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Hugh G. Lavery, Daniel J. Antoine, Craig Benson, Masautso Chaponda, Dominic Williams, B. Kevin Park

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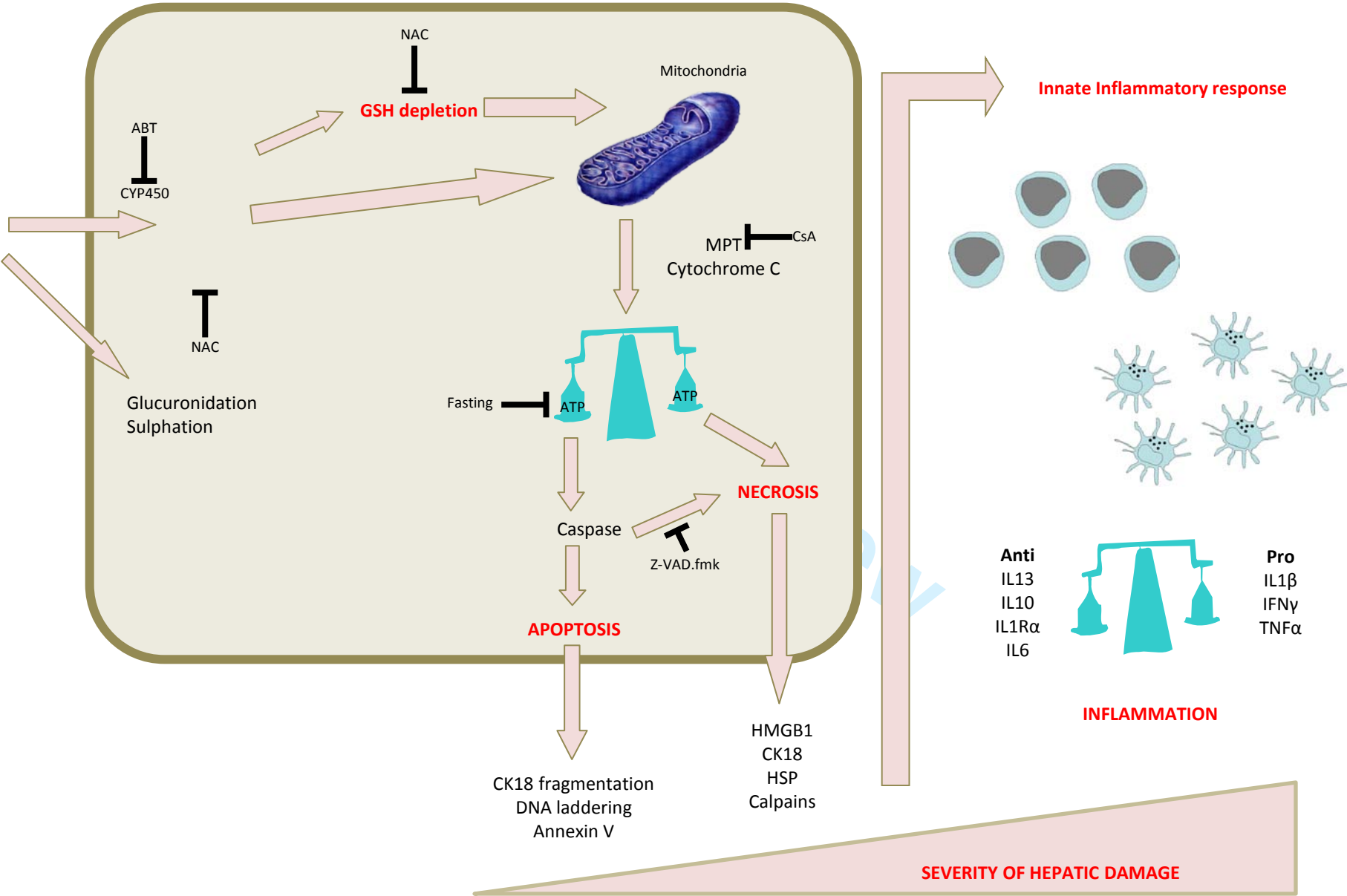


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Normal

Primary Inflammatory response

- $\text{TNF}\alpha$
- $\text{IL1}\beta$
- $\text{IFN}\gamma$

Secondary Inflammatory response

- IL17
- CXCL5
- CXCL9
- CXCL13

Injury

Viral Infection/Alcohol/Diet

Human disease

Reactive
Metabolites
/Drug
Accumulation $\text{TNF}\alpha?$

PAMPS

DAMPS
e.g., HMGB1, HSP $\text{TGF}\beta$

Cirrhosis

DILI

 HMGB1?

Necrosis/inflammation

Acute Liver Failure

 IL13 IL10 $\text{IL1R}\alpha$ IL6 Anti-
Inflammatory

For Peer Review

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The Potential of Cytokines as Safety Biomarkers for Drug-Induced Liver Injury

Laverty HG*¹, Antoine DJ¹, Benson C¹, Chaponda M¹, Williams D¹ and Park BK¹

¹MRC Centre for Drug Safety Science, Department of Pharmacology & Therapeutics, University of
Liverpool, UK. L69 3GE

Corresponding Author

*Dr Hugh Laverty

Tel: +44 (0)151 795 0383

Fax: +44 (0)151 794 5540

hugh.laverty@liv.ac.uk

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Abstract

Drug-induced liver injury (DILI) is an event that has a detrimental impact on drug development and patient safety, therefore the identification of novel biomarkers that are not only sensitive and specific to the liver would have great benefit. Inflammation is known to be associated with human cases of DILI and given the role of cytokines in modulating the inflammatory response changes in cytokine expression patterns certainly show promise as potential biomarkers of DILI. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and accurately quantifiable. In particular, recent interest has developed in mechanism, rather than tissue, specific biomarkers. However, without fully understanding the role of inflammation in DILI, and the role of cytokines in modulating the inflammatory response, they may be limited in their use, being either diagnostic of the type of injury that has occurred and/or prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify, may be better biomarkers for the prognosis of severity of injury. In both cases there is a pressing need for the development and validation of assays sensitive enough and with a sufficient dynamic range to detect changes upon drug treatment. Although promising candidates are appearing in the literature much remains to be done to understand the role of inflammation in DILI and the role that a given cytokine has in the inflammatory cascade associated with DILI before cytokines are viewed as biomarkers for DILI.

Key words

Cytokine, inflammation, pharmaceutical, biomarker, drug-induced liver injury

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Introduction

Drug Induced Liver Injury (DILI) is an adverse event that leads to regulatory action on drugs culminating in serious instances of withdrawal from the market. At a regulatory level hepatotoxicity is the single most frequent reason for removing approved medications from the market. Between 1975 and 1999, 548 new drugs were approved by the FDA of which 10 received a ‘black box warning’ for potential hepatotoxicity and an additional four were withdrawn from the market [1]. The population incidence of drug-induced liver injury is unknown as most events are often inaccurately classified [2-4]. Yet DILI is the most frequent cause of acute liver failure among those under consideration of liver transplantation in US [5], in the UK alone paracetamol (acetaminophen, APAP) overdose accounts for 200-500 deaths and 20-40 liver transplants annually [6]. In drug development animal studies detect approximately half of compounds exhibiting hepatotoxicity in man [7], whereas *in vitro* human hepatocyte testing detects 50-60% of drugs that can cause severe liver injury in man, including some not detected by animal testing [8]. So although current testing methodologies are capable of detecting some hepatotoxins in drug development there are no currently available preclinical tests that detect the potential for serious or idiosyncratic human hepatotoxicity with combined high sensitivity and specificity.

If drugs capable of causing serious DILI at a very low frequency are identified, there is a need for reliable biomarkers that could be used to predict which individuals are susceptible to develop DILI from that drug either before drug exposure, or at a very early time after drug treatment has begun (e.g., assays of substances in blood or urine). Ideally, these biomarkers would discriminate between individuals susceptible to, those capable of adapting to and those that can tolerate DILI. Upon their validation, such biomarkers could facilitate approval of drugs that would otherwise not be possible, by excluding identified susceptible people who should never be exposed to that particular drug [9].

To be able to identify novel biomarkers for evaluation of new chemical entities or determination of individual patient susceptibility requires a thorough understanding of the mechanisms involved in DILI. Although the pathogenesis of DILI is not fully understood, studies of mechanisms in animal models can be used to split DILI into a two stage process. Stage I often involves either excessive drug accumulation, biotransformation and metabolic activation, which can be followed by covalent binding and transport when bioinactivation processes become saturated or immobilized resulting in chemical stress which in turn leads to in a cellular effect e.g., mitochondrial dysfunction (Figure 1). The identification of biomarkers at this stage is difficult unless a ‘drug

related-metabolite' or a modified protein is secreted into the blood stream and/or urine at levels that can be detected. Often the earliest changes observed in safety screening are histopathological and therefore not practical for use as a biomarker in humans. Stage II represents subsequent processes of adaptation or failure of response to modification of essential cellular processes [10] which may be partially mediated by liver nonparenchymal cells, Kupffer cells and/or infiltrated macrophages [11-13]. The changes within the liver at this stage of DILI are more amenable to biomarker discovery. Injured hepatocytes release factors that are chemoattractant and subsequently activate non-parenchymal cells potentially compounding cellular damage by releasing cytotoxic mediators. Although the mediators for progression of DILI have not yet been absolutely defined, recent evidence supports the hypothesis that the types of cytokine produced in response to the initial insult influence the severity of the toxicity [14]. Can we use human liver disease and animal models, where inflammation is known to play a role to better understand the role of inflammation in DILI and in so doing identify novel biomarkers for DILI or identify potential strategies for therapeutic intervention.

Clinical features of DILI and current assessment

Due to complexity within the structure and the function of the liver, clinical symptoms of drug-induced hepatotoxicity may take many forms. DILI may mimic various forms of naturally occurring liver disease, reviewed previously [15]. The assessment of DILI is primarily based on the relatively non-invasive quantification of blood protein biomarkers and hallmarks of hepatic biochemistry. The clinical manifestations of DILI range from mild asymptomatic biochemical changes to fulminant hepatic failure. However, the most useful form of differentiation of hepatic pathology is between cholestatic or hepatocellular injury [15] which is based on the differential quantification of blood hepatic enzymes (Table 1).

Hepatic blood enzymes are roughly grouped in to hepatocellular markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the cholestatic markers alkaline phosphatase (ALP), and γ -glutamyltransferase (GGT). These enzymes are most often elevated in patients with liver disease and may reflect liver injury. However, they are present in tissues throughout the body and often physicians are faced with the problem of a wide differential diagnosis with low specificity. Currently there are no pathognomonic indicators of DILI; even liver biopsy is not diagnostic, while confounding factors such as viral hepatitis or congestive heart failure may not be recognized in the setting of hepatic illness. In certain patient groups, such as diabetic or obese

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persons, for example, liver test abnormalities are common, and other medical conditions such as coronary artery disease and renal failure may confound clear identification of affected patients.

The sensitivity of these tests is questionable. In clinical trials mild elevations of ALT or AST are used as markers of DILI. However, the thresholds used are not consistent between trials in defining DILI: variously placed at twice, thrice, five times or even ten times the upper limit of the normal reference range, but are typically below 40 IU/L. ALT is thought to be a more specific indicator than AST, but normal values vary greatly between laboratories [16]. Despite the association between greatly elevated ALT levels and hepatocellular diseases, the absolute height of the ALT elevation does not correlate with the extent of liver cell damage [17]. Transient serum enzyme activity elevations, however high, are common and can be confusing. The physician is never sure whether they would translate into potentially serious clinical symptoms of hepatic illness.

The use of the current normal range may lead to an underestimated prevalence of liver disease [18]. Because the enzyme concentration in a population forms a continuous distribution, the cut-off concentration that discriminates between healthy and diseased livers is not clearly defined [19]. Indeed several studies have recently questioned whether previously established values to define normal ALT range are accurate and have suggested that the upper limit of normal should be revised [20]. Various scoring tools have been developed to assess causality (RUCAM) usually involving complex calculation of probability based on ALT, ALP, GGT ratios weighed against time of onset, age, alcohol use, viral hepatitis infections, biliary tract disease, or hepatic ischemia. The validation, accuracy and scientific basis of these tools has been problematic. Powerful computing tools and sophisticated Bayesian algorithms [21] have been used but these are not readily available for the clinician at the bedside.

Bilirubin cannot be reliably used as a marker of early liver injury. Once again the threshold is ill-defined and once arbitrarily set can be a late marker of liver injury. Many disease processes and inherited disorders such as Gilbertt’s syndrome may lead to elevated levels of bilirubin not related to DILI and may falsely raise alarm bells to the treating physician. The appearance of jaundice is often a late marker of disease. Another commonly used test, the blood prothrombin time (or its derivative Internationalized Ratio, INR) may be useful as a liver function test (of protein synthesis), however, individual laboratories have to determine their own reference values. In paracetamol overdose the INR can be used prognostically especially on day 2 or 3 to determine deterioration or improvement. However, this too is a late indicator of liver dysfunction and initially may not predict which patients

are likely to deteriorate. Arterial blood lactate measurement rapidly and accurately identifies patients who will die from paracetamol induced acute liver failure. Its use could improve the speed and accuracy of selection of appropriate candidates for transplantation.

Consequently there is no specific non-invasive diagnosis, treatment or prevention of DILI except for the early withdrawal of a drug treatment to the individual in the case of suspected DILI. Currently, the monitoring of the parameters described in Table 1 represents the best indication of hepatic injury. Therefore newer, rapid and reflective non-invasive biomarkers are required that are not only sensitive for hepatic injury but also provide insight into the mechanism of pathology. There are a number of potential biomarkers reflective of hepatocellular damage which may offer superiority over ALT determination [22]. However, there are a limited number of candidates reflective of other pathologies observed during DILI, namely hepatitis, steatosis, fibrosis and cholestasis. Therefore, a comprehensive understanding of the mechanisms that lead to the development of these conditions is required to reveal potential biomarkers specific for established liver pathologies.

Table 1: Standard panel of currently used blood clinical chemistry parameters to assess hepatic function and injury. Taken from [23].

Biomarker	Injury type	Normal reference range
Alanine aminotransferase (ALT)	Hepatocellular	5-40 IU/L
Aspartate aminotransferase (AST)	Hepatocellular	10-40 IU/L
Alkaline phosphatase (AP)	Cholestatic	30-120 IU/L
Gamma glutamyl-transpeptidase (GGT)	Cholestatic	0-51 IU/L
Total bilirubin	Hepatic function	2-14 $\mu\text{mol/L}$
Direct (conjugated) bilirubin	Hepatic function	0-4 $\mu\text{mol/L}$
Pro-thrombin time (coagulation test)	Hepatic function	Local laboratory reference value

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Biomarkers of DILI

Biomarkers are defined as an objectively measured change in either DNA, protein or metabolite levels that is reflective of a normal biological process, pathological condition or correlates with the pharmacological action of a therapeutic agent [23, 24]. The ideal DILI biomarker would have the following characteristics:

- Reflective of the actual hepatic injury/repair status
- Rapidly assayed and can be assayed in any laboratory
- Non-invasive, i.e. present in the blood or urine compartment
- Transferable across *in vitro*, *in vivo* and clinical systems to further inform and accelerate drug safety screening
- Able to precede the onset of massive hepatic injury – secreted markers rather than leakage
- Of a low base-line variability in control samples and normal population
- Accurately identifiable and quantifiable with a suitable dynamic range to detect upon drug treatment

The current clinical standard for assessing inflammation by a blood borne biomarker is through the quantification of C-reactive protein (CRP). CRP is an acute-phase protein which is produced rapidly in response to inflammation and infection. The normal physiological role of CRP is to activate the complement system by c1q by binding phosphocholine expressed on the surface of dead / dying cell (and bacteria) which enhances phagocytosis by macrophages. CRP is synthesised in the liver, therefore CRP holds little utility as a marker of inflammation when hepatic dysfunction or DILI is suspected [25].

Taking the characteristics above in to account, cytokines as potential novel biomarkers of DILI, in particular idiosyncratic DILI, represent promising candidates. With underlying inflammation thought to be a contributing factor for the onset of idiosyncratic DILI [26-29], the quantification of cytokines as potential predictive markers of patient susceptibility holds promise. However, the large variation observed in base-line levels in normal populations renders the establishment of reference values to assay meaningful elevations difficult. There are many established and robust commercially available ELISA assays utilising non-invasive biofluids for the accurate absolute quantification of cytokines. Ease of assay use, the presence in a non-invasive biofluid and the species cross reactivity of antibodies used in ELISAs also aids the translation between pre-clinical and clinical studies. These factors represent the main advantage of cytokines as biomarkers of DILI [30]. In healthy volunteers and control pre-clinical test species with tightly controlled diets and environments cytokine

elevations often exhibit a large dynamic range upon drug treatment. However, it is important to note that added sensitivity and dynamic range may give rise to an increase in false positives, which in turn may lead to the failure to progress an otherwise safe candidate in development or the withdrawal of potential efficacious drug treatment.

When assessing novel candidates as biomarkers, it is important to have a comprehensive understanding of biomarker kinetics and distribution. The active secretion of cytokines from cells present in the liver and at distant sites indicates that blood elevations may occur prior to cell death or organ dysfunction in response to mild, non-toxic cell stress. Moreover, given the short half life of cytokines, they return to background levels rapidly and therefore may also represent markers of reversible hepatic damage, such as that shown recently for High Mobility Group Box-1 protein (HMGB-1) in a mouse model of acetaminophen hepatotoxicity [31]. Focussing upon the kinetics of blood biomarker appearance and a particular signature pattern of cytokine expression may give a real insight into hepatic function and toxicity state. However, caution should be taken in the interpretation of multiple biomarker kinetics because many of these biomarkers are not organ specific. Novel, possibly non-specific, biomarkers of damage should always be assessed alongside the gold standard biomarkers for that organ, e.g. ALT/AST for hepatic damage. The correlation between pro-inflammatory cytokine level changes in blood (increase/decrease) with the extent of liver damage or established biomarkers is not fully known and warrants further investigation.

The Role of inflammatory Cells and Cytokines in Liver Disease

Given that cytokines have several of the features required for novel, mechanism biomarkers what data is available in terms of human disease and liver injury for their use as a biomarker in drug safety. Human liver disease takes many forms and can result from viral hepatitis [31], obesity (fatty liver disease; [32]), alcohol abuse (fatty liver disease, alcoholic hepatitis, cirrhosis [33]), genetics, autoimmune disorders (primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis [34]). As the inflammatory component has been best described in the cases of viral hepatitis and alcoholic liver disease we focus on the common themes in inflammation associated with these two types of liver injury.

Viral Hepatitis

At all stages of viral hepatitis infection chemokines are key regulators of immunity and inflammation functioning within cytokine cascades that regulate the immune response to the virus. However in chronic infection their persistent expression can drive chronic inflammation leading to

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liver injury and cirrhosis [36]. The Hepatitis B Virus (HBV) itself is non-cytopathic but immune mediated liver damage often occurs in patients with both acute and chronic HBV infection. Such damage has been attributed to the killing of infected hepatocytes by virus specific toxic CD8+ T cells [37, 38]. There is increasing evidence to suggest that a non-HBV-specific inflammatory infiltration into the liver is likely responsible for hepatic pathology in patients with chronic hepatitis B [39, 40].

Upon infection with Hepatitis C Virus (HCV) hepatocytes secrete interferon (IFN) and down regulate RNA translation as a response to infection before innate immune cells, including macrophages, dendritic cells (DC), natural killer cells and natural killer T cells are activated to amplify secretion of type-1 IFNs and IFN-response genes. Activation of Toll-like receptors, triggers chemokine secretion which amplifies leukocyte recruitment [41, 42]. The activation of innate immunity by the uptake and processing of viral antigen by DCs is followed by the migration of DCs to the lymph nodes where they present viral antigen to naive T cells leading to their activation and the development of an adaptive immune response [43]. Two types of DCs have been characterised in the response to HCV infection of hepatocytes, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). pDCs secrete type-1 IFN, prime T helper 1 (Th1) responses and activate cascades of chemokine secretion in HCV infection [44]. mDCs prime Th1 responses via interleukin 12 but produce little type-1 IFN [44, 45]. In the early phases of viral infection chemokines particularly CXCL8, CXCL16, CCL2 and CCL3 promote recruitment of innate immune cells to the liver, including DCs which then initiate the adaptive immune response and it is likely that similar events shape the response to HCV infection [46-47].

TH1 immune responses dominate in the HCV-infected liver (Shields et al., 1999) and intrahepatic T cells express chemokine receptors associated with Th1 responses eg., CXCR3 [49]. Hepatic CXCR3 ligands are increased in many liver diseases and this suggests that they play a role in effector cell recruitment to the inflamed liver [50]. Expression of CXCR3 is closely linked to Th1 function and its ligands CXCL9, CXCL10 and CXCL11 are induced by the TH1 cytokines IFN γ and TNF α [51, 52]. In contrast to HCV, HBV infections are characterised by increased numbers of TH17 cells producing a cocktail of cytokines of which IL17A is characterised as a major effector cytokine [53]. IL17A can mobilise, recruit and activate neutrophils leading to massive tissue inflammation and promote the progression of autoimmune disease [54]. Peripheral and intrahepatic TH17 cells are preferentially increased in patients with chronic HBV infections, which may activate mDCs and monocytes to release inflammatory cytokines during chronic HBV infection [55].

Viral clearance is associated with a vigorous CD4⁺ and CD8⁺ T cell response again with Th1 responses dominating. Regulatory T cells (Tregs) have been found in the HCV-infected liver and appear to have a dualistic effect, they suppress HCV-specific CD8⁺ T cells and promote viral replication however they also appear to suppress collateral inflammatory damage to reduce liver injury in chronic infection [56, 57]. Successful antiviral therapy is associated with an increase in circulating CXCR3⁺CD8⁺ T cells and a reduction in CXCL10 and CXCL9 levels in blood [52, 58, 59]. Interestingly the vasculitic lesions of cryoglobulinemia in nerves and skin are characterised by upregulation of CCL3, CCL4 and CXCL10 and infiltration by CXCR3⁺ Th1 cells and CCR5⁺ monocytes. Thus the same pro-inflammatory chemokines are implicated in vasculitic complications of globuliemia and hepatitis [60].

Alcohol Liver Disease

Factors such as gender, age, nationality, weight and health can affect how a person's liver metabolizes alcohol. When the liver has too much alcohol to handle, normal liver function may be interrupted leading to a chemical imbalance. If the liver is required to detoxify alcohol continuously, liver cells may be destroyed or altered resulting in fat deposits (fatty liver) and more seriously, either inflammation (alcoholic hepatitis) and/or permanent scarring (cirrhosis). Liver cancer can also result from alcohol induced liver disease. It is increasingly evident that chronic inflammation represents the driving force in the evolution of alcohol-induced liver injury to steato-hepatitis and cirrhosis. Accordingly, the hepatic expression of pro-inflammatory cytokines precedes the development of histologic signs of necro-inflammation in alcohol fed rats [61], while steato-hepatitis is associated with leucocyte focal infiltration and elevated circulating levels cytokines/chemokines such as TNF α , IL1, IL6, IL8/CINC and macrophage chemotactic protein-1 (MCP1) [62-65].

Innate and adaptive immunity in alcohol liver disease

The interaction between alcohol and hepatic inflammation is complex. Early studies proposed the involvement of innate immunity with the increased translocation of gut-derived endotoxins to the portal circulation being recognised by Kupffer cells via the toll-like receptor-4 (TLR4 [63, 66]). Ethanol itself has been found to enhance the capacity of Kupffer cells to respond to pro-inflammatory stimuli via the up regulation of Toll-like receptors [67] and the production of pro-inflammatory cytokines and chemokines by modulation of signalling pathways such as nuclear transcription factor κ B (NF κ B), early growth response-1 (ERG1) as well as JNK, ERK and p38 protein kinases [65, 68-70].

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Histologic examination of liver biopsies from patients with alcoholic hepatitis reveals the presence of CD4+ and CD8+ T-lymphocytes [71]. The influence of adaptive immunity in the response to alcohol is supported by the predominance of Th1 cytokines (high TNF α , INF γ , IL1) in peripheral blood T-cells isolated from active drinkers with or without ALD [72]. Activated liver-infiltrating IL17-producing T Helper (TH17) cells are also responsible for neutrophil recruitment into the liver [73]. Furthermore serum IL17 levels are increased and serve as a marker of the severity of acute hepatic injury [74]. Evidence is accumulating to suggest that TH17 lymphocytes have an important role in driving inflammation during the development of other chronic inflammatory and autoimmune diseases including inflammatory bowel disease, psoriasis rheumatoid arthritis and multiple sclerosis [75] as well as viral hepatitis as has been discussed.

Alcohol may also have an effect on the immune system by effects on osteopontin, leptin and adiponectin. Osteopontin is a cytokine that enhances the pro-inflammatory Th1 response and the livers of alcohol fed rats have been found to have an associated increase in osteopontin levels [76] while patients with alcoholic hepatitis have higher levels of osteopontin mRNA in their livers compared to patients with fatty liver only [77]. Leptin has been shown to have a pro-inflammatory function while adiponectin counters pro-inflammatory cytokine production [78]. Alcohol appears to have opposite effects on these adipokines, patients with ALD have increased serum leptin [79] whereas chronic ethanol feeding decreases adiponectin secretion in the early phase of alcohol injury in rodents [80, 81].

Signal transduction pathways

In response to alcohol several different signal transduction pathways are activated. Transcription factors regulating fatty acid metabolism including sterol regulatory binding protein (SREBP) and peroxisomal proliferating factor α (PPAR α) play a pivotal role in early alcoholic liver injury [82]. One of the most well studied transcription factors is NF κ B which has a pivotal role in controlling the expression of pro-inflammatory cytokines in monocytes and macrophages. However the expression of NF κ B displayed a surprising response to ethanol treatment depending on whether the treatment is acute or chronic. Differences have been noted in the ability of acute and chronic alcohol exposure to modulate monocyte/macrophage activation. Acute alcohol exposure is associated with LPS-induced anti-inflammatory effects in monocytic cells in humans both *in vivo* and *in vitro* [83-85]. By contrast chronic alcohol induces the expression of TNF α in human and mouse models [69, 86, 87]. This switch between anti-inflammatory and pro-inflammatory responses is linked with changes in IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of LPS

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signalling and leads to decreased downstream activation of LPS signalling cascade culminating in impaired NF κ B DNA binding and transactivation and finally TNF α production. In contrast chronic alcohol exposure switches the anti-inflammatory to a pro-inflammatory response by decreasing IRAK-M and increasing IRAK-1 and IKK kinase activation followed by enhanced NF κ B DNA binding, transactivation and ERK kinase activation and TNF α production [88]. Other transcription factors such as Activator Protein-1 (AP1), PPAR γ and Egr1 have also been shown to be modulated by exposure to alcohol [89, 90, 91].

Animal models of DILI associated inflammation

There is a lot of data to support the role of inflammation in the progression of human liver disease as has been described for viral hepatitis and alcohol liver disease. Currently sufficient data to support an unequivocal role for inflammation in DILI is lacking and in many cases mechanistic details are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate immune system within the liver has been obtained from experimental *in vivo* models. The advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular and immunological backgrounds of inbred strains. This lack of variation enables a more complete exploration and determination of possible inflammatory mechanisms contributing to the overall pathophysiology. However, although animal models are useful tools with which to investigate DILI and the involvement of inflammation caution must be shown in the translation of these results to human DILI.

Inflammation in an acetaminophen (APAP) model of DILI

A widely studied model of DILI has been APAP-induced liver injury (AILI) in mice (Table 2). Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of cellular contents known as damage associated molecular patterns (DAMPs). These include HMGB-1 or Heat shock proteins (HSP) which act as signals which can activate cells of the innate immune system which reside in the liver. This in turn leads to hepatic infiltration of inflammatory cells which may contribute to the progression of liver injury by producing pro-inflammatory mediators such as cytokines and chemokines as well as reactive oxygen and nitrogen species (Figure 1, [92]). Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognised by Mitchell et al. [93] but the relevance of this accumulation to the pathophysiology is still unclear [94]. A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic injury but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these cells did not protect against AILI [95]. In a rat model of AILI, a significant reduction in neutrophil

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accumulation showed no protection of liver injury [96]. These findings are in contrast to other models in which neutrophils are involved in the course of injury where pre-treatment with an anti-neutrophil antiserum attenuated AILI in rats [97].

Other studies have demonstrated that mice treated with gadolinium chloride to inactivate KCs [98, 99] and liposome/chlodronate to deplete KCs [99, 100] are protected against APAP toxicity. Though reports suggest that KC depletion confers protection at early times after APAP treatment [100]but can lead to more severe injury at later time points [99]. The use of an anti-NK1.1 monoclonal antibody to deplete NK and NKT cells significantly protected mice from APAP toxicity probably due to the reduction in the levels of the pro-inflammatory cytokine IFN- γ [101]. However as has been observed with KC depletion, there are conflicting reports suggesting that NK/NKT cells play little role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [102, 103]. This has been supported by a recent study that suggested NK and NKT cells only contributed to liver injury when dimethyl sulfoxide (DMSO) was used to solubilise APAP [103]. Given the conflicting data available on neutrophils and KCs it is clear that more studies are required to determine their precise role in DILI.

The involvement of various pro-inflammatory mediators such as TNF- α [104-106], IL-1ra [107], IFN- γ [101, 108], in promoting tissue damage have been demonstrated whereas other cytokines such as IL-10 and IL-4 [109] have been found to be hepatoprotective in models of APAP hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor [106], p55 TNF- α receptor-1 [105], Toll-like receptors TLR4 and -9 [110, 111] and receptor for advanced glycation end-products (RAGE) [112] have been found to be important in promoting an inflammatory response and subsequent pathogenesis.

Table 2: Summary of inflammatory models used in APAP-induced hepatotoxicity

References	Inflammatory model(s)	Reported effect
[99, 100]	KC depletion	Protection against liver injury at early time points, more susceptible at later time points
[113]	Neutrophil depletion	Significantly protected against liver injury
[101]	NK/NKT cell depletion	Significantly protected against liver injury
[106]	Anti-TNF- α /IL-1- α /IL-1ra	Increased susceptibility to liver injury
[105]	Anti-TNF- α , TNF-p55 receptor	Reduced mortality and liver injury
	1 ^{-/-} mice	
[120]	TNFR1 ^{-/-} mice	Increased susceptibility to liver injury
[108]	IFN- γ ^{-/-} mice	Significantly protected against liver injury
[117]	IL-6 ^{-/-} mice	Increased susceptibility to liver injury
[109]	IL-10/4/6 ^{-/-} mice	Significantly protected against liver injury
	IL-10/4 ^{-/-} mice	Increased susceptibility to liver injury
[118]	IL-13 ^{-/-} mice	Increased susceptibility to liver injury
[111]	Anti-TLR9, TLR9 ^{-/-} , Nalp3 ^{-/-}	Reduced mortality and liver injury
	mice	
[112]	Soluble RAGE	Reduced mortality and liver injury
[115]	LPS	Increased susceptibility to liver injury

Comparison of the inflammatory response in animal models of acetaminophen-induced liver injury

Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500mg/kg, i.p) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [101, 113]. An increase of hepatic neutrophil accumulation as early as 4-6 hours with a significant 3-fold increase by 24 hours was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF- α and IFN- γ [101]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an anti-neutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice

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against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic inflammatory cell infiltration was observed over a 24 hour time course [114].

Non-toxic doses of APAP (100-400mg/kg, i.p) administered 2 hours after a dose of LPS (44×10^6 EU/kg) to fasted C57BL6 mice have been shown to produce a leftward shift of the dose-response curve for AILI [115]. Application of LPS led to significantly greater TNF- α production than APAP alone. With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 - 24 hours after 300mg/kg APAP. However neutrophils were not apparent in livers of mice treated with a non-toxic dose of APAP (175mg/kg) but significant accumulation occurred when this dose was co-administered with a non-injurious dose of LPS. Two hours after treatment with LPS (0 hours after APAP), serum TNF- α levels were large and rapidly decreased thereafter, confirming that the inflammatory response observed in this model was due to LPS [115]. These observations support the hypothesis that inflammation induced by LPS and associated elevated levels of TNF- α increase sensitivity to AILI.

Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, MIF and IL13 dosed with APAP (200-300mg/kg, i.p) displayed increased susceptibility to AILI [109,116-118]. Neutrophils were found to accumulate to a greater extent in IL13 KO mice than wild-type treated with APAP with the level of TNF- α , IL-6 and IFN- γ increased at 4 hours in WT and IL-13 KO APAP-treated animals compared to saline-treated controls [118]. These data suggest a hepatoprotective role for anti-inflammatory mediators in these models and that the balance between the Th1 and Th2 response is an important determinant for AILI. Susceptibility to AILI was compared between two mouse strains, C57BL6 (more susceptible to AILI and which develop predominantly a Th1 response) and BALB/c (less susceptible to AILI and which develop a Th2 response). 24hr after exposure to APAP C57BL6 mice produce high levels of TNF- α (pro-inflammatory) whereas levels in BALB/c mice show no difference. On the other hand IL-6 (anti-inflammatory) levels were higher in BALB/c than C57BL6 24 hours after administration. These data support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine balance and TNF- α may play a role in toxicity [117].

However, the role of TNF- α remains controversial with contradictory reports suggesting that TNF α is involved in pathogenesis of DILI [105-106]; is not involved in toxicity [104-119] or it has a role in defence against toxicity and repair [120-121]. Data suggests that like most cytokines the effects of TNF α in liver toxicity are pleiotropic with other cytokine networks being involved and it is

the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. IL1 α [106] and INF γ [108] have been shown to play a pathogenic role in APAP-induced hepatotoxicity whilst IL-6 [117] and IL10 [116] play a protective role. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for DAMPS or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [16].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during drug-induced liver injury (Table 3). Given the complexity of interplay between chemically mediated toxicity and inflammation, and the differences observed between strains of mice, species and *in vivo* model pre-treatment further investigation is required to define the precise role a cytokine has in response to the injury or as part of the inflammatory response. Once this has been defined then the value of the cytokine as a biomarker can be assessed.

Table 3: Inflammation in other models of DILI

DILI Model	Inflammatory factors investigated	References
LPS	Neutrophils, TNF- α , KCs	[122-124]
EtOH	Kupffer cells, TNF- α	[125-128]
Halothane	Macrophage inhibitory factor	[116]
Cocaine + LPS	Kupffer cells, nitric oxide	[129, 130]
Galactosamine + LPS	TNF- α , TNF-p55 receptor	[131, 132]

Cytokine expression in clinical studies of acetaminophen-toxicity

As has been discussed many studies have investigated the role of cytokines in animal models. Some of these cytokines have been reported to have hepatoprotective effects in animal models and several studies have been performed to investigate cytokine expression patterns in serum samples from different patient cohorts. In a study of 111 patients (90 female; mean age 13.6 years) plasma concentrations of IL6, IL8, IL10 and Monocyte chemoattractant protein 1 (MCP1) were analysed by enzyme-linked immunosorbent assay. Patients were stratified by toxicity severity as defined by the maximal values of hepatic transaminase elevation. Levels of IL6, IL8 and MCP1 were elevated in patients with elevated serum alanine aminotransferase and MCP1 expression had the strongest association with toxicity [133]. Other studies have found associations between elevated

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expression levels of IL8 [134, 135]; IL6 [136] and acetaminophen-induced hepatotoxicity. While the results from these studies indicate that inflammation and cytokines are associated with the progression of late stage toxicity interpretation of the data is often limited by the absence of pre-dose data, a lack of samples from early in the process of drug-induced injury and the small number of samples usually obtained in the clinic.

Several other studies have investigated the expression of various cytokines associated with various forms of acute liver failure (ALF). In a mixed cohort of patients with ALF and acutely decompensated cirrhosis it was found that not only was the degree of systemic inflammation, as measured by IL6 and TNF α , important for determining outcome but that the magnitude of the anti-inflammatory response was also important. In the whole cohort, IL10 (regarded as an anti-inflammatory cytokine) levels were found to predict outcome as accurately as IL6 [137]. However these patients are experiencing acute liver failure and while expression of the cytokine is linked with disease progression the patient cohorts are characterised by end stage severe liver failure and it is therefore difficult to relate cytokine expression patterns with the earliest stages of injury when intervention is required to prevent toxicity from developing further.

Recently a couple of studies have been reported which attempted to address the issues surrounding patient sampling by treating volunteers with a bolus of acetaminophen under controlled conditions. This has allowed the determination of a pre-acetaminophen treatment baseline for parameters and accurate timing of sampling post-acetaminophen dose. DNA microarrays and serum metabolomic methods were employed to characterise changes in the transcriptome and metabolome in serial peripheral blood (PB) samples obtained from six healthy adults treated with a 4-g bolus of acetaminophen and from three receiving placebo. Treatment did not cause liver toxicity as assessed by traditional liver chemistries. It was found that a single 4-g bolus of acetaminophen produced a transcriptomic signature in PB cells characterised by down regulation of oxidative phosphorylation genes accompanied by increased serum lactate. Similar gene expression changes were observed in rats and several patients after consuming hepatotoxic doses of acetaminophen [138]. While this study is exciting as it begins to allow an insight into the progression of acetaminophen induced liver injury in a controlled setting, it also highlights the challenges faced in identifying novel biomarkers for DILI that can be applied in the clinic. The changes reported in this paper occurred 48 hours after the acetaminophen was administered to volunteers and are therefore likely to be downstream of liver injury. The authors also recognise that the mechanism by which liver toxicity affects peripheral blood cells is unclear, are the changes observed direct or indirect effects?

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5 In a separate study it was shown that urine metabolite profiles from healthy adults each
6 receiving 4g of acetaminophen per day for 7 days profiles obtained before start of treatment were
7 not sufficient to distinguish which of the subjects would develop mild liver injury. However profiles
8 obtained shortly after the start of treatment but prior to ALT elevation, could distinguish responders
9 from non-responders [139]. This data suggests that an early-intervention pharmaco-metabolomics
10 approach could be used to identify potential toxicities early, however much more work is required to
11 validate this approach. However in both these studies it is interesting to note that few, if any, of the
12 changes described were associated with cytokines. Analysis of liver chemistries showed that liver
13 toxicity had not been caused by the dose of paracetamol used, therefore it indicates supports the
14 hypothesis that inflammation and cytokines are involved only after overt liver damage has occurred.
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24 **Immunological involvement in idiosyncratic DILI – the example of halothane**

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26 Lessons from animal studies have identified the potential role of the innate immune
27 response in dictating the overall response during APAP hepatotoxicity; however, this stills needs to
28 be proven in man. While the role of immune cells, and by extension cytokines, remains unclear
29 other approaches are been taken to identify biomarkers. Some of the characteristics of idiosyncratic
30 DILI reactions appear to have an immune component and as many autoimmune functions appear to
31 be mediated by TH17 cells, it was hypothesised that IL17 may play a role in iDILI [140]. Expression
32 patterns of cytokines were examined in the serum of 39 patients with acute liver failure (ALF) due to
33 IDILI and compared the values with those from 21 patients with acetaminophen-induced ALF and 10
34 patients with viral hepatitis-induced ALF. IL-17 levels were found to be elevated in a similar number
35 of patients IDILI and acetaminophen-induced ALF and occasionally in patients with viral hepatitis.
36 There was overlap in the level of expression of IL-21 in patients with iDILI and AILI. Auto-antibodies
37 were more frequent in patients in the IDILI group but were absent in most patients. These data
38 provide a picture of the cytokine/chemokine profile in patients with various types of ALF. The
39 pattern varies from patient to patient and not specifically by etiology. This suggests that different
40 underlying disease mechanisms may be at play in different individuals, even among those
41 demonstrating injury from the same drug. Since cytokines may originate from more than one type of
42 cell, interpretation of results of cytokine assays remains difficult in complex disease settings.
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57 However it is certainly possible that an adaptive immune response may play a role in cases
58 of human DILI. Certain drug reactions have clinical hallmarks of an immunological mechanism, the
59 liver alone may be involved or liver injury may be part of a more complex hypersensitivity syndrome.
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Unlike with APAP, where the initiation of hepatotoxicity is dependent upon the intrinsic chemistry of the molecule, these so called idiosyncratic hepatotoxicity reactions represent a function of an individual patient’s biology (Table 4).

The inhalation anaesthetic halothane is the best-studied drug causing an immunoallergic response in a subset of patients. The response ranges from asymptomatic rises in transaminases to fulminant hepatitis, which is rare but serious. Most of the patients recorded in the literature with immunoallergic hepatitis had more than one exposure [141]. Antibodies have been detected in such patients that recognise autoantigens and neoantigens created by trifluoroacetylation (TFA) of hepatic proteins. Pre-incubation of halothane pre-treated rabbit hepatocytes with sera of patients with halothane-induced fulminant hepatitis increased susceptibility to cytotoxicity of lymphocytes *in vitro* [142]. It is likely drug specific T cells play a role in the pathogenesis of hepatocyte injury but direct evidence is lacking. Halothane is metabolised by CYP450 2E1, like APAP, but forms a chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only metabolic route available. It is hypothesised that the formation of the acyl halide triggers an immune response and that mild and severe immune responses are likely to have this common initiation mechanism. Evidence for this concept of an immune trigger is both direct and indirect, with drug specific antibodies indicative that the drug has initiated an immune response in affected patients and newer metabolically inert inhaled anaesthetics being rarely associated with hepatotoxicity. Target proteins modified by the acyl halide have been identified with the principal chemical modification trifluoroacetylation of lysine residue [143-146]. Chemical modification of protein(s) is associated with the immune response observed with halothane hepatitis, however, there is no conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been observed with idiosyncratic drug reactions to Tienilic acid [147] and dihydralazine [148] but there is no unequivocal evidence that DILI such as that caused by halothane is immune mediated. Most of the information available is compatible with the hapten hypothesis where the drug undergoes bioactivation in hepatocytes leading to drug–protein conjugate formation in the liver. The current theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents interplay between a number of factors, which include the chemistry of the drug, drug metabolism and the immune system of the patient (Table 4). Whether cytokines may be useful biomarkers in such idiosyncratic cases is currently unknown as the data supporting immune involvement is equivocal and the mechanistic details are unknown. However, if the hapten hypothesis is correct what is clear is that the inflammatory response induced by chemical insult such as APAP overdose compared to that of idiosyncratic DILI may be very different.

Table 4: Idiosyncratic drug reactions with evidence of immune involvement (amended from [149])

Drug Class	Drug Name	Is there evidence to support immune involvement?
Anaesthetic	Halothane	Yes
Analgesic	Diclofenac	Yes/No
	Sulindac	Yes
Antibiotic	Amoxicillin	Yes
	Erythromycins	Yes
	Minocycline	Yes
	Nitrofluantoin	Yes
	Rifampicin	Yes/No
Anti-convulsant	Phenytoin	Yes
	Phenothiazines	Yes
Anti-epileptic	Carbamazepine	Yes/No
Antihypertensive	Dihydralazine	Yes
	Methyldopa	Yes
	Tienilic acid	Yes
Anti-thyroid	Propylthiouracil	Yes
Depression	Tricyclic antidepressants	Yes
Gout	Allopurinol	Yes
Hypertension	ACE inhibitors	Yes

Conclusions and perspectives

An analysis of the role of inflammation in human liver disease and in the *in vivo* models described offer insights into the potential role of inflammation in human DILI. Given the role of cytokines in modulating the inflammatory response changes in patterns of cytokine expression certainly show promise as potential biomarkers of DILI. However, the *context* of cytokine expression will determine their utility as biomarkers. This context will be determined by understanding the processes within DILI and the associated inflammatory cascade in which the cytokine has a role (Figure 1). One key question is whether the inflammatory response precedes the injury or is a consequence of the injury (Figure 2). Also, inflammation is associated with a lot of confounding factors experienced in the clinic (as previously described) therefore much more data is required in order to support the context of a given cytokines' qualification for use as a biomarker and the proposed application of that biomarker. Clearly prior to selecting a given cytokine as a biomarker it

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is important to accurately phenotype DILI and to have a thorough understanding of the processes and mechanisms by which injury occurs.

As discussed the balance of pro and anti-inflammatory cytokines is thought to play a significant role in dictating the overall balance of hepatic injury exacerbation or regeneration, in particular for DILI characterised as idiosyncratic in nature (Figure 2). The majority of animal models of DILI are exposed to profound, often lethal levels of chemical stress with changes in the expression of cytokines such as TNF α , HMGB-1, IL6, IL10 and IL1 β [16, 18-20]. Therefore animal model data may be misrepresentative of the majority of cases of liver injury observed clinically. The promotion of inflammation may be the most significant consequence of chemical stress in the exacerbation of DILI, in which case, cytokines remain an attractive option as biomarkers of DILI to inform the mechanism of pathology, sensitively screen for hepatic injury and predict at risk patients.

Even when a cytokine has been shown to be a candidate biomarker the benefit from use of a novel biomarker in terms of safety efficacy and or cost must be clearly established. Biomarkers must be sensitive and specific and this requires accurate definition of the context for which a biomarker should be qualified [150]. Context and qualification for new biomarkers are assessed relative to current biomarkers. If the sensitivity and specificity of current biomarkers are not perfect relative to a specific end point, the context and qualification of new biomarkers may not be accurately established. This is a particularly difficult problem if current biomarkers have pseudo-quantitative, qualitative, or subjective metrics associated with them. Even the data available for the gold standard of DILI (ALT) is continually reviewed to ensure that the context of its use is as accurate as possible [151].

Much remains to be understood about the role of inflammation in DILI and cytokines in controlling this inflammatory response. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and quantifiable. However without fully understanding the role of inflammation in DILI they may be limited in their use to being diagnostic of the type of injury that has occurred and maybe prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify may be better biomarkers for the prognosis of whether an injury will occur (Figure 1). However, further investigation is required to understand the impact of chemical stress on cellular physiology and the release of intracellular components in response to injury as well as the development of assays sensitive enough to accurately detect them.

Figure Legends

Figure 1 Paracetamol-induced hepatotoxicity: a multistep/multicellular process

The multi-step process and multi-cell type involvement during acetaminophen-induced hepatotoxicity. Acetaminophen (APAP) hepatotoxicity is dependent upon the metabolic activation by CYP450 to the reactive metabolite NAPQI. Following saturation of the phase II conjugation detoxification pathways, NAPQI becomes covalently bond to hepatic protein (in particular mitochondrial protein) which leads to cell death. Cell death can proceed through either apoptosis or necrosis depending upon the bio-energetic status of the cell. So called DAMPs (e.g HMGB-1) are released from necrotic cells which hold the potential to activate the innate immune response. Evidence from animal models suggests that the overall extent of hepatic damage is dictated by the balance between the production of pro and anti-inflammatory cytokines. The dependency upon the multiple steps has been elucidated experimentally in vitro and in vivo from CYP450 dependence (inhibitor; ABT), glutathione level (NAC; N-acetyl-cysteine), mitochondrial permeability transition (CsA; cyclosporine), and cellular ATP status (dietary restriction) to caspase activation (inhibitor; Z-VAD.fmk)

Figure 2 Inflammatory etiology of acute liver failure

Acute liver failure stimulated by a variety of inducing agents leading to the development of the pathology often share common pathways through the activation of pro-inflammatory pathways. Initial necrotic cell death from reactive metabolites, drug accumulation, diet, alcohol or viruses can lead to the release of DAMPs which in turn activate the primary and secondary inflammatory cascades. Underlying hepatitis can influence an individual's susceptibility to hepatotoxicity. Understanding the mechanisms involved from the study of liver diseases or the particular serum cytokine finger print when applied to DILI may help to predict susceptible individuals or patients likely to develop severe hepatotoxicity

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The Potential of Cytokines as Safety Biomarkers for Drug-Induced Liver Injury

Laverty HG^{*1}, Antoine DJ¹, Benson C¹, Chaponda M¹, Williams D¹ and Park BK¹

¹MRC Centre for Drug Safety Science, Department of Pharmacology & Therapeutics, University of Liverpool, UK. L69 3GE

Corresponding Author

*Dr Hugh Laverty

Tel: +44 (0)151 795 0383

Fax: +44 (0)151 794 5540

hugh.laverty@liv.ac.uk

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Abstract

Drug-induced liver injury (DILI) is an event that has a detrimental impact on drug development and patient safety, therefore the identification of novel biomarkers that are not only sensitive and specific to the liver would have great benefit. Inflammation is known to be associated with human cases of DILI and given the role of cytokines in modulating the inflammatory response changes in cytokine expression patterns certainly show promise as potential biomarkers of DILI. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and accurately quantifiable. In particular, recent interest has developed in mechanism, rather than tissue, specific biomarkers. However, without fully understanding the role of inflammation in DILI, and the role of cytokines in modulating the inflammatory response, they may be limited in their use, being either diagnostic of the type of injury that has occurred and/or prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify, may be better biomarkers for the prognosis of severity of injury. In both cases there is a pressing need for the development and validation of assays sensitive enough and with a sufficient dynamic range to detect changes upon drug treatment. Although promising candidates are appearing in the literature much remains to be done to understand the role of inflammation in DILI and the role that a given cytokine has in the inflammatory cascade associated with DILI before cytokines are viewed as biomarkers for DILI.

Key words

Cytokine, inflammation, pharmaceutical, biomarker, drug-induced liver injury

Introduction

Drug Induced Liver Injury (DILI) is an adverse event that leads to regulatory action on drugs culminating in serious instances of withdrawal from the market. At a regulatory level hepatotoxicity is the single most frequent reason for removing approved medications from the market. Between 1975 and 1999, 548 new drugs were approved by the FDA of which 10 received a 'black box warning' for potential hepatotoxicity and an additional four were withdrawn from the market [1]. The population incidence of drug-induced liver injury is unknown as most events are often inaccurately classified [2-4]. Yet DILI is the most frequent cause of acute liver failure among those under consideration of liver transplantation in US [5], in the UK alone paracetamol (acetaminophen, APAP) overdose accounts for 200-500 deaths and 20-40 liver transplants annually [6]. In drug development animal studies detect approximately half of compounds exhibiting hepatotoxicity in man [7], whereas *in vitro* human hepatocyte testing detects 50-60% of drugs that can cause severe liver injury in man, including some not detected by animal testing [8]. So although current testing methodologies are capable of detecting some hepatotoxins in drug development there are no currently available preclinical tests that detect the potential for serious or idiosyncratic human hepatotoxicity with combined high sensitivity and specificity.

If drugs capable of causing serious DILI at a very low frequency are identified, there is a need for reliable biomarkers that could be used to predict which individuals are susceptible to develop DILI from that drug either before drug exposure, or at a very early time after drug treatment has begun (e.g., assays of substances in blood or urine). Ideally, these biomarkers would discriminate between individuals susceptible to, those capable of adapting to and those that can tolerate DILI. Upon their validation, such biomarkers could facilitate approval of drugs that would otherwise not be possible, by excluding identified susceptible people who should never be exposed to that particular drug [9].

To be able to identify novel biomarkers for evaluation of new chemical entities or determination of individual patient susceptibility requires a thorough understanding of the mechanisms involved in DILI. Although the pathogenesis of DILI is not fully understood, studies of mechanisms in animal models can be used to split DILI into a two stage process. Stage I often involves either excessive drug accumulation, biotransformation and metabolic activation, which can be followed by covalent binding and transport when bioinactivation processes become saturated or immobilized resulting in chemical stress which in turn leads to in a cellular effect e.g., mitochondrial dysfunction (Figure 1). The identification of biomarkers at this stage is difficult unless a 'drug

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related-metabolite' or a modified protein is secreted into the blood stream and/or urine at levels that can be detected. Often the earliest changes observed in safety screening are histopathological and therefore not practical for use as a biomarker in humans. Stage II represents subsequent processes of adaptation or failure of response to modification of essential cellular processes [10] which may be partially mediated by liver nonparenchymal cells, Kupffer cells and/or infiltrated macrophages [11-13]. The changes within the liver at this stage of DILI are more amenable to biomarker discovery. Injured hepatocytes release factors that are chemoattractant and subsequently activate non-parenchymal cells potentially compounding cellular damage by releasing cytotoxic mediators. Although the mediators for progression of DILI have not yet been absolutely defined, recent evidence supports the hypothesis that the types of cytokine produced in response to the initial insult influence the severity of the toxicity [14]. Can we use human liver disease and animal models, where inflammation is known to play a role to better understand the role of inflammation in DILI and in so doing identify novel biomarkers for DILI or identify potential strategies for therapeutic intervention.

Clinical features of DILI and current assessment

Due to complexity within the structure and the function of the liver, clinical symptoms of drug-induced hepatotoxicity may take many forms. DILI may mimic various forms of naturally occurring liver disease, reviewed previously [15]. The assessment of DILI is primarily based on the relatively non-invasive quantification of blood protein biomarkers and hallmarks of hepatic biochemistry. The clinical manifestations of DILI range from mild asymptomatic biochemical changes to fulminant hepatic failure. However, the most useful form of differentiation of hepatic pathology is between cholestatic or hepatocellular injury [15] which is based on the differential quantification of blood hepatic enzymes (Table 1).

Hepatic blood enzymes are roughly grouped in to hepatocellular markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the cholestatic markers alkaline phosphatase (ALP), and γ -glutamyltransferase (GGT). These enzymes are most often elevated in patients with liver disease and may reflect liver injury. However, they are present in tissues throughout the body and often physicians are faced with the problem of a wide differential diagnosis with low specificity. Currently there are no pathognomonic indicators of DILI; even liver biopsy is not diagnostic, while confounding factors such as viral hepatitis or congestive heart failure may not be recognized in the setting of hepatic illness. In certain patient groups, such as diabetic or obese

persons, for example, liver test abnormalities are common, and other medical conditions such as coronary artery disease and renal failure may confound clear identification of affected patients.

The sensitivity of these tests is questionable. In clinical trials mild elevations of ALT or AST are used as markers of DILI. However, the thresholds used are not consistent between trials in defining DILI: variously placed at twice, thrice, five times or even ten times the upper limit of the normal reference range, but are typically below 40 IU/L. ALT is thought to be a more specific indicator than AST, but normal values vary greatly between laboratories [16]. Despite the association between greatly elevated ALT levels and hepatocellular diseases, the absolute height of the ALT elevation does not correlate with the extent of liver cell damage [17]. Transient serum enzyme activity elevations, however high, are common and can be confusing. The physician is never sure whether they would translate into potentially serious clinical symptoms of hepatic illness.

The use of the current normal range may lead to an underestimated prevalence of liver disease [18]. Because the enzyme concentration in a population forms a continuous distribution, the cut-off concentration that discriminates between healthy and diseased livers is not clearly defined [19]. Indeed several studies have recently questioned whether previously established values to define normal ALT range are accurate and have suggested that the upper limit of normal should be revised [20]. Various scoring tools have been developed to assess causality (RUCAM) usually involving complex calculation of probability based on ALT, ALP, GGT ratios weighed against time of onset, age, alcohol use, viral hepatitis infections, biliary tract disease, or hepatic ischemia. The validation, accuracy and scientific basis of these tools has been problematic. Powerful computing tools and sophisticated Bayesian algorithms [21] have been used but these are not readily available for the clinician at the bedside.

Bilirubin cannot be reliably used as a marker of early liver injury. Once again the threshold is ill-defined and once arbitrarily set can be a late marker of liver injury. Many disease processes and inherited disorders such as Gilbert's syndrome may lead to elevated levels of bilirubin not related to DILI and may falsely raise alarm bells to the treating physician. The appearance of jaundice is often a late marker of disease. Another commonly used test, the blood prothrombin time (or its derivative Internationalized Ratio, INR) may be useful as a liver function test (of protein synthesis), however, individual laboratories have to determine their own reference values. In paracetamol overdose the INR can be used prognostically especially on day 2 or 3 to determine deterioration or improvement. However, this too is a late indicator of liver dysfunction and initially may not predict which patients

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2 are likely to deteriorate. Arterial blood lactate measurement rapidly and accurately identifies
3 patients who will die from paracetamol induced acute liver failure. Its use could improve the speed
4 and accuracy of selection of appropriate candidates for transplantation.
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8 Consequently there is no specific non-invasive diagnosis, treatment or prevention of DILI
9 except for the early withdrawal of a drug treatment to the individual in the case of suspected DILI.
10 Currently, the monitoring of the parameters described in Table 1 represents the best indication of
11 hepatic injury. Therefore newer, rapid and reflective non-invasive biomarkers are required that are
12 not only sensitive for hepatic injury but also provide insight into the mechanism of pathology. There
13 are a number of potential biomarkers reflective of hepatocellular damage which may offer
14 superiority over ALT determination [22]. However, there are a limited number of candidates
15 reflective of other pathologies observed during DILI, namely hepatitis, steatosis, fibrosis and
16 cholestasis. Therefore, a comprehensive understanding of the mechanisms that lead to the
17 development of these conditions is required to reveal potential biomarkers specific for established
18 liver pathologies.
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25 **Table 1:** Standard panel of currently used blood clinical chemistry parameters to assess hepatic
26 function and injury. Taken from [23].
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Biomarker	Injury type	Normal reference range
Alanine aminotransferase (ALT)	Hepatocellular	5-40 IU/L
Aspartate aminotransferase (AST)	Hepatocellular	10-40 IU/L
Alkaline phosphatase (AP)	Cholestatic	30-120 IU/L
Gamma glutamyl-transpeptidase (GGT)	Cholestatic	0-51 IU/L
Total bilirubin	Hepatic function	2-14 µmol/L
Direct (conjugated) bilirubin	Hepatic function	0-4 µmol/L
Pro-thrombin time (coagulation test)	Hepatic function	Local laboratory reference value

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Biomarkers of DILI

Biomarkers are defined as an objectively measured change in either DNA, protein or metabolite levels that is reflective of a normal biological process, pathological condition or correlates with the pharmacological action of a therapeutic agent [23, 24]. The ideal DILI biomarker would have the following characteristics:

- Reflective of the actual hepatic injury/repair status
- Rapidly assayed and can be assayed in any laboratory
- Non-invasive, i.e. present in the blood or urine compartment
- Transferable across *in vitro*, *in vivo* and clinical systems to further inform and accelerate drug safety screening
- Able to precede the onset of massive hepatic injury – secreted markers rather than leakage
- Of a low base-line variability in control samples and normal population
- Accurately identifiable and quantifiable with a suitable dynamic range to detect upon drug treatment

The current clinical standard for assessing inflammation by a blood borne biomarker is through the quantification of C-reactive protein (CRP). CRP is an acute-phase protein which is produced rapidly in response to inflammation and infection. The normal physiological role of CRP is to activate the complement system by C1q by binding phosphocholine expressed on the surface of dead / dying cell (and bacteria) which enhances phagocytosis by macrophages. CRP is synthesised in the liver, therefore CRP holds little utility as a marker of inflammation when hepatic dysfunction or DILI is suspected [25].

Taking the characteristics above in to account, cytokines as potential novel biomarkers of DILI, in particular idiosyncratic DILI, represent promising candidates. With underlying inflammation thought to be a contributing factor for the onset of idiosyncratic DILI [26-29], the quantification of cytokines as potential predictive markers of patient susceptibility holds promise. However, the large variation observed in base-line levels in normal populations renders the establishment of reference values to assay meaningful elevations difficult. There are many established and robust commercially available ELISA assays utilising non-invasive biofluids for the accurate absolute quantification of cytokines. Ease of assay use, the presence in a non-invasive biofluid and the species cross reactivity of antibodies used in ELISAs also aids the translation between pre-clinical and clinical studies. These factors represent the main advantage of cytokines as biomarkers of DILI [30]. In healthy volunteers and control pre-clinical test species with tightly controlled diets and environments cytokine

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elevations often exhibit a large dynamic range upon drug treatment. However, it is important to note that added sensitivity and dynamic range may give rise to an increase in false positives, which in turn may lead to the failure to progress an otherwise safe candidate in development or the withdrawal of potential efficacious drug treatment.

When assessing novel candidates as biomarkers, it is important to have a comprehensive understanding of biomarker kinetics and distribution. The active secretion of cytokines from cells present in the liver and at distant sites indicates that blood elevations may occur prior to cell death or organ dysfunction in response to mild, non-toxic cell stress. Moreover, given the short half life of cytokines, they return to background levels rapidly and therefore may also represent markers of reversible hepatic damage, such as that shown recently for High Mobility Group Box-1 protein (HMGB-1) in a mouse model of acetaminophen hepatotoxicity [31]. Focussing upon the kinetics of blood biomarker appearance and a particular signature pattern of cytokine expression may give a real insight into hepatic function and toxicity state. However, caution should be taken in the interpretation of multiple biomarker kinetics because many of these biomarkers are not organ specific. Novel, possibly non-specific, biomarkers of damage should always be assessed alongside the gold standard biomarkers for that organ, e.g. ALT/AST for hepatic damage. The correlation between pro-inflammatory cytokine level changes in blood (increase/decrease) with the extent of liver damage or established biomarkers is not fully known and warrants further investigation.

The Role of inflammatory Cells and Cytokines in Liver Disease

Given that cytokines have several of the features required for novel, mechanism biomarkers what data is available in terms of human disease and liver injury for their use as a biomarker in drug safety. Human liver disease takes many forms and can result from viral hepatitis [31], obesity (fatty liver disease; [32]), alcohol abuse (fatty liver disease, alcoholic hepatitis, cirrhosis [33]), genetics, autoimmune disorders (primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis [34]). As the inflammatory component has been best described in the cases of viral hepatitis and alcoholic liver disease we focus on the common themes in inflammation associated with these two types of liver injury.

Viral Hepatitis

At all stages of viral hepatitis infection chemokines are key regulators of immunity and inflammation functioning within cytokine cascades that regulate the immune response to the virus. However in chronic infection their persistent expression can drive chronic inflammation leading to

liver injury and cirrhosis [36]. The Hepatitis B Virus (HBV) itself is non-cytopathic but immune mediated liver damage often occurs in patients with both acute and chronic HBV infection. Such damage has been attributed to the killing of infected hepatocytes by virus specific toxic CD8+ T cells [37, 38]. There is increasing evidence to suggest that a non-HBV-specific inflammatory infiltration into the liver is likely responsible for hepatic pathology in patients with chronic hepatitis B [39, 40].

Upon infection with Hepatitis C Virus (HCV) hepatocytes secrete interferon (IFN) and down regulate RNA translation as a response to infection before innate immune cells, including macrophages, dendritic cells (DC), natural killer cells and natural killer T cells are activated to amplify secretion of type-1 IFNs and IFN-response genes. Activation of Toll-like receptors, triggers chemokine secretion which amplifies leukocyte recruitment [41, 42]. The activation of innate immunity by the uptake and processing of viral antigen by DCs is followed by the migration of DCs to the lymph nodes where they present viral antigen to naive T cells leading to their activation and the development of an adaptive immune response [43]. Two types of DCs have been characterised in the response to HCV infection of hepatocytes, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). pDCs secrete type-1 IFN, prime T helper 1 (Th1) responses and activate cascades of chemokine secretion in HCV infection [44]. mDCs prime Th1 responses via interleukin 12 but produce little type-1 IFN [44, 45]. In the early phases of viral infection chemokines particularly CXCL8, CXCL16, CCL2 and CCL3 promote recruitment of innate immune cells to the liver, including DCs which then initiate the adaptive immune response and it is likely that similar events shape the response to HCV infection [46-47].

TH1 immune responses dominate in the HCV-infected liver (Shields et al., 1999) and intrahepatic T cells express chemokine receptors associated with Th1 responses eg., CXCR3 [49]. Hepatic CXCR3 ligands are increased in many liver diseases and this suggests that they play a role in effector cell recruitment to the inflamed liver [50]. Expression of CXCR3 is closely linked to Th1 function and its ligands CXCL9, CXCL10 and CXCL11 are induced by the TH1 cytokines IFN γ and TNF α [51, 52]. In contrast to HCV, HBV infections are characterised by increased numbers of TH17 cells producing a cocktail of cytokines of which IL17A is characterised as a major effector cytokine [53]. IL17A can mobilise, recruit and activate neutrophils leading to massive tissue inflammation and promote the progression of autoimmune disease [54]. Peripheral and intrahepatic TH17 cells are preferentially increased in patients with chronic HBV infections, which may activate mDCs and monocytes to release inflammatory cytokines during chronic HBV infection [55].

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Viral clearance is associated with a vigorous CD4+ and CD8+ T cell response again with Th1 responses dominating. Regulatory T cells (Tregs) have been found in the HCV-infected liver and appear to have a dualistic effect, they suppress HCV-specific CD8+ T cells and promote viral replication however they also appear to suppress collateral inflammatory damage to reduce liver injury in chronic infection [56, 57]. Successful antiviral therapy is associated with an increase in circulating CXCR3+CD8+ T cells and a reduction in CXCL10 and CXCL9 levels in blood [52, 58, 59]. Interestingly the vasculitic lesions of cryoglobulinemia in nerves and skin are characterised by upregulation of CCL3, CCL4 and CXCL10 and infiltration by CXCR3+ Th1 cells and CCR5+ monocytes. Thus the same pro-inflammatory chemokines are implicated in vasculitic complications of globuliemia and hepatitis [60].

Alcohol Liver Disease

Factors such as gender, age, nationality, weight and health can affect how a person’s liver metabolizes alcohol. When the liver has too much alcohol to handle, normal liver function may be interrupted leading to a chemical imbalance. If the liver is required to detoxify alcohol continuously, liver cells may be destroyed or altered resulting in fat deposits (fatty liver) and more seriously, either inflammation (alcoholic hepatitis) and/or permanent scarring (cirrhosis). Liver cancer can also result from alcohol induced liver disease. It is increasingly evident that chronic inflammation represents the driving force in the evolution of alcohol-induced liver injury to steato-hepatitis and cirrhosis. Accordingly, the hepatic expression of pro-inflammatory cytokines precedes the development of histologic signs of necro-inflammation in alcohol fed rats [61], while steato-hepatitis is associated with leucocyte focal infiltration and elevated circulating levels cytokines/chemokines such as TNF α , IL1, IL6, IL8/CINC and macrophage chemotactic protein-1 (MCP1) [62-65].

Innate and adaptive immunity in alcohol liver disease

The interaction between alcohol and hepatic inflammation is complex. Early studies proposed the involvement of innate immunity with the increased translocation of gut-derived endotoxins to the portal circulation being recognised by Kupffer cells via the toll-like receptor-4 (TLR4 [63, 66]). Ethanol itself has been found to enhance the capacity of Kupffer cells to respond to pro-inflammatory stimuli via the up regulation of Toll-like receptors [67] and the production of pro-inflammatory cytokines and chemokines by modulation of signalling pathways such as nuclear transcription factor κ B (NF κ B), early growth response-1 (ERG1) as well as JNK, ERK and p38 protein kinases [65, 68-70].

Histologic examination of liver biopsies from patients with alcoholic hepatitis reveals the presence of CD4+ and CD8+ T-lymphocytes [71]. The influence of adaptive immunity in the response to alcohol is supported by the predominance of Th1 cytokines (high TNF α , INF γ , IL1) in peripheral blood T-cells isolated from active drinkers with or without ALD [72]. Activated liver-infiltrating IL17-producing T Helper (TH17) cells are also responsible for neutrophil recruitment into the liver [73]. Furthermore serum IL17 levels are increased and serve as a marker of the severity of acute hepatic injury [74]. Evidence is accumulating to suggest that TH17 lymphocytes have an important role in driving inflammation during the development of other chronic inflammatory and autoimmune diseases including inflammatory bowel disease, psoriasis rheumatoid arthritis and multiple sclerosis [75] as well as viral hepatitis as has been discussed.

Alcohol may also have an effect on the immune system by effects on osteopontin, leptin and adiponectin. Osteopontin is a cytokine that enhances the pro-inflammatory Th1 response and the livers of alcohol fed rats have been found to have an associated increase in osteopontin levels [76] while patients with alcoholic hepatitis have higher levels of osteopontin mRNA in their livers compared to patients with fatty liver only [77]. Leptin has been shown to have a pro-inflammatory function while adiponectin counters pro-inflammatory cytokine production [78]. Alcohol appears to have opposite effects on these adipokines, patients with ALD have increased serum leptin [79] whereas chronic ethanol feeding decreases adiponectin secretion in the early phase of alcohol injury in rodents [80, 81].

Signal transduction pathways

In response to alcohol several different signal transduction pathways are activated. Transcription factors regulating fatty acid metabolism including sterol regulatory binding protein (SREBP) and peroxisomal proliferating factor α (PPAR α) play a pivotal role in early alcoholic liver injury [82]. One of the most well studied transcription factors is NF κ B which has a pivotal role in controlling the expression of pro-inflammatory cytokines in monocytes and macrophages. However the expression of NF κ B displayed a surprising response to ethanol treatment depending on whether the treatment is acute or chronic. Differences have been noted in the ability of acute and chronic alcohol exposure to modulate monocyte/macrophage activation. Acute alcohol exposure is associated with LPS-induced anti-inflammatory effects in monocytic cells in humans both *in vivo* and *in vitro* [83-85]. By contrast chronic alcohol induces the expression of TNF α in human and mouse models [69, 86, 87]. This switch between anti-inflammatory and pro-inflammatory responses is linked with changes in IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of LPS

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signalling and leads to decreased downstream activation of LPS signalling cascade culminating in impaired NFκB DNA binding and transactivation and finally TNFα production. In contrast chronic alcohol exposure switches the anti-inflammatory to a pro-inflammatory response by decreasing IRAK-M and increasing IRAK-1 and IKK kinase activation followed by enhanced NFκB DNA binding, transactivation and ERK kinase activation and TNFα production [88]. Other transcription factors such as Activator Protein-1 (AP1), PPARγ and Egr1 have also been shown to be modulated by exposure to alcohol [89, 90, 91].

Animal models of DILI associated inflammation

There is a lot of data to support the role of inflammation in the progression of human liver disease as has been described for viral hepatitis and alcohol liver disease. Currently sufficient data to support an unequivocal role for inflammation in DILI is lacking and in many cases mechanistic details are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate immune system within the liver has been obtained from experimental *in vivo* models. The advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular and immunological backgrounds of inbred strains. This lack of variation enables a more complete exploration and determination of possible inflammatory mechanisms contributing to the overall pathophysiology. However, although animal models are useful tools with which to investigate DILI and the involvement of inflammation caution must be shown in the translation of these results to human DILI.

Inflammation in an acetaminophen (APAP) model of DILI

A widely studied model of DILI has been APAP-induced liver injury (AILI) in mice (Table 2). Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of cellular contents known as damage associated molecular patterns (DAMPS). These include HMGB-1 or Heat shock proteins (HSP) which act as signals which can activate cells of the innate immune system which reside in the liver. This in turn leads to hepatic infiltration of inflammatory cells which may contribute to the progression of liver injury by producing pro-inflammatory mediators such as cytokines and chemokines as well as reactive oxygen and nitrogen species (Figure 1, [92]). Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognised by Mitchell et al. [93] but the relevance of this accumulation to the pathophysiology is still unclear [94]. A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic injury but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these cells did not protect against AILI [95]. In a rat model of AILI, a significant reduction in neutrophil

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2 accumulation showed no protection of liver injury [96]. These findings are in contrast to other
3 models in which neutrophils are involved in the course of injury where pre-treatment with an anti-
4 neutrophil antiserum attenuated ALI in rats [97].
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8 Other studies have demonstrated that mice treated with gadolinium chloride to inactivate KCs
9 [98, 99] and liposome/chlodronate to deplete KCs [99, 100] are protected against APAP toxicity.
10 Though reports suggest that KC depletion confers protection at early times after APAP treatment
11 [100] but can lead to more severe injury at later time points [99]. The use of an anti-NK1.1
12 monoclonal antibody to deplete NK and NKT cells significantly protected mice from APAP toxicity
13 probably due to the reduction in the levels of the pro-inflammatory cytokine IFN- γ [101]. However as
14 has been observed with KC depletion, there are conflicting reports suggesting that NK/NKT cells play
15 little role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [102,
16 103]. This has been supported by a recent study that suggested NK and NKT cells only contributed to
17 liver injury when dimethyl sulfoxide (DMSO) was used to solubilise APAP [103]. Given the conflicting
18 data available on neutrophils and KCs it is clear that more studies are required to determine their
19 precise role in DILI.
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27 The involvement of various pro-inflammatory mediators such as TNF- α [104-106], IL-1 α [107],
28 IFN- γ [101, 108], in promoting tissue damage have been demonstrated whereas other cytokines
29 such as IL-10 and IL-4 [109] have been found to be hepatoprotective in models of APAP
30 hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor
31 [106], p55 TNF- α receptor-1 [105], Toll-like receptors TLR4 and -9 [110, 111] and receptor for
32 advanced glycation end-products (RAGE) [112] have been found to be important in promoting an
33 inflammatory response and subsequent pathogenesis.
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Table 2: Summary of inflammatory models used in APAP-induced hepatotoxicity

References	Inflammatory model(s)	Reported effect
[99, 100]	KC depletion	Protection against liver injury at early time points, more susceptible at later time points
[113]	Neutrophil depletion	Significantly protected against liver injury
[101]	NK/NKT cell depletion	Significantly protected against liver injury
[106]	Anti-TNF- α /IL-1- α /IL-1ra	Increased susceptibility to liver injury
[105]	Anti-TNF- α , TNF-p55 receptor 1 ^{-/-} mice	Reduced mortality and liver injury
[120]	TNFR1 ^{-/-} mice	Increased susceptibility to liver injury
[108]	IFN- γ ^{-/-} mice	Significantly protected against liver injury
[117]	IL-6 ^{-/-} mice	Increased susceptibility to liver injury
[109]	IL-10/4/6 ^{-/-} mice	Significantly protected against liver injury
	IL-10/4 ^{-/-} mice	Increased susceptibility to liver injury
[118]	IL-13 ^{-/-} mice	Increased susceptibility to liver injury
[111]	Anti-TLR9, TLR9 ^{-/-} , Nalp3 ^{-/-} mice	Reduced mortality and liver injury
[112]	Soluble RAGE	Reduced mortality and liver injury
[115]	LPS	Increased susceptibility to liver injury

Comparison of the inflammatory response in animal models of acetaminophen-induced liver injury

Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500mg/kg, i.p) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [101, 113]. An increase of hepatic neutrophil accumulation as early as 4-6 hours with a significant 3-fold increase by 24 hours was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF- α and IFN- γ [101]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an anti-neutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice

against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic inflammatory cell infiltration was observed over a 24 hour time course [114].

Non-toxic doses of APAP (100-400mg/kg, i.p) administered 2 hours after a dose of LPS (44×10^6 EU/kg) to fasted C57BL6 mice have been shown to produce a leftward shift of the dose-response curve for AILI [115]. Application of LPS led to significantly greater TNF- α production than APAP alone. With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 - 24 hours after 300mg/kg APAP. However neutrophils were not apparent in livers of mice treated with a non-toxic dose of APAP (175mg/kg) but significant accumulation occurred when this dose was co-administered with a non-injurious dose of LPS. Two hours after treatment with LPS (0 hours after APAP), serum TNF- α levels were large and rapidly decreased thereafter, confirming that the inflammatory response observed in this model was due to LPS [115]. These observations support the hypothesis that inflammation induced by LPS and associated elevated levels of TNF- α increase sensitivity to AILI.

Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, MIF and IL13 dosed with APAP (200-300mg/kg, i.p) displayed increased susceptibility to AILI [109,116-118]. Neutrophils were found to accumulate to a greater extent in IL13 KO mice than wild-type treated with APAP with the level of TNF- α , IL-6 and IFN- γ increased at 4 hours in WT and IL-13 KO APAP-treated animals compared to saline-treated controls [118]. These data suggest a hepatoprotective role for anti-inflammatory mediators in these models and that the balance between the Th1 and Th2 response is an important determinant for AILI. Susceptibility to AILI was compared between two mouse strains, C57BL6 (more susceptible to AILI and which develop predominantly a Th1 response) and BALB/c (less susceptible to AILI and which develop a Th2 response). 24hr after exposure to APAP C57BL6 mice produce high levels of TNF- α (pro-inflammatory) whereas levels in BALB/c mice show no difference. On the other hand IL-6 (anti-inflammatory) levels were higher in BALB/c than C57BL6 24 hours after administration. These data support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine balance and TNF- α may play a role in toxicity [117].

However, the role of TNF- α remains controversial with contradictory reports suggesting that TNF α is involved in pathogenesis of DILI [105-106]; is not involved in toxicity [104-119] or it has a role in defence against toxicity and repair [120-121]. Data suggests that like most cytokines the effects of TNF α in liver toxicity are pleiotropic with other cytokine networks being involved and it is

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the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. IL1 α [106] and INF γ [108] have been shown to play a pathogenic role in APAP-induced hepatotoxicity whilst IL-6 [117] and IL10 [116] play a protective role. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for DAMPS or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [16].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during drug-induced liver injury (Table 3). Given the complexity of interplay between chemically mediated toxicity and inflammation, and the differences observed between strains of mice, species and *in vivo* model pre-treatment further investigation is required to define the precise role a cytokine has in response to the injury or as part of the inflammatory response. Once this has been defined then the value of the cytokine as a biomarker can be assessed.

Table 3: Inflammation in other models of DILI

DILI Model	Inflammatory factors investigated	References
LPS	Neutrophils, TNF- α , KCs	[122-124]
EtOH	Kupffer cells, TNF- α	[125-128]
Halothane	Macrophage inhibitory factor	[116]
Cocaine + LPS	Kupffer cells, nitric oxide	[129, 130]
Galactosamine + LPS	TNF- α , TNF-p55 receptor	[131, 132]

Cytokine expression in clinical studies of acetaminophen-toxicity

As has been discussed many studies have investigated the role of cytokines in animal models. Some of these cytokines have been reported to have hepatoprotective effects in animal models and several studies have been performed to investigate cytokine expression patterns in serum samples from different patient cohorts. In a study of 111 patients (90 female; mean age 13.6 years) plasma concentrations of IL6, IL8, IL10 and Monocyte chemoattractant protein 1 (MCP1) were analysed by enzyme-linked immunosorbent assay. Patients were stratified by toxicity severity as defined by the maximal values of hepatic transaminase elevation. Levels of IL6, IL8 and MCP1 were elevated in patients with elevated serum alanine aminotransferase and MCP1 expression had the strongest association with toxicity [133]. Other studies have found associations between elevated

expression levels of IL8 [134, 135]; IL6 [136] and acetaminophen-induced hepatotoxicity. While the results from these studies indicate that inflammation and cytokines are associated with the progression of late stage toxicity interpretation of the data is often limited by the absence of pre-dose data, a lack of samples from early in the process of drug-induced injury and the small number of samples usually obtained in the clinic.

Several other studies have investigated the expression of various cytokines associated with various forms of acute liver failure (ALF). In a mixed cohort of patients with ALF and acutely decompensated cirrhosis it was found that not only was the degree of systemic inflammation, as measured by IL6 and TNF α , important for determining outcome but that the magnitude of the anti-inflammatory response was also important. In the whole cohort, IL10 (regarded as an anti-inflammatory cytokine) levels were found to predict outcome as accurately as IL6 [137]. However these patients are experiencing acute liver failure and while expression of the cytokine is linked with disease progression the patient cohorts are characterised by end stage severe liver failure and it is therefore difficult to relate cytokine expression patterns with the earliest stages of injury when intervention is required to prevent toxicity from developing further.

Recently a couple of studies have been reported which attempted to address the issues surrounding patient sampling by treating volunteers with a bolus of acetaminophen under controlled conditions. This has allowed the determination of a pre-acetaminophen treatment baseline for parameters and accurate timing of sampling post-acetaminophen dose. DNA microarrays and serum metabolomic methods were employed to characterise changes in the transcriptome and metabolome in serial peripheral blood (PB) samples obtained from six healthy adults treated with a 4-g bolus of acetaminophen and from three receiving placebo. Treatment did not cause liver toxicity as assessed by traditional liver chemistries. It was found that a single 4-g bolus of acetaminophen produced a transcriptomic signature in PB cells characterised by down regulation of oxidative phosphorylation genes accompanied by increased serum lactate. Similar gene expression changes were observed in rats and several patients after consuming hepatotoxic doses of acetaminophen [138]. While this study is exciting as it begins to allow an insight into the progression of acetaminophen induced liver injury in a controlled setting, it also highlights the challenges faced in identifying novel biomarkers for DILI that can be applied in the clinic. The changes reported in this paper occurred 48 hours after the acetaminophen was administered to volunteers and are therefore likely to be downstream of liver injury. The authors also recognise that the mechanism by which liver toxicity affects peripheral blood cells is unclear, are the changes observed direct or indirect effects?

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In a separate study it was shown that urine metabolite profiles from healthy adults each receiving 4g of acetaminophen per day for 7 days profiles obtained before start of treatment were not sufficient to distinguish which of the subjects would develop mild liver injury. However profiles obtained shortly after the start of treatment but prior to ALT elevation, could distinguish responders from non-responders [139]. This data suggests that an early-intervention pharmaco-metabolomics approach could be used to identify potential toxicities early, however much more work is required to validate this approach. However in both these studies it is interesting to note that few, if any, of the changes described were associated with cytokines. Analysis of liver chemistries showed that liver toxicity had not been caused by the dose of paracetamol used, therefore it indicates supports the hypothesis that inflammation and cytokines are involved only after overt liver damage has occurred.

Immunological involvement in idiosyncratic DILI – the example of halothane

Lessons from animal studies have identified the potential role of the innate immune response in dictating the overall response during APAP hepatotoxicity; however, this stills needs to be proven in man. While the role of immune cells, and by extension cytokines, remains unclear other approaches are been taken to identify biomarkers. Some of the characteristics of idiosyncratic DILI reactions appear to have an immune component and as many autoimmune functions appear to be mediated by TH17 cells, it was hypothesised that IL17 may play a role in iDILI [140]. Expression patterns of cytokines were examined in the serum of 39 patients with acute liver failure (ALF) due to IDILI and compared the values with those from 21 patients with acetaminophen-induced ALF and 10 patients with viral hepatitis-induced ALF. IL-17 levels were found to be elevated in a similar number of patients IDILI and acetaminophen-induced ALF and occasionally in patients with viral hepatitis. There was overlap in the level of expression of IL-21 in patients with iDILI and AILI. Auto-antibodies were more frequent in patients in the IDILI group but were absent in most patients. These data provide a picture of the cytokine/chemokine profile in patients with various types of ALF. The pattern varies from patient to patient and not specifically by etiology. This suggests that different underlying disease mechanisms may be at play in different individuals, even among those demonstrating injury from the same drug. Since cytokines may originate from more than one type of cell, interpretation of results of cytokine assays remains difficult in complex disease settings.

However it is certainly possible that an adaptive immune response may play a role in cases of human DILI. Certain drug reactions have clinical hallmarks of an immunological mechanism, the liver alone may be involved or liver injury may be part of a more complex hypersensitivity syndrome.

Unlike with APAP, where the initiation of hepatotoxicity is dependent upon the intrinsic chemistry of the molecule, these so called idiosyncratic hepatotoxicity reactions represent a function of an individual patient's biology (Table 4).

The inhalation anaesthetic halothane is the best-studied drug causing an immunoallergic response in a subset of patients. The response ranges from asymptomatic rises in transaminases to fulminant hepatitis, which is rare but serious. Most of the patients recorded in the literature with immunoallergic hepatitis had more than one exposure [141]. Antibodies have been detected in such patients that recognise autoantigens and neoantigens created by trifluoroacetylation (TFA) of hepatic proteins. Pre-incubation of halothane pre-treated rabbit hepatocytes with sera of patients with halothane-induced fulminant hepatitis increased susceptibility to cytotoxicity of lymphocytes *in vitro* [142]. It is likely drug specific T cells play a role in the pathogenesis of hepatocyte injury but direct evidence is lacking. Halothane is metabolised by CYP450 2E1, like APAP, but forms a chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only metabolic route available. It is hypothesised that the formation of the acyl halide triggers an immune response and that mild and severe immune responses are likely to have this common initiation mechanism. Evidence for this concept of an immune trigger is both direct and indirect, with drug specific antibodies indicative that the drug has initiated an immune response in affected patients and newer metabolically inert inhaled anaesthetics being rarely associated with hepatotoxicity. Target proteins modified by the acyl halide have been identified with the principal chemical modification trifluoroacetylation of lysine residue [143-146]. Chemical modification of protein(s) is associated with the immune response observed with halothane hepatitis, however, there is no conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been observed with idiosyncratic drug reactions to Tienilic acid [147] and dihydralazine [148] but there is no unequivocal evidence that DILI such as that caused by halothane is immune mediated. Most of the information available is compatible with the hapten hypothesis where the drug undergoes bioactivation in hepatocytes leading to drug-protein conjugate formation in the liver. The current theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents interplay between a number of factors, which include the chemistry of the drug, drug metabolism and the immune system of the patient (Table 4). Whether cytokines may be useful biomarkers in such idiosyncratic cases is currently unknown as the data supporting immune involvement is equivocal and the mechanistic details are unknown. However, if the hapten hypothesis is correct what is clear is that the inflammatory response induced by chemical insult such as APAP overdose compared to that of idiosyncratic DILI may be very different.

Table 4: Idiosyncratic drug reactions with evidence of immune involvement (amended from [149])

Drug Class	Drug Name	Is there evidence to support immune involvement?
Anaesthetic	Halothane	Yes
Analgesic	Diclofenac	Yes/No
	Sulindac	Yes
Antibiotic	Amoxicillin	Yes
	Erythromycins	Yes
	Minocycline	Yes
	Nitrofluantoin	Yes
	Rifampicin	Yes/No
Anti-convulsant	Phenytoin	Yes
	Phenothiazines	Yes
Anti-epileptic	Carbamazepine	Yes/No
Antihypertensive	Dihydralazine	Yes
	Methyldopa	Yes
	Tienilic acid	Yes
Anti-thyroid	Propylthiouracil	Yes
Depression	Tricyclic antidepressants	Yes
Gout	Allopurinol	Yes
Hypertension	ACE inhibitors	Yes

Conclusions and perspectives

An analysis of the role of inflammation in human liver disease and in the *in vivo* models described offer insights into the potential role of inflammation in human DILI. Given the role of cytokines in modulating the inflammatory response changes in patterns of cytokine expression certainly show promise as potential biomarkers of DILI. However, the *context* of cytokine expression will determine their utility as biomarkers. This context will be determined by understanding the processes within DILI and the associated inflammatory cascade in which the cytokine has a role (Figure 1). One key question is whether the inflammatory response precedes the injury or is a consequence of the injury (Figure 2). Also, inflammation is associated with a lot of confounding factors experienced in the clinic (as previously described) therefore much more data is required in order to support the context of a given cytokines' qualification for use as a biomarker and the proposed application of that biomarker. Clearly prior to selecting a given cytokine as a biomarker it

is important to accurately phenotype DILI and to have a thorough understanding of the processes and mechanisms by which injury occurs.

As discussed the balance of pro and anti-inflammatory cytokines is thought to play a significant role in dictating the overall balance of hepatic injury exacerbation or regeneration, in particular for DILI characterised as idiosyncratic in nature (Figure 2). The majority of animal models of DILI are exposed to profound, often lethal levels of chemical stress with changes in the expression of cytokines such as TNF α , HMGB-1, IL6, IL10 and IL1 β [16, 18-20]. Therefore animal model data may be misrepresentative of the majority of cases of liver injury observed clinically. The promotion of inflammation may be the most significant consequence of chemical stress in the exacerbation of DILI, in which case, cytokines remain an attractive option as biomarkers of DILI to inform the mechanism of pathology, sensitively screen for hepatic injury and predict at risk patients.

Even when a cytokine has been shown to be a candidate biomarker the benefit from use of a novel biomarker in terms of safety efficacy and or cost must be clearly established. Biomarkers must be sensitive and specific and this requires accurate definition of the context for which a biomarker should be qualified [150]. Context and qualification for new biomarkers are assessed relative to current biomarkers. If the sensitivity and specificity of current biomarkers are not perfect relative to a specific end point, the context and qualification of new biomarkers may not be accurately established. This is a particularly difficult problem if current biomarkers have pseudo-quantitative, qualitative, or subjective metrics associated with them. Even the data available for the gold standard of DILI (ALT) is continually reviewed to ensure that the context of its use is as accurate as possible [151].

Much remains to be understood about the role of inflammation in DILI and cytokines in controlling this inflammatory response. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and quantifiable. However without fully understanding the role of inflammation in DILI they may be limited in their use to being diagnostic of the type of injury that has occurred and maybe prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify may be better biomarkers for the prognosis of whether an injury will occur (Figure 1). However, further investigation is required to understand the impact of chemical stress on cellular physiology and the release of intracellular components in response to injury as well as the development of assays sensitive enough to accurately detect them.

Figure Legends

Figure 1 Paracetamol-induced hepatotoxicity: a multistep/multicellular process

The multi-step process and multi-cell type involvement during acetaminophen-induced hepatotoxicity. Acetaminophen (APAP) hepatotoxicity is dependent upon the metabolic activation by CYP450 to the reactive metabolite NAPQI. Following saturation of the phase II conjugation detoxification pathways, NAPQI becomes covalently bond to hepatic protein (in particular mitochondrial protein) which leads to cell death. Cell death can proceed through either apoptosis or necrosis depending upon the bio-energetic status of the cell. So called DAMPs (e.g HMGB-1) are released from necrotic cells which hold the potential to activate the innate immune response. Evidence from animal models suggests that the overall extent of hepatic damage is dictated by the balance between the production of pro and anti-inflammatory cytokines. The dependency upon the multiple steps has been elucidated experimentally in vitro and in vivo from CYP450 dependence (inhibitor; ABT), glutathione level (NAC; N-acetyl-cysteine), mitochondrial permeability transition (CsA; cyclosporine), and cellular ATP status (dietary restriction) to caspase activation (inhibitor; Z-VAD.fmk)

Figure 2 Inflammatory etiology of acute liver failure

Acute liver failure stimulated by a variety of inducing agents leading to the development of the pathology often share common pathways through the activation of pro-inflammatory pathways. Initial necrotic cell death from reactive metabolites, drug accumulation, diet, alcohol or viruses can lead to the release of DAMPs which in turn activate the primary and secondary inflammatory cascades. Underlying hepatitis can influence an individual's susceptibility to hepatotoxicity. Understanding the mechanisms involved from the study of liver diseases or the particular serum cytokine finger print when applied to DILI may help to predict susceptible individuals or patients likely to develop severe hepatotoxicity

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