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**Title:** Dynamics of TNFalpha signaling and drug-related toxicity
**Issue Date:** 2017-12-06
Chapter 2

Drug-induced liver injury and TNFα signaling: from in vivo understanding to in vitro testing approaches

Highlights

- Idiosyncratic drug-induced liver injury, including diclofenac-induced liver injury, is associated with derailed proinflammatory (TNFα) signaling.
- In vitro testing methods for liver toxicity liability do not often include immune-related tests.
- Fit-for-purpose approaches could include a range of in vitro models to better predict DILI liability.

This chapter has been published as:

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Drug-Induced Liver Injury and TNFα Signaling: From In Vivo Understanding to In Vitro Testing Approaches


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For new candidate drugs the liability towards idiosyncratic drug-induced liver injury (DILI) remains difficult to predict from preclinical in vitro and in vivo studies. The DILI-inducing potential of a drug is generally only detected in the clinical testing phase or once on the market. In the last decades, research on the interaction of host factors and drug-dependent processes has produced valuable insights into the mechanisms of idiosyncratic DILI. Continued development of screening methods and prediction models has led to improved prediction of DILI. However, the immune component involved in many drug-induced toxicities is notoriously difficult to characterize. In this review, we will discuss the interaction of innate inflammatory signaling, thereby focusing on TNFα-signaling, and drug-induced processes leading to hepatotoxicity. In general, there is a clear interaction between liver-resident innate immune cells and hepatocytes during the onset of DILI. At a mechanistic, intracellular level this involves interaction of drug-induced general adaptive stress response activation and cytokine-mediated signaling, and this interaction provides a basis for hepatocyte cytotoxicity. Understanding of these complex mechanistic interactions in human idiosyncratic DILI will allow development of tailored in vitro screening approaches.

**Keywords:** diclofenac, drug-induced liver injury, hepatotoxicity, in vitro models, LPS, stress response, TNFα

**Introduction to drug-induced liver injury**

Drug-induced Liver Injury (DILI) is a complex condition with difficult diagnosis and prediction, since it displays a broad array of symptoms that resemble other chronic or acute liver conditions. In the most recent prospective studies in the US and Iceland, it was found that around 11% of all idiosyncratic acute liver failure cases were caused by drugs and that the incidence rate of DILI in the general population was 19.1 cases per 100,000 inhabitants per year. Currently, DILI remains one of the major concerns in both clinical and drug developmental practices. Since 1997, no drugs were approved by the Food and Drug Administration that had to be withdrawn later due to DILI, indicating raised awareness and improved detection of DILI in clinical trials. However, to prevent the enormous costs that accompany clinical studies for a drug with DILI-potential, the emphasis of current research lies with better prediction of DILI from animal and in vitro approaches.

Over the last decade, the over-simplified classification of DILI in intrinsic and idiosyncratic has developed in a less distinctive mapping. Acetaminophen, the classical example of intrinsic DILI, causes most acute liver failure cases in the U.S. in a predominantly dose-dependent fashion. However, the prognosis for acetaminophen-toxicity outcome seems to be dose-independent, pointing to general mechanisms starting to act the moment the threshold for toxicity is reached. Furthermore, a study in the U.S. published in 2005 found around 8% of the cases developed by therapeutic dosing, suggesting mechanistic explanations that are rather associated with idiosyncratic DILI. In this review,
idiosyncratic mechanisms of toxicity are defined as involvement of specific host factors interacting with the drug, its metabolites or responses induced by either, inducing hepatotoxicity in a few individuals.

Even when excluding acetaminophen hospitalization, drug treatment remains the major cause for acute liver failure in the U.S. Of these drug-induced cases of hepatotoxicity, most are considered idiosyncratic being relatively dose-independent and occurring in a yet unpredictable minority of people treated with the culprit drugs\textsuperscript{50}. Risk factors include a high daily dose and drug-characteristics, including lipophilicity and metabolism, but more importantly the personal environmental factors like age, pre-existing liver disease, obesity and ethnicity, all of which has been summarized in a recent review\textsuperscript{51}. One reoccurring and very interesting risk factor is gender. Although on first glance the ratio of DILI incidence might seem similar for females and males, the percentage of patients that develops severe acute liver failure is significantly higher for women\textsuperscript{45,51}. Furthermore, genetic association studies have identified various defined HLA allele variants that are associated with idiosyncratic DILI\textsuperscript{52,53}. The fact that gender background and in particular the HLA genotype are regarded as risk factors for DILI might point towards the immune system as one of the causal factors of DILI, since there are differences in immune reactions between males and females in various fields, as described in this review\textsuperscript{54}. Observations that strengthen this hypothesis are that autoimmune hepatitis upon drug exposure is rarely seen in males and that the injury pattern of DILI seems to change in females above 60 compared to younger women. However, gender has not been detected as a risk factor for DILI in all studies and has been suggested to be drug-specific\textsuperscript{51}. In mice, both halothane-induced injury and Con A-induced liver injury were more severe in females. This was regulated by sex hormones inducing an altered cytokine response and thereby modulating the subsequent adaptive immune response\textsuperscript{55,56}. Taken all together, these data suggest that the modulation of the immune system is a critical factor in developing DILI and progression to severe hepatotoxicity.

This review will discuss the role of the innate immune system in the onset and progress of idiosyncratic DILI. The central part of this review will discuss the archetypical idiosyncratic drug diclofenac and the involvement of lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF-alpha) signaling in DILI. This will be followed by an overview of the progress in the application of in vitro cell systems for the mechanistic understanding and prediction of DILI that involve innate immune signaling.

**Liver immunology and drug-induced inflammatory response**

*Immune system in the liver*

The liver has a unique immunological build-up; this is essential since it is situated at the portal vein with continuous exposure to gut-derived antigens and cellular debris from the blood stream. As peripheral immunotolerant organ, it contains a population of liver resident immune cells as Kupffer cells (KC), natural killer cells (NK) and dendritic cells (DC) that are skewed to inducing tolerance, therefore called tolerogenic\textsuperscript{6,57}. Together with hepatocytes, liver sinusoidal endothelial cells (LSEC) and stellate cells, they form an environment in
which it is difficult to invoke a tissue-damaging pro-inflammatory response (see for details\textsuperscript{3,4,58}). To sustain this tolerogenic environment, antigen presentation, clonal deletion of antigen-specific T cells and the shift from a Th2 favored response to a Th1 response must be strictly controlled by direct and paracrine signaling (reviewed by\textsuperscript{59,60}). Many of the signaling pathways in the liver that lead to tolerance mechanisms are altered and skewed towards inflammation by exposure to DILI drugs. One of the main pathways leading to activation of the innate immune system is the Toll-like receptor 4 (TLR4) signaling pathway, driven by LPS, an endotoxin from the outer membrane of Gram-negative bacteria.

**Toll-like receptor signaling in drug-induced liver injury**

In the liver, TLR4 is expressed on KCs, hepatic DCs, NK cells, LSECs, stellate cells and hepatocytes\textsuperscript{61,62}. Signaling by TLR4 is remarkably inhibited in a healthy liver, sustaining the tolerogenic environment. The continuous low exposure to LPS is essential for maintaining tolerance by affecting KCs and LSECs in a negative feedback-loop regulating expression of several proteins, miRNAs and IL10 signaling\textsuperscript{61,63–65}. Indeed, administering low doses of LPS protects against D-galactosamine/LPS and ischemia-reperfusion liver injury by downregulation of TLR4-mediated signaling\textsuperscript{66,67}. Interestingly, in many chronic liver diseases and obesity, often named risk factors for DILI, TLR4 signaling is upregulated\textsuperscript{61,68}. Furthermore, it is generally accepted that antimicrobials are amongst the drugs that most frequently and severely induce DILI\textsuperscript{69}. Altered TLR signaling might explain this DILI inducing potential. Antimicrobials change the gut microbiome, thereby increasing the transfer of commensal bacteria across the gut epithelium. Translocated bacteria can induce inflammatory responses via TLR signaling\textsuperscript{70}. Increased LPS translocation together with inflammatory signaling from the gut environment could lead to increased pro-inflammatory signaling in the liver. Combined, these data suggest a role of the gut environment and altered LPS leakage to the liver as an possible mechanism in the development of antimicrobial-induced hepatotoxicity. Finally, TLR4 signaling is involved in the several rodent models that show a synergistic increase in hepatotoxicity with co-exposure of LPS with well-known DILI drugs including diclofenac, trovafloxacin, sulindac, and chlorpromazine. In these studies, LPS was administered at different time points ranging from 2 hours pre-treatment to 16 hours post-treatment\textsuperscript{8,9,71–73}. It would be relevant to determine whether repeated low-dose LPS exposure would be able to prevent hepatotoxicity of the combined LPS/drug exposures in these animal models, as it would confirm the importance of inhibition of TLR4 signaling in the protection against aberrant inflammation during drug exposure.

The function and signaling cascades induced by TLR4 activation in the different cell types in the liver are much debated. Here, we will discuss the effects of LPS on KCs and hepatocytes. TLR4 signaling in other liver cell types is reviewed in these publications\textsuperscript{62,65}. Via the portal vein the blood enters the liver, rich in nutrients, metabolites and potentially toxic compounds. From the hepatic artery, oxygen rich blood joins this mixture. This blood mixture contains many antigens that have to be cleared from the blood before it enters the systemic circulation. The clearance of gut-derived LPS from the blood
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stream in non-inflammatory conditions depends on both hepatocytes and KCs\textsuperscript{74,75} and is based on receptor-dependent endocytosis and phagocytosis, respectively\textsuperscript{76}. In hepatocytes, binding of LPS and subsequent clearance seems to induce TLR4-induced signaling, although not via the classical MyD88 pathway but via p38/MAPK signaling\textsuperscript{77}. Furthermore, TLR4-mediated LPS clearance activates adaptive stress response pathways, like p62-regulated autophagy and the Nrf2-mediated oxidative stress response\textsuperscript{78}. Non-endocytosed LPS on the other hand, signals via the classical TLR signaling pathway, inducing NF-κB and MAPK signaling. This leads to low levels of pro-inflammatory cytokine production and induction of metabolic activity by regulation of CYP enzyme expression in hepatocytes\textsuperscript{79,80}. The induction of CYP enzyme expression upon TLR4 stimulation contradicts many studies that show an LPS-induced decrease of CYP enzyme expression and metabolic activity, both \textit{in vivo} and \textit{in vitro}\textsuperscript{81,82}. This downregulation of CYP enzymes seems to be dependent on LPS-induced cytokine production, amongst which are IL6 and TNFα. The function of down-regulated CYP enzyme expression during inflammation is currently much debated, as is discussed in this review\textsuperscript{83}.

In a recent elegant mouse study, cell-type specific TLR4 knockouts were used in a model for sepsis. TLR4 expression in the myeloid cell population (KCs and neutrophils) is essential for efficient bacterial clearance and determines the level of inflammation and liver injury in a high bacterial load model. However, in a low bacterial load model, TLR4 expression on the hepatocytes was essential to prevent LPS-induced pro-inflammatory cytokine production by KCs and hepatotoxicity, measured by increased plasma ALT levels\textsuperscript{84}. This data shows that both hepatocyte and KC TLR4 signaling is essential in clearance of LPS during sepsis. This might suggest a role for hepatocytes in LPS clearance in healthy, steady state liver as well.

LPS is not the only TLR4 ligand present upon hepatocyte dysfunction. High mobility group box-1 (HMBG1), a nuclear factor that is released from sites of hepatocellular stress, activates TLR4 signaling in the liver\textsuperscript{85}. HMBG1 resembles LPS in that preconditioning with repeated low levels of HMBG1 can protect against ischemia/reperfusion (I/R)-induced liver injury\textsuperscript{86}. TLR4-dependency of different cell type specific knockouts in mice was studied in a model of sterile inflammation caused by I/R as well. In this model, no LPS was present to stimulate TLR4 signaling. However, liver injury was dependent on effective TLR4 signaling in both KC and hepatocyte population, while the TLR4 signaling in the DC population was protective based on IL10 secretion. Furthermore, TLR4 signaling in hepatocytes was necessary for neutrophil recruitment, and via c-Jun N-terminal kinase (JNK) activation also for the excretion of HMBG1\textsuperscript{87}. Altogether these studies point to a cooperative mechanism of activated KCs and hepatocytes in the induction of both endotoxin-mediated and sterile inflammation, which could lead to liver injury.

Activated KCs upregulate the secretion of pro-inflammatory cytokines (TNFα, IL1), eicosanoids, and chemokines (e.g. CCL3, CCL4, CCL5, and CCL12)\textsuperscript{88–90}. While the exact effect of individually upregulated cytokines and chemokines on different liver cell types and hepatotoxicity is unclear, they are involved in pro-inflammatory processes as for example recruitment of immune cells, the acute phase response and increased cell death, as well as
in repair processes\textsuperscript{91}. For instance TNF\(\alpha\), as is described below, induces hepatocyte apoptosis when drugs interfere with TNF\(\alpha\)-induced cytoprotective signaling. In contrast, the expression of the TNF\(\alpha\) receptor and in some cases TNF\(\alpha\) itself, are required for liver regeneration\textsuperscript{92}.

**Drug-induced idiosyncratic liver injury**

*Diclofenac-induced idiosyncratic liver injury*

An archetypical drug that can induce idiosyncratic DILI is the common anti-inflammatory painkiller diclofenac. Currently, diclofenac is still prescribed and even sold without prescription in the UK and Europe as nonsteroidal anti-inflammatory drug (NSAID). Among the class of NSAIDs, diclofenac is one of the drugs with the highest risk on DILI\textsuperscript{93}. In the most recent study on NSAID-induced liver injury with data from the Drug-Induced Liver Injury Network in the U.S., diclofenac-induced liver injury was most frequent and most severe\textsuperscript{94}. Diclofenac-induced liver injury is in most cases hepatocellular, and in a minority of the cases cholestatic\textsuperscript{95}. This correlates with the genetic variants found in drug excretion proteins, for example in MRP2 (ABCC2), that were associated with diclofenac-induced liver injury\textsuperscript{96}. Latency times range from 6 days to a year, and the disease can present itself in some cases with an immunological phenotype with symptoms like fever and rash or with the production of autoimmune antibodies\textsuperscript{94,95}.

*Diclofenac metabolism*

Diclofenac-induced liver injury is slightly dose dependent, the risk increasing by daily doses higher than 150 mg\textsuperscript{97}. The major diclofenac metabolites are 4'-OH diclofenac mainly formed by CYP2C9, 5-OH diclofenac by CYP3A4, CYP2C8 and CYP2C19 and diclofenac acylglucuronide mainly by UGT2B7\textsuperscript{93}. Of these, especially the reactive 5-OH diclofenac-derived quinone imines and acyl-glucuronides are implicated in diclofenac-induced toxicity. Genetic variants of UGT2B7 and CYP2C8 have been correlated to diclofenac-induced hepatotoxicity\textsuperscript{96}. Furthermore, comparison between metabolically active HepaRG cells and metabolically less active HepG2 cells showed less toxicity in the HepaRG cells, pointing towards protection by increased detoxifying processes in HepaRG cells. In both cell types, TNF\(\alpha\) stimulation was able to increase cytotoxicity\textsuperscript{98}. Interestingly, the exposure of HepG2 cells to 4'-OH diclofenac in combination with TNF\(\alpha\) showed increased cytotoxicity, in contrast to the 5-OH diclofenac and diclofenac acyl-glucuronide\textsuperscript{99}. In conclusion, it is still unclear if drug metabolism actually protects against synergistic induction of cell death or plays a role in the induction of TNF\(\alpha\)-induced cytotoxicity.

*Diclofenac-induced immune activation*

Autoimmune and immunological presentation of diclofenac-induced liver injury as stated earlier, points to the activation of the adaptive immune system. The question how drug-induced activation of the immune response can occur has inspired many theories, including the hapten hypothesis, the p-i concept, and the danger hypothesis. The hapten hypothesis states that a drug on itself cannot induce an adaptive immune response unless covalently
bound to proteins in plasma or liver. Diclofenac-derived acyl-glucuronides can form metabolite adducts by binding to target proteins. These adducts can act as haptens and are mainly found in liver zone 3. In diclofenac-induced liver injury, zone 3 necrosis is a common finding, although the relation between these two observations is unclear. Indeed, acyl-glucuronide and acyl-glucuronide-induced adduct formation are found in human plasma in diclofenac users. However, antibodies recognizing diclofenac-induced protein adducts were detected in the majority of the diclofenac users that did not demonstrate DILI. This finding implicates adaptive immune responses towards acyl-glucuronide-induced protein adducts are not the single causative reason for idiosyncratic diclofenac-induced liver injury.

The p-i concept states that drugs by itself can be recognized as antigens by specific T cell clones. Although shown for several drugs including carbamazepine, this seems not to be the case for diclofenac. The danger hypothesis claims that absence of danger-related signals will lead to tolerance by hapten-presentation. The danger signals necessary to mount a full immune response are the interaction of the processed hapten with the T-cell receptor, an independent interaction of co-receptor with a stimulatory molecule and polarizing cytokine signaling. The timing and ratio of danger signals likely determines the activation profile of cytotoxic T cells. Since polymorphisms in the tolerance-inducing cytokines IL10 and IL4 were associated with diclofenac hepatotoxicity and activated cytotoxic CD8+ T cells seems to be mainly involved in DILI, it is likely this theory is at least partially applicable on diclofenac-induced liver injury.

**Animal models of inflammation in drug-induced liver injury**

*Immune-dependent latency in a mice model*

An interesting observation in clinical studies is the apparent clinical adaptation of patients with mild DILI under continuation of the drug. In these cases, patients that take the drug develop mildly abnormal liver function values. However, these abnormalities disappear while the patients continue taking the culprit drug. Very few of these patients progress towards severe DILI. This suggests that the cases of severe DILI have disturbed clinical adaptation processes, leading to severe hepatotoxicity. A mouse model in which this adaptation process is reflected has been generated by Methusi et al., in which amodiaquine treatment in PD1−/− mice induced mild liver injury, resolving despite continuous treatment. However, co-treatment with CTLA4 antibody, blocking the tolerogenic signaling induced by CTLA4, induced severe liver injury. Although CD8+ T cells were the most abundant in this model of severe injury, NK cells seem to play a more important role in the development of the mild injury upon treatment in PD1 knockout mice. This points to a role for the innate immune system in the early processes in drug-induced liver injury. It would be interesting to see the effect of treatment with diclofenac or other idiosyncratic DILI drugs in this model. This mice model nicely shows the cumulative effect of several signaling cascades that is necessary to break the tolerance and develop severe liver injury instead of adaptation. However, how these mechanisms relate to human idiosyncratic DILI remains unclear.
Animal models on the inflammatory response in diclofenac-induced liver injury

The application of rodents to understand the mechanisms of immune-related mechanisms of diclofenac toxicity shows that small non-toxic doses of LPS can greatly enhance diclofenac-induced hepatotoxicity. Although neutrophils are recruited and correlated to injury, they seem to play a minor causative role in the hepatotoxicity in LPS/diclofenac-induced injury. To mimic human treatment, Ramm et al. treated rats with very low doses of diclofenac for seven days. On the seventh day, a small non-toxic dose of LPS was administered. Significant toxicity of combined LPS/diclofenac treatment was seen compared to diclofenac or LPS treated rats. Glutathione depletion and drug metabolism were not enhanced by LPS/diclofenac treatment compared to diclofenac treatment. However, combined diclofenac/LPS treatment induced upregulation of cellular stress responses, including the oxidative stress response, the NF-κB response and hypoxic stress response. Taken together with an upregulation of pro-inflammatory cytokines and danger-associated molecules, this could lead to the hepatotoxicity seen in diclofenac-induced liver injury. Interestingly, the highest upregulation of stress responses and protein production of TNFα and HMGB1 was detected in the only two animals that died from co-treatment of LPS and diclofenac.

In vivo studies into other DILI-inducing drugs, trovafloxacin and sulindac, have showed the importance of TNFα-signaling in LPS/drug-induced liver injury. The replacement of LPS with TNFα showed a similar pattern of hepatic injury. In both cases, the increased hepatotoxicity was dependent on prolonged elevated plasma TNFα levels and altered TNFα-receptor (TNFR) signaling. Moreover, the neutralizing TNFα antibody Etanercept clearly diminished LPS-induced trovafloxin- or sulindac-mediated liver injury in rodents. However, in both human and mouse precision-cut liver slices, diclofenac downregulated LPS-induced TNFα production upon co-exposure, contradicting the trovafloxin/sulindac research. What this downregulation of LPS-induced TNFα exactly means in the context of diclofenac-induced toxicity is not clear.

Role of TNFα signaling in diclofenac-induced liver injury

Upon LPS stimulation in the liver, TNFα is mainly produced by KCs and less by other liver resident cells. In acetaminophen-induced liver injury in mice, the removal of KCs abolished the TLR4-dependent upregulation of TNFα, and led to significantly less liver injury. However, it should be noticed that the removal of KCs and their role in acetaminophen-induced liver injury has revealed contradictory results, as described in this review. Signaling by the pleiotropic cytokine TNFα regulates many processes in target cells, including proliferation, production of pro-inflammatory cytokines, cell death and cell survival. These contradictory processes are regulated by an intricate web of signaling proteins and post-translational modifications, leading to well-known key players as the transcription factors NF-κB, c-Jun, AP-1 and cell death modulators like RIP1, RIP3 and caspase-8. These complex signaling pathways leading to cell death or survival are quite recently discussed in this review by Brenner et al., and the role of these pathways in inflammatory processes by Wallach. In short, TNFα stimulation leads to conformational
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changes of the TNFR, assembling an receptor signaling complex. This complex contains TAK1, a kinase that can activate MAPK signaling cascades including JNK, and the IKK complex. The IKK complex phosphorylates IκBα, the cytoplasmic inhibitor of NF-κB. Upon subsequent proteosomal breakdown of IκBα, NF-κB translocates into the nucleus and induces signaling. Maturation of the TNFR complex based on the status of RIP1 ubiquitination can lead to formation of cell death complexes, inducing apoptosis, necrosis or necroptosis.

In *in vitro* cell culture models using HepG2 cells, primary human or rat hepatocytes, diclofenac synergizes with TNFα to induce apoptosis. This synergism has been shown to be dependent on JNK activation, inhibition of NF-κB nuclear translocation and an activated mitochondrial apoptotic pathway. In addition, diclofenac/TNFα-induced synergism has been shown to be increased by concomitant IFNγ exposure. This increased synergism is dependent on ERK-dependent STAT1 activation. Activation of the endoplasmic reticulum (ER) stress response is essential for induction of diclofenac-mediated TNFα-induced cytotoxicity. Expression of ER-stress response-related genes including transcription factor CHOP is enhanced upon diclofenac exposure alone in HepG2 cells, primary human hepatocytes (PHH) and human precision-cut liver slices. Subsequent siRNA-mediated knockdown of CHOP and its upstream translational regulator EIF4A1 in HepG2 cells confirmed the role of ER-stress in TNFα-induced hepatotoxicity.

The role of the ER-stress response in different liver diseases has recently been reviewed. In HepG2 cells, diclofenac-induced increase in intracellular calcium was increased by both TNFα and IFNγ, leading to activation of JNK and ERK and subsequent cytotoxicity. Interestingly, ER-stress in HepG2 cells seems to induce ER-stress in THP-1 macrophages via both soluble and insoluble factors. ER stress in macrophages leads to a reduction in pro-inflammatory cytokine secretion and is thereby providing a negative feedback loop for drug-induced inflammation. Also trovafloxacin-mediated TNFα-induced cell-death was characterized by ERK activation and prolonged JNK activation. However, in this case activation of these signaling pathways was mediated by DNA replication stress. Furthermore, oxidative stress and heat shock responses have been shown to interact with TLR4 and TNFα-induced signaling and NF-κB activation. Several DILI-inducing drugs that synergize with TNFα activate oxidative stress responses by themselves. Inhibition or aberrant activation of these oxidative stress responses in HepG2 cells by siRNA-mediated knockdown enhances or inhibits this TNFα-induced cytotoxicity, respectively. Together, these data suggest that several drug-induced adaptive stress response pathways can interfere with TNFα-induced hepatotoxicity.

**Idiosyncratic drug-induced liver injury prediction by *in vitro* approaches**

It is clear that the cause of idiosyncratic DILI is in all cases multifactorial. Drug-specific responses make it difficult to predict the effect of novel drug-candidates on the many host factors involved in the development of DILI. However, using improved prediction models on clinical trial results led to majorly improved prediction of DILI hazard. Currently used
vitro methods have been extensively reviewed\textsuperscript{1,122,123}. In this review, we will focus on the incorporation of inflammatory signaling in in vitro models.

DILI is characterized by massive hepatocyte cell death. Therefore, hepatocyte only models are much-used in the pharmaceutical industry to test for the hepatotoxic potential of novel compounds. For these models, PHHs are the golden standard, besides having some serious drawbacks in for example donor variability and low availability. The quite recently established hepatoma cell line HepaRG forms hepatocyte-like cells surrounded by biliary epithelial-like cells upon differentiation. This cell line is relatively easy to handle and shows enhanced metabolic capacity and hepatic transporter function, giving better prediction of especially drug-induced cholestasis\textsuperscript{124}. Immortalized cell lines, for instance HepG2, are much used for their easy handling, high availability and stable phenotype. However, severely decreased drug-metabolism capacity makes this model less applicable in cases of drug metabolite-induced toxicity. Functionality and phenotypes of these cell lines for toxicity research have been frequently compared and the results are not conclusive\textsuperscript{125,126}, introducing the pragmatic “fit for purpose” approach\textsuperscript{1,127,128}. PHHs, HepaRG cells and HepG2 cells have all been shown to greatly improve their hepatocyte phenotype by sandwich culture or 3D culture, together with prolonged sustainability and the possibility of repeated dosing. However, high-throughput screening, especially with other than endpoint measurements, remains difficult with this culture method\textsuperscript{1}, although some progress has been made (unpublished data;\textsuperscript{129}). Mimicking activated liver immune cells in hepatocyte only cultures occurs mainly by manual addition of effector proteins or cytokines. This approach can be used in high-throughput screening methods and in prediction models (unpublished data;\textsuperscript{41,130}).

Co-culture models with non-parenchymal cell types in 2D and 3D systems greatly improve hepatocyte function and phenotype\textsuperscript{1}. In 2D, KC and hepatocyte co-culture showed in general upregulated hepatocyte CYP enzyme expression and drug metabolism in hepatocytes, and increased KC cytokine production and concurrent hepatotoxicity\textsuperscript{12,31,32,35}.

**Fig. 1.** The applicability of experimental model systems for hepatotoxicity screening and understanding cytokine-induced hepatocyte toxicity Model systems used to detect immune signaling in human idiosyncratic drug-induced liver injury are assessed, both on the reflection of the human immune response in the model system and the possibility to detect specific hepatocyte signaling on immune activation. All depicted cell model systems can be implemented with either cell lines or pri- mary cells. The models are evaluated on the complexity level of immune signaling that is possible, the hepatocyte phenotype, the donor variability, the possibility of hepatocyte-specific measurements, the possibility of genomic interference, the availability of the model for high-throughput approaches, the sustainability of the cell culture model over time, and the possibility of interference of the system (in, for instance, measuring or adding a specific cytokine). Specifically mentioned is the possibility to use fluorescent reporter cell lines in complex models as 3D models and cocultures of cell lines. The use of these fluorescent reporters is extensively reviewed in Wink et al. (2014); the use of these reporter cell lines confers the possibility to detect hepatocyte-specific immune signaling on the mentioned models, increasing model value for mechanistic and predictive toxicity research. LPS, lipopolysaccharide; PHHs, primary human hepatocytes. Color images available online at www.liebertpub.com/aivt

www.liebertpub.com/aivt
The applicability of research models for screening and understanding cytokine-induced hepatotoxicity

Human
+ best immune/non parenchymal cell environment
+ best functional liver phenotype
+ high donor variability
- hepatocyte measurements not possible
- genomic interference techniques not possible
- need of reliable biomarkers
- low controllability of the system interferences during research

Human liver ex vivo and human precision-cut liver slices
+ good immune/non parenchymal cell environment
- no recruited immune cells
+ good functional liver phenotype
+/- high donor variability
- no interaction with body-derived input (e.g. gut-derived LPS)
- hepatocyte measurements not possible
- genomic interference techniques not possible
+ low/medium availability
- low sustainability
+/- higher controllability of the system interferences during research

Human 3D spheroids/sandwich co-culture
+/- relatively good immune environment
- no recruited immune cells
- artifical ratio hepatocytes/immune cells
+/- relatively high-differentiated hepatocyte phenotype
+/- less donor variability (applicable for cell lines and PHHs)
- direct hepatocyte measurements not possible
+ genomic interference techniques possible in cell lines
+ medium availability
+ high sustainability
+/- higher controllability of the system interferences during research

Human 2D co-culture/micropatterned culture
+/- medium level immune environment
- no recruited immune cells
- artifical ratio hepatocytes/immune cells
+/- less donor variability (applicable for cell lines and PHHs)
- direct hepatocyte measurements not possible
+ genomic interference techniques possible
+ high availability
+/- relatively low sustainability
+/- higher controllability of the system interferences during research

Human 3D hepatocyte spheroids/sandwich culture
- no representative immune environment
+/- relatively low differentiated hepatocyte phenotype
+/- less donor variability (applicable for cell lines and PHHs)
+ direct hepatocyte measurements possible
+ genomic interference techniques possible
+/- medium availability
+/- medium sustainability
+ high controllability of the system interferences during research

Human 2D hepatocyte culture
- no representative immune environment
- low differentiated hepatocyte phenotype
+/- less donor variability (applicable for cell lines and PHHs)
+ direct hepatocyte measurements possible
+ genomic interference techniques possible
+ high availability
- low sustainability
+ high controllability of the system interferences during research
Culture systems in which the proximity of KCs and hepatocytes can be controlled, as is the case in micropatterned culturing methods, have demonstrated an increased hepatocyte function compared to random co-culture\(^1\). Furthermore, it has been shown that the ratio of KCs to hepatocytes, cell-cell contact and the relative proximity between these cell types determine the functionality of hepatocytes\(^{131}\). However, to our best knowledge, no co-exposure experiments with LPS and drugs have been performed in this model. However, co-culture of KCs with hepatocytes in a 3D culture model, shows that KC activation by LPS decreased the cytotoxicity threshold for trovafloxacin threefold compared to only trovafloxacin treatment\(^{132}\). Taken together, these co-culture models show promising results for the assessment of inflammatory stress in high throughput approaches.

Another, more complex, multicellular approach eventually feasible for low/medium throughput purposes are human precision-cut liver slices. This model can show LPS-induced synergistic cytotoxicity and can be used to classify hepatotoxicants, although it has to be further developed for screening purposes\(^{112,113,133}\). A general overview of the most-used cell culture methods in toxicity screening and their applicability for the detection of inflammation-induced signaling and hepatotoxicity is displayed in figure 1. Advantages and disadvantages of each model are listed, clearly showing that these models have their own restricted values. The advantages and shortcomings of each model make serious consideration of the purpose of the screening or mechanistic study essential in choosing a fitting model system. Currently, the most exciting progress in model development in the idiosyncratic DILI research field is made with human-induced pluripotent stem cells. These cells can be developed into donor-specific hepatocytes, enabling the research on donor-specific cells from DILI-susceptible donors\(^1,134\). Once the induction of stem cells into hepatocytes and especially in non-parenchymal cells is optimized, this model will revolutionize toxicity research.

The models described above can be used not only for screening and prediction purposes, but also to gain mechanistic understanding. Fluorescent HepG2 reporter cell lines allow the quantitative, time-resolved detection of adaptive stress response activation on a single-cell basis\(^{135}\). These data, in combination with phosphoproteomics\(^{130}\), genomics approaches\(^{16}\), and co-culture system proteomics\(^{35}\) point towards the important role of adaptive stress responses interfering with cytokine-induced signaling. These insights in cytokine-induced toxicity from \textit{in vitro} models might help unravel human DILI. They also show how immune/cytokine signaling is reflected, or lacks, in \textit{in vitro} high throughput predictive models when compared to human and animal \textit{in vivo} data..

**Summary and future perspective**

Idiosyncratic DILI remains one of the most unpredictable adverse drug reactions, often leading to discontinuation of drug development or restricted use of effective drugs. In this review, the inflammatory signaling as a host factor that increases DILI potential has been discussed, highlighting the recent progress in this field. This with a main focus on the role of TNF\(\alpha\) signaling, TLR4 signaling, and diclofenac. Taken together, these data suggest that drug-induced adaptive stress response pathways in hepatocytes and non-parenchymal
cells could sensitize cells to drug-independent host factors. It would explain the idiosyncratic nature of many drug-induced liver injury, since a critical amount of host factors would be involved in both drug-dependent and -independent cellular adaptive processes. The typically unpredictable length of latency time suggests that many processes are involved in the adaptive processes before the threshold is reached for induction of hepatotoxicity. Furthermore, the clinical adaptation of most of the mild DILI cases despite continuation of drug treatment implies inherent negative feedback signaling. Finally, understanding idiosyncratic DILI in human, together with the understanding of the potential and limitations of our in vitro screening approaches will lead to improved prediction of idiosyncratic DILI in an early drug development stage.

Acknowledgements
This work was supported by the Innovative Medicine Initiative MIP-DILI project (grant agreement no 115336) and the European Horizon2020 EU-ToxRisk project (grant agreement no. 681002).

Disclosure Statement
No competing financial interests exist.