**Immune Drug-induced Liver Disease and Drugs**

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**Abstract**

Idiosyncratic drug-induced liver injury (DILI) represents a major public health concern and impediment to drug development. The mechanisms of DILI are not completely understood; both non-immune and immune mediated mechanisms have been proposed. Non-immune mediated mechanisms including direct damage to hepatocytes, mitochondrial toxicity, interference with transporters, and alteration of bile ducts, are well known to be associated with drugs such as acetaminophen and diclofenac; whereas immune-mediated mechanisms involving activation of both adaptive and innate immune cells, and the interactions of these cells with parenchymal cells have been proposed. A variety of hypotheses for immune-mediated DILI and the roles of liver immune cells in the development of DILI are discussed with respect to recent scientific advances. Additionally, understanding the mechanisms of how circulating leukocytes home to the liver upon injury, and their nature and location in the liver, is critical for the pathogenesis of DILI. Continuous research in understanding the molecular and cellular mechanisms of DILI and identifying risk factors will provide an opportunity to develop better test systems for the diagnosis, prediction, and prevention of DILI.

**Keywords:** DILI; immune-mediated; tolerance; antigen; immune cells homing

**Abbreviations:** DILI (drug-induced liver injury), HLA (human leukocyte antigen), APCs (antigen presenting cells), DCs (dendritic cells) LSECs (liver sinusoidal endothelial cells), KCs (Kupffer cells), APAP (acetaminophen), NAPQI (N-acetyl-p-benzoquinone imine), DAMPs (damage-associated molecular patterns), HMGB1 (high mobility group box 1), HSCs (hepatic stellate cells)TNF (tumour necrosis factor), IC (immune complex), ADA (anti-drug antibodies)

**Introduction**

Drug-induced liver injury (DILI) represents a major public health concern and impediment to drug development. In particular, idiosyncratic DILI, which is rare and often recognized only late in drug development, remains problematic for clinicians, the pharmaceutical industry, and regulatory agencies. The mechanisms underlying the pathogenesis of idiosyncratic DILI are not completely understood. However, there is now compelling evidence that activation of the immune system is involved in some cases. Immune-mediated DILI usually has a prolonged latency and can be characterised by the presence of a rash, fever, eosinophilia, and a rapid positive rechallenge in the clinic. The presence of antibodies directed against native or drug-modified hepatic proteins in DILI patients provides convincing evidence of the involvement of the adaptive immune system [1]. Furthermore, the infiltration of cytotoxic CD8 T-cells in the liver [2] and circulating drug-specific T-cells [3-5] in patients with DILI demonstrates the critical role of cell-mediated immunity in the underlying mechanisms. In addition, the finding of an association of specific alleles of human leukocyte antigen (HLA) class I and II with liver injury caused by certain drugs [6], including amoxicillin-clavulanate [7], ticlopidine [8], ximelagatran [9], flucloxacillin [10], lumiracoxib [11] and lapatinib [12], further supports the involvement of adaptive immunity in DILI (Table 1). Despite intensive research in this field, the precise cascade events that leads to activation of the immune system and how this manifests in liver injury remains to be fully defined.

The liver contains large populations of immune cells, including resident innate immune cells (Kupffer cells, dendritic cells, and natural killer cells) and intrahepatic lymphocytes (natural killer T-cells, CD4+ and CD8+ T-cells). Apart from the resident immune cells, the unique vascularization of the liver also allows the rapid recruitment of circulating leukocytes during tissue damage and inflammation (Figure 1). The co-residence of these immune cells in the liver creates a unique environment that regulates liver homeostasis to maintain a delicate balance between tolerance and immunity. Under basal conditions, the liver is able to regulate immune responses against pathogens from the gut and the circulation, but also maintain a tolerogenic environment. However, this tolerance can be broken, e.g. during infection and inflammation, or in the presence of immune check points inhibitors such as ipilimumab, nivolumab, and pembrolizumab [13-15], leading to activation of immune cells. Following the initial immune activation, additional mechanisms including transporter inhibition, oxidative stress, and the potential involvement of the innate immune system can further amplify or reduce the injury, and thereby determine the progression and severity of DILI. Therefore, understanding the complex interactions between immune cells and the local parenchymal or non-parenchymal cells in the liver, particularly those factors that determine tolerance and immunity, will inform the various immunological mechanisms of DILI. In doing so, models can be developed for predicting and preventing the occurrence of DILI in susceptible patients. This review will focus on recent advances in understanding the role of immune reactions in the development and progression of DILI.

1. **Adaptive immune responses**

Adaptive immunity, including both humoral and cellular immune responses, can play an important role in immune-mediated DILI. Humoral immune responses are mediated primarily by antibodies secreted by B lymphocytes; whereas cellular immune responses involve activation of antigen-specific T lymphocytes and the release of signalling molecules (e.g. cytokines). Several types of T cells are involved in cellular immunity including CD4+, CD8+, and γδ-T cells, which can be further categorized into several subsets. The roles of both humoral and cellular immunity in DILI are discussed in the following sections.

**Humoral immune responses.** Antigens with molecular weight less than 1000 Da are generally not capable of inducing humoral immune responses. However, small molecule drugs or their reactive metabolites can covalently bind to proteins to form neoantigens which can be recognized by B cells as non-self (hapten hypothesis). Examples include anti-drug antibodies (ADA), anti-CYP 2C9 autoantibodies in tienilic acid-induced hepatitis [16], and anti-isoniazid and anti-CYP (2E1, 2C9, and 3A4) antibodies in patients with severe isoniazid liver injury [1]. These antibodies can recognize antigens presented on the surface of hepatocytes and mediate cytotoxic effects through either complement activation or antibody-dependent cytotoxicity. Additionally, if an antibody-antigen immune complexes (IC) is internalized, processed, and presented by B cells, it can promote further B cell activation and antibody generation. It is worth noting that the formation of neoantigens and the generation of antibodies can occur in patients without clinical manifestation of DILI. Therefore the roles of these antibodies in DILI require further investigation. However, there is compelling evidence that ADA elicited *in vivo* to a biotherapeutic is central to both the therapeutic efficacy and adverse effects. As a large protein, biotherapeutics have the potential to be recognized as non-self by the host immune system, therefore, generation of ADA is a potential outcome to almost all biotherapeutics [17]. Although the roles of ADA in the immunogenicity of such therapeutics are not fully understood, the formation of a drug/ADA immune complex is believed to be able to mediate adverse effects through cross-linking of Fc receptors, activating the complement cascade, and promoting B cell maturation [18]. For example, hepatotoxicity associated with ADA formation has been a major limitation for the clinical use of the anti-tumour necrosis factor (TNF) superfamily, such as infliximab, adalimumab, and recombinant human Apo2L/TRAIL [19-21]. Crosslinking of IC can trigger hepatocyte apoptosis through clustering death receptors at the surface of the hepatocytes. However, given the diversity of biotherapeutics and the complexity of potential immune responses, the mechanisms of DILI induced by such class of drugs require further research.

**Cellular immune responses**. It is now widely believed that certain populations of lymphocytes play an important role in DILI (Figure 1). IFN-γ and granzyme-B secreting flucloxacillin-specific T-cells have been identified in the blood of patients with flucloxacillin-induced liver injury [5]. Also, T-cells isolated from patients with amoxicillin/clavulanic acid and isoniazid DILI have been shown to proliferate and secreted IFN-γ in response to drug treatment [3,4]. Although the identification of peripheral drug-specific T-cells in DILI patients provided strong evidence for the involvement of the adaptive immune system, the precise molecular mechanisms whereby these peripheral drug-specific T-cells cause injury to the liver remain unknown. It is worth noting that the resident lymphocytes in the liver are phenotypically different from those in peripheral blood. Analysis of leukocytes from healthy human donors revealed that there was an increase in CD8+ T-cells (CD4+/CD8+, 1:3.5) in human liver compared with that in peripheral blood (2:1), and an increase in the CD3+ cells expressing γδ TCR [22]. In addition, the lymphocyte populations of the liver may change significantly in DILI patients during inflammation. Indeed, a comparative analysis of portal hepatic infiltrating leukocytes from patients with acute DILI revealed that the predominant cell types are cytotoxic T-cells (CD8+), rather than T helper cells (CD4+), and the number of CD8+ T-cells was associated with the degree of liver damage regardless of the causative drugs and clinical manifestations [23]. These cytotoxic CD8+ cells either migrate from peripheral blood or are expanded locally upon antigen stimulation and can attack and/or damage hepatocytes/cholangiocytes, leading to liver injury (Figure 1). Indeed, in a mouse model, depletion of CD8+T-cells was found to protect mice from amodiaquine-induced liver injury, strongly suggesting that they are responsible for the liver damage [24]. Apart from CD8+ T-cells, Th17 cells have also been shown to be involved in DILI in mouse models [25]. Extensive research within this filed has been focused on characterisation of circulating drug-specific T-cells, however, an investigation of resident drug-specific immune cells, which has been hindered by the scarcity of tissue biopsies, is urgently needed.

**Activation of immune cells by multiple mechanisms.** Lymphocytes can be activated by drugs, and/or their metabolites through covalent binding to proteins to generate neoantigens (hapten hypothesis, Figure 2), which are processed and presented by antigen presenting cells (APCs). Neoantigens may be formed in the liver due to the metabolic functions of liver, but can be formed with circulating proteins if the metabolites are stable enough to escape from the site(s) of formation. For example, circulating carbamazepine protein adducts derived from the stable 10,11 epoxide metabolite have been detected in carbamazepine exposed patients [26]. Alternatively, T-cells can be activated by direct interactions of drugs (metabolites) with immune cells (PI hypothesis), or even drug-altered self-peptides (altered peptides hypothesis) (Figure 2). Recent studies revealed the heterogeneity of drug-specific T-cells from patients with DILI that can recognize both drug-modified proteins and drug itself, indicating multiple mechanisms could be involved [3,27]. The advances in proteomics have allowed identification of circulating drug-modified proteins; however, the hepatic protein targets and the exact location of neoantigens formed in the liver remains to be investigated. It is worth noting that the formation of neoantigens may be important for initiating an immune response, but this process alone will not always result in liver injury. Indeed, circulating drug-modified proteins have been detected in patients without DILI [28-31]. Therefore, the critical question remains, what determines immunogenicity? Is the actual level of drug-modified proteins important? If so, the nature and location of proteins that are prone to modification by reactive drugs (metabolites) may be important in determining the overall immune response.

Neoantigens are generally processed and presented by professional APCs such as dendritic cells (DCs) for recognition by T-cells. Human liver contains large populations of resident APCs including DCs, liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs). Furthermore, in some circumstances, hepatocytes can also act as APCs, but with low presentation capacity due to the low expression of MHC I molecules [32]. However, under immunostimulatory conditions, such as exposure to IFNα, the expression of MHC Class I on the surface of hepatocytes can be markedly increased, making hepatocytes more susceptible to T cell toxicity [33]. Details of the antigen presentation functions of these cells are described in several recent reviews [13,14,34]. Liver APCs are well known to create bias towards tolerance rather than immunity [35]. These cells can mediate immunosuppression by generating anti-inflammatory cytokines (IL22, IL10, TGFβ) and ligands (PDL1). In addition, the liver is provided with an abundance of immunosuppressive cells such as CD4+CD25+Foxp3+ regulatory T-cells to ensure that local antigens do not stimulate immune responses. For example, amodiaquine DILI can be suppressed by T regulatory cells expressing PD1, CTLA4 and the transcription factor Fox p3 [36]. Recent studies have also demonstrated that IL22 prevents hepatocytes from damage, resulting in inhibition of liver inflammation [37]. It is therefore convincible that neoantigens formed in the liver generally do not stimulate immune responses. However, during inflammation, APCs can rapidly switch to effective APCs in response to inflammatory signals, e.g. upon exposure to chemokines (Figure 2). In addition, the unique trafficking and interactions between lymphoid DCs and KCs have allowed efficient cross-presentation of local antigens trapped by KCs [34].

1. **Innate immune responses**

Within the liver, innate immune cells including KCs, NK cells, neutrophils, DCs, and NKT-cells are critical for maintaining liver homeostasis by inducing both immunogenic and tolerogenic immune responses. There is now compelling evidence that the innate immune response is critical in promoting or inhibiting the extent of inflammation and thereby determining the progression and severity of DILI. The role of the innate immune response in DILI has been characterized for a small number of drugs such as acetaminophen (APAP) and diclofenac. Hepatocyte necrosis due to APAP metabolism and N-acetyl-p-benzoquinone imine (NAPQI)-mediated cell death releases damage-associated molecular patterns (DAMPs) that can interact with the TLR4 to induce inflammation [38]. For example, the increased total circulating high mobility group box 1 (HMGB1) protein, as well as the acetylated HMGB1 that is produced during the activation of immune cells, have been associated with APAP DILI in both mice and in humans [39,40]. Using a mouse model, Oda *et al* demonstrated that diclofenac acyl glucuronide, a phase II reactive metabolite, was partly involved in the pathogenesis of diclofenac DILI by activating innate immunity and neutrophils [41].

The factors that activate innate immune cells are not completely understood. It has been hypothesized that reactive metabolites, hepatocytes-derived exosomes [9], or DAMP molecules released by apoptotic and necrotic cells can activate these cells. For example, a recent study has shown that hepatocyte conditioned medium has a profound effect on both the phenotype and function of dendritic cells [42]. In addition, investigations have demonstrated that DAMP molecules such as HMGB1, heat shock proteins, and hyaluronan can stimulate KCs and HSCs in response to the initial liver injury [43]. Activation of KCs by DAMPs is believed to proceed via the complex TLR singling cascade, though the precise mechanisms need further investigation. The activated innate immune cells, e.g. KCs, can in turn produce reactive oxygen species and other inflammatory mediators, perpetuating further inflammation (Figure 1). Furthermore, these cells play a crucial role in recruiting other cells such as neutrophils, DCs and circulating T-cells, to the site of injury. The precise role of each innate immune cell in the pathogenesis of DILI remains controversial. Due to the complex and overlapping inflammatory mediators released by these cells and many of the experimental techniques used to deplete or inhibit a cell type, it has been difficult to define the contribution of these cells to DILI and how the innate and adaptive immune systems interact in the liver of a susceptible patient.

1. **Genetic associations with DILI**

An important aspect of immune-mediated DILI is individual susceptibility which can be partially explained by genetic predisposition. Recent advances in genomic analyses have revealed significant associations between DILI and specific HLA alleles, as well as certain innate immune genes (Table 1). This has been reviewed elsewhere and detailed information can also be found at <https://www.pharmgkb.org/>. These discoveries have paved the way for predicting DILI in certain patient populations. Almost all associations have high negative predictive values, but, importantly a low positive predictive value. Thus, while such genetic associations present strong evidence for the role of the immune system in particular forms of DILI, the generally low positive predictive values show that factors other than genetic disposition may also contribute to the pathogenesis of DILI. For example, a recent study demonstrated that flucloxacillin caused bile canaliculi dilatation and subsequent disruption of canalicular bile flow, leading to non-immune mediated cholestasis [44]. As the same neoantigens could be formed in both healthy human and DILI patients, of which only 0.1% will be presented by specific MHC molecules [45]. The critical question remains, what determines the nature of the antigenic peptides that are presented? Despite extensive research within this field, the bona fide antigenic peptides that are able to stimulate T-cells remain unknown. In addition, the observation that drug-specific T-cells were able to kill antigen-bearing hepatocytes in HLA restricted manner adds further complexity [46]. Little is known about the antigenic peptides presented on the surface of target cells, e.g. hepatocytes that can be seen by the antigen specific T-cells. Are these peptides the same ones that stimulate T-cells or peptides with partial homology that cross react with the same TCRs? The latter seems more likely because a single TCR could bind dozens of peptides with similar TCR contact amino acids but very different MHC anchor residues [47]. Advanced mass spectrometry has allowed profiling peptides presented by HLA-B\*57:01 in the presence of abacavir. These studies led to the discovery of self-peptides that might have the potential to stimulate abacavir-specific T-cells and thereby cause abacavir hypersensitivity [48,49]. Continuing efforts to analyse liver antigenic peptides presented by specific HLA alleles expressed on target cells will certainly shed light on the mechanisms underlying immune-mediated DILI.

1. **Immune cells homing to the liver**

Upon liver injury, aside from the resident immune cells, the anatomical position and substantial vascularization of the liver allows rapid recruitment of additional circulating leukocytes including neutrophils, monocytes, and lymphocytes. Their nature and the intrahepatic localization may determine the pattern of DILI, for example, lymphocytes surrounding and infiltrating bile ducts generally lead to the cholestatic DILI [50]. Thus, understanding how immune cells are recruited and positioned in the liver is critical for understanding of the pathogenesis of DILI.

Selective recruitment at the level of the endothelium and subsequent compartmentalization within the liver determines the intrahepatic distribution of specific lymphocyte populations. The homing of lymphocytes to the liver involves multiple cells present in the hepatic sinusoids, including hepatic sinusoidal endothelial cells, KCs, and hepatocytes. The cellular mechanisms of T-cell infiltration into tissue are poorly understood, but believed to be driven by the complex classic adhesion cascade which involves intergrins, VAP-1, ICAM-1 and chemokines. Indeed, flucloxacillin-responsive CD8+ T-cells detected in DILI patients and healthy volunteers expressed chemokine receptors such as CCR2, CCR4 and CCR9. These chemokines are implicated in liver homing [5]. An investigation of live-infiltrating monocytes in patients with chronic inflammatory/fibrotic liver diseases found significant accumulation of CD14+CD16+ monocytes within the inflamed liver [51]. The accumulation of these cells in the liver was believed to be a consequence of either transmigration from blood across hepatic endothelium or local differentiation from classical CD14+CD16- monocytes in the presence of TGFβ and IL-10. Interestingly, a recent investigation found that platelets also played an important role in hepatic CD8+ homing, highlighting the complex mechanisms involved in the cascade [52].

**Conclusion**

Considerable progress has been made in understanding the molecular and cellular mechanisms underlying DILI. However, due to the low incidence of DILI, the lack of *in vitro* models, and definitive prognostic biomarkers, prediction and prevention of immune-mediated DILI has been extremely challenging. Recent advances in developing more complex *in vitro* models such as long-term 3D co-culture systems and liver-on-a-chip [53,54], which have allowed for a more *in vivo*-like physiological microenvironment, has made the future prediction of DILI more promising. In particular, the co-culture of immune cells with target cells such as hepatocytes, has allowed us to at least consider investigating the complex immunological mechanisms, for example, the danger signals and antigens that can activate immune cells and the cellular mechanisms involved in killing target cells. More importantly, co-culturing immune cells with hepatocytes expressing specific HLA risk alleles will provide a valuable system for addressing patient-specific factors contributing to DILI. From a clinical perspective it is important that there is a better understanding of how both the innate and adaptive immune response integrate to cause immune mediated DILI in a particular patient. From a chemical perspective we need to be aware of the multiple mechanisms by which drugs (metabolites) can initiate various events that activate the immune system in the liver. The use of novel *in silico* models together with new biomarkers such as MicroRNA-122, HMGB1 and keratin-18, alongside markers of drug-specific immunoglobulins does provide an opportunity to develop better predictive test systems for the prediction and prevention of DILI.

Considerable progress has been made in elucidating DILI caused by small molecules. Further research is warranted to understand the complex mechanisms underlying biotherapeutics DILI. As a large protein, such therapeutics can induce both antibody-mediated and cellular immune responses, which may target multiple tissues and manifest complex clinical presentation. Of particular importance, the lack of quantitative assays for assessing the immunogenicity of biotherapeutics in patients makes it difficult to make clinical decisions in routine practice. A deeper understanding of mechanisms and effective information sharing between the biopharmaceutical industry, academia, clinicians, and regulatory agencies is essential to identify the potential safety hazards that might be associated with biotherapeutics and the ensuring risk assessment.

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**Notes**

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Table 1. Drug-specific immune responses detected in DILI patients

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Drug | HLA restriction | Odd ratio |  Detection of T-cells | Covalent binding |
| phenotype | patient T-cells | In vitro | In vivo |
| Amoxicillin /clavulante | A\*02:01/DRB1\*15:01 | 2.2/2.8 [7] | CD4>CD8 | Yes | HSA [29] | HSA [29] |
| Flucloxacillin | B\*57:01 | 72 [10] | CD8>CD4 | Yes | HSA [30] | HSA [21] |
| Isoniazid | NK | NK | CD4>CD8 | Yes | HSA [28] | HSA [28], mouse liver S9 proteins [55] |
| Ticlopidine | A\*33:01/03 | 13 [8] | NK | No | CYP2C19 [56] | NK |
| Terbinafine | A\*33:01 | 40.5 [57] | NK | No | HSA, GSTP | NK |
| Ximelagatran | DRB1\*07:01 | 4.4 [9] | NK | No | NO | NO |
| Lumiracoxib | DRB1\*15:01 | 7.5 [11] | NK | No | NK | NK |
| Lapatinib | DQA1\*02:01 | 9.0 [12] | NK | No | HSA, GSTP [58] | NK |
| Fenofibrate | A\*33:01 | 58.7 [57] | NK | No | NK | NK |
| Nevirapine | DRB1\*01 | 3.0 [59] | NK | No | HSA [31], Hb [60] | HSA [31], Hb [60] |

**Figure Legend**

**Figure 1. Immune-mediated drug-induced liver injury.** Innate immune cells (NK cell, Kupffer cells, and dendritic cells) can be activated by damage-associated molecular patterns molecules released by apoptotic and necrotic cells in the liver. The innate immune cells can promote either tolerance /liver regeneration by production of IL-10, IL-13, and TGFβ, or immunity by secreting TNFα, IL-β, and IFN-γ. The adaptive immune cells can be activated by a drug and its metabolites, drug-modified proteins, or drug altered self –peptides. The drug specific T cells that are either expanded in the liver or migrated from blood can damage hepatocytes or other cells in the liver, leading to liver injury.

**Figure 2. Antigen presentation in the liver.** (A) Liver APCs generally express low levels of MHC molecules and high levels of inhibitory molecules such as PDL1, thereby creating bias towards tolerance rather than immunity. During infection and inflammation, APCs can rapidly switch to effective APCs in response to inflammatory signals, leading to activation of adaptive immune cells. (B) An immune response can be initiated by drug-altered self-peptides (altered peptides hypothesis), direct interactions with immune cells (PI hypothesis), or the drug or its metabolites through covalent binding to peptides (hapten hypothesis).

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