, P. C., Harrison, D. J., Bickmore, W. A., and Plevris, J. N. (2003). Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology* 124, 1891–1900.

Oertel, M., Menthena, A., Dabeva, M. D., and Shafritz, D. A. (2006). Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. *Gastroenterology* 130, 507–520.

Oertel, M., and Shafritz, D. A. (2008). Stem cells, cell transplantation and liver repopulation. *Biochim. Biophys. Acta* 1782, 61–74.

O'Grady, J. G. (2005). Acute liver failure. *Postgrad. Med. J.* 81, 148–154.

Ostapowicz, G., and Lee, W. M. (2000). Acute hepatic failure: a Western perspective. *J. Gastroenterol. Hepatol.* 15, 480–488.

Overturf, K., Al-Dhalimy, M., Finegold, M., and Grompe, M. (1999). The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion. *Am. J. Pathol.* 155, 2135–2143.

Overturf, K., al-Dhalimy, M., Ou, C. N., Finegold, M., and Grompe, M. (1997). Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. *Am. J. Pathol.* 151, 1273–1280.

Palmes, D., Skawran, S., and Spiegel, H.-U. (2005). Acute liver failure: from bench to bedside. *Transplant. Proc.* 37, 1628–1631.

Parekkadan, B., van Poll, D., Megeed, Z., Kobayashi, N., Tilles, A. W., Berthiaume, F., and Yarmush, M. L. (2007a). Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* 363, 247–252.

Parekkadan, B., van Poll, D., Suganuma, K., Carter, E. A., Berthiaume, F., Tilles, A. W., and Yarmush, M. L. (2007b). Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS ONE* 2, e941. doi:10.1371/journal.pone.0000941

Ponder, K. P., Gupta, S., Leland, F., Darlington, G., Finegold, M., DeMayo, J., Ledley, F. D., Chowdhury, J. R., and Woo, S. L. (1991). Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. *Proc. Natl. Acad. Sci. U.S.A.* 88, 1217–1221.

Puppi, J., and Dhawan, A. (2009). Human hepatocyte transplantation overview. *Methods Mol. Biol.* 481, 1–16.

Rahman, T., and Hodgson, H. (2001). Clinical management of acute hepatic failure. *Intensive Care Med.* 27, 467–476.

Rajvanshi, P., Kerr, A., Bhargava, K., Burk, R., and Gupta, S. (1996). Efficacy and safety of repeated hepatocyte transplantation for significant liver repopulation in rodents. *Gastroenterology* 111, 1092–1102.

Reuben, A., Koch, D. G., and Lee, W. M. (2010). Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. *Hepatology* 52, 2065–2076.

Rhim, J. A., Sandgren, E. P., Degen, J. L., Palmiter, R. D., and Brinster, R. L. (1994). Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 263, 1149–1152.

Sancho-Bru, P., Najimi, M., Caruso, M., Pauwelyn, K., Cantz, T., Forbes, S., Roskams, T., Ott, M., Gehling, U., Sokal, E., Verfaillie, C. M., and Muraca, M. (2009). Stem and progenitor cells for liver repopulation: can we standardise the process from bench to bedside? *Gut* 58, 594–603.

Sandgren, E. P., Palmiter, R. D., Heckel, J. L., Daugherty, C. C., Brinster, R. L., and Degen, J. L. (1991). Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. *Cell* 66, 245–256.

Sandhu, J. S., Petkov, P. M., Dabeva, M. D., and Shafritz, D. A. (2001). Stem cell properties and repopulation of the rat liver by fetal liver epithelial progenitor cells. *Am. J. Pathol.* 159, 1323–1334.

Santoni-Rugiu, E., Jelnes, P., Thorgeirsson, S. S., and Bisgaard, H. C. (2005). Progenitor cells in liver regeneration: molecular responses controlling their activation and expansion. *APMIS* 113, 876–902.

Sato, Y., Araki, H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., Sato, T., Miyanishi, K., Takayama, T., Takahashi, M., Takimoto, R., Iyama, S., Matsunaga, T., Ohtani, S., Matsuura, A., Hamada, H., and Niitsu, Y. (2005). Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood* 106, 756–763.

Schwartz, R. E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W. S., and Verfaillie, C. M. (2002). Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J. Clin. Invest.* 109, 1291–1302.

Sell, S. (2001). Heterogeneity and plasticity of hepatocyte lineage cells. *Hepatology* 33, 738–750.

Seo, M. J., Suh, S. Y., Bae, Y. C., and Jung, J. S. (2005). Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem. Biophys. Res. Commun.* 328, 258–264.

Seppen, J., Filali, E. E., and Elferink, R. O. (2009). Small animal models of hepatocyte transplantation. *Methods Mol. Biol.* 481, 75–82.

Shafritz, D. A., and Oertel, M. (2011). Model systems and experimental conditions that lead to effective repopulation of the liver by transplanted cells. *Int. J. Biochem. Cell Biol.* 43, 198–213.

Shafritz, D. A., Oertel, M., Menthena, A., Nierhoff, D., and Dabeva, M. D. (2006). Liver stem cells and prospects for liver reconstitution by transplanted cells. *Hepatology* 43, S89–S98.

Shi, X.-L., Zhang, Y., Gu, J.-Y., and Ding, Y.-T. (2009). Coencapsulation of hepatocytes with bone marrow mesenchymal stem cells improves hepatocyte-specific functions. *Transplantation* 88, 1178–1185.

Smets, F., Najimi, M., and Sokal, E. M. (2008). Cell transplantation in the treatment of liver diseases. *Pediatr. Transplant.* 12, 6–13.

Soto-Gutierrez, A., Navarro-Alvarez, N., Yagi, H., and Yarmush, M. L. (2009). Stem cells for liver repopulation. *Curr. Opin. Organ Transplant.* 14, 667–673.

Steiner, J. W., Perz, Z. M., and Taichman, L. B. (1966). Cell population dynamics in the liver. A review of quantitative morphological techniques applied to the study of physiological and pathological growth. *Exp. Mol. Pathol.* 5, 146–181.

Stock, P., Bruckner, S., Ebensing, S., Hempel, M., Dollinger, M. M., and Christ, B. (2010). The generation of hepatocytes from mesenchymal stem cells and engraftment into murine liver. *Nat. Protoc.* 5, 617–627.

Stock, P., Ebensing, S., Hempel, M., Dollinger, M. M., Seufferlein, T., and Christ, B. (2009). Hepatocyte-like cells derived from human mesenchymal stem cells support regeneration of the mouse liver after acetaminophen-induced injury. *Regen. Med.* 4, S79.

Taléns-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J. V., and Gómez-Lechón, M. J. (2006). Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World J. Gastroenterol.* 12, 5834–5845.

van Poll, D., Parekkadan, B., Cho, C. H., Berthiaume, F., Nahmias, Y., Tilles, A. W., and Yarmush, M. L. (2008). Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology* 47, 1634–1643.

Vassilopoulos, G. P., Wang, R., and Russell, D. W. (2003). Transplanted bone marrow regenerates liver by cell fusion. *Nature* 422, 901–904.

Wang, P. P., Wang, J. H., Yan, Z. P., Hu, M. Y., Lau, G. K., Fan, S. T., and Luk, J. M. (2004). Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction. *Biochem. Biophys. Res. Commun.* 320, 712–716.

Wang, X., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. (2001). Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. *Am. J. Pathol.* 158, 571–579.

Wang, X., Montini, E., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. (2002). Kinetics of liver repopulation after bone marrow transplantation. *Am. J. Pathol.* 161, 565–574.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. (2003). Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422, 897–901.

Weber, A., Groyer-Picard, M.-T., Franco, D., and Dagher, I. (2009). Hepatocyte transplantation in animal models. *Liver Transpl.* 15, 7–14.

Yagi, H., Soto-Gutierrez, A., Kitagawa, Y., Tilles, A. W., Tompkins, R. G., and Yarmush, M. L. (2010). Bone marrow mesenchymal stromal cells attenuate organ injury induced by lps and burn. *Cell Transplant.* 19, 823–830.

Yan, C., Zhou, L., and Han, Y.-P. (2008). Contribution of hepatic stellate cells and matrix metalloproteinase 9 in acute liver failure. *Liver Int.* 28, 959–971.

Yukawa, H., Noguchi, H., Oishi, K., Takagi, S., Hamaguchi, M., Hamajima,

N., and Hayashi, S. (2009). Cell Transplantation of adipose tissue-derived stem cells in combination with heparin attenuated acute liver failure in mice. *Cell Transplant.* 18, 611–618.

Zagoura, D. S., Roubelakis, M. G., Bitsika, V., Trohatou, O., Pappa, K. I., Kapelouzou, A., Antsaklis, A., and Anagnou, N. P. (2011). Therapeutic potential of a distinct population of human amniotic fluid mesenchymal stem cells and their secreted molecules in mice with acute hepatic failure. *Gut.* doi:10.1136/gutjnl-2011–300908. [Epub ahead of print].

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 February 2012; accepted: 16 March 2012; published online: 02 April 2012.

Citation: Christ B and Brückner S (2012) Rodent animal models for surrogate analysis of cell therapy in acute liver failure. Front. Physio. ***3****:78. doi: 10.3389/fphys.2012.00078*

frontiers in PHYSIOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 21 December 2012
doi: 10.3389/fphys.2012.00476

Circulating microRNAs: promising candidates serving as novel biomarkers of acute hepatitis

Natalia Elfimova[1], Martin Schlattjan[2], Jan-Peter Sowa[2], Hans Peter Dienes[1], Ali Canbay[2] and Margarete Odenthal[1]*

[1] *Laboratory of Molecular Hepatology, Institute for Pathology, University Hospital of Cologne, Cologne, Germany*
[2] *Laboratory Ali Canbay, Department for Gastroenterology and Hepatology, University Hospital of Essen, Essen, Germany*

Edited by:
Heike Bantel, Hannover Medical School, Germany

Reviewed by:
Takashi Joh, Nagoya City University Graduate School of Medical Sciences, Japan
Rolf Gebhardt, University of Leipzig, Germany
Heike Bantel, Hannover Medical School, Germany

Correspondence:
Margarete Odenthal, Laboratory of Molecular Hepatology, Institute for Pathology, University Hospital of Cologne, Kerpener Str. 62, 50924 Koeln, Germany.
e-mail: m.odenthal@uni-koeln.de

Acute liver failure as life threatening condition comprises a difficult diagnostic situation to evaluate potential outcomes and therapeutic options. Thus, prognostic indicators are urgently needed for evaluation of progression of liver injury, clinical outcome, prognosis, and for therapeutic response. Recently, circulating microRNA, in particular miR-122, was described as a potential biomarker of acute liver injury after intoxication of mice. Circulating microRNA (miRNA) molecules are very stable and RNase-resistant due to protein aggregation and vesicle enclosure. Since miRNA species are known to be associated with chronic liver damage or with liver cancer, circulating miRNA patterns are suggested to serve also as reporters for progression of acute liver failure. miRNA profiling analyses using PCR arrays or next generation sequencing, may achieve identification of miRNA species that are linked to the rapid progression of acute liver injury, to the outcome of liver failure, or to the therapeutic response. Therefore, circulating miRNAs are promising, non-invasive biomarkers of future diagnostic approaches. However, normalisation of circulating miRNA levels is essential and further standardisation of miRNA quantification assays is needed.

Keywords: microRNA, extracellular miRNA, miR-122, acute liver failure, spike-in RNA, miRNA quantification

INTRODUCTION

Acute liver failure is a life-threatening liver disease characterized by a rapid and fulminant loss of liver function. Common causes of acute liver failure are viral hepatitis infection, mainly hepatitis A and B, and drug-induced liver intoxication (Canbay et al., 2005; Hadem et al., 2008; Ichai and Samuel, 2008, 2011; Lee, 2012). Acute liver failure demands urgent medical care, but initial symptoms of acute liver failure such as diarrhea, fatigue, and loss of appetite are rather unspecific and difficult to interpret (Renner, 2007). Rapid progression of liver malfunction is then accompanied by serious symptoms as ascites, hepatic encephalopathy, and coma. Sometimes, the acute liver failure can be reversed by valiant clinical intervention, but in many cases liver transplantation might be the only option of cure (Bernal et al., 2008; Ichai and Samuel, 2008; Lee, 2012). Thus, prognostic indicators are urgently needed for evaluation of progression of liver injury, clinical outcome, prognosis, and for therapeutic response. Recently, circulating microRNA is described as a novel tool to diagnose and monitor various diseases [summarized by Cortez and Calin (2009)].

MicroRNAs (miRNAs) are short noncoding, endogenous RNAs that regulate posttranscriptional gene expression by either RNA interference or inhibition of translational initiation and elongation (Bartel, 2004). Therefore, miRNAs are implicated in a widespread variety of cellular processes like differentiation, cell proliferation, and apoptosis (Miska, 2005; Bushati and Cohen, 2007). In human, more than thousand miRNAs are known (Kozomara and Griffiths-Jones, 2011). Mature miRNAs are formed in a step-wise process from larger primary transcripts (pri-miRNA), which are further processed, folding to hairpin structured precursor miRNA (pre-miRNA). The pre-miRNAs are exported from the nucleus, serving as substrates for the Dicer family of RNase III enzymes. One strand of the resulting short dsRNA guides the RNA-induced silencing complex (RISC) to its target mRNA (Zhao and Srivastava, 2007). Then, perfect complementary interaction of miRNAs with the untranslated region (UTR) of transcripts results in transcript degradation, whereas imperfect base pairing of miRNAs with the targeted UTR leads to translational repression (Zhao and Srivastava, 2007; Bartel, 2009).

In addition to the cellular function of miRNA in posttranscriptional gene repression, recent data collect evidence that miRNA also occur in extracellular compartments (Hunter et al., 2008; Mitchell et al., 2008). In our present review, we summarize the perspectives of circulating miRNAs to function as novel promising biomarkers of acute hepatitis.

DYSREGULATION OF HEPATIC miR-122 AFTER LIVER INJURY

miRNA expression profiles appear to be tissue-specific. Thus, miR-122 is highly expressed in hepatocytes, due to its liver-specific transcriptional regulation by hepatocyte nuclear transcription factors (HNF1α, HNF3β, and HNF4α) (Coulouarn et al., 2009; Xu et al., 2010). miR-122, comprising approximately 70% of total miRNA in the healthy liver, takes part in liver-specific functions such as cholesterol metabolism (Esau et al., 2006; Jopling, 2012). Interestingly, miR-122 is transcribed in a circadian fashion affecting gene expression pattern of a wide range of proteins (Gatfield et al., 2009). Whereas miR-122 interaction with the 3′ untranslated region (UTR) of various transcripts

leads to gene repression, miR-122 binding to two target sites in the 5′-UTR of the HCV genome results in HCV-RNA genome stabilization and enhanced replication. Hence, the liver-specific miR-122 may contribute to HCV liver tropism at the level of translation (Henke et al., 2008; Jopling, 2008).

Dedifferentiation of hepatocytes during hepatocellular carcinogenesis is associated with the loss of miR-122 (Coulouarn et al., 2009; Burchard et al., 2010; Negrini et al., 2011). In addition, during, both, acute and chronic liver damages in response to various noxa such as viral infection, drug or alcohol intoxication, or heriditary disorders, miR-122 is markedly decreased in the injured liver. Thus, after non-alcoholic fat liver diseases (Cheung et al., 2008) as well as after chronic hepatitis C infection reduced hepatic miR-122 levels were observed (Sarasin-Filipowicz et al., 2009; Morita et al., 2011). However, whereas miRNA is decreased in the injured liver, recent reports pointed to increased levels of circulating miR-122 in the blood stream after acute liver injury-induced by paracetamol intoxication of mice (Wang et al., 2009).

miRNA RELEASED INTO THE BLOOD STREAM AFTER LIVER DISEASE

In serum from human patients suffering from prostate cancer, it was first described that miRNA occur also extracellularily (Hunter et al., 2008; Mitchell et al., 2008). Circulating miRNA are highly stable in serum and also RNase resistant (**Figure 1A**), due to protein aggregation and vesicle enclosure (Mitchell et al., 2008; Cortez and Calin, 2009; Chen et al., 2010). As vesicular structures embedding extracellular miRNA, apoptotic bodies, microvesicles (Hunter et al., 2008; Skog et al., 2008), and exosomes (Taylor and Gercel-Taylor, 2008) have been discussed (**Figure 1B**). Due to their high stability, they are ideal candidates considered as non-invasive diagnostic markers indicating progression and therapy outcome of disease. Accordingly, circulating miRNAs were detected in some other tumorigenic diseases such as ovarian (Lodes et al., 2009; Resnick et al., 2009), lung, and colorectal cancer (Chen et al., 2008; Ng et al., 2009) (for review please see Cortez and Calin, 2009). Furthermore, Vasilescu et al. revealed circulating miR-150 as a new prognostic marker of patients with sepsis and Wang et al. described prominent upregulation of serum

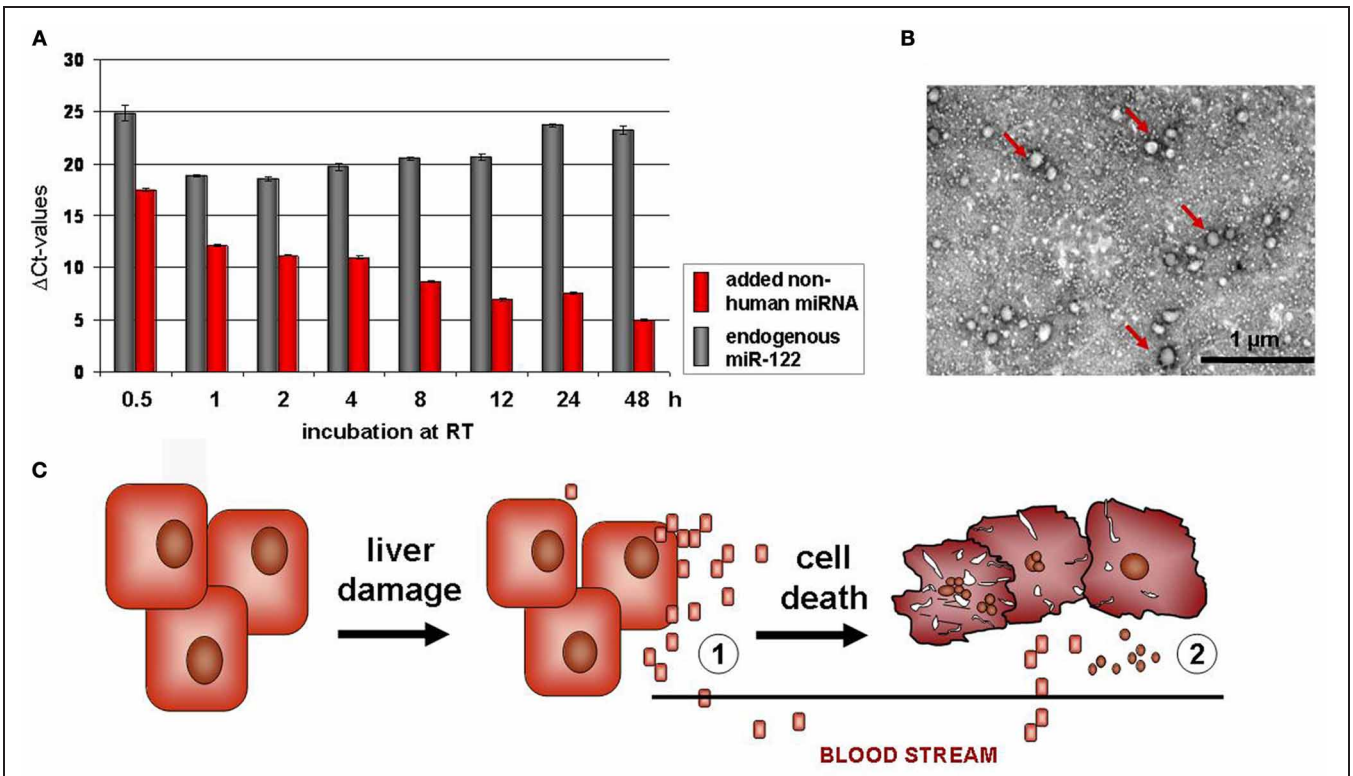


FIGURE 1 | Highly stable miRNA is released from hepatocytes after acute liver damage. (A) High stability of endogenous miRNA in serum samples. Non-human chemically synthesized miRNA was added to human serum samples. Then the samples were incubated for 0.5 up to 48 h at room temperature (RT). Endogenous miR-122 (red) and spiked RNA (gray) was quantified by Real Time PCR, demonstrating high stability of endogenous miR-122, but degradation of the added non-human RNA. **(B)** Vesicular enclosure of circulating serum miRNA. Vesicles of around 50 nm in diameter are found in the serum, as shown by uranyl acetate negative-staining and subsequent transmission electronmicroscopy (TEM) **(B)**. The vesicle fraction carries miRNA as proven by Real Time PCR (Hunter et al., 2008; Skog et al., 2008; Chen et al., 2010). Interestingly, Bala et al. pointed out that after acute paracetamol intoxication in mice, circulating miR-122 is not predominantly associated to vesicles, but to protein aggregates (Bala et al., 2012). **(C)** Hepatic miRNA release after acute liver injury. miRNA is released from hepatocytes after acute liver damage. Interestingly, circulating miR-122 levels are increased in serum samples before levels of transaminases (ALT) were elevated. Therefore, miR-122 might not only be released after hepatocellular damage and death (2), but also by other mechanisms e.g., inflammatory processes, not yet described (1).

miR-122 and miR-192 after acute hepatic intoxication by paracetamol in mice (Wang et al., 2009). In addition to miR-122 and miR-192, Zhou et al. identified miR-21, miR-223, miR-26a, miR-27a, and miR-801 in serum of patients with hepatocellular carcinoma (HCC) and proposed this miRNA panel as predictive markers of HCC. A comprehensive study on a wide cohort of patients with HBV or HCV based HCC revealed that high miR-25, let7f and primary miR-375 profiles only occurred in HCC-positive patients. Herein, increased miR-375 levels are shown to be specifically associated to HBV positive HCC (Li et al., 2010).

Importantly, miRNA panels in serum could not only be applied to differentiate between HCC and normal healthy donors, but also between HCC and cirrhosis (Zhou et al., 2011). Although miR-122 quantification of 68 serum samples of chronic HCV-positive patients was not normalized, the findings of Bihrer et al. definitively demonstrate that miR-122 correlated with alanine aminotransferase (ALT) values indicating liver inflammatory activity (Bihrer et al., 2011). Thus, circulating miRNAs are proposed as new biomarkers not only for tumorigenic, but also for inflammatory liver diseases.

EXTRACELLULAR miRNAs IN THE BLOOD STREAM ARE PROMISING BIOMARKES OF ACUTE HEPATITIS

Previous findings in mice after acute intoxication revealed that miR-122 increased markedly in serum samples before liver transaminases were raised (Wang et al., 2009; Zhang et al., 2010). This is of particular interest, because miRNA might not only be released by hepatocellular destruction processes, but also by active secretory delivery into the blood stream (**Figure 1C**). Consequently, early increased levels of serum miR-122 in response to inflammatory stimuli have to be considered as better indicators of liver failure than determination of liver-specific enzymes such as ALT (Zhang et al., 2010; Wang et al., 2012). Interestingly, Bala et al. found that after acute paracetamol intoxication of mice, miR-122 and miR-155 were predominantly associated in protein aggregates, whereas after alcoholic liver disease these two miRNAs were mainly found in the vesicular fraction (Bala et al., 2012). In addition, Novellino et al. suggested that in 13 serum samples of patients with HBV infection, miRNA is mainly complexed with the Ago2 protein, which in turn binds the hepatitis B surface protein (Novellino et al., 2012).

In addition to the experiments on acutely intoxicated mice, first data are now available on human, showing high miR-122 levels after acute hepatitis in man (Starkey Lewis et al., 2011; Ding et al., 2012). The extracellular miR-122 levels in serum from patients with acetaminophen based acute liver injury were normalized using small nuclear (sn)U6 spliceosomal RNA which is so far the most commonly applied internal reference of circulating miRNA quantification (**Table 1**). Though snU6 RNA is proposed to be also released into the blood stream after cellular damage

Table 1 | miRNAs as potential biomarkers for liver disease.

Potential miRNA biomarker	Human liver disease	Normalisation	Detection/quantification method	Message	References[b]
High: miR-21, miR-192, −801 **Low:** miR-26a, −27a, **miR-122**, −223,	HBV HCC HBV chronic ($N \sim 1000$)	miR-1228	Microarray, Real Time PCR	Differentiation between healthy donors, chronic HBV, and HCC	Zhou et al., 2011
High: miR-25, −92a, let7f, miR-375	HBV chronic, HCC ($N \sim 150$)	/	NGS, Real Time PCR	miR-375 is HBV specific and a HCC predictor	Li et al., 2010
High: miR-122, miR-21, 223	HBV chronic, HCC ($N \sim 150$)	miR-181a[a] miR-181c[a]	Relative Real Time PCR	Increase in chronic HBV and HCC	Xu et al., 2011
High: miR-122	HBV chronic ($N = 83$)	U6 RNA	Relative Real Time PCR	Increase of miR-122	Zhang et al., 2010
High: miR-885-5p	HBV chronic, cirrhotic, HCC ($N > 100$)	U6 RNA	Relative Real Time PCR	Increase in chronic, cirrhotic HBV and HCC	Gui et al., 2011
High: miR-122	HCV chronic ($N = 68$)	/	Relative Real Time PCR	Increase correlated with ALT	Bihrer et al., 2011
High: miR-571 **Low:** miR-652	Chronic HCV and alcohol ($N = 67$)	Spike-in RNA	Relative Real Time PCR	miR-571 reflects progression	Roderburg et al., 2012
High: miR-122, −34	HCV chronic ($N = 34$), NAFLD ($N = 35$)	Spike-in RNA	Absolute Real Time PCR	Correlation with ALT, inflammatory activity and fibrosis	Cermelli et al., 2011
High: miR-122, −192	Acute (POD) ($N = 53$)	U6 RNA	Real Time PCR	Increase correlated with ALT	Starkey Lewis et al., 2011

[a] according to geNorm; POD: acetominophen overdose.

[b] Unfortunately, we could not refer to all literature.

miRNA shown in bold: miRNA identified in serum samples by various reports.

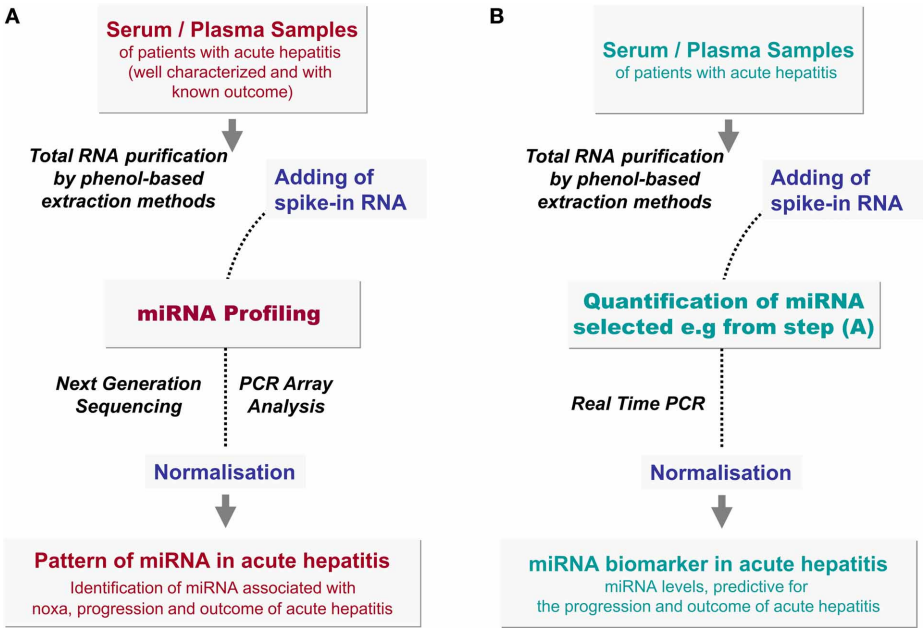


FIGURE 2 | Identification of predictive miRNAs in acute hepatitis. **(A)** Retrospective studies of miRNA pattern during acute hepatitis (Discovery). For identification of putative miRNA biomarkers, well defined serum or plasma samples of patients suffering from acute hepatitis are used for total RNA isolation by means of a phenol-based extraction method. In order to normalize the levels of circulating miRNAs spike-in RNA, highly dissimilar to human miRNAs e.g., *C. elegans*, *SV-40* virus, or *Arabidopsis thaliana* or an artificial miRNA sequence, should be added to the sample before extraction. Quantitative miRNA pattern analyses can be performed by next generation sequencing (NGS) or by PCR array analyses. The correlation of miRNA profiles with clinical parameters, with disease progression and outcome will suggest a panel of miRNAs as putative indicators of hepatitis. **(B)** Analysis of selected miRNAs during acute hepatitis (Training and Validation). miRNA, identified by NGS or PCR array screening approaches, have to be validated on a wide cohort of patients with acute hepatitis by retrospective and prospective studies. For validation and future diagnostic analyses, selected miRNA are quantified by Real Time PCR (**Figure 3**). Normalisation of miRNA levels by spike-in RNA is essential as described in the text.

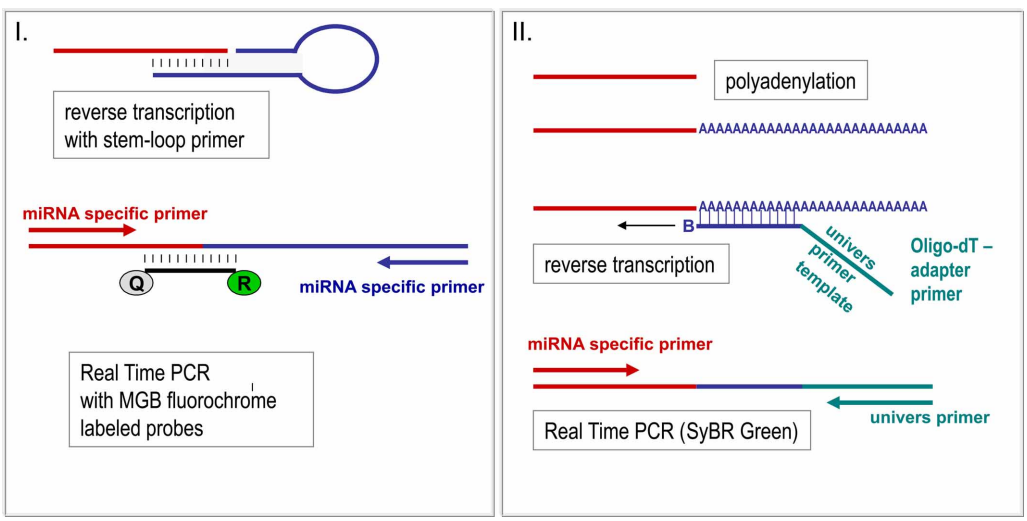


FIGURE 3 | miRNA quantification by Real Time PCR. For PCR amplification the short miRNA molecules have to be prolonged first. Elongation of miRNA takes place simultaneously to the reverse transcription reaction by hair looped primer sets recognizing the miRNA **(I)** (Chen et al., 2005) or by unspecific polyadenylation of RNA molecules **(II)** (Shi and Chiang, 2005). Whereas in the hairpin-loop primed cDNA two specific primers are used for PCR amplification **(I)**, polyadenylated RNA, which is reversely transcribed by an oligo-dT primer carrying an universal template sequence, is amplified by the universal and only one specific primer. Real-time monitoring can be performed by integration of fluorochrome labeled probes or by interaction of fluorescent dyes with the templates. Both methods **(I** and **II)** are highly effective, though having different advantages. Whereas the usage of miRNA-specific hairpin-looped primers results in very robust and highly specific miRNA quantification, polyadenylation provides the opportunity to use cDNA from one reverse transcription reaction for analyses of several miRNAs.

including liver parenchymal injury, this data interpretation may provide a primary impression of the role of miR-122 in human acute liver failure. However, Ji et al. found no change of miR-122 in 21 patients with HBV-induced acute-on-chronic liver failure, whereas miR-122 in patients with chronic HBV infection was even decreased in comparison to 12 healthy controls (Ji et al., 2011). These conflicting results might be due to diversity of study designs and technical approaches.

FOR PREDICTIVE USE, STANDARDISATION IN miRNA QUANTIFICATION IS NEEDED

Different methods are used to identify the pattern of miRNA in serum. Due to the very high sensitivity required for detection of circulating miRNA, Real Time PCR approaches are mostly carried out for screening as well as for validation of miRNA levels (**Figure 2**). Since miRNA are small templates, PCR needs a careful primer design and an elongation step of miRNA templates has to be combined to the reverse transcription before PCR amplification is started (**Figure 3**). For elongation of miRNA targets, either polyadenylation of miRNA is carried out (Shi and Chiang, 2005) or hairpin-looped primers are applied to the reverse transcription step leading to elongation of cDNA by the hairpin sequence (Chen et al., 2005) (**Figure 3**).

However, next generation sequencing (NGS) is also a valuable method to detect the pattern of circulating miRNAs followed by PCR quantification to validate data on a wide cohort of patients (**Figure 2**).

Although miR-122, miR-192, miR-21, and miR-34a are shown by most reports to be increased after experimental or human liver injury (**Table 1**), high variance and conflicting data exist about miRNA incidence in the blood stream upon different liver diseases. Kim et al. pointed out, that blood components that are co-purified with miRNA from serum or plasma highly affect efficiency of miRNA quantification by PCR (Kim et al., 2012). It is well-known that anti-coagulants in blood samples strongly inhibit Taq-polymerase, but plasma or serum sample volume, time until serum or plasma is prepared might also affect miRNA accessibility by Real Time PCR assays.

In addition, the accuracy of extracellular miRNA quantification highly depends on normalisation using an appropriate reference RNA. Thus, Xu et al. identified increased levels of circulating miR-122 and mir-92a as putative markers of chronic HBV infection after normalisation to endogenous miR-181 values, whereas Ji et al. observed decreasing levels of both miRNA after normalisation using snU6-RNA (Ji et al., 2011; Xu et al., 2011). snU6-RNA is mostly used as a reference. It is ubiquitously expressed in cells, but highly differs in the blood stream of different individuals (Qi et al., 2012). Furthermore, Ding et al. found that snU6-RNA is decreased after hepatocarcinogenesis (Ding et al., 2012). Hence, an internal standard for miRNA quantification is missing, so far. Therefore, the application of spike-in RNA is recommended in order to normalize errors in sample handling and extraction (**Figure 2**). Though miRNA are very stable molecules in serum or plasma, for better comparability and reproducibility in future studies, a well-standardized protocol is needed, in order to evaluate miRNAs as biomarkers for acute hepatitis.

REFERENCES

Bala, S., Petrasek, J., Mundkur, S., Catalano, D., Levin, I., Ward, J., et al. (2012). Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology* 56, 1946–1957.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233.

Bernal, W., Auzinger, G., Sizer, E., and Wendon, J. (2008). Intensive care management of acute liver failure. *Semin. Liver Dis.* 28, 188–200.

Bihrer, V., Friedrich-Rust, M., Kronenberger, B., Forestier, N., Haupenthal, J., Shi, Y., et al. (2011). Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. *Am. J. Gastroenterol.* 106, 1663–1669.

Burchard, J., Zhang, C., Liu, A. M., Poon, R. T., Lee, N. P., Wong, K. F., et al. (2010). microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol. Syst. Biol.* 6:402. doi: 10.1038/msb.2010.58

Bushati, N., and Cohen, S. M. (2007). microRNA functions. *Annu. Rev. Cell Dev. Biol.* 23, 175–205.

Canbay, A., Chen, S. Y., Gieseler, R. K., Malago, M., Karliova, M., Gerken, G., et al. (2005). Overweight patients are more susceptible for acute liver failure. *Hepatogastroenterology* 52, 1516–1520.

Cermelli, S., Ruggieri, A., Marrero, J. A., Ioannou, G. N., and Beretta, L. (2011). Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS ONE* 6:e23937. doi: 10.1371/journal.pone.0023937

Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 33:e179. doi: 10.1093/nar/gni178

Chen, C., Skog, J., Hsu, C. H., Lessard, R. T., Balaj, L., Wurdinger, T., et al. (2010). Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab. Chip* 10, 505–511.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., et al. (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 18, 997–1006.

Cheung, O., Puri, P., Eicken, C., Contos, M. J., Mirshahi, F., Maher, J. W., et al. (2008). Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 48, 1810–1820.

Cortez, M. A., and Calin, G. A. (2009). MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin. Biol. Ther.* 9, 703–711.

Coulouarn, C., Factor, V. M., Andersen, J. B., Durkin, M. E., and Thorgeirsson, S. S. (2009). Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* 28, 3526–3536.

Ding, X., Ding, J., Ning, J., Yi, F., Chen, J., Zhao, D., et al. (2012). Circulating microRNA-122 as a potential biomarker for liver injury. *Mol. Med. Rep.* 5, 1428–1432.

Esau, C., Davis, S., Murray, S. F., Yu, X. X., Pandey, S. K., Pear, M., et al. (2006). miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab.* 3, 87–98.

Gatfield, D., Le Martelot, G., Vejnar, C. E., Gerlach, D., Schaad, O., Fleury-Olela, F., et al. (2009). Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes Dev.* 23, 1313–1326.

Gui, J., Tian, Y., Wen, X., Zhang, W., Zhang, P., Gao, J., et al. (2011). Serum microRNA characterization identifies miR-885-5p as a potential marker for detecting liver pathologies. *Clin. Sci. (Lond.)* 120, 183–193.

Hadem, J., Stiefel, P., Bahr, M. J., Tillmann, H. L., Rifai, K., Klempnauer, J., et al. (2008). Prognostic implications of lactate, bilirubin, and etiology in German patients with acute liver failure. *Clin. Gastroenterol. Hepatol.* 6, 339–345.

Henke, J. I., Goergen, D., Zheng, J., Song, Y., Schuttler, C. G., Fehr, C., et al. (2008). microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J.* 27, 3300–3310.

Hunter, M. P., Ismail, N., Zhang, X., Aguda, B. D., Lee, E. J., Yu,

L., et al. (2008). Detection of microRNA expression in human peripheral blood microvesicles. *PLoS ONE* 3:e3694. doi: 10.1371/journal.pone.0003694

Ichai, P., and Samuel, D. (2008). Etiology and prognosis of fulminant hepatitis in adults. *Liver Transpl.* 14(Suppl. 2), S67–S79.

Ichai, P., and Samuel, D. (2011). Epidemiology of liver failure. *Clin. Res. Hepatol. Gastroenterol.* 35, 610–617.

Ji, F., Yang, B., Peng, X., Ding, H., You, H., and Tien, P. (2011). Circulating microRNAs in hepatitis B virus-infected patients. *J. Viral. Hepat.* 18, e242–e251.

Jopling, C. (2012). Liver-specific microRNA-122: biogenesis and function. *RNA Biol.* 9, 137–142.

Jopling, C. L. (2008). Regulation of hepatitis C virus by microRNA-122. *Biochem. Soc. Trans.* 36, 1220–1223.

Kim, D. J., Linnstaedt, S., Palma, J., Park, J. C., Ntrivalas, E., Kwak-Kim, J. Y., et al. (2012). Plasma components affect accuracy of circulating cancer-related microRNA quantitation. *J. Mol. Diagn.* 14, 71–80.

Lee, W. M. (2012). Acute liver failure. *Semin. Respir. Crit. Care Med.* 33, 36–45.

Kozomara, A., and Griffiths-Jones, S. (2011). miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157.

Li, L. M., Hu, Z. B., Zhou, Z. X., Chen, X., Liu, F. Y., Zhang, J. F., et al. (2010). Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. *Cancer Res.* 70, 9798–9807.

Lodes, M. J., Caraballo, M., Suciu, D., Munro, S., Kumar, A., and Anderson, B. (2009). Detection of cancer with serum miRNAs on an oligonucleotide microarray. *PLoS ONE* 4:e6229. doi: 10.1371/journal.pone.0006229

Miska, E. A. (2005). How microRNAs control cell division, differentiation and death. *Curr. Opin. Genet. Dev.* 15, 563–568.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10513–10518.

Morita, K., Taketomi, A., Shirabe, K., Umeda, K., Kayashima, H., Ninomiya, M., et al. (2011). Clinical significance and potential of hepatic microRNA-122 expression in hepatitis C. *Liver Int.* 31, 474–484.

Negrini, M., Gramantieri, L., Sabbioni, S., and Croce, C. M. (2011). microRNA involvement in hepatocellular carcinoma. *Anticancer Agents Med. Chem.* 11, 500–521.

Ng, E. K., Chong, W. W., Jin, H., Lam, E. K., Shin, V. Y., Yu, J., et al. (2009). Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 58, 1375–1381.

Novellino, L., Rossi, R. L., Bonino, F., Cavallone, D., Abrignani, S., Pagani, M., et al. (2012). Circulating hepatitis B surface antigen particles carry hepatocellular microRNAs. *PLoS ONE* 7:e31952. doi: 10.1371/journal.pone.0031952

Qi, R., Weiland, M., Gao, X. H., Zhou, L., and Mi, Q. S. (2012). Identification of endogenous normalizers for serum microRNAs by microarray profiling: U6 small nuclear RNA is not a reliable normalizer. *Hepatology* 55, 1640–1642; author reply 1642–1643.

Renner, E. L. (2007). How to decide when to list a patient with acute liver failure for liver transplantation? Clichy or King's College criteria, or something else? *J. Hepatol.* 46, 554–557.

Resnick, K. E., Alder, H., Hagan, J. P., Richardson, D. L., Croce, C. M., and Cohn, D. E. (2009). The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol. Oncol.* 112, 55–59.

Roderburg, C., Mollnow, T., Bongaerts, B., Elfimova, N., Vargas Cardenas, D., Berger, K., et al. (2012). Micro-RNA profiling in human serum reveals compartment-specific roles of miR-571 and miR-652 in liver cirrhosis. *PLoS ONE* 7:e32999. doi: 10.1371/journal.pone.0032999

Sarasin-Filipowicz, M., Krol, J., Markiewicz, I., Heim, M. H., and Filipowicz, W. (2009). Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat. Med.* 15, 31–33.

Shi, R., and Chiang, V. L. (2005). Facile means for quantifying microRNA expression by real-time PCR. *Biotechniques* 39, 519–525.

Skog, J., Wurdinger, T., Van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., et al. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476.

Starkey Lewis, P. J., Dear, J., Platt, V., Simpson, K. J., Craig, D. G., Antoine, D. J., et al. (2011). Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology* 54, 1767–1776.

Taylor, D. D., and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* 110, 13–21.

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., et al. (2009). Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4402–4407.

Wang, S., Qiu, L., Yan, X., Jin, W., Wang, Y., Chen, L., et al. (2012). Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1) -modulated P53 activity. *Hepatology* 55, 730–741.

Xu, H., He, J. H., Xiao, Z. D., Zhang, Q. Q., Chen, Y. Q., Zhou, H., et al. (2010). Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* 52, 1431–1442.

Xu, J., Wu, C., Che, X., Wang, L., Yu, D., Zhang, T., et al. (2011). Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol. Carcinog.* 50, 136–142.

Zhang, Y., Jia, Y., Zheng, R., Guo, Y., Wang, Y., Guo, H., et al. (2010). Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin. Chem.* 56, 1830–1838.

Zhao, Y., and Srivastava, D. (2007). A developmental view of microRNA function. *Trends Biochem. Sci.* 32, 189–197.

Zhou, J., Yu, L., Gao, X., Hu, J., Wang, J., Dai, Z., et al. (2011). Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. *J. Clin. Oncol.* 29, 4781–4788.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 September 2012; paper pending published: 22 September 2012; accepted: 05 December 2012; published online: 21 December 2012.

*Citation: Elfimova N, Schlattjan M, Sowa J-P, Dienes HP, Canbay A and Odenthal M (2012) Circulating microRNAs: promising candidates serving as novel biomarkers of acute hepatitis. Front. Physio. **3**:476. doi: 10.3389/fphys.2012.00476*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 12 June 2012
doi: 10.3389/fphys.2012.00193

Acute alcohol-induced liver injury

Veronica L. Massey and Gavin E. Arteel*

Department of Pharmacology and Toxicology, University of Louisville Alcohol Research Center, University of Louisville Health Sciences Center, Louisville, KY, USA

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Wing-Kin Syn, Foundation for Liver Research, UK
Christian Rust, University of Munich, Germany

****Correspondence:***
Gavin E. Arteel, Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, 505 South Hancock Street, CTRB Room 506, Louisville, KY 40292, USA.
e-mail: gavin.arteel@louisville.edu

Alcohol consumption is customary in most cultures and alcohol abuse is common worldwide. For example, more than 50% of Americans consume alcohol, with an estimated 23.1% of Americans participating in heavy and/or binge drinking at least once a month. A safe and effective therapy for alcoholic liver disease (ALD) in humans is still elusive, despite significant advances in our understanding of how the disease is initiated and progresses. It is now clear that acute alcohol binges not only can be acutely toxic to the liver, but also can contribute to the chronicity of ALD. Potential mechanisms by which acute alcohol causes damage include steatosis, dysregulated immunity and inflammation, and altered gut permeability. Recent interest in modeling acute alcohol exposure has yielded new insights into potential mechanisms of acute injury, which also may well be relevant for chronic ALD. Recent work by this group on the role of PAI-1 and fibrin metabolism in mediating acute alcohol-induced liver damage serve as an example of possible new targets that may be useful for alcohol abuse, be it acute or chronic.

Keywords: alcohol, acute, hepatic, inflammation, steatosis

INTRODUCTION

Alcohol consumption is customary in most cultures and alcohol abuse is common worldwide. For example, more than 50% of Americans consume alcohol, with an estimated 23.1% of Americans participating in heavy and/or binge drinking at least once a month (Substance Abuse and Mental Health Services Administration, 2011). The detrimental effects of drinking alcohol, particularly heavy and/or chronic consumption, are well established. The most commonly recognized symptoms of alcohol consumption are associated with chronic alcoholism, and it is a causal/risk factor in over 60 major types of diseases. These and other effects of alcohol consumption have made alcohol the third leading risk factor globally for disease and disability (World Health Organization, 2011). The cost of medical consequences of alcohol abuse have been projected to be more than 26 billion dollars in the US in 1998, making it a significant financial burden for the health care system (U. S. Department of Health and Human Services, 2000). These estimates take into account only direct medical expenses; it is estimated that the cost of lost productivity due to alcohol-related illness was ∼90 billion dollars in the US in 1998. Thus, the medical consequences of alcohol consumption have significant societal consequences in addition to the effects on the individual.

The liver is the main site of alcohol metabolism and a major target organ of alcohol-induced injury. The susceptibility of the liver to alcohol-induced toxicity is due to both the high concentrations of alcohol found in the portal blood (versus systemic), as well as the metabolic consequences of ethanol metabolism. Alcoholic Liver Disease (ALD) is a spectrum of disease states that includes steatosis (fatty liver), steatohepatitis, and in severe cases, fibrosis and/or cirrhosis. Steatosis, characterized by fat accumulation in hepatocytes, develops in 90% of individuals who drink more than 16 g of alcohol/day (Crabb, 1999), but resolves upon cessation of alcohol consumption (Bergheim et al., 2005). In only a minority of even heavy drinkers, steatosis progresses to steatohepatitis, which is characterized by the persistence of fatty liver accompanied by inflammation. In later stages of ALD, collagen deposition and regenerative nodules can result in the development of fibrosis and cirrhosis, respectively. Abstinence from alcohol is beneficial for patients in all stages of ALD and is necessary to prevent progression of liver injury in those with early stages of disease (Bergheim et al., 2005). Unfortunately, a high rate of recidivism among chronic alcohol abusers greatly reduces the opportunity for disease remission via abstinence. Therefore, better understanding of the mechanisms by which alcohol damages the liver may yield new pharmacologic strategies to blunt, halt, or reverse disease progression, potentially even in inveterate alcoholics.

Although alcohol exposure has been studied for decades, most work has focused on chronic exposure and our understanding of the mechanisms and effects of acute alcohol exposure are lacking. Acute alcohol exposure, commonly known as "binge drinking," is defined by the National Survey on Drug Use and Health (NSDUH) as five or more drinks on a single occasion, within a time period of 3 h. However, acute alcohol exposure can also include a period of heavy drinking that may span several days or periods of intermittent, repeated episodes of heavy drinking. For example, in the clinical setting an episode of "binge" drinking often describes a period of alcohol consumption and intoxication that lasts upward of 2 days (Weschsler and Austin, 1998). The NSDUH in 2010 showed that more than 58 million Americans have participated in binge drinking on at least one occasion within 30 days (Substance Abuse and Mental Health Services Administration, 2011). Due to the increasing prevalence of binge drinking in people aged 18–25, greater interest has turned toward the effects of acute alcohol exposure. Although chronic alcohol abuse is the most commonly recognized source of illness caused by alcohol consumption, acute alcohol abuse is also detrimental. Many acute alcohol-related

deaths are due to severe CNS depression, which slows motor coordination and respiratory rates. Acute alcohol exposure also impairs recovery from infection and trauma and delays wound healing (Radek et al., 2005, 2012). As such, acute alcohol consumption is a major underlying cause of morbidity and mortality during hospital admittance (Jones et al., 1991).

ACUTE ALCOHOL-INDUCED LIVER INJURY

The current understanding of the effects of binge drinking on liver injury is not as complete as our knowledge regarding the effects of chronic ethanol exposure. There are, however, some parallels between acute and chronic alcohol exposure. Potential mechanisms by which acute alcohol causes liver injury are discussed below.

MECHANISMS OF STEATOSIS

As mentioned above, the first and most common hepatic change caused by alcohol consumption is steatosis, or fatty liver. Fat accumulation can be both macrovesicular (having one large fat droplet per hepatocyte and lateral displacement of the nucleus) or microvesicular (many small fat droplets per hepatocyte; Ishak et al., 1991). One mechanism by which alcohol exposure causes steatosis is directly via alcohol metabolism. Concentrations of alcohol can easily reach the mM range in the portal/hepatic circulation during alcohol consumption. In the process of metabolizing ethanol to acetate, two equivalents of reduced NADH are generated per equivalent of ethanol oxidized. This metabolism robustly increases the ratio of NADH:NAD^+ within the cell, which then inhibits the β-oxidation of fatty acids in the liver. Furthermore, ethanol metabolism also increases the rate of esterification of fatty acids (Ontko, 1973). The net effect is to favor fat accumulation in the hepatocytes.

While alcohol metabolism may be sufficient to cause fat accumulation, it is not the only mechanism by which steatosis develops. Blocking lipopolysaccharide (LPS) signaling (Yin et al., 2001) and pro-oxidant producing enzymes (Kono et al., 2000; McKim et al., 2003) in rodents via genetic alterations also protect against steatosis, without altering ethanol metabolism. Fat accumulation can also be stimulated by mediators that alter lipid metabolism, such as pro-inflammatory cytokines (Grunfield et al., 1990). Signaling downstream of these cytokines contributes to hepatic fat accumulation in two ways – by increasing the deposition of fatty acids, and decreasing the breakdown and secretion of lipids into the circulation. For example, TNFα increases free fatty acid release from peripheral adipocytes (Hardardottir et al., 1992), increases *de novo* lipid synthesis (Feingold and Grunfeld, 1987), and inhibits β-oxidation of fatty acids (Nachiappan et al., 1994). Ultimately, these changes stimulate fat accumulation in the liver by increasing fatty acid supply, and concomitantly impairs the liver's capacity for fatty acid metabolism and secretion.

Hepatic steatosis develops acutely in the majority of individuals consuming even moderate amounts of alcohol. Steatotic changes are also seen in rodent models of binge drinking (Kaiser et al., 2009; Donohue et al., 2011). Steatosis is considered an asymptomatic disease state, which readily reverses with abstinence (Bergheim et al., 2005). Indeed, at the level of the organism, hepatic steatosis can be viewed as a protective measure, as it partitions lipids away from the blood and stores them for potential later use (van Ginneken, 2008). However, although steatosis is an inert pathology *per se*, it sensitizes the liver to injury caused by a second insult (Day and James, 1998). According to this "two-hit hypothesis," alcohol consumption alone does not cause progression of ALD from steatosis to steatohepatitis, cirrhosis, or fibrosis; rather, a second risk factor or insult is required for development of later stages of disease. Such a second insult could include an inflammatory response, reactive oxygen species, or hypoxia, among others. For example, acute ethanol exposure is well known to enhance liver pathology induced by xenobiotics, such as bacterial cell wall products (e.g., lipopolysaccharide). Furthermore, acute alcohol consumption itself could provide the second hit; for example, an episode of binge drinking can trigger entry into acute alcoholic hepatitis in a chronic alcoholic (Rivara et al., 1993; Barrio et al., 2004).

DYSREGULATION OF IMMUNITY AND INFLAMMATION

The two-hit hypothesis is mirrored on a molecular level by the concepts of "priming" and "sensitization" which are now considered to be fundamental to alcohol-induced liver injury (Tsukamoto et al., 2001). Here, "priming" refers to the ability of ethanol pre-exposure to cause inflammatory cells of the liver (e.g., Kupffer cells) to more robustly release pro-inflammatory cytokines in response to a second stimulus, such as LPS. Alcoholic hepatitis patients have larger amounts of circulating TNFα both basally and after stimulus (McClain and Cohen, 1989). Additionally, ethanol pre-exposure can prime Kupffer cells to LPS stimulation, resulting in enhanced TNF-α release (Enomoto et al., 1998). Ethanol can also sensitize cells, causing cell populations downstream of inflammatory cytokine signaling to respond more robustly. Patients with fatty liver are more sensitive to LPS-induced liver injury (Yang et al., 1997). This sensitization to LPS is recapitulated in rodent models of acute alcohol exposure (Bergheim et al., 2006; Beier et al., 2009).

Although some studies have shown that alcohol exposure primes macrophages to induce more inflammatory tissue damage, others have shown that acute alcohol intoxication (i.e., when alcohol is present in the system) actually impairs the immune response. For example, binge drinking increases the risk of infection (e.g., Gentilello et al., 1993; Gamble et al., 2006; Griffin et al., 2009). Furthermore, acute ethanol consumption worsens the prognosis of sepsis (Lin et al., 2009). A major mechanism by which alcohol can impair the immune response is via altering Toll-like receptor (TLR) signaling, which serve as pattern recognition receptors on macrophages and other cells of the innate immune system (Beutler, 2009). TLRs are involved in both direct cytotoxic and effector responses of macrophages, and therefore serve as key early initiators of an appropriate immune response. Importantly, ethanol intoxication has been shown to blunt the stimulation of macrophages by a number of TLR ligands, including zymosan A (TLR2) and LPS (TLR4; Pruett et al., 2004; Goral and Kovacs, 2005).

Although it may seem that these effects of acute ethanol on macrophage responsiveness are contradictory (i.e., both impairing and priming the macrophages), the timing of the inflammatory stimulus relative to ethanol exposure seems to be important. For example, acute ethanol (4–6 g/kg) blunted inflammatory liver damage caused by LPS, when LPS was injected 3 h after ethanol

exposure (during intoxication), but enhanced damage if the LPS was administered 24 h after ethanol (Enomoto et al., 1998). The mechanisms by which acute ethanol causes both tolerance and priming of the innate immune system are still incompletely understood. However, it is clear that acute alcohol exposure produces a dangerous situation in which the likelihood of infection is increased during acute intoxication, but liver damage is enhanced by the inflammatory response to that infection.

GUT PERMEABILITY AND ENDOTOXIN

Even in the absence of infection, alcohol can alter the response to inflammatory stimuli. The association between increased circulating bacterial endotoxin (i.e., LPS) and liver injury has been established since the 1970s (Nolan, 2010). Circulating levels of LPS are increased in ALD patients (Bode et al., 1987; Fukui et al., 1991) as well as in experimental models of both acute (Lambert et al., 2003) and chronic (Nanji et al., 1994; Keshavarzian et al., 2001) alcohol consumption. Increased circulating LPS in response to alcohol consumption is due, at least in part, to increased gut permeability, which thereby allows translocation of LPS into the portal blood. In fact, elimination of intestinal bacteria reduces alcohol-induced liver injury via reduced circulating endotoxin levels in mice (Nanji et al., 1994). Experimental models of both chronic (Mathurin et al., 2000; Tamai et al., 2002) and acute (Tamai et al., 2000; Lambert et al., 2003) alcohol exposure showed that alcohol increased permeation of LPS. Acute alcohol consumption increased circulating levels of LPS in both healthy volunteers and alcoholics with ALD (Bode et al., 1987; Fukui et al., 1991).

Under normal conditions, the spaces between epithelial cells that make up the intestinal barrier are sealed by tight junctions. These tight junctions prevent transfer of toxic compounds, like LPS, into the circulation (Purohit et al., 2008). Changes in intestinal permeability caused by ethanol may be due in part to the disruption of apical junctional proteins in the small intestine. *In vitro* studies using Caco-2 enterocytes showed that alcohol can disrupt ZO-1 tight junctions, ultimately causing gaps in the paracellular space (Ma et al., 1999). Furthermore, chronic alcohol exposure reduces ZO-1 and occludin in the ileum of mice (Zhong et al., 2010). The same studies also demonstrated that the reduction in tight junction proteins was associated with oxidative stress in the intestine. Potential sources of oxidative stress in the GI tract after alcohol exposure include acetaldehyde and nitric oxide (Purohit et al., 2008). Similar mechanisms may be involved in gut permeability due to acute alcohol exposure.

SUMMARY

Taken together, acute alcohol exposure can cause a "perfect storm" that favors inflammatory liver damage. Acute alcohol exposure enhances the risk of infection and permeability of the GI tract. Either or both mechanisms will increase the delivery of TLR ligands to macrophages in the liver. At later times after alcohol exposure, the inflammatory response of macrophages to TLR ligands is primed, and more cytotoxic cytokines (e.g., TNFα) are produced. Furthermore, steatosis in parenchymal cells sensitizes them to cytotoxic killing by the cytokines released by macrophages.

MODELS OF ACUTE ETHANOL EXPOSURE

The majority of research on alcoholic liver injury has investigated the effects of chronic alcohol consumption. However, clinical evidence highlighting the detrimental effects of acute alcohol consumption has spurred recent interest in experimental models of binge drinking, intermittent heavy drinking, and other acute alcohol exposures. These models include *in vitro* and *in vivo* paradigms, with the latter spanning many species including rodents, dogs, primates, and micropigs (Dolganiuc and Szabo, 2009). The majority of acute alcohol research is performed in rodent models. Such models achieve pathological states (e.g., steatosis, inflammation) that resemble the early stages of liver injury seen in humans. The use of rodents by the research community has increased since the development of a variety of knockout and other genetically modified mouse strains that allow for more convenient and specific mechanistic studies.

One of the major obstacles in rodent models of alcohol exposure is their aversion to ethanol. Rodents do not voluntarily consume alcohol at concentrations that will recapitulate liver disease found in humans. Therefore, forced bolus administration by intragastric gavage is the most common approach to modeling acute alcohol abuse (Siegmund et al., 2005). Rodent models of acute alcohol exposure often fall into one of three categories: single bolus dose models, multiple bolus dose models, and "2-hit" models. Ethanol is administered at bolus doses of up to 6 g/kg, which takes into account differences in ethanol kinetics in rodents versus humans (Gershman and Steeper, 1991; Carson and Pruett, 1996). The peak concentrations of alcohol in rodents are similar to those achievable in humans during an acute alcohol binge.

Chronic models are costly, and it is often difficult to distinguish between effects and proximate causes in chronic models of liver damage. In addition to studying acute hepatotoxicity, acute models are therefore also useful as a screening tool and/or mechanistic analysis to complement chronic studies. This approach is supported by the observation that animals exposed to acute alcohol administration develop steatosis and inflammation in a manner similar to animals in chronic ethanol studies. Further strengthening this idea of "a model of a model" is the mechanistic overlap of protective agents in both acute and chronic alcohol exposure models. For example, Enomoto et al. (2000) demonstrated that compounds known to protect against liver damage in chronic exposure models [e.g., $GdCl_3$ (Adachi et al., 1994), antibiotics (Adachi et al., 1995), nimodipine (Iimuro et al., 1996)] were also protective against steatosis in a rat model of acute ethanol exposure. Because of their use as models of chronic alcohol exposure, acute models of alcohol exposure are particularly valuable for screening compounds that may offer therapeutic benefit, as well as for the investigation of new mechanisms downstream of therapeutic compounds. Thus far, acute alcohol exposure models have lent few false positives when used to screen for efficacy of therapeutic molecules for chronic study.

PAI-1 AND FIBRIN – NEW MEDIATORS IN ACUTE ALCOHOL-INDUCED LIVER INJURY

PLASMINOGEN ACTIVATOR INHIBITOR-1

Plasminogen activator inhibitor-1 (PAI-1) is an acute phase protein that plays an important role in injury and inflammation, at

least in part via regulation of fibrinolysis. PAI-1 prevents the activation of plasmin and subsequent degradation of fibrin into fibrin degradation products (FDP) by inhibiting both the urokinase-type and tissue-type plasminogen activators (uPA and tPA; **Figure 1**; Kruithof, 1988). A role for PAI-1 in alcohol-induced liver injury was first seen in cirrhotic patients, who had an increased PA:PAI-1 ratio (Violi et al., 1992; Hu et al., 2001), resulting in hyperfibrinolysis. In contrast, during the development of ALD, levels of circulating PAI-1 are elevated and the PA:PAI-1 is lower, favoring hypofibrinolysis (Marques-Vidal et al., 1995; Dimmitt et al., 1998; Mukamal et al., 2001). Levels of PAI-1 correlate with the degree of lipid accumulation in experimental models of alcohol-induced liver steatosis (Alessi et al., 2003), and may be used as an index of severity of later stages of ALD (Tran-Thang et al., 1989).

While its importance in some diseases (e.g., atherosclerosis) has been established, the role of PAI-1 in ALD is still unclear. Studies using knockout mice strains, particularly PAI-$1^{-/-}$ mice, have begun to clarify the role of PAI-1 in lipid accumulation. Ma et al. (2004) showed that PAI-$1^{-/-}$ mice were protected from hepatic lipid accumulation in a model of high fat/high carbohydrate diet induced obesity. Additionally, our group has shown that both acute and chronic ethanol-induced steatosis correlate with hepatic PAI-1 expression, and that blocking PAI-1, either pharmacologically or genetically, confers protection against steatosis (Bergheim et al., 2006). In this study, TNFR$1^{-/-}$ mice were also protected against steatosis with concomitant blunting of PAI-1 (Bergheim et al., 2006), which suggests that the mechanism of PAI-1 induction after alcohol exposure is downstream of TNFα, possibly via MAPK signaling (Fearns and Loskutoff, 1997). PAI-1 also appears to be critical in mediating organ inflammation. For example, mice lacking PAI-1 were protected against leukocyte infiltration and tissue damage in a model of glomerulonephritis (Kitching et al., 2003). Work in this laboratory has shown that PAI-$1^{-/-}$ mice were nearly completely protected against inflammation caused by chronic alcohol (Arteel, 2008). How PAI-1 mediates inflammation, particularly after alcohol exposure, remains unclear. One potential mechanism is that PAI-1 mediates changes in the proteolytic activation/deactivation of cytokines important in the inflammatory response. These effects could include prevention of the activation of cytokines including transforming growth factor-β (Espevik et al., 1987; Sato et al., 1990), or stabilization of pro-inflammatory cytokines, such as the chemoattractant IL-8 (Marshall et al., 2003).

FIBRIN

Another potential mechanism underlying the inflammatory effects of PAI-1 involves impaired fibrinolysis and subsequent fibrin accumulation (Holdsworth et al., 1979; Loike et al., 1995). Fibrin(ogen) is a structural component of the extracellular matrix (ECM). Upon activation of the coagulation cascade, the latent form, fibrinogen, is cleaved by thrombin into the active form, fibrin (**Figure 1**). Fibrin can crosslink to create clots or be degraded by plasmin into fibrinogen degradation products. In addition to its role in hemostasis, fibrin can also function as a signaling molecule via interaction with cell surface receptors. Fibrin's role in inflammation has been highlighted by its ability to exert a myriad of pro-inflammatory effects. For example, Qi et al. (1997) showed that fibrin induces IL-8 expression in vascular endothelial cells. Fibrin matrices can regulate the migration of inflammatory cells

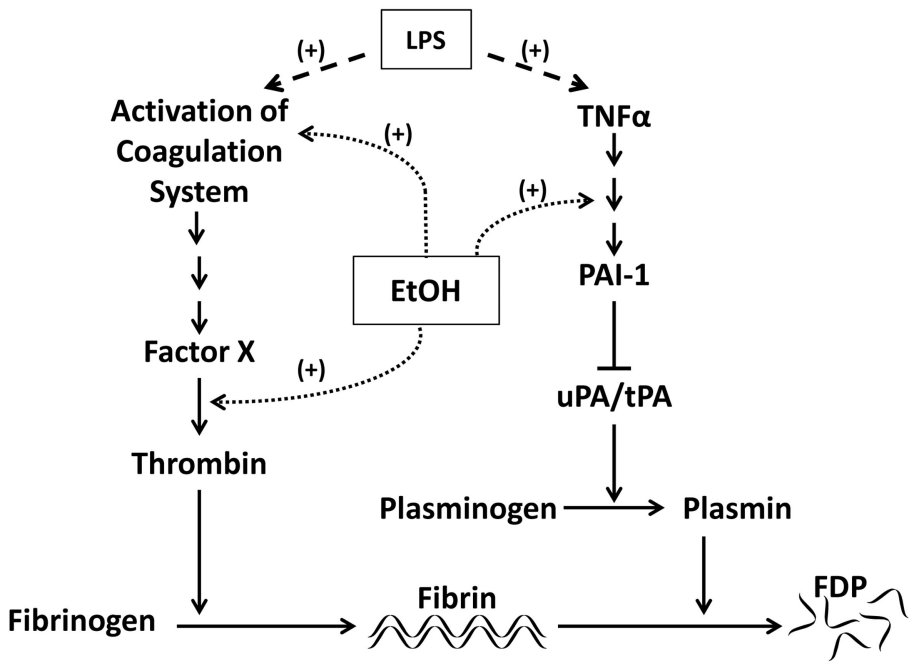


FIGURE 1 | Effects of LPS and ethanol on PAI-1 and fibrinolysis. Activation of the coagulation cascade by LPS via thrombin results in accumulation of cross-linked fibrin. Cross-linked fibrin is degraded by plasmin. PAI-1 blocks fibrinolysis by inhibiting the plasminogen activators uPA and tPA and therefore the activation of plasmin. Both ethanol and LPS can modulate signaling downstream of the coagulation cascade, increasing fibrin deposition. Ethanol may also blunt fibrin degradation by altering signaling downstream of TNFα. LPS can have similar effects on fibrinolysis by stimulating release of TNFα.

in vitro, including IL-8-stimulated neutrophils (Loike et al., 1995). Additionally, defibrillation reduces macrophage migration in the kidney (Holdsworth et al., 1979).

Fibrin has been shown to have a direct role in experimental ALD as well. Our group has shown that fibrin deposition in hepatic sinusoids is increased by chronic ethanol consumption (**Figure 2**) and that fibrin accumulation correlates with inflammation in an acute-exposure model of alcohol-induced liver injury. Furthermore, blocking fibrin deposition either directly, using the thrombin inhibitor Hirudin, or indirectly, using the MEK-inhibitor U0126, protected against enhanced liver injury caused by alcohol pre-exposure (Beier et al., 2009). Blocking fibrin accumulation also protected against inflammation (Beier et al., 2009). These data implicate fibrin as an important player in enhanced inflammation and injury mediated by PAI-1 after alcohol exposure.

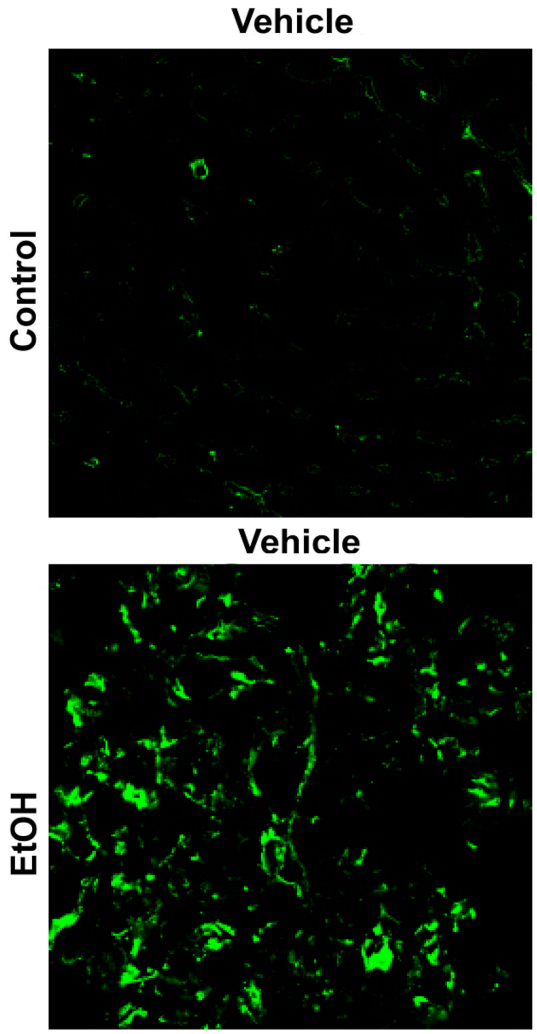


FIGURE 2 | Chronic ethanol increases hepatic fibrin(ogen) deposition. Representative photomicrographs (40×) depicting immunofluorescent detection of fibrin(ogen) ECM are shown. Liver tissue is from mice fed either control diet or enteral ethanol diet for 4 weeks.

INTEGRINS – MEDIATORS OF CELL-TO-ECM COMMUNICATION

While this group demonstrated a strong correlation between alcohol-induced fibrin deposition and hepatic inflammation and injury, the mechanism(s) by which fibrin is mediating its inflammatory effects remain unclear. One mechanism by which fibrin accumulation mediates inflammatory effects is via interaction with cell surface receptors such as integrins. Integrins are heterodimeric receptors expressed on many cell types including endothelial cells, inflammatory cells including macrophages and neutrophils, and platelets. Upon interaction with extracellular components, including surrounding matrices and cells, the beta subunit of an integrin undergoes a rapid conformational change. This change in integrin structure allows for transfer of information across the plasma membrane by activation of intracellular signaling pathways. Adapter proteins, including Integrin-Linked Kinase (ILK), are important mediators in signal transduction downstream of integrin activation and play a role in specifying which signaling cascades are activated within the cell. The integrins represent a diverse superfamily with at least 24 different heterodimers that are important in a variety of cell processes including proliferation, angiogenesis, and inflammation.

Fibrin(ogen) ECM is capable of interacting with the integrin family of cell surface adhesion receptors, such as the integrin $\alpha_v\beta_3$ (Cheresh et al., 1989). The role of integrin $\alpha_v\beta_3$ in tumor angiogenesis and metastasis is well documented, and stimulation of the $\alpha_v\beta_3$ integrin has been shown to contribute to inflammation (Zhou et al., 2009). Integrin $\alpha_v\beta_3$ is strongly expressed by endothelial cells (Carloni et al., 1996), including those that line the hepatic sinusoids (HSECs), which is a region that colocalizes with fibrin accumulation after alcohol exposure (**Figure 2**). Integrin $\alpha_v\beta_3$ binds fibrin(ogen) via an RGD sequence on the protein (Cheresh et al., 1989), which is pharmacologically targetable with small peptide antagonists. Studies by this group show that inhibition of $\alpha_v\beta_3$ using the small peptide antagonist, CycloRGDfV, protects against enhanced LPS-induced liver injury caused by acute alcohol in a "2-hit" model of acute alcohol-induced liver injury (**Figure 3**) without any effects on fibrin accumulation. Additionally, protection from liver injury by CycloRGDfV was concomitant with the blunting of LPS-induced neutrophil infiltration in this model (**Figure 3**). These data suggest that inflammation and liver injury due to LPS after acute alcohol exposure may be due to interaction between sinusoidal fibrin and integrin $\alpha_v\beta_3$.

SUMMARY AND CONCLUSION

A safe and effective therapy for ALD in humans is still elusive, despite significant advances in our understanding of how the disease is initiated and progresses. It is clear that alcohol binges not only can be acutely toxic to the liver, but also can contribute to the chronicity of ALD. Potential mechanisms by which acute alcohol causes damage include steatosis, dysregulated immunity and inflammation, and altered gut permeability. Recent interest in modeling acute alcohol exposure has yielded new insights into potential mechanisms of acute injury, that also may well be relevant for chronic ALD. Recent work by this group on the role of PAI-1 and fibrin metabolism in mediating acute alcohol-induced liver damage serve as an example of possible new targets that may be useful for alcohol abuse, be it acute or chronic.

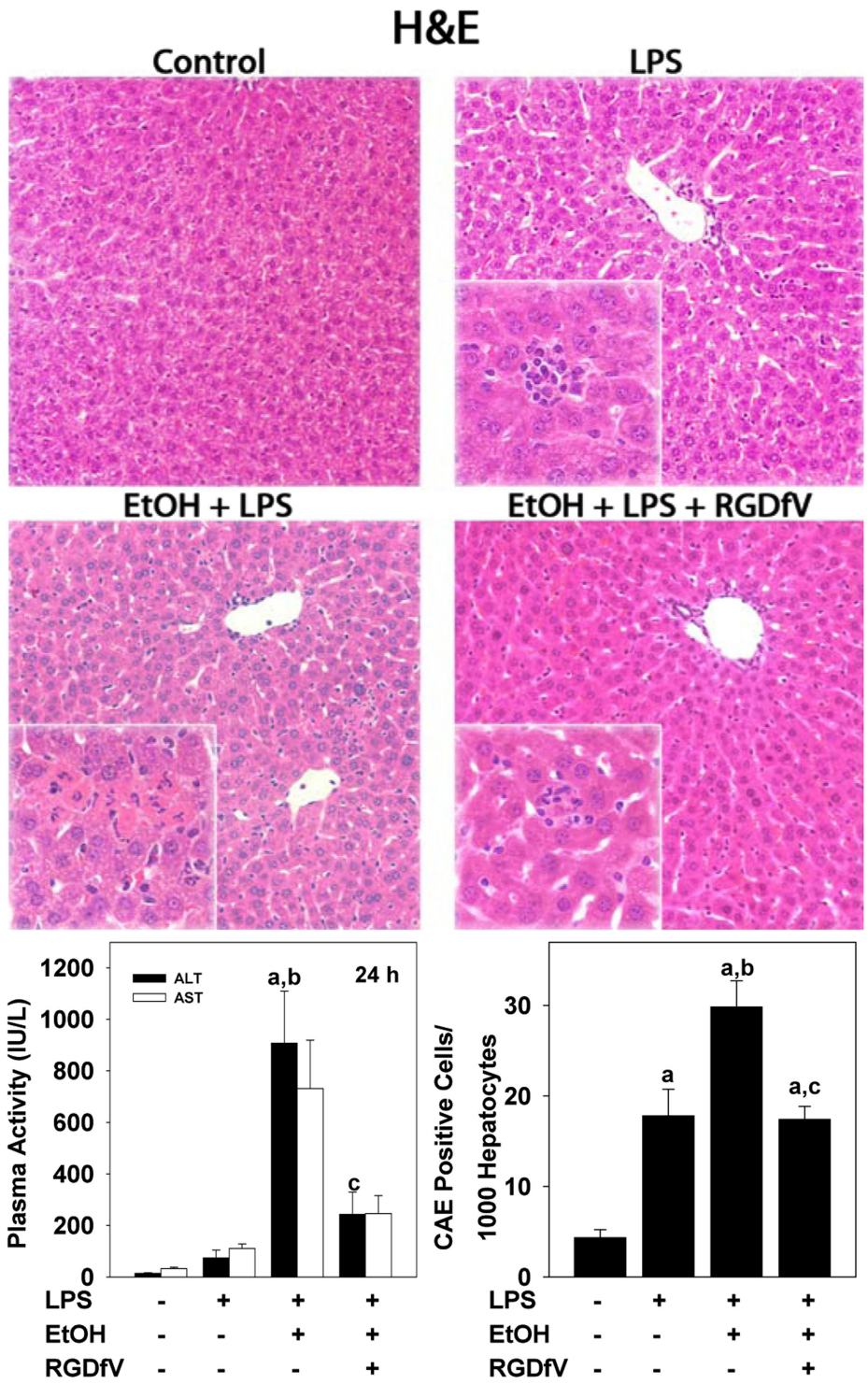


FIGURE 3 | Inhibition of Integrin $\alpha_v\beta_3$ protects against enhanced LPS-induced liver injury caused by ethanol pre-exposure. Representative photomicrographs (100×; insets 400×) of hematoxylin and eosin staining are shown in upper panel. Circulating ALT levels and neutrophil migration (determined by CAE staining) are in the lower left and right panels, respectively. Animals were exposed to alcohol (6 g/kg i.g.) for 3 days, followed by LPS administration (10 mg/kg; i.p) 24 h after last dose of ethanol and were sacrificed 24 h after ethanol administration. Some animals received CycloRGDfV (3 mg/kg i.p.) 1 h prior to LPS.

ACKNOWLEDGMENTS

This work was supported, in part, by a grant from the National Institute of Alcohol Abuse and Alcoholism (AA003624). Veronica L. Massey was supported by a predoctoral (T32) fellowship from National Institute of Environmental Health Science (ES011564).

REFERENCES

Adachi, Y., Bradford, B. U., Gao, W., Bojes, H. K., and Thurman, R. G. (1994). Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology* 20, 453–460.

Adachi, Y., Moore, L. E., Bradford, B. U., Gao, W., and Thurman, R. G. (1995). Antibiotics prevent liver injury in rats following long-term exposure to ethanol. *Gastroenterology* 108, 218–224.

Alessi, M. C., Bastelica, D., Mavri, A., Morange, P., Berthet, B., Grino, M., and Juhan-Vague, I. (2003). Plasma PAI-1 levels are more strongly related to liver steatosis than to adipose tissue accumulation. *Arterioscler. Thromb. Vasc. Biol.* 23, 1262–1268.

Arteel, G. E. (2008). New role of plasminogen activator inhibitor-1 in alcohol-induced liver injury. *J. Gastroenterol. Hepatol.* 23, S54–S59.

Barrio, E., Tome, S., Rodriguez, I., Gude, F., Sanchex-Leira, J., Perez-Becerra, E., and Gonzalez-Quintela, A. (2004). Liver disease in heavy drinkers with and without alcohol withdrawal syndrome. *Alcohol. Clin. Exp. Res.* 28, 131–136.

Beier, J. I., Luyendyk, J. P., Guo, L., von Montfort, C., Staunton, D. E., and Arteel, G. E. (2009). Fibrin accumulation plays a critical role in the sensitization to lipopolysaccharide-induced liver injury caused by ethanol in mice. *Hepatology* 49, 1545–1553.

Bergheim, I., Guo, L., Davis, M. A., Lambert, J. C., Beier, J. I., Duveau, I., Luyendyk, J. P., Roth, R. A., and Arteel, G. E. (2006). Metformin prevents alcohol-induced liver injury in the mouse: critical role of plasminogen activator inhibitor-1. *Gastroenterology* 130, 2099–2112.

Bergheim, I., McClain, C. J., and Arteel, G. E. (2005). Treatment of alcoholic liver disease. *Dig. Dis.* 23, 275–284.

Beutler, B. (2009). Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunol. Rev.* 227, 248–263.

Bode, C. H., Kugler, V., and Bode, J. C. H. (1987). Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J. Hepatol.* 4, 8–14.

Carloni, V., Romanelli, R. G., Pinzani, M., Laffi, G., and Gentilini, P. (1996). Expression and function of integrin receptors for collagen and laminin in cultured human hepatic stellate cells. *Gastroenterology* 110, 1127–1136.

Carson, E. J., and Pruett, S. B. (1996). Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol. *Alcohol. Clin. Exp. Res.* 20, 132–138.

Cheresh, D. A., Berliner, S. A., Vicente, V., and Ruggeri, Z. M. (1989). Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell* 58, 945–953.

Crabb, D. W. (1999). Pathogenesis of alcoholic liver disease: newer mechanisms of injury. *Keio. J. Med.* 48, 184–188.

Day, C. P., and James, O. F. (1998). Steatohepatitis: a tale of two "hits?" *Gastroenterology* 114, 842–845.

Dimmitt, S. B., Rakic, V., Puddey, I. B., Baker, R., Oostryck, R., Adams, M. J., Chesterman, C. N., Burke, V., and Beilin, L. J. (1998). The effects of alcohol on coagulation and fibrinolytic factors: a controlled trial. *Blood Coagul. Fibrinolysis* 9, 39–45.

Dolganiuc, A., and Szabo, G. (2009). In vitro and in vivo models of acute alcohol exposure. *World J. Gastroenterol.* 15, 1168–1177.

Donohue, T. M. Jr., Osna, N., Trambly, C., Whitaker, N., Thomes, P., Todero, S., and Davis, J. S. (2011). Early growth response-1 contributes to steatosis development after acute ethanol administration. *Alcohol. Clin. Exp. Res.* 36, 759–767.

Enomoto, N., Ikejima, K., Bradford, B. U., Rivera, C. A., Kono, H., Brenner, D. A., and Thurman, R. G. (1998). Alcohol causes both tolerance and sensitization of rat Kupffer cells via mechanisms dependent on endotoxin. *Gastroenterology* 115, 443–451.

Enomoto, N., Ikejima, K., Yamashina, S., Enomoto, A., Nishiura, T., Nishimura, T., Brenner, D. A., Schemmer, P., Bradford, B. U., Rivera, C. A., Zhong, Z., and Thurman, R. G. (2000). Kupffer cell-derived prostaglandin E(2) is involved in alcohol-induced fat accumulation in rat liver. *Am. J. Physiol.* 279, G100–G106.

Espevik, T., Figari, I. S., Shalaby, M. R., Lackides, G. A., Lewis, G. D., Shepard, H. M., and Palladino, M. A. Jr. (1987). Inhibition of cytokine production by cyclosporin A and transforming growth factor beta. *J. Exp. Med.* 166, 571–576.

Fearns, C., and Loskutoff, D. J. (1997). Induction of plasminogen activator inhibitor 1 gene expression in murine liver by lipopolysaccharide. Cellular localization and role of endogenous tumor necrosis factor-alpha. *Am. J. Pathol.* 150, 579–590.

Feingold, K. R., and Grunfeld, C. (1987). Tumor necrosis factor-alpha stimulates hepatic lipogenesis in the rat in vivo. *J. Clin. Invest.* 80, 184–190.

Fukui, H., Brauner, B., Bode, J. C., and Bode, C. (1991). Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay. *J. Hepatol.* 12, 162–169.

Gamble, L., Mason, C. M., and Nelson, S. (2006). The effects of alcohol on immunity and bacterial infection in the lung. *Med. Mal. Infect.* 36, 72–77.

Gentilello, L. M., Cobean, R. A., Walker, A. P., Moore, E. E., Wertz, M. J., and Dellinger, E. P. (1993). Acute ethanol intoxication increases the risk of infection following penetrating abdominal trauma. *J. Trauma.* 35, 669–674.

Gershman, H., and Steeper, J. (1991). Rate of clearance of ethanol from the blood of intoxicated patients in the emergency department. *J. Emerg. Med.* 9, 307–311.

Goral, J., and Kovacs, E. J. (2005). In vivo ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inflammatory response by limiting p38 and ERK1/2 activation. *J. Immunol.* 174, 456–463.

Griffin, R., Poe, A. M., Cross, J. M., Rue, L. W., and McGwin, G. (2009). The association between blood alcohol level and infectious complications among burn patients. *J. Burn Care Rehabil.* 30, 395–399.

Grunfield, C., Soued, M., Adi, S., Moser, A. H., Dinarello, C. A., and Feingold, K. R. (1990). Evidence for two classes of cytokines that stimulate hepatic lipogenesis: relationships among tumor necrosis factor, interleukin-1 and interferon-alpha. *Endocrinology* 127, 46–54.

Hardardottir, I., Doerrler, W., Feingold, K. R., and Grunfeld, C. (1992). Cytokines stimulate lipolysis and decrease lipoprotein lipase activity in cultured fat cells by a prostaglandin independent mechanism. *Biochem. Biophys. Res. Commun.* 186, 237–243.

Holdsworth, S. R., Thomson, N. M., Glasgow, E. F., and Atkins, R. C. (1979). The effect of defibrination on macrophage participation in rabbit nephrotoxic nephritis: studies using glomerular culture and electronmicroscopy. *Clin. Exp. Immunol.* 37, 38–43.

Hu, K. Q., Yu, A. S., Tiyyagura, L., Redeker, A. G., and Reynolds, T. B. (2001). Hyperfibrinolytic activity in hospitalized cirrhotic patients in a referral liver unit. *Am. J. Gastroenterol.* 96, 1581–1586.

Iimuro, Y., Ikejima, K., Rose, M. L., Bradford, B. U., and Thurman, R. G. (1996). Nimodipine, a dihydropyridine-type calcium channel blocker, prevents alcoholic hepatitis due to chronic intragastric ethanol exposure in the rat. *Hepatology* 24, 391–397.

Ishak, K. G., Zimmerman, H. J., and Ray, M. B. (1991). Alcoholic liver disease: pathologic, pathogenetic and clinical aspects. *Alcohol. Clin. Exp. Res.* 15, 45–66.

Jones, J. D., Barber, B., Engrav, L., and Heimback, D. (1991). Alcohol use and burn injury. *J. Burn Care Rehabil.* 12, 148–152.

Kaiser, J. P., Beier, J. I., Zhang, J., David, H. J., von Montfort, C., Guo, L., Zheng, Y., Monia, B. P., Bhatnagar, A., and Arteel, G. E. (2009). PKCepsilon plays a causal role in acute ethanol-induced steatosis. *Arch. Biochem. Biophys.* 482, 104–111.

Keshavarzian, A., Choudhary, S., Holems, E. W., Yong, S., Banan, A., Jakate, S., and Fields, J. Z. (2001). Preventing gut leakiness by oats supplementation ameliorates alcohol-induced liver damage in rats. *J. Pharmacol. Exp. Ther.* 299, 442–448.

Kitching, A. R., Kong, Y. Z., Huang, X. R., Davenport, P., Edgtton, K. L., Carmeliet, P., Holdsworth, S. R., and Tipping, P. G. (2003). Plasminogen activator inhibitor-1 is a significant determinant of renal injury in experimental crescentic glomerulonephritis. *J. Am. Soc. Nephrol.* 14, 1487–1495.

Kono, H., Rusyn, I., Yin, M., Gabele, E., Yamashina, S., Dikalova, A., Kadiiska, M. B., Connor, H. D., Mason, R. P., Segal, B. H., Bradford, B. U., Holland, S. M., and Thurman,

R. G. (2000). NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J. Clin. Invest.* 106, 867–872.

Kruithof, E. K. (1988). Plasminogen activator inhibitors – a review. *Enzyme* 40, 113–121.

Lambert, J. C., Zhou, Z., Wang, L., Song, Z., McClain, C. J., and Kang, Y. J. (2003). Prevention of alterations in intestinal permeability is involved in zinc inhibition of acute ethanol-induced liver damage in mice. *J. Pharmacol. Exp. Ther.* 305, 880–886.

Lin, J. N., Tsai, Y. S., Lai, C. H., Chen, Y. H., Tsai, S. S., Lin, H. L., Huang, C. K., Lin, H. H. (2009). Risk factors for mortality of bacteremic patients in the emergency department. *Acad. Emerg. Med.* 16, 749–755.

Loike, J. D., el Khoury, J., Cao, L., Richards, C. P., Rascoff, H., Mandeville, J. T., Maxfield, F. R., and Silverstein, S. C. (1995). Fibrin regulates neutrophil migration in response to interleukin 8, leukotriene B4, tumor necrosis factor, and formyl-methionyl-leucyl-phenylalanine. *J. Exp. Med.* 181, 1763–1772.

Ma, L. J., Mao, S. L., Taylor, K. L., Kanjanabuch, T., Guan, Y., Zhang, Y., Brown, N. J., Swift, L. L., McGuinness, O. P., Wasserman, D. H., Vaughan, D. E., and Fogo, A. B. (2004). Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. *Diabetes* 53, 336–346.

Ma, T. Y., Nguyen, D., Bui, V., Nguyen, H., and Hoa, N. (1999). Ethanol Modulation of intestinal epithelial tight junction barrier. *Am. J. Physiol.* 276, G965–G974.

Marques-Vidal, P., Cambou, J. P., Nicaud, V., Luc, G., Evans, A., Arveiler, D., Bingham, A., and Cambien, F. (1995). Cardiovascular risk factors and alcohol consumption in France and Northern Ireland. *Atherosclerosis* 115, 225–232.

Marshall, L. J., Ramdin, L. S., Brooks, T., DPhil, P. C., and Shute, J. K. (2003). Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. *J. Immunol.* 171, 2057–2065.

Mathurin, P., Deng, Q. G., Keshavarzian, A., Choudhary, S., Holmes, E. W., and Tsukamoto, H. (2000). Exacerbation of alcoholic liver injury by enteral endotoxin in rats. *Hepatology* 32, 1008–1017.

McClain, C. J., and Cohen, D. A. (1989). Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. *Hepatology* 9, 349–351.

McKim, S. E., Gabele, E., Isayama, F., Lambert, J. C., Tucker, L. M., Wheeler, M. D., Connor, H. D., Mason, R. P., Doll, M. A., Hein, D. W., and Arteel, G. E. (2003). Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. *Gastroenterology* 125, 1834–1844.

Mukamal, K. J., Jadhav, P. P., D'Agostino, R. B., Massaro, J. M., Mittleman, M. A., Lipinska, I., Sutherland, P. A., Matheney, T., Levy, D., Wilson, P. W., Ellison, R. C., Silbershatz, H., Muller, J. E., and Tofler, G. H. (2001). Alcohol consumption and hemostatic factors: analysis of the Framingham offspring cohort. *Circulation* 104, 1367–1373.

Nachiappan, V., Curtiss, D., Corkey, B. E., and Kilpatrick, L. (1994). Cytokines inhibit fatty acid oxidation in isolated rat hepatocytes: synergy among TNF, IL-6 and IL-1. *Shock* 1, 123–129.

Nanji, A. A., Khettry, U., and Sadrzadeh, S. M. H. (1994). *Lactobacillus* feeding reduces endotoxemia and severity of experimental alcoholic liver disease. *Proc. Soc. Exp. Biol. Med.* 205, 243–247.

Nolan, J. P. (2010). The role of intestinal endotoxin in liver injury: a long and evolving history. *Hepatology* 52, 1829–1835.

Ontko, J. A. (1973). Effects of ethanol on the metabolism of free fatty acids in isolated liver cells. *J. Lipid Res.* 14, 78–86.

Pruett, S. B., Zheng, Q., Fan, R., Matthews, K., and Schwab, C. (2004). Ethanol suppresses cytokine responses induced through Toll-like receptors as well as innate resistance to *Escherichia coli* in a mouse model for binge drinking. *Alcohol* 33, 147–155.

Purohit, V., Bode, J. C., Bode, C., Brenner, D. A., Choudhry, M. A., Hamilton, F., Kang, Y. J., Keshavarzian, A., Rao, R., Sartor, R. B., Swanson, C., and Turner, J. R. (2008). Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. *Alcohol* 42, 349–361.

Qi, J., Goralnick, S., and Kreutzer, D. L. (1997). Fibrin regulation of interleukin-8 gene expression in human vascular endothelial cells. *Blood* 90, 3595–3602.

Radek, K. A., Kovacs, E. J., and Dipietro, L. A. (2012). Matrix proteolytic activity during wound healing: modulation by acute ethanol exposure. *Alcohol. Clin. Exp. Res.* 31, 1045–1052.

Radek, K. A., Matthies, A. M., Burns, A. L., Heinrich, S. A., Kovacs, E. J., and Dipietro, L. A. (2005). Acute ethanol exposure impairs angiogenesis and the proliferative phase of wound healing. *Am. J. Physiol. Heart Circ. Physiol.* 289, 1084–1090.

Rivara, F. P., Jurkovich, G. J., Gurney, J. G., Seguin, D., Fligner, C. L., Ries, R., Raisys, V. A., and Copass, M. (1993). The magnitude of acute and chronic alcohol abuse in trauma patients. *Arch. Surg.* 128, 907–912.

Sato, Y., Tsuboi, R., Lyons, R., Moses, H., and Rifkin, D. B. (1990). Characterization of the activation of latent TGF-beta by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J. Cell Biol.* 111, 757–763.

Siegmund, S. V., Haas, S., and Singer, M. V. (2005). Animal models and their results in gastrointestinal alcohol research. *Dig. Dis.* 23, 181–194.

Substance Abuse and Mental Health Services Administration. (2011). *Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings.* Rockville, MD: Substance Abuse and Mental Health Services Administration.

Tamai, H., Horie, Y., Kato, S., Yokoyama, H., and Ishii, H. (2002). Long-term ethanol feeding enhances susceptibility of the liver to orally administered lipopolysaccharides in rats. *Alcohol. Clin. Exp. Res.* 26, 75S–80S.

Tamai, H., Kato, S., Horie, Y., Ohki, E., Yokoyama, H., and Ishii, H. (2000). Effect of acute ethanol administration on the intestinal absorption of endotoxin in rats. *Alcohol. Clin. Exp. Res.* 24, 390–394.

Tran-Thang, C., Fasel-Felley, J., Pralong, G., Hofstetter, J. R., Bachmann, F., and Kruithof, E. K. (1989). Plasminogen activators and plasminogen activator inhibitors in liver deficiencies caused by chronic alcoholism or infectious hepatitis. *Thromb. Haemost.* 62, 651–653.

Tsukamoto, H., Takei, Y., McClain, C. J., Joshi-Barve, S., Hill, D., Schmidt, J., Deaciuc, I., Barve, S., Colell, A., Garcia-Ruiz, C., Kaplowitz, N., Fernandez-Checa, J. C., Yokoyama, H., Okamura, Y., Nakamura, Y., Ishii, H., Chawla, R. K., Barve, S., Joshi-Barve, S., Watson, W. H., Nelson, W. J., Lin, M., Ohata, M., Motomura, K., Enomoto, K., Ikejima, K., Kitamura, T., Oide, H., Hirose, M., Bradford, B. U., Rivera, C. A., Kono, H., Peter, S., Yamashina, S., Konno, A., Ishikawa, H., Shimizu, H., Sato, N., and Thurman, R. (2001). How is the liver primed or sensitized for alcoholic liver disease? *Alcohol. Clin. Exp. Res.* 25, 171S–181S.

U. S. Department of Health and Human Services. (2000). *10th Special Report to the U. S. Congress on Alcohol and Research.*

van Ginneken, V. J. (2008). Liver fattening during feast and famine: an evolutionary paradox. *Med. Hypotheses* 70, 924–928.

Violi, F., Ferro, D., Basili, S., Quintarelli, C., Saliola, M., Alessandri, C., Cordova, C., and Balsano, F. (1992). Hyperfibrinolysis increases the risk of gastrointestinal hemorrhage in patients with advanced cirrhosis. *Hepatology* 15, 672–676.

Weschsler, H., and Austin, S. B. (1998). Binge drinking: the five/four measure. *J. Stud. Alcohol* 59, 122–124.

World Health Organization. (2011). *Global Status Report on Alcohol and Health.* Geneva: WHO Press.

Yang, S. Q., Lin, H. Z., Lane, M. D., Clemens, M., and Diehl, A. M. (1997). Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2557–2562.

Yin, M., Bradford, B. U., Wheeler, M. D., Uesugi, T., Froh, M., Goyert, S. M., and Thurman, R. G. (2001). Reduced early alcohol-induced liver injury in cd14-deficient mice. *J. Immunol.* 166, 4737–4742.

Zhong, W., McClain, C. J., Cave, M., Kang, Y. J., and Zhou, Z. (2010). The role of zinc deficiency in alcohol-induced intestinal barrier dysfunction. *Am. J. Physiol.* 298, G625–G633.

Zhou, H. F., Chan, H. W., Wickline, S. A., Lanza, G. M., and Pham, C. T. (2009). Alphavbeta3-targeted nanotherapy suppresses inflammatory arthritis in mice. *FASEB J.* 23, 2978–2985.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 April 2012; accepted: 22 May 2012; published online: 12 June 2012.
*Citation: Massey VL and Arteel GE (2012) Acute alcohol-induced liver injury. Front. Physio. **3**:193. doi: 10.3389/fphys.2012.00193*
This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 20 February 2012
doi: 10.3389/fphys.2012.00024

Hepatoprotectant ursodeoxycholyl lysophosphatidylethanolamide increasing phosphatidylcholine levels as a potential therapy of acute liver injury

Walee Chamulitrat[1]*, Wujuan Zhang[2], Weihong Xu[1], Anita Pathil[1], Kenneth Setchell[2] and Wolfgang Stremmel[1]

[1] *Department of Internal Medicine IV, University Heidelberg Hospital, Heidelberg, Germany*
[2] *Mass Spectrometry Laboratory, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Ali Canbay, University Hospital Essen, Germany
Matias Antonio Avila, Universidad de Navarra, Spain

****Correspondence:***
Walee Chamulitrat, Forschungsgruppen, Department of Internal Medicine IV, University Heidelberg Hospital, Im Neuenheimer Feld 345, EG, Lab 054, 69120 Heidelberg, Germany.
e-mail: walee.chamulitrat@med.uni-heidelberg.de

It has been long known that hepatic synthesis of phosphatidylcholine (PC) is depressed during acute such as carbon tetrachloride-induced liver injury. Anti-hepatotoxic properties of PC as liposomes have been recognized for treatment of acute liver damage. Ursodeoxycholate (UDCA) is a known hepatoprotectant in stabilizing cellular membrane. For therapeutic management of liver injury, we coupled UDCA with a phospholipid known as ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE). UDCA-LPE has been shown to first-in-class hepatoprotectant being superior to UDCA or PC. It inhibits mitochondrial damage and apoptosis, elicits survival signaling pathway, and promotes regeneration of hepatocytes. We herein report that a unique contribution of UDCA-LPE in increasing concentrations of PC *in vitro and in vivo*. UDCA-LPE-treated hepatocytes contained significantly increased PC levels. UDCA-LPE underwent the hydrolysis to LPE which was not the precursor of the increased PC. The levels of PC in the liver and blood were increased rapidly after intraperitoneally administration UDCA-LPE, and were found to be sustained even after 24 h. Among PC synthesis genes tested, UDCA-LPE treatment of mouse hepatocytes increased transcription of CDP-diacylglycerol synthase 1 which is an enzyme catalyzing phosphatidic acid to generate intermediates for PC synthesis. Thus, UDCA-LPE as a hepatoprotectant was able to induce synthesis of protective PC which would supplement for the loss of PC occurring during acute liver injury. This property has placed UDCA-LPE as a candidate agent for therapy of acute hepatotoxicity such as acetaminophen poisoning.

Keywords: acute liver injury, cytoprotection, drug poisoning, bile acid–phospholipid conjugate, PC homeostasis

INTRODUCTION

Phospholipids including lecithins and phosphatidylcholine (PC) have been used and tested in humans for treatment of liver disease since the 1970s (Wallnofer and Hanusch, 1973). PC is the major membrane phospholipids and thus their hepatoprotective effects are due to membrane stabilization (Miyazaki et al., 1991) as well as enhancement of cell membrane integrity (Li et al., 2006). Research on phospholipid biochemistry in the past three decades has revealed physiological role of PC which is required for hepatocellular division and survival (Jackowski, 1994), and that alterations of PC homeostasis could result in development of liver diseases (Noga and Vance, 2003; Li et al., 2005).

Hepatoprotective role of PC is applicable not only for chronic liver disease but also for acute liver damage. It was found nearly 50 years ago that carbon tetrachloride (CCl_4) intoxication is associated with changes in composition of PC and phosphatidylethanolamine (PE; Sgoutas, 1967; Shimizu, 1969; Sugano et al., 1970). Total phospholipids in hepatic microsomes (Sgoutas, 1967), endoplasmic reticulum (James et al., 1982), and plasma membrane (Camacho and Rubalcava, 1984) are markedly depressed following CCl_4 administration of experimental animals. This is due to decreased syntheses of both phospholipids and triglycerides readily observed 4–5 h after treatment (Gebhart and Brabec, 1985). CCl_4-induced injury *in vivo* produces an early peroxidative event from the generation of trichloromethyl radical as a result of cytochrome P450-mediated metabolism of CCl_4 (Trudell et al., 1982). These free radicals react with cellular PC forming PC-derived lipid radicals which subsequently react with oxygen to form PC hydroperoxides (Miyazawa et al., 1990). The formation of PC hydroperoxides is indicative of hepatic injury causing alterations in membrane structure. For treatment, it is conceivable that a supplementation of PC during acute liver injury replenishes

Abbreviations: BSA, bovine serum albumin; CDS1, CDP-diacylglycerol synthase 1; LC/MS, liquid chromatography mass spectrometry; LPE, 1-18:1 lysophosphatidylethanolamine; Pal, palmitate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPAR, peroxisome proliferator-activated receptor; PS, phosphatidylserine; UDCA, ursodeoxycholic acid; UDCA-LPE, ursodeoxycholyl lysophosphatidylethanolamide.

PC levels which can serve as both structural components, and targets for trichloromethyl free radical binding (Jaeschke et al., 1987). Indeed, administration of PC to experimental animals intoxicated with acetaminophen (Jaeschke et al., 1987), CCl_4 (Aleynik et al., 1997), alcohol (Okiyama et al., 2009), or infected with sepsis-induced bacteria (Yan et al., 2004), has been proven to be effective in alleviating liver injury.

To date, therapeutic use of PC to treat liver disease in humans has however not been successful (Lieber et al., 2003). As exogenously added PC can provide only ∼50% of the PC required for membrane biogenesis (Esko et al., 1982), the amount of PC taken up into hepatocytes may be limited thus providing not enough PC needed for membrane protection. For treatment of acute liver injury, antioxidants such as acetylcysteine to treat acetaminophen overdose have been commonly used (Green et al., 2010). Alternatively, manipulation of metabolic pathways to increase PC levels has been adopted for treatment of acute liver injury. These strategies include the use of PC precursors, such as, betaine (Kharbanda et al., 2007), fish oils (Speck and Lauterburg, 1991), dietary saturated and monounsaturated fats (Hwang et al., 2011), and agonists of the peroxisome proliferator-activated receptor (PPAR) alpha (Lee et al., 2004). At least in experimental settings, both administration of PC and the modulation of pathways to increase PC have currently provided positive results in ameliorating acute hepatotoxicity.

We had synthesized a hepatoprotectant ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE, Chemical structure shown in **Figure 1A**), which as an intact compound provides cytoprotection against TNF-alpha-induced apoptosis (Chamulitrat et al., 2009). UDCA-LPE lowers aminotransferases and elicits anti-inflammatory response in mice treated with galactosamine/endotoxin (Pathil et al., 2011a) or fed with high-fat diet (Pathil et al., 2011b). UDCA-LPE as a hepatoprotectant may thus potentially be used to treat acute liver injury. Herein, we provided evidence that UDCA-LPE induced elevation of hepatocellular PC levels under *in vitro* and *in vivo* conditions. This is in part due to UDCA-LPE's ability to elicit transcriptional control of a PC synthesis gene phosphatidate cytidylyltransferase or another name CDP-diacylglycerol synthase 1 (CDS1). The ability of UDCA-LPE to rapidly increase hepatic PC levels renders it suitable to treat

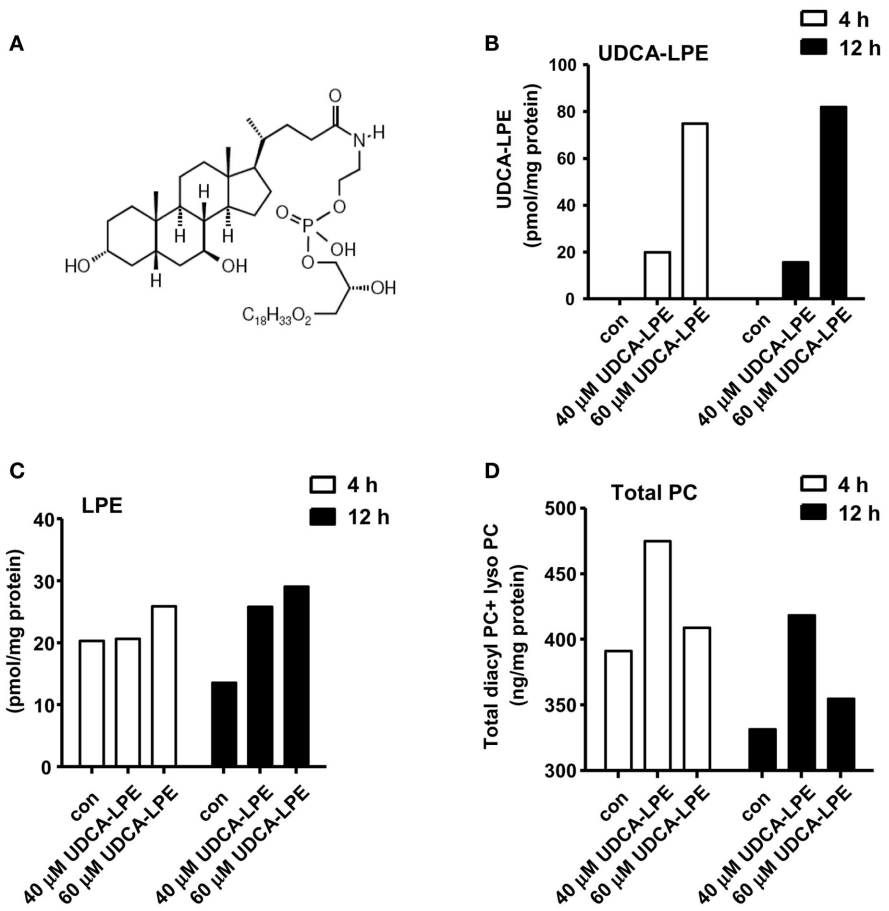


FIGURE 1 | Ursodeoxycholyl lysophosphatidylethanolamide hydrolysis to LPE and increases PC concentrations in mouse hepatocytes. (A) Chemical structure of UDCA-LPE (UDCA-18:1-LPE). **(B)** Upon treatment with 50 μM UDCA-LPE, UDCA-LPE was taken to hepatocytes over 4–12 h. **(C)** Upon treatment with 50 μM UDCA-LPE, LPE concentrations were increased after 12 h treatment indicating that UDCA-LPE hydrolysis occurred at 12 h but not at 4 h. **(D)** Total PC (diacyl PC and lyso PC) concentrations were increased mouse hepatocytes treated with 50 μM UDCA-LPE for 4 and 20 h. "Con" represents control hepatocytes treated with ethanol used as a vehicle. Data are mean ± SD, $N = 4$; $^*p < 0.05$ versus control.

acute hepatotoxicity in clinical settings, such as, acetaminophen poisoning.

MATERIALS AND METHODS

MATERIALS

Ursodeoxycholyl lysophosphatidylethanolamide was synthesized by ChemCon GmbH (Freiburg, Germany). UDCA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phospholipid standards (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (18:1-lyso PE), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (16:0-lyso PC), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (18:0-lyso PC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho choline (18:1-lyso PC), 1-arachidonyl-2-hydroxy-*sn*-glycero-3-phosphocholine (20:4-lyso PC) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-choline (16:0, 18:1-PC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0, 18:2-PC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0, 20:4-PC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18:0, 18:1-PC), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (18:0, 18:2-PC), and 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phos-phocholine (d_{31}-16:0, 18:1-PC) were obtained from Avanti Lipids, Inc. (Alabaster, AL, USA) and were at least 99% pure. All other solvents and chemicals used were of either high-performance-liquid-chromatography grade or of known analytical purity and obtained from Sigma-Aldrich (St. Louis, MO, USA).

ANIMAL EXPERIMENTS

Male C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) at 10 weeks old received 30 mg/kg UDCA-LPE by intraperitoneal injections. UDCA-LPE was dissolved in 0.5% carboxymethylcellulose. At designated time, each mouse for each time point was sacrificed. Blood, liver, and intestine were collected for lipid extraction and LC–MS/MS analysis. All experiments were approved by the Animal Care and Use Committee of the University Heidelberg.

HEPATOCYTE ISOLATION AND CULTURES

Hepatocytes were isolated from 7- to 10-week-old C57/BL6 mice (Charles Rivers Laboratories, Sulzfeld, Germany) by using two-step collagenase perfusion technique and purified by Percoll gradient centrifugation. Freshly isolated hepatocytes were plated on multi-well collagen-coated plates and cultured for 4 h in M199 medium containing Hank' salts and L-glutamine (PAA, Cölbe, Germany) supplement with 1% penicillin and streptomycin, 100 nM dexamethasone, 0.5 nM insulin, and 5% neonatal calf serum. Dead hepatocytes were removed, and the adhered cells were treated with serum-free medium. After overnight in culture, hepatocytes were treated with UDCA-LPE in serum-free M199 medium for 4 or 12 h. Ethanol as a vehicle of UDCA-LPE was added in the control groups.

PALMITATE-INDUCED APOPTOSIS

Pal stock solution in BSA was prepared. Briefly, 250 μL of 200 mM Pal in ethanol was mixed with 4.5 mL of 27% BSA in PBS. The total 5 mL volume was adjusted to pH to 7.4 with 0.1 N NaOH until the mixture became clear. The appropriate control for Pal treatment contained 0.5% BSA and 0.1% ethanol. For apoptosis assays, caspase $3/7^{glo}$ assay kits (Promega, Mannheim, Germany) were used as previously described (Chamulitrat et al., 2009). Incubation time for apoptosis assay was 20 h.

PREPARATION OF STANDARDS AND SAMPLES FOR LC–MS/MS ANALYSIS

Phosphatidylcholine standards obtained from Avanti Lipids are in chloroform at a concentration of 10 mg/mL. They were diluted to 100 ng/μL with methanol and used as working solutions. Stock solutions of 18:1-lyso PE (1 mg/mL) was prepared in chloroform:methanol (1:4, v/v) and stored at −20°C. UDCA and d_4-UDCA stock solution (1 mg/mL) was prepared in methanol and stored at −20°C. Working solutions were prepared by appropriate dilution of the stock solutions with methanol. Calibration curves were constructed for UDCA-LPE, UDCA, and LPE in PBS solution using d_4-UDCA as internal standard and curve range was in 0–50 ng. Calibration curve of each individual PC was built using d_{31}-16:0–18:1-PC as an internal standard. The linear response for PC and lyso PC was in the range of 0–200 and 0–100 ng, respectively.

Hepatocytes treated with UDCA-LPE (in 20 μL suspension) was added with 180 μL H_2O, and 20 μL of 0.5 ng/μL d_4-UDCA, and then extracted with 1 mL $CHCl_3$:MeOH (2:1) twice. The chloroform layer was dried down and reconstituted into 200 μL MeOH, and 20 μL was injected onto the HPLC column. Mouse plasma and urine samples were treated with the same procedure as for hepatocytes. For analyses of tissue samples, homogenates were prepared in PBS to give a final concentration of 1 g/mL d_4-UDCA (0.5 ng/μL, 20 μL) and d_{31}-16:0–18:1-PC (2 ng/μL, 20 μL) were added to the homogenates and subsequent lipid extraction with $CHCl_3$:MeOH was performed.

LC–MS/MS ANALYSIS OF UDCA-LPE AND PHOSPHOLIPIDS

LC–MS/MS analysis was carried out on a Waters Quattro Micro API triple quadrupole mass spectrometer (Milford, MA, USA) interfaced with Acquity UPLC system. Nitrogen was used as nebulizer and argon was used as collision gas. Optimized mass spectrometry parameters were determined with individual standard compounds. UDCA-LPE and LPE analysis were performed under negative mode with multiple reactions monitoring (MRM) mass transitions set at $852.5 > 281.3, 478.2 > 281.2, 391.1 > 391.1$ respectively. Quantification of diacyl PC and lyso PC were performed under positive mode, monitoring transition pair of the individual protonated parent ions for PCs and their common daughter ion m/z 184. Total PC was the sum of diacyl PC and lyso PC.

Online chromatographic separation was achieved using a 100-mm × 2.0-mm i.d. Luna 3 μ C18 column (Phenomenex, CA, USA). Binary solvents used for analysis were 95% H_2O/MeOH with 2 mM ammonium acetate (solvent A) and 95% MeOH/H_2O with 2 mM ammonium acetate (solvent B). For analysis of UDCA-LPE and LPE, gradient started with 90% solvent A for 1 min, changed to 100% solvent B in 0.5 min, held for 5 min, and finally switched back to initial condition in 0.1 min. The total analysis time was 8 min. Gradient for PC analysis started with 85% solvent B and was maintained for 1 min. Then rose in 0.5 min linearly to 100% solvent B. The elution was held at 100% B for 10 min, then reversed to 85% methanol in 0.5 min. The 3-min holding at 85% methanol equilibrated the column efficiently. Flow rate for both analyses was 0.2 mL/min.

GENE EXPRESSION BY REAL-TIME RT-PCR

Total RNA of treated mouse hepatocytes was isolated using QIAGEN RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2 μg RNA using a Maxima First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). The mRNA expression was analyzed in quadruplets by real-time PCR using Applied Biosystems TaqMan® gene expression assays with TaqMan® Universal PCR Master Mix (Applied Biosystems) and run on an Applied Biosystem 7500 Fast real-time PCR machine by using assay-on-Demand TaqMan® primers. The expression level of targets was calculated using $\Delta - C_t$ transformation method, and showed as ratio after having been normalized to house-keeping gene GAPDH. PCR results were obtained from at least three independent experiments.

DATA ANALYSIS

Statistical significance at $p > 0.05$ was determined with ANOVA or a Student's *t*-test using Primer Biostatistics. Results were expressed as mean ± SD.

RESULTS

UDCA-LPE INCREASES PC CONCENTRATIONS IN MOUSE HEPATOCYTES

Intracellular concentrations of UDCA-LPE (chemical structure shown in **Figure 1A**) in mouse hepatocytes were increased from 20 to 80 pmol/mg protein with 40 or 60 μM UDCA-LPE (**Figure 1B**). At the same time, intracellular concentrations of LPE were not yet increased at 4 h but markedly increased after 12 h by ∼15 pmol/mg protein (**Figure 1C**).

Cellular concentrations of PC were elevated in mouse hepatocytes treated with 40 or 60 μM UDCA-LPE for 4 or 12 h (**Figure 1D**). Compared with 4 h, the total PC levels were decreased after 12 h incubation likely due to PC export into the medium. It is observed that the increases of total PC (diacyl PC and lyso PC) concentrations at 4 and 12 h were on the order of 52–54 ng/mg protein or ∼150 pmol/mg protein. The increases of LPE observed after 12 h at ∼15 pmol/mg protein did not correspond to the increases of PC because the increased LPE concentrations were ∼10-folds lower than those of the observed increased PC. This excludes the possibility that PC could arise from LPE as a result of UDCA-LPE hydrolysis (to UDCA and LPE). Pathways for LPE conversion to PC include acylation of LPE to PE and subsequent PE metabolism to PC by PE methyltransferase (DeLong et al., 1999). Taken together, increased PC did not arise from UDCA-LPE hydrolysis, and may likely be from the effects of UDCA-LPE on certain metabolic signaling pathways which increase PC abundance.

UDCA-LPE ADMINISTRATION INCREASES PC CONCENTRATIONS *IN VIVO*

We further confirmed UDCA-LPE effects on PC levels under *in vivo* conditions. Intraperitoneal injections of UDCA-LPE at 30 mg/kg 1 h prior to the challenge with galactosamine/endotoxin in mice were found to be effective in alleviating acute liver injury observed at 8 h (Pathil et al., 2011a). We therefore performed intraperitoneal injections of 30 mg/kg UDCA-LPE over 2, 4, 8, and 24 h, and determined diacyl PC and total PC (as the sum of diacyl PC and lyso PC). We observed that the levels of PC in plasma were gradually increased after 2 h and stayed elevated even after 24 h (**Figure 2A**). The levels of PC found in urine were increased significantly 2 h after injection, and quickly decreased to basal levels after 4 h (**Figure 2B**). As urinary PC arises from PC secreted from tissues, PC pharmacokinetics in urine indicated that PC clearance from tissues was significantly slowed down at the later time, i.e., longer than 4 h.

Similar to plasma, the levels of PC species in the liver were significantly elevated after 2 h and sustained at these concentrations even at 24 h after administration (**Figure 2C**). However, PC concentrations in the intestine were slightly increased at 6 h, and drastically decreased at 8 and 24 h (**Figure 2D**). Our data clearly demonstrated sustained increases of PC in blood and liver following UDCA-LPE intraperitoneal injection concomitant with reduced PC clearance from these tissues to the urine. Significant increases of PC were found in the liver but not the intestine indicating UDCA-LPE liver targeting, and hence demonstrating the suitability of UDCA-LPE for treatment of liver disease.

UDCA-LPE UPREGULATES CDS1 mRNA IN MOUSE HEPATOCYTES

To determine possible activation of PC synthesis by UDCA-LPE, we analyzed PC synthesis genes in mouse hepatocytes which had been treated with UDCA-LPE for 4 or 20 h. UDCA-LPE treatment for 4 h did not modulate expression of the genes under investigation (data not shown). These genes included CDP-diacylglycerol synthase 1 (CDS1), choline kinase alpha, CDP-diacylglycerol synthase 2 (CDS2), choline/ethanolamine phosphotransferase 1 (Cept1), choline phosphate cytidylyltransferase 1, alpha isoform (CCTalpha or Pcyt1a), and phosphatidylethanolamine *N*-methyltransferase (PEMT). For most genes tested, UDCA-LPE had only marginal effects (**Figure 3A**). However, UDCA-LPE treatment for 20 h significantly increased mRNA expression of CDS1 by ∼1.4-fold. CDS1 is an enzyme which converts phosphatidic acid to phosphatidylserine (PS) intermediate and then PE which is further metabolized to generate PC by PE methyltransferase (**Figure 3B**). Under apoptosis induction by palmitate exposure for 20 h, CDS1 mRNA was slightly downregulated by palmitate, and UDCA-LPE again weakly upregulated CDS1 mRNA. (**Figure 3C**, left). UDCA-LPE co-treatment with palmitate however significantly increased CDS1 mRNA expression by ∼5-folds. UDCA-LPE co-treatment with palmitate did not upregulate other PC synthesis genes listed in **Figure 3A** (data not shown). The increases of CDS1 mRNA expression were concomitant with UDCA-LPE protection against palmitate-induced apoptosis as determined by caspase 3 activity (**Figure 3C**, right). Hence UDCA-LPE protection was associated with an upregulation of CDS1 mRNA for increased PC synthesis.

DISCUSSION

Ursodeoxycholyl lysophosphatidylethanolamide is a first-in-class hepatoprotectant being superior to UDCA or PC capable of inhibiting hepatocellular apoptosis (Chamulitrat et al., 2009) and inflammation *in vivo* under acute liver injury (Pathil et al., 2011a). PC controls liver homeostasis by membrane stabilization and the loss of PC levels results in an on-set of development of liver disease. It is of a question whether UDCA-LPE could increase PC to replenish PC for cytoprotection. We herein demonstrated that UDCA-LPE enhanced PC concentrations *in vitro* and *in vivo*, and these increases were associated with upregulation of CDS1. This ability

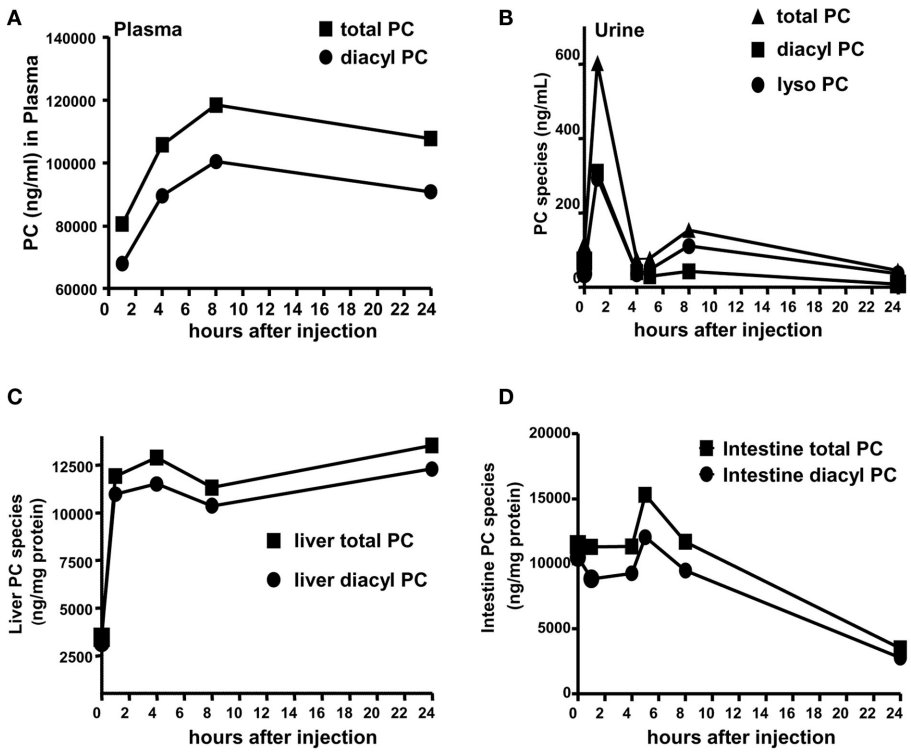


FIGURE 2 | Ursodeoxycholyl lysophosphatidylethanolamide increases PC levels *in vivo*. Mice were injected with 30 mg/kg UDCA-LPE by an intraperitoneal injection. **(A)** Concentrations of PC species (total or diacyl PC) in ng/mL in plasma were increased after 2 h and stayed elevated even after 24 h. **(B)** Concentrations of total PC, diacyl PC, and lyso PC in ng/mL in urine were increased after 2 h and drastically decayed to control levels after 4 h. **(C)** Concentrations of PC species in nanogram per milligram protein in the liver were increased after 2 h and stayed elevated even after 24 h. **(D)** Concentrations of total PC, and diacyl PC in ng/mg protein in the intestine were slightly increased after 6 h and drastically decayed to control levels after 8 h.

of UDCA-LPE to increase PC can enhance hepatoprotection and renders it appropriate to treat acute hepatotoxicity.

We have shown that UDCA-LPE is able to induce survival signaling pathway by activating PI3K/Akt and MAPK/ERK1/2 pathways within 0.5–7 h in cultured hepatocytes (Chamulitrat et al., 2009). At the rapid time point of 4 h where increased PC was observed (**Figures 1** and **2**), it is unlikely that UDCA-LPE could induce PC synthesis genes on the transcriptional and translational levels. In cultured hepatocytes, the early PC response obtainable at 4-h treatment with UDCA-LPE may thus likely due to some unknown signaling pathways triggered by UDCA-LPE which result in increases of PC abundance. For such rapid activation, it is possible that UDCA-LPE may interact with G-protein coupled receptors on plasma membrane or nuclear receptors intracellularly. The immediate PC increases were also observed *in vivo* 2 h after intraperitoneal injection. Specific signaling pathways in regulating PC metabolism responsible for a rapid rise of PC have not been identified. PPAR alpha and gamma as well as glucocorticoid receptors are among nuclear receptors proposed to be affected by UDCA-LPE. These receptors are known to modulate the syntheses of phospholipids (Lee et al., 2004) including PC (Pan et al., 2006). After prolonged hepatocyte treatment with UDCA-LPE for 20 h, we found that CDS1 mRNA was upregulated. CDS1 generates PC from phosphatidic acid. CDS1 upregulation could explain the increased and sustained PC levels observed even after 24 h under *in vivo* conditions. The immediate and prolonged increases of hepatic PC levels by UDCA-LPE may provide feasibility for its use in the clinics, for an example, for effective reduction of acute severe hepatotoxicity from acetaminophen overdose. UDCA-LPE may be used as an antidote against hepatotoxins in humans.

Ursodeoxycholyl lysophosphatidylethanolamide is better than UDCA or PC in inhibiting apoptosis and liver injury (Chamulitrat et al., 2009; Pathil et al., 2011a). PC synthesis is known to be down-regulated during apoptosis, and that PC supplementation provides protection (Cui and Houweling, 2002). In addition to UDCA-LPE's ability to inhibit apoptosis, the PC generated by UDCA-LPE by ways of CDS1 upregulation could also be a mechanism for its anti-apoptosis effects (**Figure 3C**). In support of this notion, the contents of product of CDS1 CDP-diacylglycerol contents are found to be decreased in livers of diabetic rats (Whiting et al., 1977), and increased CDS1 is found during the development of heart failure and identified to be involved in compensatory mechanisms (Saini-Chohan et al., 2009). These data suggest protective function of CDS1 and consistent with UDCA-LPE protection.

In addition to UDCA (Simko and Michael, 1994), other protective bile acids, such as 5 beta-scymnol (Slitt et al., 2004), have been shown to reduce liver injury and acetaminophen toxicity. UDCA-LPE may be hepatoprotective in these settings as UDCA-LPE is superior to UDCA. Furthermore, UDCA-LPE inhibits activities of hepatic phospholipase A2 thus decreasing the levels of cytotoxic

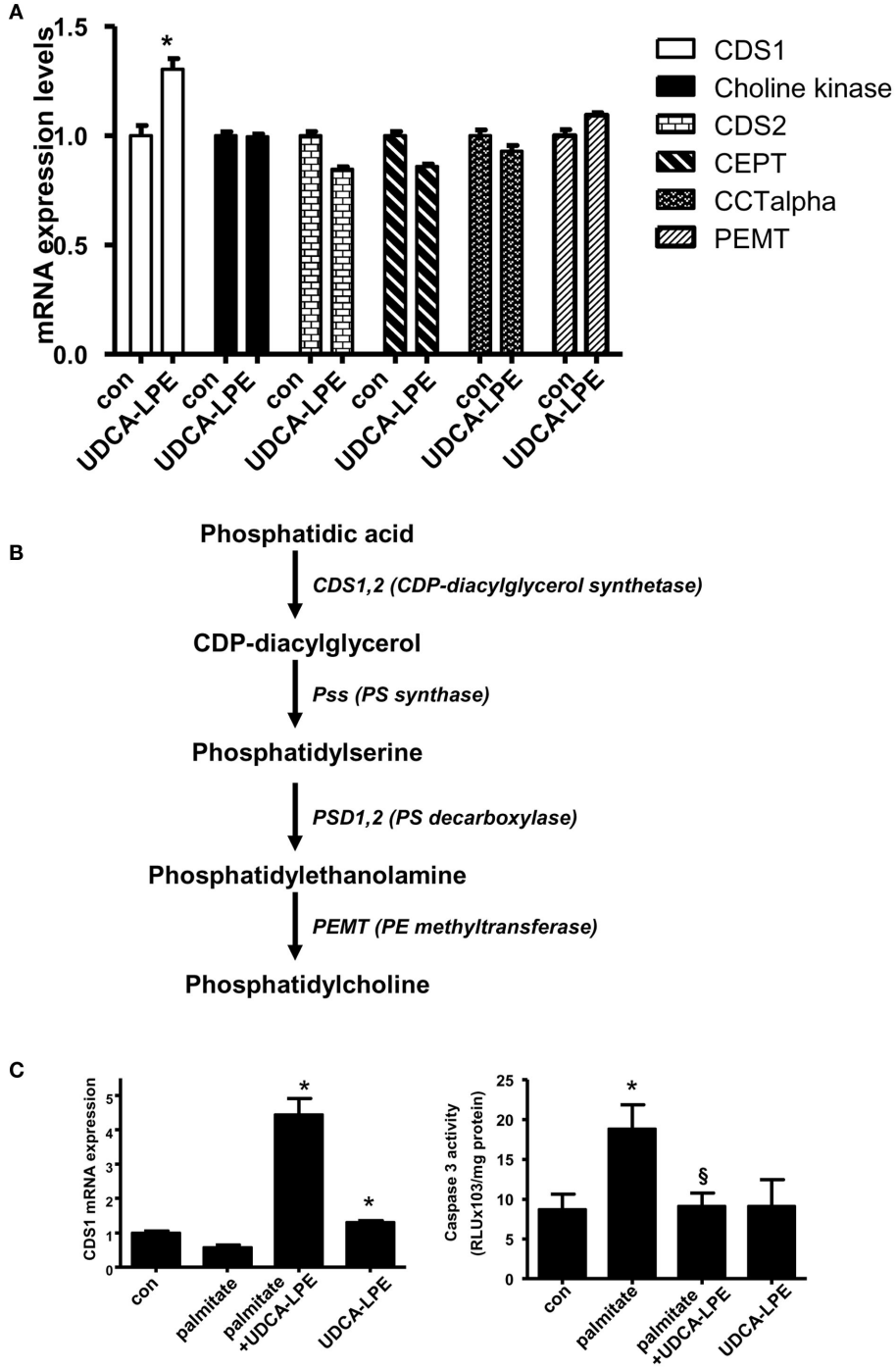


FIGURE 3 | Ursodeoxycholyl lysophosphatidylethanolamide upregulated CDS1 gene concomitant with inhibition of apoptosis induced by palmitate. (A) Treatment of mouse hepatocytes with 60 μM UDCA-LPE for 20 h increased expression of CDS1 but failed to increase other PC synthesis genes including choline kinase, CDS2, CEPT2, CEPT, CCTalpha, and PEMT. Quantitative RT-PCR was performed by TaqMan® RT-PCR with relative expression (ΔRn) of the target gene versus GAPDH mRNA. Data were representative data obtained from two different experiments. Data were mean ± SD, $N = 4$; $^{*}p < 0.05$ versus con. **(B)** A scheme demonstrates metabolism of phosphatidic acid by CDS1. Intermediate lipids were metabolized by PS synthase, PS decarboxylase, and finally PE methyltransferase to result in increased PC abundance. **(C)** Co-treatment of UDCA-LPE with palmitate markedly upregulated expression of CDS1 after 20 h treatment (left panel). Quantitative RT-PCR was carried out as in **(A)**. Concomitantly, palmitate-induced apoptosis as determined by caspase 3 activity was significantly inhibited by UDCA-LPE (right panel). Data are mean ± SD, $N = 6$; $^{*}p < 0.05$ versus control; $^{\S}p < 0.05$ versus palmitate. "Con" represents control hepatocytes treated with 0.5% BSA used as a vehicle.

lyso PC (Pathil et al., 2011a,b). It has been shown that CDS1 activity is inhibited by lyso PC *in vitro* (Lin et al., 1991). UDCA-LPE's ability to lower lyso PC concentrations may lead to increases in CDS1 activity and hence increased PC. Increased CDS1 and PC abundance by UDCA-LPE could also provide dilinoleyl- or polyenyl PC (Aleynik et al., 1997; Lieber et al., 2003) as well as PC-containing medium-chain fatty acids, such as dilauroyl PC, which is a new PC molecule recently identified to elicit protective antidiabetic effects (Lee et al., 2011). Alternatively, UDCA-LPE may prevent PC export to extracellular space thus allowing more PC for maintaining cell integrity during stress. Further analyses of PC export genes affected by UDCA-LPE are still yet to be determined.

It is possible that increases of PC may indicate increased availability of free fatty acids due to upregulation of lipogenic genes by drug treatment (Anthérieu et al., 2011). This possibility may be applicable for treatment longer than 4 h to allow protein translation. UDCA-LPE's ability to increase lipogenic genes such as fatty acid synthase and elongase genes has previously been observed (unpublished data). These *de novo* lipogenic lipids including PC in fact reflect the protection against cell death during palmitate stress after 20 h (Collins et al., 2010; Green and Olsen, 2011). It has been shown that CDS1 expression is concomitantly increased with lipogenesis genes fatty acid synthase during fetal lung development (Zhang et al., 2004), it is surmised that CDS1 may be similarly regulated by *de novo* lipogenesis transcription factors. As UDCA-LPE elicits lipoprotection after 20 h treatment, UDCA-LPE may be found most effective in treatment of pathological acute hepatotoxicity as to compare with normal physiological conditions. While many cationic amphiphilic drugs such as chlorpromazine and tamoxifen are known to be cytotoxic to hepatocytes by inducing phospholipidosis (Chatman et al., 2009), it is still to be determined whether UDCA-LPE could accumulate phospholipids in lysosomes (which are the organelles for phospholipidosis). As UDCA-LPE targets mitochondria by inhibiting the loss of mitochondrial membrane potentials (Chamulitrat et al., 2009), UCDA-LPE may elicit protection during acute liver injury at this organelle (Pathil et al., 2011a). PC could likely be accumulated in the mitochondrial membrane for protection.

We herein demonstrated that UDCA-LPE rapidly caused accumulation of hepatic PC likely by stimulation of specific signaling pathways of PC metabolism. Further experiments are still yet to be performed to investigate UDCA-LPE protective effects against severe hepatotoxicity induced by drugs such as acetaminophen.

ACKNOWLEDGMENTS

We thank Nenad Katava and Sabine Tuma for technical assistance. This study was supported by DFG (STR216/15-3) to Wolfgang Stremmel.

REFERENCES

Aleynik, S. I., Leo, M. A., Ma, X., Aleynik, M. K., and Lieber, C. S. (1997). Polyenylphosphatidylcholine prevents carbon tetrachloride-induced lipid peroxidation while it attenuates liver fibrosis. *J. Hepatol.* 27, 554–561.

Anthérieu, S., Rogue, A., Fromenty, B., Guillouzo, A., and Robin, M. A. (2011). Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in HepaRG cells. *Hepatology* 53, 1895–1905.

Camacho, J., and Rubalcava, B. (1984). Lipid composition of liver plasma membranes from rats intoxicated with carbon tetrachloride. *Biochim. Biophys. Acta* 776, 97–104.

Chamulitrat, W., Burhenne, J., Rehlen, T., Pathil, A., and Stremmel, W. (2009). Bile salt-phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamine as a hepatoprotective agent. *Hepatology* 50, 143–154.

Chatman, L. A., Morton, D., Johnson, T. O., and Anway, S. D. (2009). A strategy for risk management of drug-induced phospholipidosis. *Toxicol. Pathol.* 37, 997–1005.

Collins, J. M., Neville, M. J., Hoppa, M. B., and Frayn, K. N. (2010). De novo lipogenesis and stearoyl-CoA desaturase are coordinately regulated in the human adipocyte and protect against palmitate-induced cell injury. *J. Biol. Chem.* 28, 6044–6052.

Cui, Z., and Houweling, M. (2002). Phosphatidylcholine and cell death. *Biochim. Biophys. Acta* 1585, 87–96.

DeLong, C. J., Shen, Y. J., Thomas, M. J., and Cui, Z. (1999). Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J. Biol. Chem.* 274, 29683–29688.

Esko, J. D., Nishijima, M., and Raetz, C. R. (1982). Animal cells dependent on exogenous phosphatidylcholine for membrane biogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 79, 1698–1702.

Gebhart, A. M., and Brabec, M. J. (1985). Carbon tetrachloride depresses hepatic phospholipid synthesis in rats. *Toxicol. Lett.* 24, 71–78.

Green, C. D., and Olsen, L. K. (2011). Modulation of lipogenesis in hepatocytes by SCD-1 and Elovol6. *Am. J. Physiol. Endocrinol. Metab.* 300, E640–E649.

Green, T. J., Sivilotti, M. L., Langmann, C., Yarema, M., Juurlink, D., Burns, M. J., and Johnson, D. W. (2010). When do the aminotransferases rise after acute acetaminophen overdose? *Clin. Toxicol. (Phila.)* 48, 787–792.

Hwang, J., Chang, Y. H., Park, J. H., Kim, S. Y., Chung, H., Shim, E., and Hwang, H. J. (2011). Dietary saturated and monounsaturated fats protect against acute acetaminophen hepatotoxicity by altering fatty acid composition of liver microsomal membrane in rats. *Lipids Health Dis.* 10, 184.

Jackowski, S. (1994). Coordination of membrane phospholipid synthesis with the cell cycle. *J. Biol. Chem.* 269, 3858–3867.

Jaeschke, H., Werner, C., and Wendel, A. (1987). Disposition and hepatoprotection by phosphatidyl choline liposomes in mouse liver. *Chem. Biol. Interact.* 64, 127–137.

James, J. L., Moody, D. E., Chan, C. H., and Smuckler, E. A. (1982). The phospholipids of the hepatic endoplasmic reticulum. Structural change in liver injury. *Biochem. J.* 206, 203–210.

Kharbanda, K. K., Mailliard, M. E., Baldwin, C. R., Beckenhauer, H. C., Sorrell, M. F., and Tuma, D. J. (2007). Betaine attenuates alcoholic steatosis by restoring phosphatidylcholine generation via the phosphatidylethanolamine methyltransferase pathway. *J. Hepatol.* 46, 314–321.

Lee, J. M., Lee, Y. K., Mamrosh, J. L., Busby, S. A., Griffin, P. R., Pathak, M. C., Ortlund, E. A., and Moore, D. D. (2011). A nuclear-receptor-dependent phosphatidylcholine pathway with antidiabetic effects. *Nature* 474, 506–510.

Lee, S. S., Chan, W. Y., Lo, C. K., Wan, D. C., Tsang, D. S., and Cheung, W. T. (2004). Requirement of PPARalpha in maintaining phospholipid and triacylglycerol homeostasis during energy deprivation. *J. Lipid Res.* 45, 2025–2037.

Li, Z., Agellon, L. B., Allen, T. M., Umeda, M., Jewell, L., Mason, A., and Vance, D. E. (2006). The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab.* 3, 321–331.

Li, Z., Agellon, L. B., and Vance, D. E. (2005). Phosphatidylcholine homeostasis and liver failure. *J. Biol. Chem.* 280, 37798–37802.

Lieber, C. S., Weiss, D. G., Groszmann, R., and Paronetto, F. II. (2003). Veterans affairs cooperative study of polyenyl-phosphatidylcholine in alcoholic liver disease. *Alcohol. Clin. Exp. Res.* 27, 1765–1772.

Lin, C. H., Lin, J., and Strickland, K. P. (1991). Bovine brain microsomal CDP-diacylglycerol synthetase: solubilization and properties. *Biochem. Int.* 25, 299–306.

Miyazaki, M., Bai, L., and Namba, M. (1991). Extending effects of phospholipids, cholesterol, and ethanolamines on survival of adult rat hepatocytes in serum-free primary culture. *Res. Exp. Med. (Berl.)* 191, 77–83.

Miyazawa, T., Suzuki, T., Fujimoto, K., and Kaneda, T. (1990). Phospholipid hydroperoxide accumulation in liver of rats intoxicated with carbon tetrachloride and its inhibition by dietary alpha-tocopherol. *J. Biochem.* 107, 689–693.

Noga, A. A., and Vance, D. E. (2003). Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J. Lipid Res.* 44, 1998–2005.

Okiyama, W., Tanaka, N., Nakajima, T., Tanaka, E., Kiyosawa, K., Gonzalez, F. J., and Aoyama, T. (2009). Polyene-phosphatidylcholine prevents alcoholic liver disease in PPARalpha-null mice through attenuation of increases in oxidative stress. *J. Hepatol.* 50, 1236–1246.

Pan, H. J., Lin, Y., Chen, Y. E., Vance, D. E., and Leiter, E. H. (2006). Adverse hepatic and cardiac responses to rosiglitazone in a new mouse model of type 2 diabetes: relation to dysregulated phosphatidylcholine metabolism. *Vascul. Pharmacol.* 45, 65–71.

Pathil, A., Warth, A., Chamulitrat, W., and Stremmel, W. (2011a). The synthetic bile acid-phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamine suppresses TNFα-induced liver injury. *J. Hepatol.* 54, 674–684.

Pathil, A., Mueller, J., Warth, A., Chamulitrat, W., and Stremmel, W. (2011b). Ursodeoxycholyl lysophosphatidylethanolamine improves steatosis and inflammation in murine models of nonalcoholic fatty liver disease. *Hepatology* doi:10.1002/hep.25531. [Epub ahead of print].

Saini-Chohan, H. K., Holmes, M. G., Chicco, A. J., Taylor, W. A., Moore, R. L., McCune, S. A., Hickson-Bick, D. L., Hatch, G. M., and Sparagna, G. C. (2009). Cardiolipin biosynthesis and remodeling enzymes are altered during development of heart failure. *J. Lipid Res.* 50, 1600–1608.

Sgoutas, D. S. (1967). Phospholipid changes during hepatic injury caused by carbon tetrachloride. *Metab. Clin. Exp.* 16, 382–391.

Shimizu, Y. (1969). Effect of carbon tetrachloride administration on the synthesis of triglycerides and phospholipids in rat liver. *J. Lipid Res.* 10, 479–486.

Simko, V., and Michael, S. (1994). Effect of ursodeoxycholic acid on in vivo and in vitro toxic liver injury in rats. *Aliment. Pharmacol. Ther.* 8, 315–322.

Slitt, A. L., Naylor, L., Hoivik, J., Manautou, J. E., Macrides, T., and Cohen, S. D. (2004). The shark bile salt 5 beta-scymnol abates acetaminophen toxicity, but not covalent binding. *Toxicology* 203, 109–121.

Speck, R. F., and Lauterburg, B. H. (1991). Fish oil protects mice against acetaminophen hepatotoxicity in vivo. *Hepatology* 13, 557–561.

Sugano, M., Cho, S., Imaizumi, K., and Wada, M. (1970). Hepatotoxicity and lipid metabolism. 3. Changes in phosphatidylcholine and phosphatidylethanolamine during hepatic injury caused by carbon tetrachloride. *Biochem. Pharmacol.* 19, 2325–2333.

Trudell, J. R., Bösterling, B., and Trevor, A. J. (1982). Reductive metabolism of carbon tetrachloride by human cytochromes P-450 reconstituted in phospholipid vesicles: mass spectral identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine. *Proc. Natl. Acad. Sci. U.S.A.* 79, 2678–2682.

Wallnofer, H., and Hanusch, M. (1973). Essential phospholipids for therapy in liver diseases. *Med. Monatsschr.* 27, 131–136.

Whiting, P. H., Bowley, M., Sturton, R. G., Pritchard, P. H., Brindley, D. N., and Hawthorne, J. N. (1977). The effect of chronic diabetes, induced by streptozotocin, on the activities of some enzymes of glycerolipid synthesis in rat liver. *Biochem. J.* 168, 147–153.

Yan, J. J., Jung, J. S., Lee, J. E., Lee, J., Huh, S. O., Kim, H. S., Jung, K. C., Cho, J. Y., Nam, J. S., Suh, H. W., Kim, Y. H., and Song, D. K. (2004). Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nat. Med.* 10, 161–167.

Zhang, F., Pan, T., Nielsen, L. D., and Mason, R. J. (2004). Lipogenesis in fetal rat lung: importance of C/EBPalpha, SREBP-1c, and stearoyl-CoA desaturase. *Am. J. Respir. Cell Mol. Biol.* 30, 174–183.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 January 2012; accepted: 03 February 2012; published online: 20 February 2012.

Citation: Chamulitrat W, Zhang W, Xu W, Pathil A, Setchell K and Stremmel W (2012) Hepatoprotectant ursodeoxycholyl lysophosphatidylethanolamide increasing phosphatidylcholine levels as a potential therapy of acute liver injury. Front. Physio. ***3****:24. doi: 10.3389/fphys.2012.00024*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 13 December 2013
doi: 10.3389/fphys.2013.00351

Hepatitis E in liver biopsies from patients with acute hepatitis of clinically unexplained origin

Uta Drebber[1†], Margarete Odenthal[1†], Stephan W. Aberle[2], Nadine Winkel[1,3], Inga Wedemeyer[1], Jutta Hemberger[1], Heidemarie Holzmann[2] and Hans-Peter Dienes[2]*

[1] *Institute for Pathology, University of Cologne, Koeln, Germany*
[2] *Department of Virology, Medical University of Vienna, Vienna, Austria*
[3] *Department of Gastroneterology, University of Cologne, Koeln, Germany*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Carolin Lackner, Medical University of Graz, Austria
Hideo Andreas Baba, University of Duisburg-Essen, Germany

***Correspondence:**
Hans-Peter Dienes, Institute for Pathology, University Hospital of Cologne, Kerpener Str. 62, 50924 Koeln, Germany
e-mail: hp.dienes@gmail.com
† *These authors have contributed equally to this work.*

Hepatitis E virus (HEV) is a small RNA virus and the infectious agent of hepatitis E that occurs worldwide either as epidemics in Asia caused by genotype 1 and 2 or as sporadic disease in industrialized countries induced by genotype 3 and 4. The frequency might be underestimated in central Europe as a cause of acute hepatitis. Therefore, we analyzed on liver biopsies, if cases of acute hepatitis with clinically unknown or obscure diagnosis were actually caused by the infection with HEV. We included 221 liver biopsies retrieved from the files of the institute of pathology during the years 2000 till 2010 that were taken from patients with acute hepatitis of obscure or doubtful diagnosis. From all biopsies RNA was extracted, prepared, and subjected to RT-PCR with specific primers. Amplified RNA was detected in 7 patients, sequenced and the genotype 3 could be determined in four of the seven of positive specimens from 221 samples. Histopathology of the biopsies revealed a classic acute hepatitis with cholestatic features and in some cases confluent necrosis in zone 3. Histology in a cohort of matched patients was less severe and showed more eosinophils. The analysis of the immune response by subtyping of liver infiltrating lymphocytes showed circumstantial evidence of adaptive immune reaction with CD 8 positive CTLs being the dominant lymphocyte population. In conclusion, in doubtful cases of acute hepatitis of unknown origin, HEV infection should be considered as etiology in central Europe. We demonstrate for the first time that the diagnosis can be made in paraffin-embedded liver biopsies reliably when no serum is available and also the genotype can be determined. The analysis of the immune response by subtyping of liver infiltrating lymphocytes indicates an adaptive mechanism suggesting in analogy with HAV, HBV and HCV that the virus itself is not cytopathic but liver damage is due to immune reaction.

Keywords: acute hepatitis, HEV, HEV genotype 3, immune response, FFPE material

INTRODUCTION

Hepatitis E is an endemic disease in many developing countries and occurs both as sporadic cases but also in endemic outbreaks (Purcell and Emerson, 2010). According to a WHO report from 2012 there are over 20 million infections of hepatitis E every year with more than 60% taking place in east and south Asia in waterborne epidemic manifestations (Who Fact Sheet, 2012). In central Europe hepatitis E was regarded as a typical travel associated disease. In recent years, however, there are increasing numbers of reports of sporadic cases in industrialized countries UK (Dalton et al., 2008), the Netherlands (Herremans et al., 2007), France (Kamar et al., 2012), and Germany (Wichmann et al., 2008) of patients who acquired the disease at home. Almost all of the European patients were infected by genotype 3 whereas in cases from developing countries genotypes 1 and 2 could be isolated. In central Europe, animals as pigs, boars, and deer especially in rural areas could be identified as a reservoir of the infectious agent (Herremans et al., 2007; Wichmann et al., 2008). Reports from the Robert-Koch Institute, center of infectious diseases in Germany, showed an increased number of cases from the year 2001 (first year of registration of hepatitis E as an notifiable disease) up to 2012. The number increased from 30–338 patients with presumably a majority of autochthonous cases. The present data confirm that hepatitis E is a disease in Germany with a prevalence of antibodies of up to 14% in general population (Faber et al., 2012; Juhl et al., 2013).

In practice, clinicians are not always aware of this disease and many patients are biopsied with acute hepatitis of unknown clinical etiology. The diagnosis can be made by serology or more reliable by direct determination of HEV-RNA by RT- PCR. In order to see if the diagnosis can also be established in the liver biopsy and above that how many cases of acute hepatitis of clinical unknown etiology are caused by HEV, we examined liver biopsies from our file from 2000–2010. We assessed histopathology by the Ishak scoring system and isolated HEV-RNA from the paraffin sections and examined the virus genotype.

Additionally, we performed subtyping of infiltrating inflammatory cells because immunopathology of acute hepatitis E has so far not been documented in the tissue. From 221 patients with acute hepatitis we found seven cases which were HEV positive.

MATERIAL AND METHODS

PATIENTS, HISTOLOGY, AND IMMUNOLOGICAL CHARACTERIZATION

221 cases with clinical diagnosis of hepatitis of unknown etiology were taken from the files of the Institute of Pathology, University Clinic of Cologne, Germany. All patients were negative for serology of HAV, HBV, HDV, HCV as well as EBV and CMV. Autoantibodies were not detected. Intake of alcohol and drugs were excluded clinically. Transaminase levels varied between 600 units per ml and 1400 units per ml. Bilirubin was above 5 mg/dl, gamma GT and alkaline phosphatase were below three times of upper normal limit. We matched this cohort of positive patients with the cohort of seven HEV- negative patients regarding age, gender and biochemical data. The liver biopsies were prepared according to standard protocols and stained for H and E, Prussian blue, Van Gieson for connective tissue as well as PASD and Gomori. Additionally, immunohistochemistry was performed for lymphocyte subtyping by applying antibodies against CDla, CD3, CD4, CD8, CD20, CD56, CD 57, CD68, and TIA according to the instructions of the manufacturers. The absolute number of positive infiltrating cells was determined by counting the cells in 20 areas with high power field (hpf) in the microscope including at least 10 portal tracts.

RNA EXTRACTION FROM FORMALIN-FIXED, PARAFFIN-EMBEDDED BIOPSIES

Three 5–7 μm sections from formalin-fixed and paraffin-embedded samples were prepared and deparaffinized in xylene by incubation at 65°C for a total of 20 min, substituting xylene twice. After two washes with 100% ethanol, samples were lysed in 200 ml proteinase K buffer [500 mg/ml proteinase K (*Invitrogen*) 50 mM Tris-HCl pH 7.4 and 5mM EDTA pH 8] overnight. Subsequently, total RNA was extracted by phenol/chloroform and precipitated with 200 mM sodium acetate and isopropanol as previously described (Dries et al., 1999). RNA yield was quantified by A260/280 measurement using a ND-1000 NanoDrop spectrophometer (NanoDrop, Wilmington, DE).

HEV RNA DETERMINATION BY REAL TIME PCR

Extracts of total RNA (10–50 ng) were reverse transcribed in a 10 μl volume using Superscript reverse transcriptase (*Invitrogen*, Darmstadt, Germany), 5 μmol random primer (Roche Diagnostics, Mannheim, Germany), 1x First strand buffer (*Invitrogen*), 0.5 micro mol dNTP, and 0.5 U RNase Inhibitor (Roche Diagnostics). Incubation was performed according to the recommendations of *Invitrogen*. Then 1 μl of the reverse transcription reaction was used in each of the real-time PCR assays by means with the TaqMan®RNA Assay-Kit (Applied Biosystems) following the manufacturer's instructions. The sequence of primers and a HEV specific LNA probe were modified according to Jothikumar (Jothikumar et al., 2006) and are listed in **Table 1**.

Real-time PCR amplification was carried out by a two-step incubation protocol (20 s of each:a 95°C step for denaturation and a 60°C step for annealing and synthesis). PCR assays of all samples were performed in triplicates on a CFX 96 thermocycler from Biorad Laboratories (Munich, Germany).

In order to identify the HEV genotype of specimens in which HEV-positive amplification was shown, a qualitative HEV-PCR was performed using M13 universe or reverse tailed HEV-specific primers, respectively, (**Table 1**) (10). The corresponding M13 primer set was then taken to sequence both directions of the amplicons using the BigDyeTerminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was then performed by capillary electrophoresis using a ABI 3730 platform (Life Technologies, Darmstadt, Germany).

RESULTS

IDENTIFICATION OF HEV-RNA IN THE TISSUE

From a total of 221 biopsies representing histology of acute hepatitis of unknown clinical etiology RNA was analysed for the HEV genome by high sensitivity real-time PCR. RNA quality was proven by intron-spanning β-actin amplication (**Figure 1A**). By HEV specific real-time PCR seven liver biopsies were shown to be positive for HEV genomic RNA (**Figure 1B**). Subsequent sequence analysis could demonstrate that four patients had an infection with HEV genotype 3.

HISTOPATHOLOGIC FEATURES OF ACUTE HEV INFECTION

Next, the seven cases of HEV positive cases were matched with a group of biopsies representing acute hepatitis from patients of similar age, gender and biochemistry but negative HEV-RNA. The mean age of the patients was 58 years (40–78y) (**Table 2**). The histopathology of HEV positive biopsies showed the classical picture of acute hepatitis with spotty necrosis and portal expansion with inflammatory infiltrates mostly lymphocytes, but also a considerable number of polymorphnuclear leucocytes (**Figure 2**). Bile ducts were involved with cholangitis and lymphocytic infiltrates of biliary epithelia. Portal tracts were devoid of connective tissue and the features of chronic hepatitis were absent. In the lobules spotty necroses were present in all biopsies to a varying

Table 1 | Primers and Probes.

Oligonucleotide	Sequence	Assay
Hep-E-F	CGGTGGTTTCTGGGGTGAC	Real-time PCR (primer)
Hep-E-R	GGRTTGGTTGGATGAATATAGG	Real-time PCR (primer)
Hep-E-P	Fam- TGATTCTCAGCCCTTCGC-BHQ–1	Real-time PCR (probe)
β-actin-F	TTGGCAATGAGCGGTTCCGCTG	Real-time PCR (primer)
β-actin-R	CACGTCACACTTCATGATGGAG	Real-time PCR (primer)
β-actin-P	Fam-tccagccttccttcctgggcatg-BHQ–1	Real-time PCR (probe)
HEV-M13uF	TGTAAAACGACGGCCAGTCTACGGTGGTTTCTGGGGTGAC	Sequencing
HEV-M13rR	CAGGAAACAGCTATGACCGGRTTGGTTGGATGAATATAGG	Sequencing

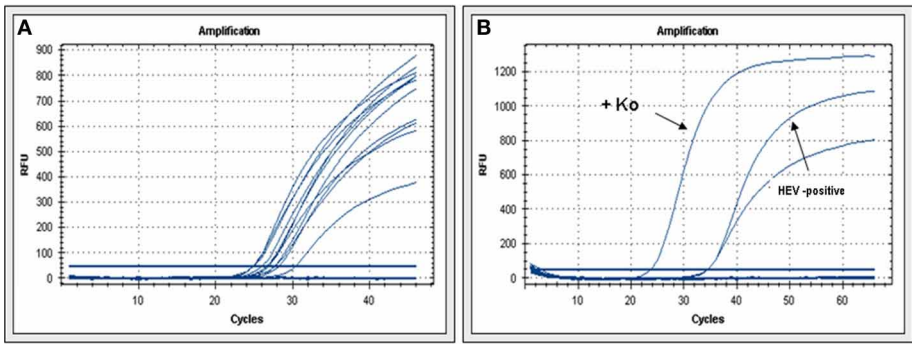


FIGURE 1 | HEV real-time PCR. (A) β-actin real-time PCR demonstrates that extracted RNA is accessible for PCR. **(B)** Examples of real-Time PCR for HEV-cDNA from a positive reference (Ko) or from one of the human biopsies with acute hepatitis.

Table 2 | Characteristics of Patients with Acute Hepatitis E.

No.	Age	Gender	Transaminases	Stays in abroad	EBV	CMV
1	60	Female	AST 1300 U/L, ALT 2400 U/L	n.d.	Negative	Negative
2	61	Male	>1000 U/L	n.d.	Negative	Negative
3	65	Female	AST 2200 U/L, ALT 2300 U/L	n.d.	Negative	IgM: positive PCR: Negative
3	78	Female	max: AST 1573 U/L, ALT 1586 U/L at the date of bíopsy: AST 343 U/L, ALT 724 U/L	n.d.	Negative	IgM: positve / Negative PCR: Negative
4	40	Male	AST 541 U/L, ALT 474 U/L	Greece	Negative	Negative
5	42	Female	max: ALT > 600 U/L at the date of bíopsy: ALT 70 U/L	n.d.	Negative	Negative
6	57	Male	n.d.	n.d.	Negative	Negative

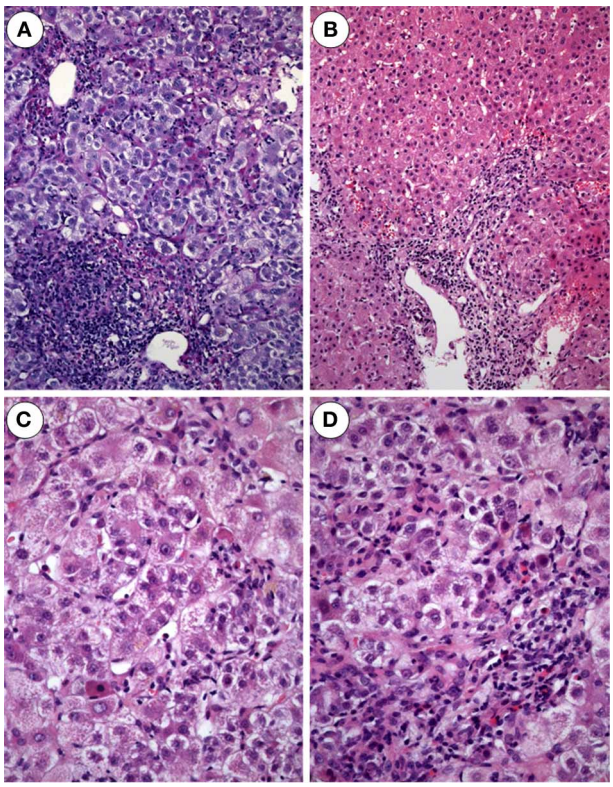


FIGURE 2 | Acute hepatitis E. (A) Expanded portal tract with dense inflammatory infiltrates mostly lymphocytes. Bile ducts display mild accompanying cholangitis (H and E × 100). **(B)** Acute hepatitis E with enlarged portal tract densely infiltrated by lymphocytes and some PMN leukocytes as well as some spotty necroses in the lobule (H and E × 80). **(C)** Acute hepatitis E with areas of spotty necrosis, aptotic bodies and infiltrates of lymphocytes, Kupffer cells and few polymorphnuclear leukocytes (H and E m× 240). **(D)** Biopsy from a patient with acute hepatitis E: the lobule shows foci of spotty necrosis, ballooning of hepatocytes and infiltrates with lymphocytes and polymorphnuclear leukocytes (H and E × 240)

degree. Four of the biopsies showed confluent necrosis and in one patient central necrosis with portal bridging was observed. Interestingly, cholestasis was noted with bile plugs in dilated canaliculi but also with intracellular bile pigment. The staining for iron was negative in all cases.

In the HEV-negative group, we found more eosinophilic leucocytes and the incidence of confluent necrosis was less frequent (see **Table 3**). Only one biopsy displayed bridging hepatic necrosis. **Table 3** summarizes the comparison of HEV histology characteristics.

SUBTYPING OF THE INFILTRATING INFLAMMATORY CELLS BY IMMUNOHISTOCHEMISTRY

In order to see if the analysis of the infiltrating inflammatory infiltrates especially the lymphocytes could give some information of underlying immune mechanisms we performed immunohistological subtyping of the lymphocytes.

The exact data are given in **Table 4** with the absolute number of positive cells when counted in 20 high power

Table 3 | Histopathology in 7 HEV patients and matched cohort of 7 non-HEV patients scoring according to the hepatitis activity index (HAI).

	HAI Score (Mean Values)	
	HEV group	**Non-HEV group**
Confluent necrosis	13	10
Spotty necro-sis,apoptosis, and local inflammation	25	25
Portal inflammation	18	8

Comment: Cholestasis and reactive cholangitis was more prominent in patients with HEV. In the non-HEV group 3 of 7 showed the presence of many eosinophils in portal tracts and lobules.

Table 4 | The number of liver infiltrating inflammatory cell[s#].

Immune cell marker epitope	HEV Biopsies	Matched biopsies
CDla	82 (*SD* = 9.6)	95 (*SD* = 7.5)
CD3	420 (*SD* = 14.7)	370 (*SD* = 15.5)
CD4	138 (*SD* = 11.9)	122 (*SD* = 9.1)
CD8	287 (*SD* = 20.3)	230 (*SD* = 12.1)
CD20	65 (*SD* = 6.5)	55 (*SD* = 6.7)
CD56	29 (*SD* = 3.3)*	49 (*SD* = 5.5)*
CD57	25 (*SD* = 3.8)*	52 (*SD* = 7.1)*
CD68	71 (*SD* = 9.7)	75 (*SD* = 7.5)
TIA	97 (*SD* = 9.2)	102 (*SD* = 12.6)

Given in absolute numbers per 20 hpf's at a mean range and standard deviation (SD) in brackets.

**Differences in number of marker cells observed in HEV positive vs. HEV negative tissue was significant ($p < 0.05$).*

fields. The numbers give the mean range for each cell-population.

T-lymphocytes, positive for CD 3 made up the majority of the infiltrating cells. CD 8 positive lymphocytes were the second most numerous cellpopulation with higher numbers in HEV patients but not statistically significant from non-HEV patients. CD 4 positive lymphocytes made up the third frequent cell population whereas B-lymphocytes, positive for CD 20, were less numerous. There was quite a highportion of CD 68 positive macrophages.in both HEV-positive and negative acute hepatitis. However, in non-HEV patients we observed asignificant trend for a higher NK activity (**Table 4**).

DISCUSSION

Reports on autochthonous hepatitis E are increasing in industrialized countries (Purcell and Emerson, 2010; Who Fact Sheet, 2012). Transmission of genotype 3 has been implicated as the infectious agent also from animals like pigs, deer and boars which seem to be the reservoir and the consumption of meat of these animals has been identified as the source of infection. The prevalence of the infection in the general population has been estimated between one and 14 percent in several countries of Europe (Wichmann et al., 2008; Kamar et al., 2012; Juhl et al., 2013). In clinical practice, without a history of traveling to developing countries the hepatitis is often not diagnosed correctly thus the patients are biopsied to evaluate the cause of acute hepatitis of unexplained etiology.

In order to see how many biopsies from patients with clinically unknown etiology maybe due to infection with HEV and also to evaluate if the virus can be detected in the liver tissue, we performed an analysis on liver biopsies from the files of our institute between the years 2000–2010. We selected 221 biopsies with the clinical diagnosis of acute hepatitis of unknown origin. Infection with other hepatitis viruses as HAV, HBV, HDV, HCV, CMV, and EBV was excluded as well as consumption of drugs, alcohol and previous travels to endemic areas. In our cases the serology of anti HEV was not available. Seven of the 221 biopsies proved to contain HEV-RNA after extraction of the RNA and application of real-time-PCR with specific primers. Whereas Gupta et al. suggested that immunohistology might be superior to RNA based detection methods (Gupta et al., 2012), our PCR approach benefits from a primer design allowing detection of low copy HEV numbers and the differentiation of HEV gentoypes. Furthermore, low amplicon length (69 bp) reduces amplification problems of fragmented nucleic acids derived from FFPE material. Although Real Time PCR has the advantage to be comparable in sensivity to nested PCR (Ratcliff et al., 2007; Drebber et al., 2011), formalin artifacts due to fragmentation and polymerase synthesis errors are limitations of the pathogen detection in archived material. Multiple polymerase reading errors due to formalin caused base mismatching might have also been the reason for three HEV amplicons failing to be sequenced. However, the virus could be specified as genotype 3 in four of seven patients after sequencing. This is in accordance with other reports from UK (Dalton et al., 2008), France (Kamar et al., 2012), Netherlands (Herremans et al., 2007) and Germany (Wichmann et al., 2008).

The histopathology of acute hepatitis E has been reported in animals and few liver biopsies from patients with acute infection mostly in endemic areas. There is less information on histology in hepatitis E when compared to other virotypes of hepatitis. A short description is given in the textbook of MacSween by These, however, referring to infection with all genotypes of HEV (Theise et al., 2011). Only few reports deal with histopathology of the indigenous form of hepatitis E in Europe namely infection with genotype 3 (Malcolm et al., 2007; Peron et al., 2007). The study of Malcolm et al describes histopathology of sporadic hepatitis E in more details and underlines the cholestatic appearance of the lesion with ductular proliferation and cholangiolitis in the portal tracts (Malcolm et al., 2007). So cholangitic-cholestatic features seem to be characteristic for acute hepatitis E. Another case report published by Wendum et al. (2005) of acute sporadic hepatitis E even showed lymphocytic destructive cholangitis. Our cases confirm this observation with prominent cholestasis and cholangitis in four of the seven biopsies. We could not find any pathognomonic features, but a histopathology that resembles a classic hepatitis with spotty necrosis, extending to confluent necrosis in four of the seven biopsies. In the non-HEV group confluent

necrosis was less severe, cholestasis was also less prominent and in three biopsies the presence of eosinophils was obvious. In clinical practice HEV infection can be reliably established by serology with IgM and IgG antibodies and PCR techniques in the serum. Our reports demonstrate for the first time that the diagnosis can be made also in the liver tissue when no serum is available. Extracted RNA can be sequenced to determine the genotype which turned out to be genotype 3 in all our cases confirming other reports from industrialized areas as the disease as an autochthonous infection. The immunopathology of liver damage seems to be T-cell mediated with CD 8 positive CTL's being the major population of infiltrating lymphocytes. The subtyping of inflammatory infiltrating cells by immunohistochemistry (see **Table 3**) showed CD 3/CD 8 positive lymphocytes as a predominant population suggesting an adaptive immune response to the virus as a major mechanism of defense and liver damage and supporting results obtained from analysis of peripheral blood lymphocytes (Suneetha et al., 2012). In the non-HEV group the number of CD 56/CD 57 lymphocytes was higher than in the patients perhaps due to another mechanism. In combination with the presence of eosinophils the findings suggest that these patients may have undergone drug induced liver injury without reporting drug intake which can be an important differential diagnosis to acute hepatitis E (Davern et al., 2011).

In conclusion we show that in cases of clinically unexplained acute hepatitis in central Europe the cause may be HEV. For the first time we document that the diagnosis can be made reliably on liver biopsies by PCR. Furthermore, this is the first report on histopathology of HEV infection exclusively- by genotype 3. Immunopathology with predominant CD 3/CD 8 positive lymphocytes indicate an adaptive immune response as defense and the cause of liver damage.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Heidemarie Holzmann, Hans-Peter Dienes. Performed the experiments: Margarete Odenthal, Inga Wedemeyer, Stephan W. Aberle, Jutta Hemberger. Analyzed the data: Heidemarie Holzmann, Uta Drebber, Inga Wedemeyer, Nadine Winkel, Stephan W. Aberle. Contributed reagents/materials/analysis tools: Hans-Peter Dienes, Margarete Odenthal. Wrote the paper: Heidemarie Holzmann, Hans-Peter Dienes. Performed the illustrations of the data: Margarete Odenthal, Uta Drebber.

REFERENCES

Dalton, H. R., Stableforth, W., Thurairajah, P., Hazeldine, S., Remnarace, R., Usama, W., et al. (2008). Autochthonous hepatitis E in Southwest England: natural history, complications and seasonal variation, and hepatitis E virus IgG seroprevalence in blood donors, the elderly and patients with chronic liver disease. *Eur. J. Gastroenterol. Hepatol.* 20, 784–790. doi: 10.1097/MEG.0b013e3282f5195a

Davern, T. J., Chalasani, N., Fontana, R. J., Hayashi, P. H., Protiva, P., Kleiner, D. E., et al. (2011). Acute hepatitis E infection accounts for some cases of suspected drug-induced liver injury. *Gastroenterology* 141, 1665-72.e1–1665-72.e9. doi: 10.1053/j.gastro.2011.07.051

Drebber, U., Hardt, A., Dienes, H. P., and Odenthal, M. (2011). Cytomegalovirus. Pathological-anatomical manifestations and detection methods. *Pathologe* 32, 418–427. doi: 10.1007/s00292-011-1449-8

Dries, V., Von Both, I., Muller, M., Gerken, G., Schirmacher, P., Odenthal, M., et al. (1999). Detection of hepatitis C virus in paraffin-embedded liver biopsies of patients negative for viral RNA in serum. *Hepatology* 29, 223–229. doi: 10.1002/hep.510290118

Faber, M. S., Wenzel, J. J., Jilg, W., Thamm, M., Hohle, M., and Stark, K. (2012). Hepatitis E virus seroprevalence among adults, Germany. *Emerging Infect. Dis.* 18, 1654–1657. doi: 10.3201/eid1810.111756

Gupta, P., Jagya, N., Pabhu, S. B., Durgapal, H., Acharya, S. K., and Panda, S. K. (2012). Immunohistochemistry for the diagnosis of hepatitis E virus infection. *J. Viral Hepat.* 19, e177–e183. doi: 10.1111/j.1365-2893.2011.01498.x

Herremans, M., Vennema, H., Bakker, J., Van Der Veer, B., Duizer, E., Benne, C. A., et al. (2007). Swine-like hepatitis E viruses are a cause of unexplained hepatitis in the Netherlands. *J. Viral Hepat.* 14, 140–146. doi: 10.1111/j.1365-2893.2006.00786.x

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J., and Hill, V. R. (2006). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J. Virol. Methods* 131, 65–71. doi: 10.1016/j.jviromet.2005.07.004

Juhl, D., Baylis, S. A., Blumel, J., Gorg, S., and Hennig, H. (2013). Seroprevalence and incidence of hepatitis E virus infection in German blood donors. *Transfusion.* doi: 10.1111/trf.12121. [Epub ahead of print].

Kamar, N., Bendall, R., Legrand-Abravanel, F., Xia, N. S., Ijaz, S., Izopet, J., et al. (2012). Hepatitis E. *Lancet* 379, 2477–2488. doi: 10.1016/S0140-6736(11)61849-7

Malcolm, P., Dalton, H., Hussaini, H. S., and Mathew, J. (2007). The histology of acute autochthonous hepatitis E virus infection. *Histopathology* 51, 190–194. doi: 10.1111/j.1365-2559.2007.02756.x

Peron, J. M., Danjoux, M., Kamar, N., Missoury, R., Poirson, H., Vinel, J. P., et al. (2007). Liver histology in patients with sporadic acute hepatitis E: a study of 11 patients from South-West France. *Virchows Arch.* 450, 405–410. doi: 10.1007/s00428-007-0382-y

Purcell, R. H., and Emerson, S. U. (2010). Hidden danger: the raw facts about hepatitis E virus. *J. Infect. Dis.* 202, 819–821. doi: 10.1086/655900

Ratcliff, R. M., Chang, G., Kok, T., and Sloots, T. P. (2007). Molecular diagnosis of medical viruses. *Curr. Issues Mol. Biol.* 9, 87–102.

Suneetha, P. V., Pischke, S., Schlaphoff, V., Grabowski, J., Fytili, P., Gronert, A., et al. (2012). Hepatitis E virus (HEV)-specific T-cell responses are associated with control of HEV infection. *Hepatology* 55, 695–708. doi: 10.1002/hep.24738

Theise, N., Bodenheimer, H., and Feerel, L. (2011). "Acute and chronic viral hepatitis," in *MacSween's Pathology of the Liver*, eds A. Burt, B. Portman, and L. Ferrel (New York, NY: Elsevier, Churchill Livingston), 361–401.

Wendum, D., Nachury, M., Yver, M., Lemann, M., Flejou, J. F., Janin, A., et al. (2005). Acute hepatitis E: a cause of lymphocytic destructive cholangitis. *Hum. Pathol.* 36, 436–438. doi: 10.1016/j.humpath.2005.01.006

Who Fact Sheet, H. E. (2012). Hepatitis E. *WHO Fact Sheet* No:280, Geneva.

Wichmann, O., Schimanski, S., Koch, J., Kohler, M., Rothe, C., Plentz, A., et al. (2008). Phylogenetic and case-control study on hepatitis E virus infection in Germany. *J. Infect. Dis.* 198, 1732–1741. doi: 10.1086/593211

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 August 2013; paper pending published: 01 October 2013; accepted: 13 November 2013; published online: 13 December 2013.

*Citation: Drebber U, Odenthal M, Aberle SW, Winkel N, Wedemeyer I, Hemberger J, Holzmann H and Dienes H-P (2013) Hepatitis E in liver biopsies from patients with acute hepatitis of clinically unexplained origin. Front. Physiol. **4**:351. doi: 10.3389/fphys.2013.00351*

This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 28 August 2012
doi: 10.3389/fphys.2012.00340

Prediction of outcome and selection of the liver transplantat candidate in acute liver failure

***Johannes Hadem*[1]*, *Christian P. Strassburg*[2] *and Michael P. Manns*[1]**

[1] *Department of Gastroenterology, Hepatology, and Endocrinology, Medical School Hannover, Hannover, Germany*
[2] *Medical Clinic I – General Internal Medicine, Gastroenterology, Hepatology, Nephrology, Infectious Diseases, and Endocrinology, University Clinic Bonn, Bonn, Germany*

Edited by:
Ali Canbay, University Hospital Essen, Germany

Reviewed by:
Pavel Strnad, University Medical Center Ulm, Germany
Matthias J. Bahr, Sana Kliniken Lübeck GmbH, Germany
Christoph Schramm, University Medical Center Hamburg-Eppendorf, Germany

***Correspondence:**
Johannes Hadem, Department of Gastroenterology, Hepatology, and Endocrinology, Medical School Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany.
e-mail: hadem.johannes@mh-hannover.de

Acute liver failure (ALF) is characterized by a sudden and severe deterioration of liver function, typically mirrored by a marked increase of the international normalized ratio (INR) and hepatic encephalopathy (HE). Due to various possible causes hepatocytes get damaged via either apoptotic or necrotic pathways. Anticipating the natural prognosis of a patient with ALF is one of the most challenging tasks in hepatology critical care. Important factors that influence the chance of spontaneous recovery are the underlying etiology of acute liver failure, the acuity of disease, and the severity of HE. Once an estimation of the prognosis in the individual patient has been made, this quickly has to be integrated in the discussion whether high-urgency liver transplantation is necessary and justifiable. This decision has to cover several medical, social, and organizational issues. Well organized liver transplantation programs around the world have achieved an impressive improvement of the 1 year survival rate in ALF from around 40% without transplantation up to nearly 80% with transplantation. The recent debate on whether severe acute alcoholic hepatitis could represent a new candidate eligible for high-urgency liver transplantation shows that the topic is still open for discussion.

Keywords: acute hepatitis, biomarker, emergency liver transplantation, hepatic encephalopathy, hepatic failure, outcome, prognosis

INTRODUCTION

The overall incidence of acute liver failure (ALF) in developed countries is estimated to be 1–6 per million people every year (Bower et al., 2007; Bernal et al., 2010). Emergency liver transplantation (ELT) is a potentially life-saving procedure in patients with ALF, but carries relevant long-term morbidity and mortality. Appropriate selection of patients for ELT therefore requires tools allowing to accurately prognosticate spontaneous recovery (Renner, 2007). The prediction of outcome in an individual patient with ALF is among the most challenging tasks in hepatology critical care. Many retrospective observational studies have tried to identify particular clinical and laboratory markers or prognostic models capable of predicting prognosis and need of ELT. This article addresses some of the questions that arise when facing a patient who progressively deteriorates from severe acute hepatitis to ALF.

ACUTE OR ACUTE-ON-CHRONIC—DIFFICULT TO DISTINGUISH

A deteriorating liver function that comes to attention for the first time often triggers the assumption of an ALF. But by definition, ALF implies the absence of chronic liver disease (Trey and Davidson, 1970; Bernal et al., 2010; Lee et al., 2011), and the majority of patients that present with worsening liver function suffer from acute-on-chronic liver failure (i.e., an acute deterioration superimposed on chronic liver disease). Non-alcoholic and alcoholic fatty liver disease with varying degrees of fibrosis are among the most common causes of pre-existing liver injury in those patients. Patients with acute-on-chronic liver failure can often be identified by thorough history taking and physical examination. The role of abdominal ultrasound is more complex. Undoubtedly, ultrasound will detect overt liver cirrhosis presenting with reduced liver volume, irregular liver surface, distorted liver veins, and nodular liver parenchyma (Caselitz, 1999). But cirrhotic parenchymal changes can be difficult to detect in steatosis, and signs of portal hypertension may also develop in subacute liver failure. Furthermore, acute hepatitis triggers inflammatory infiltration, hepatocyte swelling, and early regenerative fibrotic parenchymal changes. These processes have been shown to increase liver stiffness when measured by transient elastography and newer ultrasound technologies and thereby can hamper the distinction between acute and chronic liver injury (Arena et al., 2008; Sagir et al., 2008; Dechêne et al., 2010).

A transjugular liver biopsy might aid in confirming certain ALF etiologies (Lee et al., 2011) and even in defining the degree of liver necrosis (Beckmann et al., 2009). But liver biopsy is not recommended and probably of minor help in distinguishing acute-on-chronic from acute liver failure. Moreover, it remains an open question which degree of pre-existing liver injury should be regarded as a contraindication for ELT. Whereas a significant chronic component of deteriorated liver function is generally regarded a contraindication for ELT, there are at least three exceptions to this rule: ALF in

autoimmune hepatitis, Wilson's disease, and Budd-Chiari syndrome can arise from a relapsing or chronic course of disease. Those patients are suitable ELT candidates despite cirrhotic hepatic changes.

ROLE OF HEPATIC ENCEPHALOPATHY AND ETIOLOGY IN OUTCOME PREDICTION

Not too surprisingly, the prospective observational study by the US ALF Study Group underlined the major prognostic role of hepatic encephalopathy (HE) and etiology in ALF. HE grade possibly reflects the degree of cerebral edema. Whereas ALF patients with a baseline HE grade 1/2 had a transplant-free 21-day survival of 52%, this was only 33% in patients initially presenting with HE grade 3/4 (Ostapowicz et al., 2002). A hyperacute course (interval from icterus to encephalopathy <7 days) of liver failure (O'Grady et al., 1989) that is typically seen in acetaminophen, hepatitis A or herpes simplex virus (Graham et al., 2009; Bernal et al., 2010) is associated with a sudden rise in international normalized ratio (INR), an increased risk of cerebral edema and a rather high transplant-free survival rate. A subacute course (interval from icterus to encephalopathy >21 days) (O'Grady et al., 1989), as seen in indeterminate ALF implies a low transplant-free survival rate despite initially moderate increases in INR and low rates of cerebral herniation (Ostapowicz et al., 2002; Hadem et al., 2008; Bernal et al., 2010).

Data from Europe and the US ALF Study Group show that transplant-free survival rates in ALF patients are strongly associated with the underlying ALF etiology: 60–80% in acetaminophen-induced ALF, 30–40% in severe acute hepatitis B, 10–20% in indeterminate ALF, 20–50% in non-acetaminophen drug-induced ALF, 0–20% in Wilson's disease and around 70–80% in amanita ingestion (Ostapowicz et al., 2002; Escudié et al., 2007; Hadem et al., 2008). Only 10% of patients with drug-induced hepatitis progress to ALF which then implies a bad prognosis even after the provoking medication has been stopped (Andrade et al., 2005; Reuben et al., 2010). Phenprocoumon, a coumadin derivate used in Germany can cause drug-induced ALF associated with low transplant-free survival rates (Hadem et al., 2008; Canbay et al., 2009). Mortality rate in ALF caused by hepatitis E is assumed to be 8–11% in Western countries, but can be as high as 50% when ELT is not available, and may also be higher when acquired during pregnancy (Bernuau et al., 2008). Pregnancy-related ALF is a heterogeneous disease entity which has not sufficiently been studied. A recent observational study reported on 54 patients (18 acute fatty liver of pregnancy, 32 eclampsia-associated) of whom 13% died and 7% received ELT (Westbrook et al., 2010). Budd-Chiari syndrome and early graft dysfunction are among other causes of ALF with high risk of a fatal outcome. Although transjugular intrahepatic portosystemic shunting (TIPS) has undoubtedly decreased the case fatality rate of Budd-Chiari syndrome presenting with ALF, data on the use of TIPS in this setting are still scarce. In the important paper on TIPS in Budd-Chiari syndrome by Garcia-Pagan et al. only 9/124 (7%) had ALF prior to TIPS, only 27/124 (22%) received TIPS due to (acute or chronic) liver failure, and a significant proportion of these patients needed ELT or died (Garcia-Pagán et al., 2008).

PROGNOSTIC MARKERS IN ACUTE LIVER FAILURE

RATIONALE BEHIND PROGNOSTIC MARKERS

Many prognostic markers have been proposed to overcome the prognostic uncertainty that is a major problem in the early phase of ALF. One common approach is to deduce the outcome from the degree of aminotransferase increase. These enzymes are—like many other components of the hepatocellular content—released into the circulation in apoptosis and necrosis. However, there is no reliable association between aminotransferases and outcome. Ischemic hepatitis and acetaminophen toxicity regularly present with huge aminotransferase increases, but often are associated with favorable prognosis. Another approach is to measure serum markers of liver regeneration (e.g., alpha-fetoprotein, cytokines, growth factors, and also phosphate) to get an idea of the hepatic regenerative capacity. These parameters have mainly been studied in patients with hyperacute liver failure in the setting of acetaminophen toxicity. A reasonable way to quantify the severity of liver damage is to extrapolate the course of ALF according to serial hepatocellular function measurements. Synthetic liver capacity is best observed by tracking the INR that reflects the synthesis of clotting factors II, V, VII, and X. Rapid deteriorations can be recognized by decreases of factor V due to its short half time of 12–15 h. Detoxification liver capacity can be monitored by bilirubin, ammonia, indocyanin green, and other serum parameters. Many of these parameters have been incorporated in a number of prognostic criteria in an attempt to predict ALF outcome as accurately as possible.

ALPHA-FETOPROTEIN

Among the early investigations on prognostic markers in ALF is that of Murray-Lyon et al. from King's College Hospital. Sixty-four patients with fulminant hepatic failure were examined regarding their serum alpha-fetoprotein (AFP) levels. AFP levels >50 ng/ml were observed in 48% of the 23 survivors, but only in 10% of the 41 ALF patients with a fatal outcome (Murray-Lyon et al., 1976). In a subsequent study on 239 patients with acetaminophen intoxication, a threshold AFP of $\geq$3.9 μg/l on day + 1 after peak ALT to identify nonsurvivors had a sensitivity of 100% and a specificity of 74% (Schmidt and Dalhoff, 2005). A retrospective analysis of 206 patients from the US ALF study (80 acetaminophen, 30 indeterminate) showed that an AFP day 3-to-day1 ratio >1 predicted survival with a sensitivity of 65% and a specificity of 84% (Schiødt et al., 2006).

LACTATE

Arterial blood lactate levels can be measured rapidly by point-of-care testing and reflect both increased production from peripheral tissues and the injured liver and reduced clearance from the circulation as a consequence of impaired hepatic metabolic capacity (Bernal, 2010). In a mixed prospective/retrospective study of over 200 patients with acetaminophen-induced ALF, high blood lactate levels were found to be closely related to a fatal outcome (Bernal et al., 2002). Subsequent studies found arterial lactate (baseline or 12 h after admission) to be associated strongly and independently with death or transplantation in both acetaminophen and non-acetaminophen-induced ALF (Macquillan et al., 2005; Schmidt and Larsen, 2006; Hadem et al., 2008; Bernal et al., 2010). Baseline

lactate levels were higher (median 4.7 vs. 2.9 mmol/L) and steadily increased in ALF patients with an unfavorable outcome (Hadem et al., 2008). Others have argued that lactate lacks the specificity required to a sole criterion for ELT listing (Schmidt and Larsen, 2010). Different views on the prognostic value of lactate levels in ALF are likely to reflect variability in the timing and degree of fluid resuscitation in early ALF management. The greatest prognostic value will therefore be gained from those patients in whom no correction of initially high levels occurs following volume resuscitation, and these patients should probably be considered for ELT (Bernal, 2010).

INR

A number of studies illustrating the time course of daily INR values nicely show that a single INR level at the onset of HE (i.e., the overt manifestation of ALF) do not necessarily mirror the severity of liver damage and prognosis. This is particularly true for hyperacute ALF courses such as in acetaminophen or amanita toxicity (Escudié et al., 2007; Schmidt and Larsen, 2007). It is particularly in the subacute ALF setting that sustained elevations of INR become important for decision-making.

FACTOR V AND CLICHY-VILLEJUIF CRITERIA

Early investigations of factor V in patients with fulminant hepatitis B have proven this marker to be an independent predictor of survival (Bernuau et al., 1986). Factor V, HE, and patients' age were later incorporated into the so called Clichy-Villejuif criteria which have been established particularly in France (Bismuth et al., 1995), but also form part of the German transplantation law (Richtlinien Organtransplantation, 2011). An unfavorable outcome can be expected in >90% of cases with HE plus a factor V level less than 20% of normal (in patients younger than 30 years of age) or less than 30% of normal (in patients older than 30 years of age), respectively (**Table 1**) (Bismuth et al., 1995).

AMMONIA

In ALF, ammonia escapes hepatic metabolism, leading to high arterial ammonia concentrations. Clemmesen et al. showed that ALF patients who developed clinical signs of cerebral herniation ($n = 14$ out of 44) had higher arterial plasma ammonia levels compared to those who did not (230 vs. 118 μmol/L, $p < 0.001$) (Clemmesen et al., 1999). This finding was later validated in a group of 165 patients with ALF and HE. An arterial ammonia level greater than 100 μmol/L was an independent risk factor for severe HE (sensitivity 59%, specificity 78%) and intracranial hypertension (sensitivity 73%, specificity 44%). The combination of arterial ammonia with the Model of End-stage Liver Disease (MELD) further increased specificity in prediction of HE (Bernal et al., 2007).

Table 1 | Clichy-Villejuif criteria for non-acetaminophen ALF.

Hepatic encephalopathy AND
Factor V <20%, if <30 years of age OR
Factor V <30%, if >30 years of age

PHOSPHATE

Schmidt and Dalhoff published data on serum phosphate levels in 95 patients with severe acetaminophen poisoning. Phosphate concentrations were significantly higher in nonsurvivors than in survivors 48 h after overdose. A threshold phosphate concentration of 1.2 mmol/L at 48–96 h after overdose specifically and sensitively identified a group of patients with very little chance of spontaneous survival. The phosphate criteria had higher predictive values than the King's College criteria (KCC). The authors proposed that hyperphosphatemia is caused by renal dysfunction in the absence of hepatic regeneration, whereas the latter appears to be associated with lowering of serum phosphate. Possible limitations of this study were the relatively low numbers of patients with HE ($n = 30$) and a fatal outcome ($n = 16$) (Schmidt and Dalhoff, 2002).

KING'S COLLEGE CRITERIA

The most commonly used prediction models are the KCC that were initially based on a retrospective study on 588 patients with ALF managed medically during 1973–1985. The model was validated in an independent cohort of 175 ALF patients treated between 1986 and 1987. The KCC distinguish between acetaminophen and non-acetaminophen-induced ALF and include pH, HE, INR, creatinine, etiology, bilirubin, age, and acuity of symptom onset (**Table 2**) (O'Grady et al., 1989). Several case series and meta-analyses have confirmed that KCC have clinically acceptable specificity of 80–90%, with survival without transplantation in patients meeting criteria of less than 15%. However, sensitivity of KCC has been reported to be as low as 60–70%, indicating that KCC may fail to detect patients facing a fatal outcome without ELT (Anand et al., 1997; Hadem et al., 2008; Bernal et al., 2010; Craig et al., 2010; McPhail et al., 2010). Despite this draw-back, KCC form the basis for ELT registration in many countries (Neuberger et al., 2008; Bernal et al., 2010; Richtlinien Organtransplantation, 2011).

MODEL OF END-STAGE LIVER DISEASE (MELD) SCORE

Originally developed for allocation in patients with chronic liver disease, the MELD score has been evaluated as prognostic marker

Table 2 | King's college criteria.

ACETAMINOPHEN-INDUCED ALF
Arterial pH <7.3 (regardless of HE)
OR all 3 of the following
– INR >6.5
– Creatinine >300 μmol/l
– HE grade 3–4
NON-ACETAMINOPHEN-INDUCED ALF
INR >6.5 (regardless of HE)
OR 3 of 5 of the following (regardless of HE)
– Age <10 or >40 years
– Etiology: indeterminate, drug-induced
– Time interval icterus to encephalopathy > 7 days
– INR >3.5
– Bilirubin >300 μmol/l

in ALF. Yantorno et al. investigated 64 adult ALF patients of mixed etiology and found MELD to provide better predictive value than KCC in a Cox model, defining a MELD of 30 as prognostic cut-off (Yantorno et al., 2007).

Another study examined MELD in 124 patients with acetaminophen-induced ALF. A threshold MELD score of 33 on the day after the onset of HE had a sensitivity of 60%, and a specificity of 69% in predicting death. However, the discriminative power of MELD score was not superior to that of INR alone or of the KCC (Schmidt and Larsen, 2007). Katoonizadeh et al. evaluated 99 patients with non-acetaminophen-induced ALF and found the best MELD cut-off to be >35 (sensitivity 86%, specificity 75%). Again, MELD was not superior to KCC (Katoonizadeh et al., 2007). A retrospective observational study on 134 German ALF patients of mixed etiology showed MELD to be an independent prognostic factor with a median MELD of 33 in the fatal prognostic group (Canbay et al., 2009). Another retrospective study on 102 ALF patients of mixed etiology from northern Germany demonstrated that MELD-based outcome prediction was comparable to that by KCC (Hadem et al., 2008). Finally, a modification of classic MELD by substituting the level of the M65 epitope of cytokeratin-18 (CK 18) for bilirubin, significantly improved the positive predictive value in a cohort of mainly 68 acetaminophen-induced ALF (Bechmann et al., 2010). The M65 epitope of CK 18 is exposed on all intact and fragmented CK18 variants released from destroyed hepatocytes, has been studied in several chronic liver disease entities (Bantel et al., 2001), and might thus allow a better quantification of liver damage in ALF (Volkmann et al., 2008).

BILIRUBIN-LACTATE-ETIOLOGY (BiLE) SCORE

Bilirubin-Lactate-Etiology (BiLE) score was empirically developed based on 102 ALF patients of mixed etiology (predominantly indeterminate ALF) from northern Germany and found to be slightly superior to KCC, MELD, and SAPS-III in predicting death or need of ELT with a sensitivity of 79% and a specificity of 84%. BiLE score is calculated by adding bilirubin and lactate with an etiology-specific summand (**Table 3**) (Hadem et al., 2008). In an external retrospective evaluation of BiLE in 422 consecutive ALF patients from the UK (57% with acetaminophen-induced ALF), BiLE lacked sensitivity (55%), but had a good specificity of 89% (Bernal et al., 2009). As with other models, BiLE score has therefore not yet proven superiority over established models such as KCC, and it appears premature to base transplant decisions on BiLE at this point of time.

Table 3 | Bilirubin Lactate Etiology (BiLE) score.

BiLE score =
Bilirubin (μmol/l)/100
+ Lactate (mmol/l)
+ 4 [in case of indeterminate ALF, Budd-Chiari syndrome or phenprocoumon toxicity]
− 2 [in case of acetaminophen toxicity]
+ 0 [in case of any other ALF etiology]

RECENT ADVANCES IN ALF OUTCOME PREDICTION

An interesting prognostic scoring model for non-acetaminophen ALF has been proposed by colleagues from Japan. Four parameters (unfavorable etiology, HE grade 3/4, presence of systemic inflammatory response syndrome, and ratio of total to direct bilirubin >2) were evaluated retrospectively in 80 patients, and prospectively validated in another 26 patients on days 1, 4, 8, and 15. The scoring model predicted 2-week survival rates with high sensitivity and specificity, when all four time points of scoring were taken into account. However, the earliest score on day 1 which is the one of greatest clinical interest tended to underestimate the probability of a fatal outcome (Miyake et al., 2005).

As in liver cirrhosis, there has been a growing interest in applying scores of multiple organ dysfunction to ALF patients. Sequential Organ Failure Assessment (SOFA) score is an ordinal variable that covers six organ systems and has been established as dynamic disease severity measurement tool in intensive care (Vincent et al., 1996). Craig et al. investigated SOFA in 138 acetaminophen overdoses (only 48% with HE) and found that SOFA provided superior outcome discrimination compared to MELD. The authors stated, however, that due to limited specificity, SOFA would not be capable of replacing KCC as definite listing criteria (Craig et al., 2012). Moreover, organ dysfunction as documented by SOFA, typically develops >72 h following ALF onset and therefore does probably not represent a very early predictive tool.

Angiopoietin-2 (Angpt2), a mediator of endothelial activation and capillary leakage, is known to be up-regulated in sepsis, but its role in ALF has just recently been investigated. Angpt2 serum levels were examined in 37 patients with ALF of mixed etiology and 20 healthy controls. Angpt2 revealed to be a predictor of the composite end-point of death or ELT and correlated strongly with surrogate markers of organ dysfunction. Immunohistological studies showed that Angpt2 was upregulated in ALF explants (Hadem et al., 2012). Angpt2 might therefore explain the regularly observed sepsis-like clinical picture of ALF patients and also serve as a marker of impending multiple organ failure.

ACUTE LIVER FAILURE—WHOM AND WHEN TO TRANSPLANT IN CLINICAL PRACTICE

Current criteria for selection of patients with ALF for ELT are far from being perfect (Renner, 2007). The ideal means for identification of patients who are likely to benefit from ELT still remains controversial (Bernal et al., 2010). A patient who would have survived with medical management, incorrectly transplanted will be subjected to unnecessary surgery, life-long immunosuppression, and an increased risk of death. Furthermore, a graft that could be used in a more appropriate candidate will be lost (Bernal, 2010). Decisions regarding ELT have to be made early-on during the course of ALF because of impending complications such as cerebral herniation or multiple organ failure. In a retrospective study from King's College Hospital of 310 ALF patients listed for ELT, the median time from listing to ELT was 1 day. However, 24% of listed patients did not undergo surgery, mainly because of death on the waiting list at a median of 2 days after listing (Bernal et al., 2009). Similar observations were made in the US ALF study

cohort with a median time from listing to ELT of 3.5 days and a median time from admission to death of 5 days (Ostapowicz et al., 2002).

The difficult questions whom and when to perform ELT in an individual patient are best answered in a multiple step approach.

First, the focus is on ALF etiology and HE grade to ensure that the ELT candidate will have a grim prognosis without ELT. Other predictive markers such as acuity of symptom onset, sustained increase in INR, or persisting elevation of lactate after adequate fluid resuscitation can be incorporated in the decision. Sometimes, daily ultrasound monitoring of progressing liver dystrophy can complete the physician's judgment on which therapeutic path to follow. Established prognostic models such as the KCC and the Clichy Villejuif criteria are certainly of value. All these factors should rather be regarded as pieces of a mosaic than definite criteria. It is rather the rule than an exception that a clear-sighted decision has to rely on a certain amount of physician's gut feeling. For example, slow clinical deterioration, moderately elevated INR and undulating bilirubin levels in a young female with subacute indeterminate ALF should not deceive the team about her likely bad prognosis. On the other hand, a patient with acetaminophen-induced ALF and hyperacute clinical presentation may have a high spontaneous survival rate even though experiencing advanced stages of disease (Schmidt and Larsen, 2010). A useful summary of the UK criteria for registration as high-urgent ELT candidate has recently been published by Neuberger et al. (2008).

Second, potential contraindications against ELT have to be excluded. Among them are (1) advanced biological patient age, (2) pre-existing chronic comorbidities (major psychiatric disorders probably influencing patient's post-transplant compliance, heavy alcohol abuse, cardiopulmonary disorders), (3) unfavorable severity of pretransplant illness (reflected by high-dose vasopressor support or multiple organ dysfunction), and (4) secondary ALFs in the setting of severe sepsis or generalized ischemia (e.g., after cardiopulmonary resuscitation). Patients with acetaminophen-induced ALF are at high risk of developing pre-ELT complications. Additionally, ELT recipients >50 years face a doubled postoperative mortality (Bernal et al., 2009).

Third, the quality of the liver graft influences post-ELT outcome and has to be matched in each individual case. It is very demanding to find the right balance between risking delay of ELT until an ideal graft is available and early acceptance of a suboptimum graft that might be associated with a poor outcome (Bernal et al., 2009, 2010).

SEVERE ACUTE ALCOHOLIC HEPATITIS—A NEW CANDIDATE FOR EMERGENCY LIVER TRANSPLANTATION?

Mathurin et al. recently reported on 26 patients with a first episode of severe alcoholic hepatitis that were not responding to medical therapy and selected to receive ELT. Six-month survival was markedly improved compared with patients not receiving ELT (78% vs. 24%). Three out of 26 patients (12%) experienced alcohol relapse (Mathurin et al., 2011). As these patients were highly selected involving the whole primary care team and focusing on the patient's social background, it remains open, if a broader consideration of alcoholic hepatitis for ELT would result in similarly good outcomes. The current discussion about alcoholic hepatitis as potential candidate for ELT focuses on the issue of justice among liver graft recipients, possible biases in selecting suitable candidates, the 12% relapse rate, the rather arbitrarily defined 6-month abstinence rule, and the high rate of perioperative infectious complications (Brown, 2011; John and Chung, 2012; Moreno et al., 2012).

CONCLUSION

In conclusion, selecting the ALF patient in need of ELT, determining the right point of time for wait-listing, and matching patient and liver graft remains challenging. This demands experience as well as a high grade of suspicion. Available prognostic parameters and models can be of help, but should be regarded as pieces of a mosaic rather than definite therapeutic algorithms. Proper management of ALF patients have now achieved an increase in overall survival rates from 40% without availability of ELT to over 80% post ELT (Ostapowicz et al., 2002; Lee et al., 2011).

ACKNOWLEDGMENTS

This work was kindly supported by a grant from the Collaborative Research Centre 738 ("Optimierung konventioneller und innovativer Transplantate") of the German Research Foundation.

REFERENCES

Anand, A. C., Nightingale, P., and Neuberger, J. M. (1997). Early indicators of prognosis in fulminant hepatic failure: an assessment of the King's criteria. *J. Hepatol.* 26, 62–68.

Andrade, R. J., Lucena, M. I., Fernández, M. C., Pelaez, G., Pachkoria, K., García-Ruiz, E., García-Muñoz, B., González-Grande, R., Pizarro, A., Durán, J. A., Jiménez, M., Rodrigo, L., Romero-Gomez, M., Navarro, J. M., Planas, R., Costa, J., Borras, A., Soler, A., Salmerón, J., Martin-Vivaldi, R., and Spanish Group for the Study of Drug-Induced Liver Disease. (2005). Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 129, 512–521.

Arena, U., Vizzutti, F., Corti, G., Ambu, S., Stasi, C., Bresci, S., Moscarella, S., Boddi, V., Petrarca, A., Laffi, G., Marra, F., and Pinzani, M. (2008). Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 47, 380–384.

Bantel, H., Ruck, P., Gregor, M., and Schulze-Osthoff, K. (2001). Detection of elevated caspase activation and early apoptosis in liver diseases. *Eur. J. Cell Biol.* 80, 230–239.

Bechmann, L. P., Jochum, C., Kocabayoglu, P., Sowa, J. P., Kassalik, M., Gieseler, R. K., Saner, F., Paul, A., Trautwein, C., Gerken, G., and Canbay, A. (2010). Cytokeratin-18 based modification of the MELD score improves prediction of spontaneous survival after acute liver failure. *J. Hepatol.* 53, 639–647.

Beckmann, M. G., Bahr, M. J., Hadem, J., Bredt, M., Wedemeyer, H., Schneider, A. S., Kubicka, S., Manns, M. P., Strassburg, C. P., and Wedemeyer, J. (2009). Clinical relevance of transjugular liver biopsy in comparison with percutaneous and laparoscopic liver biopsy. *Gastroenterol. Res. Pract.* 2009, 947014.

Bernal, W. (2010). Lactate is important in determining prognosis in acute liver failure. *J. Hepatol.* 53, 209–210.

Bernal, W., Auzinger, G., Dhawan, A., and Wendon, J. (2010). Acute liver failure. *Lancet* 376, 190–201.

Bernal, W., Auzinger, and Wendon, J. A. (2009). Prognostic utility of the BiLE score. *Clin. Gastroenterol. Hepatol.* 7, 249.

Bernal, W., Cross, T. J., Auzinger, G., Sizer, E., Heneghan, M. A., Bowles, M., Muiesan, P., Rela, M., Heaton, N., Wendon, J., and O'Grady, J. G. (2009). Outcome after wait-listing for emergency liver transplantation in acute liver failure: a single centre experience. *J. Hepatol.* 50, 306–313.

Bernal, W., Donaldson, N., Wyncoll, D., and Wendon, J. (2002). Blood lactate as an early predictor of outcome in paracetamol-induced acute liver failure: a cohort study. *Lancet* 359, 558–563.

Bernal, W., Hall, C., Karvellas, C. J., Auzinger, G., Sizer, E., and Wendon J. (2007). Arterial ammonia and clinical risk factors for encephalopathy and intracranial hypertension in acute liver failure. *Hepatology* 46, 1844–1852.

Bernuau, J., Goudeau, A., Poynard, T., Dubois, F., Lesage, G., Yvonnet, B., Degott, C., Bezeaud, A., Rueff, B., and Benhamou, J. P. (1986). Multivariate analysis of prognostic factors in fulminant hepatitis B. *Hepatology* 6, 648–651.

Bernuau, J., Nicand, E., and Durand, F. (2008). Hepatitis E-associated acute liver failure in pregnancy: an Indian puzzle. *Hepatology* 48, 1380–1382.

Bismuth, H., Samuel, D., Castaing, D., Adam, R., Saliba, F., Johann, M., Azoulay, D., Ducot, B., and Chiche, L. (1995). Orthotopic liver transplantation in fulminant and subfulminant hepatitis. The Paul Brousse experience. *Ann. Surg.* 222, 109–119.

Bower, W. A., Johns, M., Margolis, H. S., Williams, I. T., and Bell, B. P. (2007). Population-based surveillance for acute liver failure. *Am. J. Gastroenterol.* 102, 2459–2463.

Brown, R. S. (2011). Transplantation for alcoholic hepatitis – time to rethink the 6-month rule. *N. Engl. J. Med.* 365, 1836–1838.

Canbay, A., Jochum, C., Bechmann, L. P., Festag, S., Gieseler, R. K., Yüksel, Z., Lütkes, P., Saner, F. H., Paul, A., and Gerken G. (2009). Acute liver failure in a metropolitan area in Germany: a retrospective study (2002–2008). *Z. Gastroenterol.* 47, 807–813.

Caselitz, M. (1999). "Cirrhosis of the liver," in *Ultrasound in Gastroenterology and Hepatology*, ed M. Gebel (Berlin: Springer), 40–41.

Clemmesen, J. O., Larsen, F. S., Kondrup, J., Hansen, B. A., and Ott, P. (1999). Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. *Hepatology* 29, 648–653.

Craig, D. G., Ford, A. C., Hayes, P. C., and Simpson, K. J. (2010). Systematic review: prognostic tests of paracetamol-induced acute liver failure. *Aliment. Pharmacol. Ther.* 31, 1064–1076.

Craig, D. G., Reid, T. W., Wright, E. C., Martin, K. G., Davidson, J. S., Hayes, P. C., and Simpson, K. J. (2012). The sequential organ failure assessment (SOFA) score is prognostically superior to the model for end-stage liver disease (MELD) and MELD variants following paracetamol (acetaminophen) overdose. *Aliment. Pharmacol. Ther.* 35, 705–713.

Dechêne, A., Sowa, J. P., Gieseler, R. K., Jochum, C., Bechmann, L. P., El Fouly, A., Schlattjan, M., Saner, F., Baba, H. A., Paul, A., Dries, V., Odenthal, M., Gerken, G., Friedman, S. L., and Canbay, A. (2010). Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 52, 1008–1016.

Escudié, L., Francoz, C., Vinel, J. P., Moucari, R., Cournot, M., Paradis, V., Sauvanet, A., Belghiti, J., Valla, D., Bernuau, J., and Durand, F. (2007). *Amanita phalloides* poisoning: reassessment of prognostic factors and indications for emergency liver transplantation. *J. Hepatol.* 46, 466–473.

Garcia-Pagán, J. C., Heydtmann, M., Raffa, S., Plessier, A., Murad, S., Fabris, F., Vizzini, G., Abraldes, J. G., Olliff, S., Nicolini, A., Luca, A., Primignani, M., Janssen, H. L., Valla, D., Elias, E., Bosch, J. and Budd-Chiari Syndrome-Transjugular Intrahepatic Portosystemic Shunt Group. (2008). TIPS for Budd-Chiari syndrome: long-term results and prognostic factors in 124 patients. *Gastroenterology* 135, 808–815.

Graham, B. B., Kaul, D. R., Saint, S., and Janssen, W. J. (2009). Kiss of death. *N. Engl. J. Med.* 360, 2564–2568.

Hadem, J., Bockmeyer, C. L., Lukasz, A., Pischke, S., Schneider, A. S., Wedemeyer, H., Jonigk, D., Manns, M. P., and Kümpers, P. (2012). Angiopoietin-2 in acute liver failure. *Crit. Care Med.* 40, 1499–1505.

Hadem, J., Stiefel, P., Bahr, M. J., Tillmann, H. L., Rifai, K., Klempnauer, J., Wedemeyer, H., Manns, M. P., and Schneider, A. S. (2008). Prognostic implications of lactate, bilirubin and etiology in German patients with acute liver failure. *Clin. Gastroenterol. Hepatol.* 6, 339–345.

John, S., and Chung, R. T. (2012). Letter to the editor. *N. Engl. J. Med.* 366, 477–478.

Katoonizadeh, A., Decaestecker, J., Wilmer, A., Aerts, R., Verslype, C., Vansteenbergen, W., Yap, P., Fevery, J., Roskams, T., Pirenne, J., and Nevens, F. (2007). MELD score to predict outcome in adult patients with non-acetaminophen-induced acute liver failure. *Liver Int.* 27, 329–334.

Lee, W. M., Stravitz, R. T., and Larson, A. M. (2011). AASLD position paper: the management of acute liver failure: update 2011. *Hepatology* 55, 965–967.

Macquillan, G. C., Seyam, M. S., Nightingale, P., Neuberger, J. M., and Murphy, N. (2005). Blood lactate but not serum phosphate levels can predict patient outcome in fulminant hepatic failure. *Liver Transpl.* 11, 1073–1079.

Mathurin, P., Moreno, C., Samuel, D., Dumortier, J., Salleron, J., Durand, F., Castel, H., Duhamel, A., Pageaux, G. P., Leroy, V., Dharancy, S., Louvet, A., Boleslawski, E., Lucidi, V., Gustot, T., Francoz, C., Letoublon, C., Castaing, D., Belghiti, J., Donckier, V., Pruvot, F. R., and Duclos-Vallée, J. C. (2011). Early liver transplantation for severe alcoholic hepatitis. *N. Engl. J. Med.* 365, 1790–1800.

McPhail, M. J. W., Wendon, J. A., and William, B. (2010). Meta-analysis of performance of Kings's College Hospital Criteria in prediction of outcome in non-paracetamol-induced acute liver failure. *J. Hepatol.* 53, 492–499.

Moreno, C., Duclos-Vallee, J. C., and Mathurin, P. (2012). Author reply. *N. Engl. J. Med.* 366, 479.

Miyake, Y., Sakaguchi, K., Iwasaki, Y., Ikeda, H., Makino, Y., Kobashi, H., Araki, Y., Ando, M., Kita, K., and Shiratori, Y. (2005). New prognostic scoring model for liver transplantation in patients with non-acetaminophen-related fulminant hepatic failure. *Transplantation* 80, 930–936.

Murray-Lyon, I. M., Orr, A. H., Gazzard, B., Kohn, J., and Williams, R. (1976). Prognostic value of serum alpha-fetoprotein in fulminant hepatic failure including patients treated by charcoal haemoperfusion. *Gut* 17, 576–580.

Neuberger, J., Gimson, A., Davies, M., Akyol, M., O'Grady, J., Burroughs, A., Hudson, M., and Liver Advisory Group; UK Blood and Transplant. (2008). Selection of patients for liver transplantation and allocation of donated livers in the UK. *Gut* 57, 252–257.

O'Grady, J. G., Alexander, G. J., Hayllar, K. M., and Williams, R. (1989). Early indicators of prognosis in fulminant hepatic failure. *Gastroenterology* 97, 439–445.

Ostapowicz, G., Fontana, R. J., Schiødt, F. V., Larson, A., Davern, T. J., Han, S. H., McCashland, T. M., Shakil, A. O., Hay, J. E., Hynan, L., Crippin, J. S., Blei, A. T., Samuel, G., Reisch, J., Lee, W. M., and U.S. Acute Liver Failure Study Group. (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann. Int. Med.* 137, 947–954.

Renner, E. L. (2007). How to decide when to list a patient with acute liver failure for liver transplantation? Clichy or King's College criteria, or something else? *J. Hepatol.* 46, 554–557.

Reuben, A., Koch, D. G., Lee, W. M., and Acute Liver Failure Study Group. (2010). Drug-induced acute liver failure: results of a US multicenter, prospective study. *Hepatology* 52, 2065–2076.

Richtlinien Organtransplantation gem. §16 Abs. 1 S. 1 Nrn. 2 und 5 TPG. (2011). *Deutsches Ärzteblatt International* 12, A662–A673.

Sagir, A., Erhardt, A., Schmitt, M., and Häussinger, D. (2008). Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. *Hepatology* 47, 592–595.

Schiødt, F. V., Ostapowicz, G., Murray, N., Satyanarana, R., Zaman, A., Munoz, S., and Lee, W. M. (2006). Alpha-fetoprotein and prognosis in acute liver failure. *Liver Transpl.* 12, 1776–1781.

Schmidt, L. E., and Dalhoff, K. (2002). Serum phosphate is an early predictor of outcome in severe acetaminophen-induced hepatotoxicity. *Hepatology* 36, 659–665.

Schmidt, L. E., and Dalhoff, K. (2005). Alpha-fetoprotein is a predictor of outcome in acetaminophen-induced liver injury. *Hepatology* 41, 26–31.

Schmidt, L. E., and Larsen, F. S. (2006). Prognostic implications of hyperlactatemia, multiple organ failure, and systemic inflammatory response syndrome in patients with acetaminophen-induced acute liver failure. *Crit. Care Med.* 34, 337–343.

Schmidt, L., and Larsen, F. S. (2007). MELD score as a predictor of liver failure and death in patients with acetaminophen-induced liver injury. *Hepatology* 45, 789–796.

Schmidt, L. E., and Larsen, F. S. (2010). Is lactate concentration of major value in determining the

prognosis in patients with acute liver failure? Hardly. *J. Hepatol.* 53, 211–212.

Trey, C., and Davidson, C. S. (1970). "The management of fulminant hepatic failure," in *Progress in Liver Diseases*, eds H. Popper and F. Shaffner (New York, NY: Grune and Stratton), 282–298.

Vincent, J. L., Moreno, R., Takala, J., Willatts, S., De Mendonça, A., Bruining, H., Reinhart, C. K., Suter, P. M., and Thijs, L. G. (1996). The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the working group on Sepsis-related problems of the European society of intensive care medicine. *Intensive Care Med.* 22, 707–710.

Volkmann, X., Anstaett, M., Hadem, J., Stiefel, P., Bahr, M. J., Lehner, F., Manns, M. P., Schulze-Osthoff, K., and Bantel, H. (2008). Caspase activation is associated with spontaneous recovery from acute liver failure. *Hepatology* 47, 1624–1633.

Westbrook, R. H., Yeoman, A. D., Joshi, D., Heaton, N. D., Quaglia, A., O'Grady, J. G., Auzinger, G., Bernal, W., Heneghan, M. A., and Wendon, J. A. (2010). Outcomes of severe pregnancy-related liver disease: refining the role of transplantation. *Am. J. Transplant.* 10, 2520–2526.

Yantorno, S. E., Kremers, W. K., Ruf, A. E., Trentadue, J. J., Podestá, L. G., and Villamil, F. G. (2007). MELD is superior to King's College criteria and Clichy's criteria to assess prognosis in fulminant hepatic failure. *Liver Transpl.* 13, 822–828.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 May 2012; accepted: 06 August 2012; published online: 28 August 2012.

Citation: Hadem J, Strassburg CP and Manns MP (2012) Prediction of outcome and selection of the liver transplantat candidate in acute liver failure. Front. Physio. ***3****:340. doi: 10.3389/fphys.2012.00340*

This article was submitted to Frontiers in Gastrointestinal Sciences, a specialty of Frontiers in Physiology.