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Definition of the Chemical and Immunological Signals Involved in Drug-Induced Liver Injury

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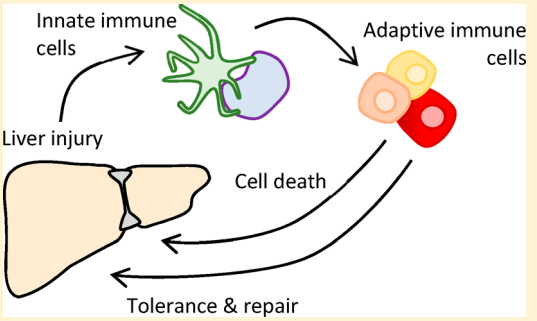
ABSTRACT: Idiosyncratic drug-induced liver injury (iDILI), which is rare and often recognized only late in drug development, poses a major public health concern and impediment to drug development due to its high rate of morbidity and mortality. The mechanisms of DILI are not completely understood; both 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. The chemical signals involved in activation of both innate and adaptive immune responses are discussed with respect to recent scientific advances. In addition, the immunological signals including cytokine and chemokines that are involved in promoting liver injury are also reviewed. Finally, we discuss how liver tolerance and regeneration can have profound impact on the pathogenesis of iDILI. Continuous research in developing *in vitro* systems incorporating immune cells with liver cells and animal models with impaired liver tolerance will provide an opportunity for improved prediction and prevention of immune-mediated iDILI.



1. INTRODUCTION

Drug-induced liver injury (DILI) is one of the most common causes of clinical trial failures of new therapeutic agents (33%) and post-marketing withdrawals.[1] In particular, idiosyncratic drug-induced liver injury (iDILI), which is rare and often only recognized late in drug development, poses a major clinical challenge due to its high rate of morbidity and mortality coupled with its unpredictable nature. iDILI can be characterized as hepatocellular, cholestatic, or mixed type of liver injury depending on the ratio of alanine aminotransferase to alkaline phosphatase relative to their respective upper limits of normal.[2] Antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs) namely amoxicillin-clavulanate, flucloxacillin, diclofenac, and isoniazid are most often associated with iDILI.[3] Under basal conditions, the liver not only is capable of regulating immune responses against pathogens from the gut and the circulatory system but also maintaining a tolerogenic environment. Moreover, the liver is a unique organ with an extraordinary capacity to regenerate after damage. The regeneration is surprisingly fast and plays a pivotal role in maintaining body homeostasis.[4] However, impaired liver tolerance and insufficient regeneration and repair, for example, during infection, inflammation, and inhibition of immune modulators, can lead to the activation of immune cells.[5,6] 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, thereby determining the progression and severity of DILI. Therefore, understanding the complex interactions between immune cells and the local parenchymal or nonparenchymal cells in the liver, particularly those factors that determine tolerance and immunity, will inform the various immunological mechanisms of DILI.

Although the mechanisms underlying the pathogenesis of iDILI are not completely understood, many cases show features of immune-mediated reactions such as the presence of a rash, fever, eosinophilia, and a rapid positive rechallenge in the clinic. The detection of antibodies directed against native or drug-modified hepatic proteins in DILI patients,[7] the infiltration of cytotoxic CD8+ T-cells in the liver,[8] and circulating drug-specific T-cells[9−11] in patients support immune-based mechanisms. The association between specific alleles of human leukocyte antigen (HLA) class I and II and susceptibility to liver injury further supports the involvement of adaptive immunity in iDILI. A growing number of HLA alleles have been identified to be associated with drugs including amoxicillin-clavulanate,[12] ticlopidine,[13] ximelagatran,[14] flucloxacillin,[15] lumiracoxib,[16] and lapatinib.[17] Despite intensive research in this field, the precise cascade events that lead to the activation of the immune system and how this manifests into liver injury remain to be fully defined. This review focuses on the chemical signals that activate immune cells and the molecular pathways that promote liver injury.

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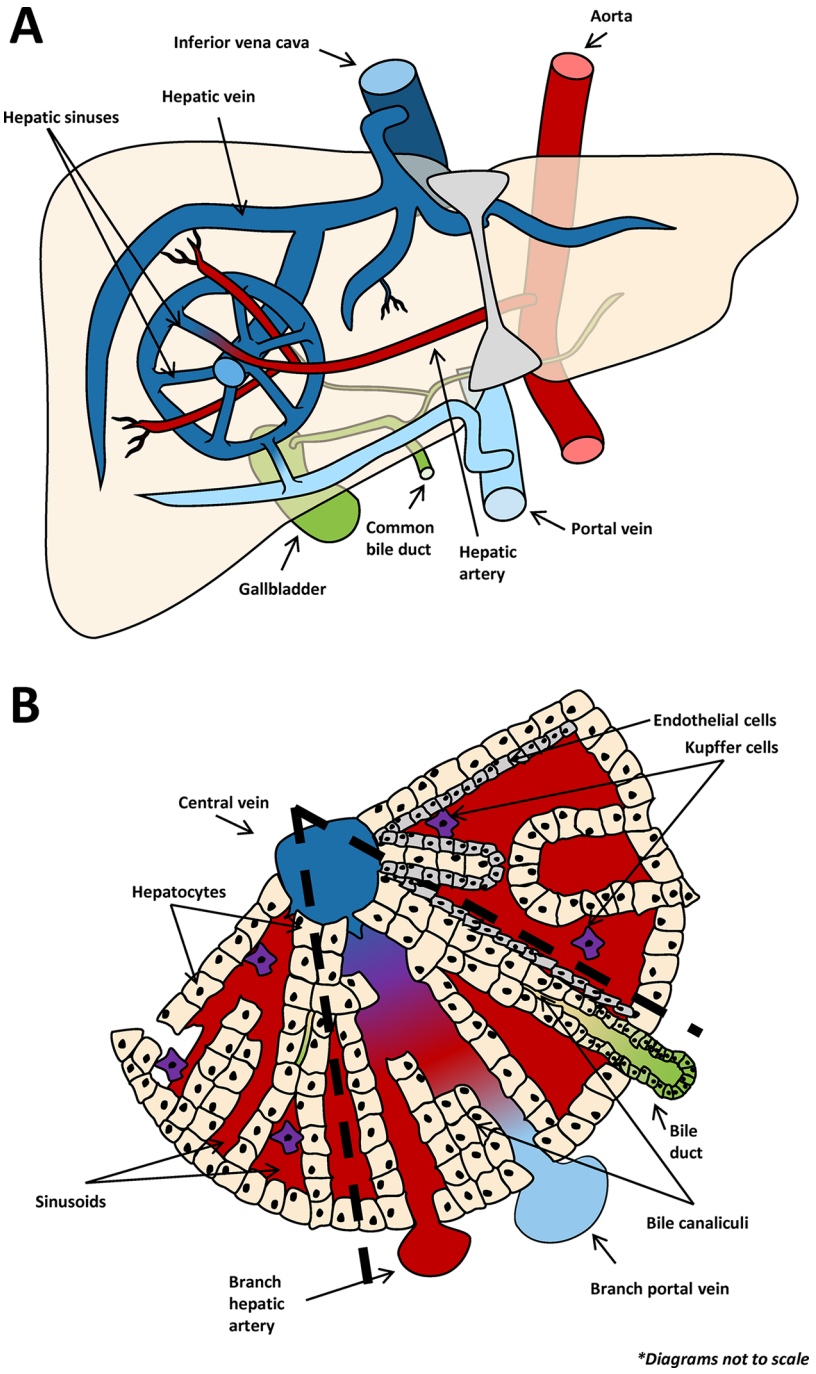

Figure 1. Structure of the liver. (A) The liver is a large organ supplied by multiple arteries and veins. Oxygenated blood is supplied to the liver via the hepatic artery, a branch of the aorta. It exits via the hepatic vein to the inferior vena cava. The portal vein supplies blood from the digestive system for the liver to undertake its primary functions of metabolism, detoxification, and others. (B) Close up schematic of the lobules of the liver. Blood enters the lobules through branches of the portal vein and the hepatic artery where they are mixed in the sinusoids. Excretion products are transported into the bile canaliculi where they enter the bile duct and are subsequently excreted into the duodenum. Resident immune cells, such as KCs and T-cells, are present in the sinusoids of the liver.

2. IMMUNOLOGICAL FUNCTIONS WITHIN THE LIVER

2.1. The Anatomical Structure and Physiological Functions of the Liver. The liver is the largest solid organ in the body and is critical to metabolic processes and immune functions (Figure 1). Lobules comprised of canaliculi flowing toward the common bile duct exist alongside small sinusoids flowing from branches of the hepatic portal vein and hepatic artery to a "central" vein, a branch of the hepatic vein.[18] Through this network, all of the blood flow from the gastrointestinal tract passes through the sinusoids, exiting into the central vein. The liver contains a mixture of cell types, including parenchymal cells, that is, hepatocytes, and multiple nonparenchymal cells located around the sinusoids, creating a unique network for cellular communication. Hepatocytes perform the majority of hepatic metabolic functions, which are highly regulated by substances released from the neighboring nonparenchymal cells such as liver sinusoidal endothelial cells (LSECs), biliary epithelial cells (cholangiocytes), hepatic stellate cells (HSCs),

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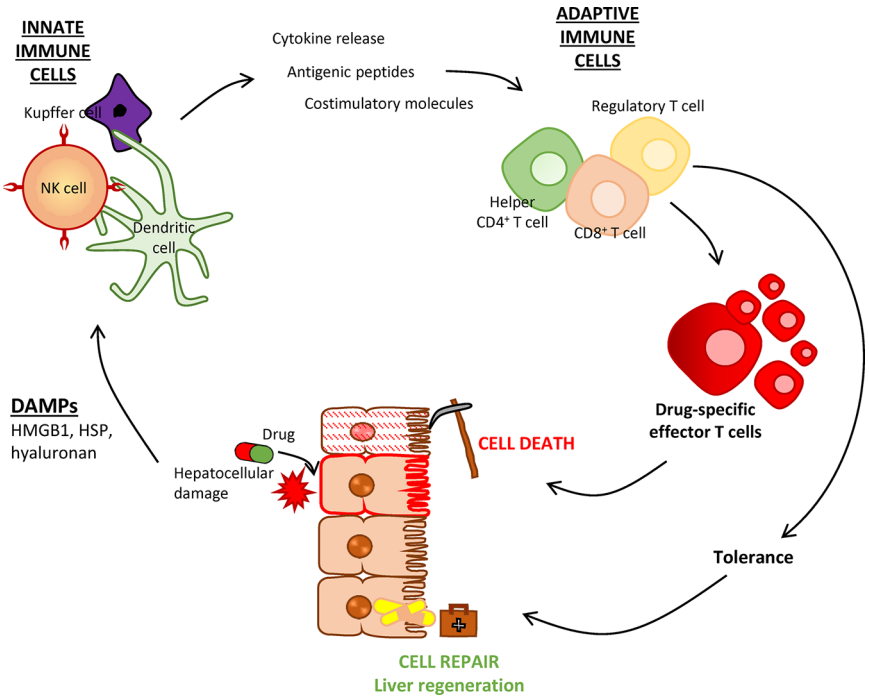

Figure 2. Interactions between the innate and adaptive immune cells and liver cells. Damaged liver cells can release DAMPs, which are important in activating innate immune cells. The activated innate immune cells can either release cytokines or present drug-associated antigens, leading to activation of adaptive immune cells. The cytotoxic T-cells can kill the hepatocytes, leading to liver injury when the liver regenerative capacity is impaired.

and large populations of immune cells.[19] Apart from resident innate immune cells such as Kupffer cells (KCs), dendritic cells (DCs), and natural killer cells (NKs), and intrahepatic lymphocytes (natural killer T-cells, CD4+ and CD8+ T-cells), the unique vascularization of the liver also allows the rapid recruitment of circulating leukocytes during tissue damage and inflammation. 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.

2.2. The Role of the Innate Immune System in DILI. Within the liver, innate immune cells including KCs, NKs, neutrophils, DCs, and NK T-cells are critical for maintaining liver homeostasis by inducing both immunogenic and tolerogenic immune responses. However, the precise role of each innate immune cell in the pathogenesis of DILI remains ill defined. It has been shown that innate immunity plays an important role in responding to drug-induced stress. The factors that activate innate immune cells are not completely understood. It has been hypothesized that reactive metabolites, hepatocytes-derived exosomes,[20,21] or danger-associated molecular pattern (DAMP) molecules released by apoptotic and necrotic cells can activate these cells. Especially, DAMPs including high mobility group box 1 (HMGB1) protein, ATP, mitochondrial DNA, nuclear DNA fragments, RNA, purines, uric acid, heat-shock proteins (HSPs), and bile acids have been shown to activate KCs, neutrophils, and DCs via toll-like receptors (TLRs). DAMPs released by necrotic hepatocytes due to overdoses of acetaminophen (APAP) can activate KCs and neutrophils, which may enhance inflammation by secreting various pro-inflammatory cytokines.[22] The release of HSPs has also been shown to mature DCs and promote the secretion of pro-inflammatory cytokines through TLR activation.[23] HMGB1, forming relatively stable complexes with promiscuous substrates, can promote the maturation of DCs through TLR-4-dependent signaling. Primary human hepatocytes cultured with SMX-NO and flucloxacillin resulted in a drug-specific and concentration-dependent release of HMGB1. Culturing DCs with HMGB1 conditioned medium resulted in the secretion of IL-1α, IL-1β, TNF-α, and IL-6, leading to the enhanced priming of naïve T-cells.[24] Reactive acyl glucuronide metabolite of diclofenac has been shown to activate neutrophils *in vitro* and *in vivo*, and the activated neutrophils partly contributed to the pathogenesis of diclofenac-induced acute liver injury.[25]

Activated innate immune cells can in turn produce reactive oxygen species and other inflammatory mediators, perpetuating further inflammation (Figure 2). Due to the complex and overlapping inflammatory mediators released by these cells and many of the experimental techniques used to inhibit a cell type, it has been difficult to define the contribution of these cells to iDILI and how the innate and adaptive immune systems interact in the liver of a susceptible patient. It should be noted that activation of innate immunity does not necessarily lead to adaptive immune responses. Overwhelmed innate immunity that resulted in extensive liver injury may activate the regulatory pathways, leading to inhibition of adaptive immune responses to prevent further injury. Indeed, although extensive protein adducts were formed following APAP overdoses, the lack of APAP-specific lymphocytes could be partially due to the immunosuppression following APAP hepatotoxicity.[26] However, activation of innate immune system is a prerequisite for adaptive immunity.

2.3. The Role of Adaptive Immunity in DILI. Adaptive immunity including both humoral and cellular immune responses play an important role in immune-mediated iDILI. Humoral immune responses, mediated primarily by antibodies, may cause hepatotoxicity through either complement activation or antibody-dependent cytotoxicity. Cellular immune responses involve activation of antigen-specific T lymphocytes, followed by subsequent T-cell-mediated cytotoxicity. Although the exact

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role of humoral immunity in iDILI remains undefined, both antidrug antibodies (ADAs) and autoantibodies detected in the sera of iDILI patients have been shown to induce significant cytotoxicity to hepatocytes. For example, IgG3 anti-isoniazid antibodies and anticytochrome P450 antibodies were detected in the sera of patients with isoniazid DILI. These antibodies were found to be associated with a Th1-type immune response that may play a pathogenic role in isoniazid DILI.[7,27] Moreover, ADA formation has been a major limitation for the clinical use of therapeutic proteins (TPs), such as the antitumor necrosis factor (TNF) superfamily including infliximab, adalimumab, and recombinant human Apo2L/TRAIL.[28,29] ADAs directed against TPs are believed to contribute to the hepatotoxicity through the formation of a drug/ADA immune complex, which activates the complement cascade and promotes B-cell maturation.[30]

A growing number of cases have shown that both circulating and liver resident lymphocytes are involved in DILI. IFN-γ and granzyme-B secreting flucloxacillin-specific T-cells have been identified in the blood of patients with flucloxacillin-induced liver injury.[9] Also, T-cells isolated from patients with amoxicillin/clavulanic acid and isoniazid DILI have been shown to proliferate and secrete IFN-γ in response to drug treatment.[10,11] 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, in that they generally lack the cytotoxic functions. 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. Apart from CD8+ T-cells, Th17 cells have also been implicated in DILI in mouse models.[31]

Importantly, a number of HLA class I and II alleles have been shown to play a significant role in iDILI susceptibility, indicating the involvement of adaptive immune responses in the pathogenesis of iDILI. Details of HLA associations with specific drugs can be found in recent reviews.[32−34] These discoveries provided mechanistic insight into the activation of adaptive immunity and paved the way for predicting iDILI in certain patient populations. Although almost all associations have high negative predictive values, the low incidence of iDILI leads to a low positive predictive value for the majority of iDILI-associated HLA alleles. For example, strong association was observed between HLA-B*57:01 and flucloxacillin iDILI, however, the low positive predictive value of this association (0.12%) makes the prospective screening tests for identification of patients at risk of flucloxacillin iDILI economically unpractical.[35] While HLA associations may not provide any clinical value in predicting DILI per se, they may be one of several factors which can influence drug-T-cell interactions at the molecular level, which may potentially lead to different clinical outcomes. The common associations of some haplotypes with structurally unrelated drugs are extremely interesting, for example, the association of HLA-DRB1*15:01-DQB1*06:02 haplotype with amoxicillin-clavulanate[12] and lumiracoxib[16] DILI and HLA-B*57:01 with flucloxacillin and pazopanib[36] DILI. Abacavir, a retroviral treatment used in HIV infection, is also strongly associated with the carriage of HLA-B*57:01. Abacavir hypersensitivity syndrome (AHS) occurs in 2−5% of patients, resulting in fever, rash, nausea, and vomiting. A number of studies identified the genetic association between abacavir and HLA-B*57:01, giving a positive predictive value of 48% and a negative predictive value of 100%. Carriage of HLA-B*57:01 results in a 48% chance of developing AHS, while 100% of cases are from patients carrying the allele. While all three share the same genetic predisposition, it is not fully understood why flucloxacillin and pazopanib result in DILI when abacavir manifests as a skin reaction. Furthermore, abacavir has a much stronger association, resulting in 1 in 2 HLA-B*57:01 carriers developing AHS. This is in contrast to 1 in 1000 carriers developing flucloxacillin iDILI.[15] The strong association of abacavir with HLA-B*57:01 could be related to the unique binding of abacavir to the F pocket of the peptide binding groove, which has not been observed with any other drug molecules. Due to the structural difference, these drugs may bind differently to the peptide binding groove, leading to altered presentation of drug associated self-peptides, which can potentially result in autoimmune-like reactions.

3. CHEMICAL SIGNALS FOR THE ACTIVATION OF DRUG-SPECIFIC T-CELL RESPONSES

3.1. Mechanisms of T-Cell Activation. Activation of naïve T-cells generally requires multiple signals presented by antigen presenting cells (APCs). The first signal, which is essential for initiating T-cell activation, is the interaction between T-cell receptors (TCRs) and antigenic peptides presented by HLA molecules on the surface of APCs. The second signal is the interaction between the co-stimulatory molecules on T-cells (e.g., CD28) and their ligands. The third signal, which suppresses T-cell activation, is the interactions between the coinhibitory molecules on the T-cells and their ligands on APCs, for example, programmed cell death protein 1 (PD-1) and PD-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and CD86/CD80. In addition, other signals including danger signals, cytokines and chemokines may also influence the outcome of T-cell activation.

Lymphocytes can be activated by drugs/metabolites through multiple mechanisms. Covalent binding of drugs/metabolites to proteins, which are processed and presented as drug-modified peptides by APCs, can stimulate drug-specific T-cells (hapten hypothesis, Figure 3). 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). Recent studies revealed the heterogeneity of drug-specific T-cells from patients with DILI that can recognize both drug-modified proteins and the drug itself, indicating multiple mechanisms could be involved.[37] Advanced structural biology coupled with proteomic techniques has allowed investigation of the detailed interactions between antigens and immune receptors, however, how activated drug-specific immune cells induce liver injury has still not been fully elucidated

3.2. Drug Protein Conjugates As Potential Antigens. Reactive drugs such as β-lactam antibiotics and covalent tyrosine kinase inhibitors, and reactive metabolites (RMs), can covalently bind to macromolecules. Particularly, RMs that have extremely short half-lives or great affinity toward proteins may preferentially bind to intracellular proteins. The resulted drug protein conjugates can induce either drug-specific antibody or T-cell responses, leading to unwanted immunological reactions. Characterization of the precise structure of epitopes formed on proteins is essential for understanding the interaction between drugs and immune receptors, thus providing insights into both the efficacy and safety of drug design. The emergence

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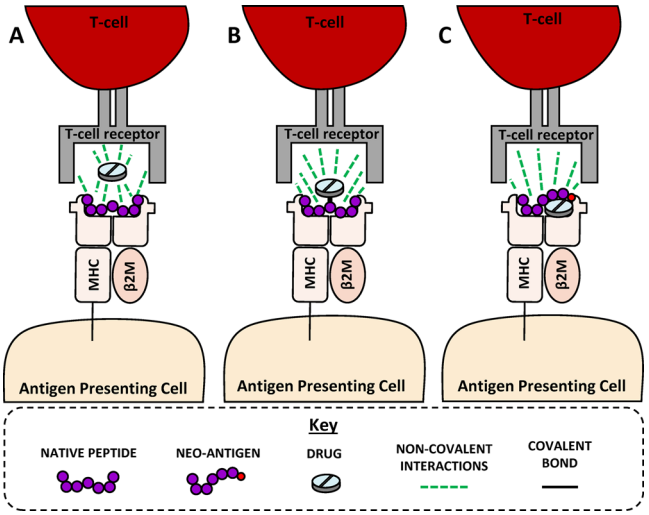

Figure 3. Interactions between the peptide, MHC, drug, and TCR follow three different hypotheses. MHC class I molecules are comprised of three α chains and a β-2-microglobulin subunit. Peptides are presented in the binding groove located between two of the α chains. (A) The drug interacts noncovalently with the peptide, MHC, and the TCR. (B) The drug covalently binds to the peptide presenting a neo-antigen to the TCR. (C) The drug interacts with the MHC, altering the conformation of the peptide binding groove to accommodate nondrug-modified neo-antigens. In all cases, these lead to T-cell activation and subsequent cytokine release.

of sensitive bioanalytical and proteomic techniques has allowed for a more precise identification of reactive metabolites and their protein targets in both *in vitro* systems and *in vivo* (Figure 4).

Nowadays, screening and characterization of RM formation of lead compounds during early drug discovery have been well established. Because of the high chemical reactivity, trapping agents including glutathione (GSH), cyanide, and amino acids are commonly used to detect them readily *in vitro*. Owing to the high sensitivity, LC-MS-based methods have been developed for rapid screening of RM formation in complex matrices, while NMR can be used for the absolute structural elucidation (Figure 4A). Multiple MS scan methods including the neutral loss, precursor ion, and multiple reaction monitoring scans have been traditionally used for characterization of GSH conjugates formed by RMs.[38] A recent study using a high-resolution MS platform coupled with polarity switching has enabled rapid screening and characterization of GSH-trapped RMs formed in liver microsomes and hepatocytes.[39] The structural elucidation of trapped-RMs will undoubtedly provide insights into the mechanisms of how protein adducts are formed. However, not all RMs can be trapped with common trapping agents. Both peptide-based trapping agents and model proteins such as GSH S-transferase π and human serum albumin (HSA) that contain multiple reactive nucleophilic residues have also been used to map the global reactivity of RMs.[40,41] It should be noted that the formation of reactive metabolites does not necessarily lead to toxicity, and extensive safety evaluation is required to determine whether or not a compound that forms RMs should progress through drug development.[42]

HSA has been identified as the major target for many reactive drugs. For example, circulating HSA adducts formed by a number of β-lactams were detected in the blood of patients taking β-lactam antibiotics.[43−45] These adducts have been shown to activate both ADAs and drug-specific T-cells. Cross-reactive ADAs and T-cells are common between penicillins due to the conserved thiazolidine ring across the family of penicillins. However, ADAs and T-cells that recognize the side chains of penicillins are highly specific. In recent years, covalent drugs have been developed for the treatment of cancer or hepatitis C infections (e.g., ibrutinib and afatinib).[46] These drugs can covalently bind to cysteine residues in a number of tyrosine kinases and display great clinical efficacy against cancer with mutations. For example, afatinib is capable of forming Michael adducts with cysteine residues within the catalytic sites of EGFR (Cys797), HER2 (Cys805), and HER4 (Cys803).[47] Despite promising efficacy across a broad range of disease, there have been great concerns that covalent drugs might cause potential off-target toxicity. Rare but severe livery injury has been reported to be associated with the use of ibrutinib and afatinib. Although the exact mechanisms of the liver injury are not fully understood, hepatocellular injury caused by lymphocytes infiltrate and additional canalicular cholestasis suggests an immune mechanism.[48,49] How these drugs activate the immune system remains unknown, however, covalent binding to off-target proteins to form neoantigens could partly contribute to the immune-mediated liver injury. Mass spectrometric (MS) analysis of ibrutinib-modified HSA revealed that ibrutinib formed lysine adducts (K190) via Michael addition with lysine attacking the β-carbon of the amide moiety.[50] Whether and how ibrutinib-HSA adducts contribute to the observed toxicity require further investigation. However, ibrutinib was shown to irreversibly bind to Cys424 in interleukin-2-inducible kinase, leading to inhibition of downstream activation of Th2-polarized CD4+ T-cells *in vitro* and *in vivo*.[51] Recent studies on covalent drugs have focused on identification of both target and off-target proteins to improve the efficacy, thereby reducing the toxicity associated with this class of drugs. Novel chemical proteomic methods to globally map the reactivity of functional cysteines in proteomes have enabled accelerated identification of novel protein targets and binding sites for kinase inhibitors.[52−54] For example, THZ1, a cyclin-dependent kinase inhibitor, also bound to Cys840 on PKN3.[54] Osimertinib, the third-generation T790M-EGFR inhibitor, was found to react with multiple cathepsins in cells and animal models due to the accumulation of the drug in lysosomes.

HSA is also a target for many RMs because of its high abundance in the liver. Both phase I and phase II metabolites have been shown to form HSA adducts in *in vitro* system and *in vivo*. A classic example of reactive phase I metabolites is NAPQI formed by APAP. APAP protein adducts were detected even at therapeutic doses, however, overdose resulted in extensive covalent binding to proteins that may contribute to hepatotoxicity.[55,56] The levels of APAP protein adducts in patients with acute liver failure (ALF) correlated well with the severity of toxicity, supporting their use as specific biomarkers for APAP toxicity in patients with ALF.[57] However, the role of APAP-HSA adducts in the activation of adaptive immunity remains undefined. It should be emphasized that drugs with aniline structural alerts, including lapatinib, diclofenac, nevirapine (NVP), and amodiaquine, can all form HSA adducts through the formation of quinone imine intermediates. Both ADAs and drug-specific T-cells may contribute to liver injury associated with these drugs. A number of phase II metabolites have also been shown to form protein adducts both *in vitro* and *in vivo*. NVP was shown to form protein adducts in the liver through its phase II metabolite, NVP-12-sulfate.[58] In addition, many carboxylic acid-containing drugs such as NSAIDs can form protein adducts through the reactive β-1-*O*-acyl glucuronides

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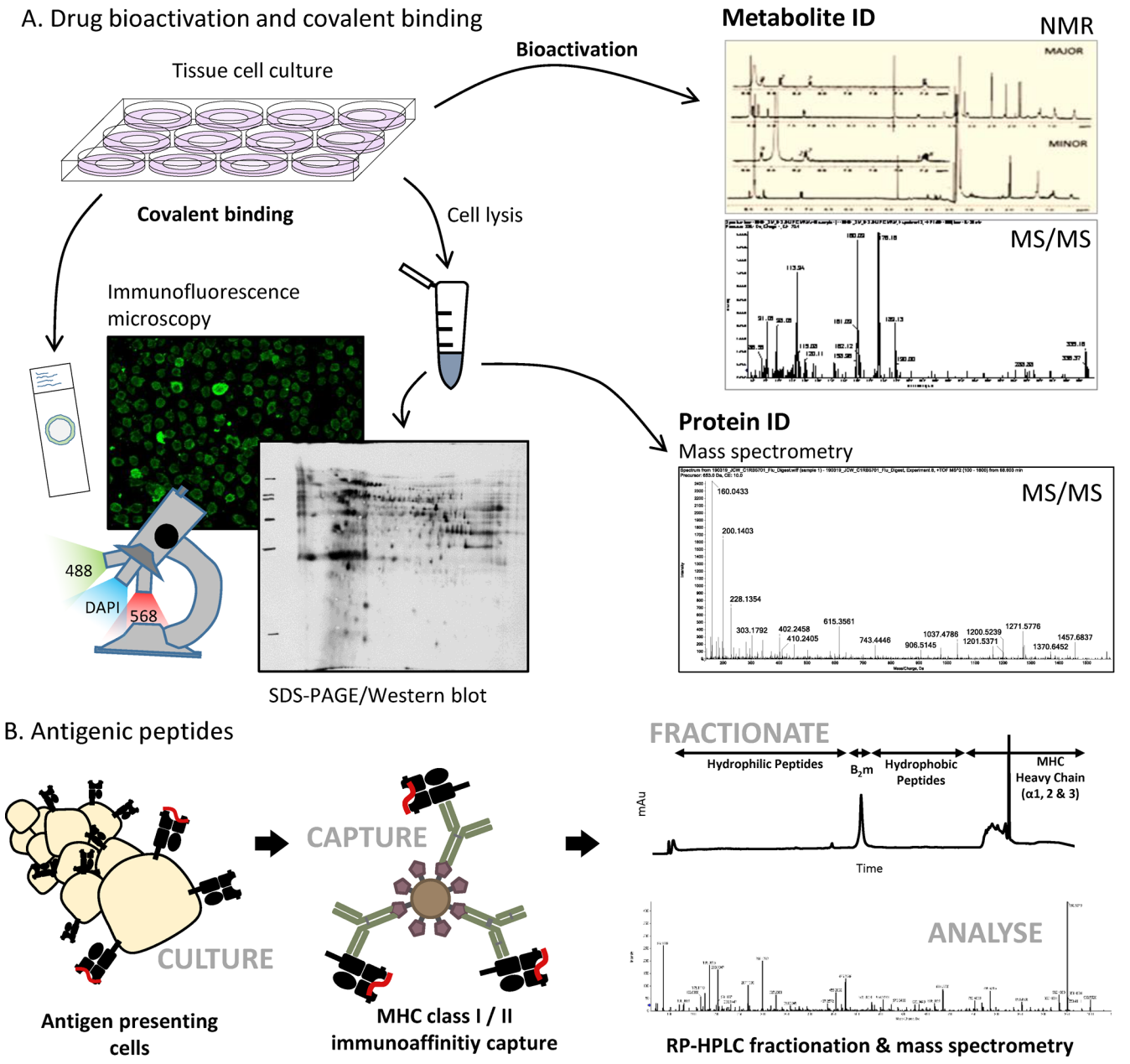

Figure 4. Characterization of drug associated antigens. (A) Drug metabolism in the liver leads to the formation of both stable and reactive metabolites; these metabolites can be characterized by advanced bioanalytical techniques including LC-MS/MS and NMR. Drug protein adducts can be characterized by immuno-histochemistry or Western blots using ADAs; the precise structure of epitopes can be determined by LC-MS/MS analysis. (B) Drug associated antigenic peptides presented by specific HLA molecules can be characterized using immunopeptidomic studies; peptides are eluted from antigen presenting cells using immuno-affinity columns, followed by HPLC purification and LC/MS/MS analysis.

(AGs). Two distinct types of adducts could be formed: transacylation adducts through direct reactions with certain amino acid residues on proteins, such as lysine, cysteine, and arginine. Alternatively, a Schiff base adduct retaining the glucuronide moiety could be formed through the reaction of isomeric glucuronides with amine nucleophiles followed by an Amadori rearrangement.[59,60] ADAs against acylation adducts have been detected in patients with liver injury, however, it remains unknown whether acyl glucuronides are exclusively responsible for antibody response in humans, as acylation adducts could be formed through other pathways (e.g., Coenzyme A pathway). Interestingly, the AG of diclofenac, and many other carboxylic acid-containing NSAIDs, has been found to selectively bind to several proteins located on the apical (bile canaliculi) domain of the hepatocyte plasma membrane.[61] These drug-modified proteins may be responsible for the accumulation of CD8+ T-cells around the bile ducts, which may contribute to the cholestasis associated with many NSAIDs.

3.3. Antigenic Peptide Presentation. T-cell activation is mediated through the interactions between the TCR and HLA-peptide complex displayed by APCs. However, for the majority of drugs, how drug molecules/metabolites are incorporated into the HLA-peptide complex for T-cell recognition remains undefined. Recently, advanced immunopeptidomic analysis of HLA-peptide complexes eluted from APCs (Figure 4B) has enabled the identification of thousands of peptides naturally presented on the cell surface by HLA molecules. Coupled with X-ray crystallographic analysis of HLA peptide complexes,

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immunopeptidomics has provided new insights into the mechanisms of immune-mediated disease. For example, recent studies have shown that abacavir interacts with the peptide binding cleft of HLA-B*57:01, altering the shape and chemistry of the HLA molecule and the array of peptides that bind.[62−64] Further structural elucidation of the drug peptide-HLA complex revealed that abacavir bound noncovalently in the vicinity of the F pocket of the HLA binding groove. Up to a thousand abacavir unique HLA binding peptides were identified, however, their contribution to the CD8+ T-cell response seen in abacavir hypersensitive patients is yet to be defined.

In terms of activation of drug-specific CD8+ T-cells involved in liver injury, it is possible that reactive metabolites formed within the liver can covalently bind to intracellular proteins followed by presentation of drug-modified peptides or directly bind to peptide-HLA complexes and the HLA molecule itself, leading to presentation of altered self-peptides. However, identification of drug-associated antigenic peptides presented by liver cells is more complicated due to the diverse subsets of APCs in the liver. Several liver cell types located in the sinusoids can serve as APCs to activate naïve CD8+ T-cells. These include hepatocytes, LSECs, KCs, and HSCs. Hepatocytes generally express low levels of class I MHC molecules but can respond with upregulation of heavy chain and β_2m under immune-stimulatory conditions, such as exposure to IFN-γ.[65] Interestingly, MHC-1 and ICAM-1 expression by hepatocytes was found to be polarized to the basolateral surface facing the sinusoids and is critical for CD8+ T-cell retention in the liver.[66] However, the nature of antigenic peptides presented by liver APCs remains undefined.

4. IMMUNOLOGICAL SIGNALS PROMOTE LIVER INJURY

4.1. Cytokines. The release of pro-inflammatory cytokines from damaged hepatocytes and innate immune cells can influence DC activation and T-cell phenotype. In addition, upon differentiation of naïve T-cells to effector T-cells, different cytokines are released by effector T-cells to carry out their function. Pro-inflammatory cytokines such as TNF-α and IL-1β can enhance inflammation and liver injury, whereas anti-inflammatory cytokines including IL-10, IL-6, and IL-13 have a protective function and prevent liver injury. TNF-α plays a major role in the pathogenesis of DILI, and elevated plasma levels were found to be associated with severity of DILI and prognosis both in patients and animal models.[67,68] Hepatotoxic drugs such as APAP, troglitazone, and trovafloxacin significantly increased LPS-induced IL-1β release in KCs, leading to an imbalance between pro- and anti-inflammatory cytokines.[69] IFN-γ has been shown to activate KCs and increase adhesion of leukocytes through activation of the intracellular JAK signaling and transcription activator STAT pathways, leading to promotion of an inflammatory response and ultimately cell death.[70] In addition, the synergetic effects of two key cytokines, IFN-γ and TNF-α, are known to cause the death of hepatocytes and activation of the immune system.[71] Distinct cytokine expression and release profiles are strongly attributed to the clinical features, prognosis, and progression of DILI.[72] Global metabolomics and proteomics approaches have now enabled systemic evaluation of the cytokines associated with DILI, providing new insight into the roles of these signals in immune-mediated DILI.[73]

IL-10 is a key immuno-regulator to prevent the excessive cytotoxic T-cell responses that are responsible for liver injury. Substantially elevated IL-10 levels were observed in mice with APAP overdose, and knockout of IL-10 in mice substantially increases lethality of APAP.[74] As a member of the IL-10 family, IL-22 also has profound tissue-protective properties by increasing tissue robustness and stress resistance, possibly through activation of STA3 and the subsequent MAPK and Atk pathways.[75] Severe liver inflammation was observed in IL-22-deficient mice treated with Concanavalin A (ConA, a known cause of DILI). Blockage of IL-22 also results in increased liver injury in an IL-22/CXCL10-dependent manner.[76] IL-6 is also well-known as a heptato-protective cytokine.[77] The levels of IL-6 are highly correlated with hepatocyte regeneration and severity of liver injury. Serum elevated IL-6 levels were observed in patients with liver diseases and mice treated with APAP.[73,78] IL-6 KO mice showed delayed regeneration after APAP, however, enhanced regeneration and reduced APAP-induced liver injury were observed by treatment with recombinant IL-6.[74]

It should be noted that the infection and inflammatory disease can significantly influence the severity of DILI through effects on the circulating cytokines. Several conditions of infection and inflammation including influenza, HIV infection, and hepatitis B and C have long been known to alter cytokine release. As reviewed recently, increased pro-inflammatory cytokines such as IFNγ, IL-6, IL-1β, and TNF-α can downregulate metabolizing CYPs, drug transporters, and conjugative enzymes subsequently affecting drug binding, transport, and bioavailability.[79,80] P450 depression in the liver was at the highest upon treatment with IL-1β, IL-6, and TNF-α.[81] Likewise a Th2 cytokine response (IL 4, 5, and 13) resulted in downregulation of P450 mRNAs in mice with *Schistosoma mansoni* infection.[82] Hepatic CYP down-regulation is important in the elaboration or resolution of the inflammatory response because it can prevent further oxidative stress caused by drugs that are bioactivated by these enzymes. Similarly, downregulation of UDP-glucuronosyltransferases, sulfotransferases, GSH S-transferases, and transporters can reduce the hepatic accumulation of cholesterol and its derivatives, bile acids, and steroids, preventing further stress.[80]

It has been shown that the balance between the pro- and anti-inflammatory cytokines determines the susceptibility and severity of liver injury.[83] However, the cytokine profiles in patients with DILI have been shown to be extremely complex, in particular, the complex interactions between individual cytokines, which makes the interpretation of their roles in DILI extremely difficult.[84] Over a third of patients in a cytokine release study of DILI patients did not fall into a unique defined immune group, this is particularly due to numerous external factors such as difference in causative drugs, underlying conditions, gender differences, age, and ethnicity, demonstrating the difficulty in determining cytokine profiles.[72] *In silico* modeling is required to evaluate the relationship between cytokine networks and patient prognosis and severity of DILI.

4.2. Leukocytes Home to the Liver. Before T-cells can exert a cytotoxic immune response in a particular location, they must first be recruited or "homed" from the systemic circulation. The inflammatory state is regulated by chemokines and their subsequent receptors present on lymphocytes, which allow for distinct immune populations to enter the liver.[85] Chemokines are characteristically small molecular weight proteins and can be categorized into four different families (CC, CXC, CX3C, C) based on the arrangement of the first two of four conserved cysteine residues. These arrangements give rise to 50 chemokine ligands and 20 cognate receptors, a number of which are associated with DILI.[86,87] However, before chemokines can be

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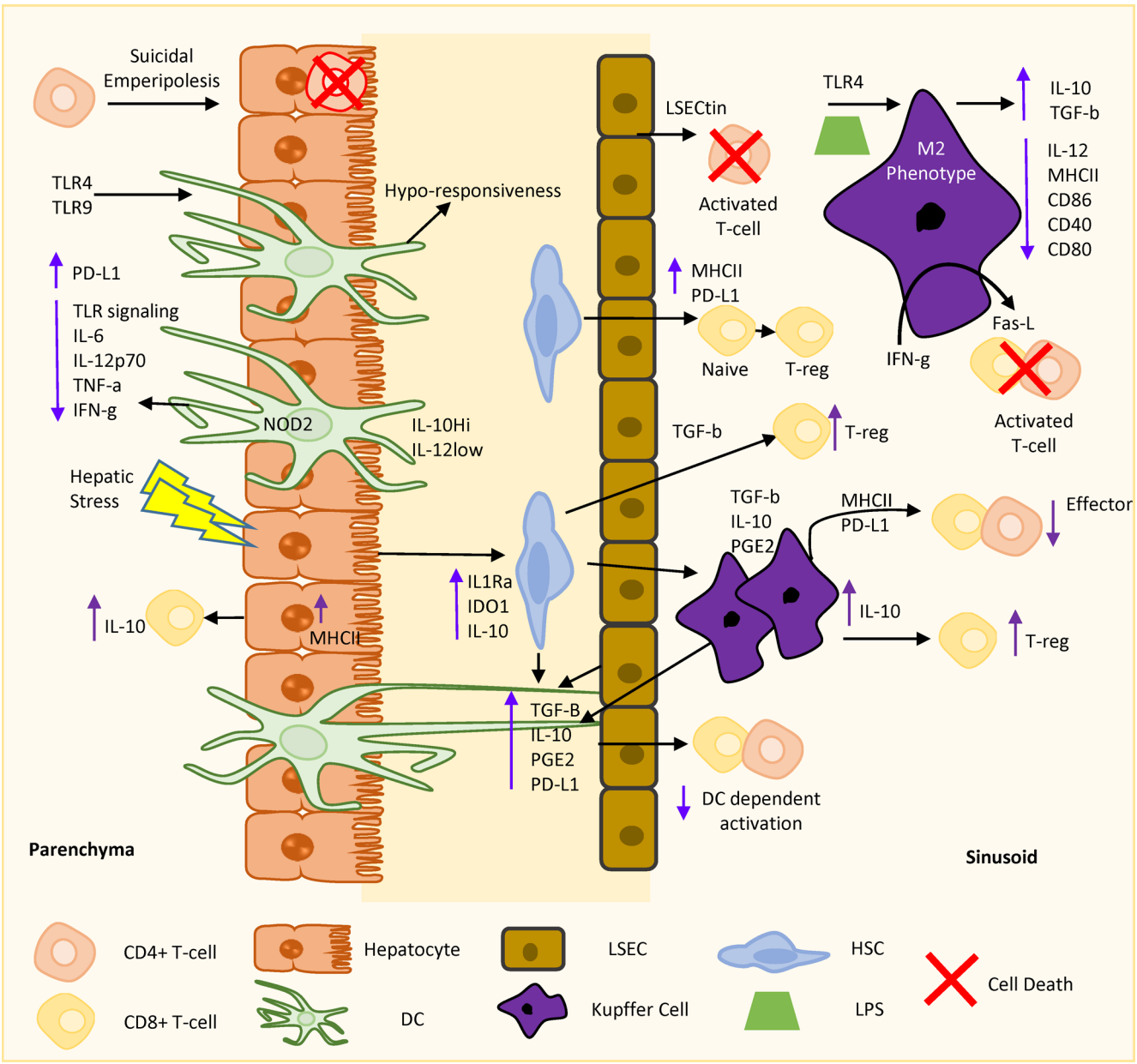

Figure 5. Hepatocytes are intrinsically tolerogenic. Through suicidal emperipolesis, hepatocytes can prevent proliferation of reactive T-cells. Hepatocytes with an increased MHCII expression have also been shown to induce IL-10 secretion in naive CD4+ T-cells. KCs are known to constitutively express the IL-10 receptor as well as excreting TGF-β and PGE2. KCs also exhibit an M2 anti-inflammatory phenotype upon stimulation from TLR4 and LPS, resulting in the down regulation of MHCII, CD86, CD80, and CD40. In addition, the upregulation of Fas-L on KCs through IFN-g induction has resulted in the elimination of activated T-cells. The upregulation of PD-L1 and release of IL-10, TGF-B, and PGE2 result in the suppression of DC-mediated T-cells. LSECs have shown to actively suppress CD4+ T-cell effector function and cell contact-dependent suppression of Th1 and Th17 responses but not proliferation. LSECs also have a number of bystander properties; one distinctive property is the expression of the ligand for CD44 and LSECtin, inhibiting T-cell activation and proliferation. LSCEs can also prohibit inflammation by priming regulatory CD4+ T-cells, shifting antigen-dependent immune responses to tolerance, especially under inflammatory conditions. The perisinusoidal space between LSECs and hepatocytes is populated by HSCs. It has been shown that after inflammatory stress, HSCs release IL1Ra, IDO1, and IL-10, preventing downstream pro-inflammatory signaling and microbial proliferation. HCSs show a marked increase in expression of PD-L1 which results in the decreased responsiveness of T-cells associated with a high T-cell apoptotic rate. The production of IL-10 by HSCs has an inhibitory effect on DCs, macrophages, and Th1 lymphocytes, and NKs promoting tolerance. DCs are key players in liver tolerance through inhibition of T-cell proliferation and apoptosis of activated T-cells. Hepatic DCs promote tolerance though priming IL-10 producing T-regs, inducing the generation of CD4+CD25+ Tregs and through utilization of PD-L1 to induce and maintain T-cell unresponsiveness.

employed, immune cells must be slowed down in the circulation to allow for sufficient interactions with chemokines presented by endothelial cells; a process known as tethering and rolling.[88] The release of danger signals results in chemokine control and management by hepatocytes, KCs, LSECs and HSCs. A number of chemokines have been associated with the homing of T-cells to the liver in inflammatory diseases including CCL25, CCL21 CCL3-5, CXCL9-11, CXCL16, and CCL4.[89−92] However, the role of chemokines in DILI is less well-defined. Monshi et al. described the distinct chemokine receptor expression profile in

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flucloxacillin-specific T-cell clones isolated from DILI patients. CCR2, CCR4, CCR9, and CXCR3 were found to be highly expressed and resulted in migration of CD8+ drug clones *in vitro*. The chemokine receptors CCR1, CCR3, CCR5, and CXCR6 were expressed at low levels on a limited number of clones.[9]

This distinct chemokine release profile has also been demonstrated in KCs. Treatment with hepatotoxic compounds results in the release of CCL3 and CXCL2 which have a key role in filtration of various leukocytes and neutrophils into the liver.[69] This is supported by the fact that there is a marked increase in hepatic CCL3 expression after ConA treatment, resulting in the recruitment of CD4+ T-cells to the liver, ultimately leading to hepatic injury. Disruption of the CCL3 gene significantly reduced concanavalin-induced hepatitis.[93] A study assessing the profiles of serum cytokines and chemokines in acute DILI found that the chemokines CCL3 and RANTES (CCL5) were marginally associated with mortality within 6 months of DILI onset based on univariate analyses.[72] This study highlights the role of chemokine receptors in the homing of immune cells to the liver, the promotion of an inflammatory environment, and eventually toxicity. CCL5 has also been found to be in higher abundance in DILI patients compared to those in the ALF group.[73] Furthermore, the treatment of DCs with SMX-NO treated hepatocyte conditioned medium found an increase in CCR1 and CD40 expression.[24] The increase of CCR1, possibly in response to an increased expression of CCL16 released by SMX-NO stressed hepatocytes, is vital for the migration of DCs to the liver.

4.3. The Interaction of Immune Cells with Hepatocytes. How circulating drug-specific T-cells interact with hepatocytes after they have extravagated into the liver is less well understood. The liver sinusoids, lined by LSECs, play an important role in cellular communications. LESCs, which lack tight junctions as well as a basal membrane, allow direct interaction of circulating and liver residential leukocytes with underlying hepatocytes.[66,94,95] Dynamic imaging revealed that CD8+ T-cells crawl along liver sinusoids independent of blood direction, surveying hepatocytes for antigens through sinusoidal fenestrae. Following antigen recognition on hepatocytes, CD8+ T-cells slowed down and eventually arrested. In general, hepatocyte-activated CD8+ T-cells developed poor cytotoxic function and subsequently died by Bim-dependent apoptosis.[96] However, antigen-specific CD8+ T-cells, particularly those activated by high affinity antigens, can kill hepatocytes through perforin and Fas ligand (FasL)-mediated mechanisms.[97] Although hepatocytes express high levels of Fas and are highly susceptible to apoptosis, hepatocyte damage by infiltrating T-cells is not exclusively caused by the FasL-mediated killing pathway. Additional effector molecules such as TNF-α, IFN-γ, and granzyme B may also be involved due to the heterogeneity of T-cells. Since drug-specific T-cells can be activated by multiple mechanisms, how these T-cells damage hepatocytes remains largely unknown. However, recent studies demonstrated that flucloxacillin-specific CD8+ T-cells can kill hepatocytes through the release of cytolytic mediators such as IFN-γ and granzyme B.[8,9] Interestingly, MHC molecules also play an important role in the interactions between T-cells and hepatocytes. Confocal immunofluorescence and flow cytometric analyses demonstrated that only MHC-matched CD8+ T-cells isolated from the liver of HBV replication-competent transgenic mice eventually extravasate into the liver parenchyma.[98] In addition, flucloxacillin-specific T-cells can kill hepatocytes in a HLA-B*57:01 restricted manner.[8]

5. THE ROLES OF IMMUNE TOLERANCE AND ADAPTATION IN DILI

The liver is routinely exposed to foreign antigens that are ingested and metabolized or excreted via the biliary system. Therefore, the liver has been privileged with a large capacity for peripheral immune tolerance. Immune tolerance is a critical adaptation to protect hepatocytes from suffering a constant state of inflammation. Experimental studies have been able to establish and maintain allogeneic liver grafts in animal models without the need for immunosuppression.[99] Likewise the ability to transplant the liver without HLA matching and rejection has led to the phenomena known as spontaneous operational tolerance.[100,101] The liver maintains this tolerance through a number or processes (Figure 5): the control of antigen activation and presentation, clonal deletion (apoptosis of antigen-specific T-cells), and immune deviation (switching from Th2 and Th1).[102]

5.1. Hepatocytes Are Intrinsically Tolerogenic. Hepatocytes have the capability of inducing activation and proliferation of naïve CD8+ T-cells. However, without the presence of co-stimulatory molecules, these hepatocyte-activated CD8+ T-cells demonstrate poor effector function and early BIM-dependent apoptosis, representing an acquired tolerant phenotype.[96] Interestingly, the thresholds of antigen presentation by hepatocytes appear to be vital in balancing a tolerogenic versus an effector immune response: It has been shown that the low levels of hepatocellular antigens lead to an effector CD8+ T-cell response, whereas the abundance of antigens causes CD8+ T-cell exhaustion and silence.[103] Another mechanism by which the liver promotes a tolerogenic environment is through peripheral deletion of self-reactive T-cells.[104] Hepatocytes also determine the nonapoptotic destruction of activated CD8+ T-cells through lysosomal proteolytic enzyme degradation; a process known as suicidal emperipolesis (SE). This process is rapid and prevents proliferation and expansion of reactive T-cells. Inhibition of SE is associated with preclusion of tolerance and promotes liver damage.[105] Hepatocytes with an increased MHC II expression have been shown to induce IL-10 secretion in naïve CD4+ T-cells *in vitro* in a Notch receptor-dependent manner, playing a central role in tolerance.[106] It is well understood that the liver competes with lymphoid tissue to determine T-cell activation and fate, with the former promoting tolerance.[107] All of these mechanisms denote that the liver, under steady-state conditions, prefers and promotes a tolerogenic environment.

5.2. Tolerance Promoted by APCs in the Liver. The tolerogenic environment in the liver is also partly attributed to the effects of KCs, LSECs, or HSCs as APCs and the distinctive liver-resident DCs.[108,109] Liver APCs can promote tolerance through multiple pathways, including secretion of a range of anti-inflammatory cytokines, suppression of effector CD4+ and CD8+ T-cells, and induction of Tregs.

KCs are liver resident macrophages that play a critical role in modulating tolerance in the liver. KCs are known to secret IL-10 and a range of anti-inflammatory and immunosuppressive factors such as nitric oxide, TGFβ or arachidonic acid metabolite prostaglandin E2.[110] One remarkable feature of KCs is their ability to exhibit an anti-inflammatory M2-like macrophage polarization state upon signals from LPS and TLR4, protecting the liver from injury by inhibiting pro-inflammatory cytokine

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production and attraction of Tregs to the liver.[111] It is thought that this shift from M1 to M2 phenotype is a result of the interaction between mesenchymal cells and KCs. The expression of MHC II, CD86, CD80, and CD40 was significantly decreased in the presence of mesenchymal cells. In addition to the cytokine release and APC properties, KCs can modulate tolerance in the liver through either immune inhibition by increased expression of PD-L1 or elimination of activated T-cells by upregulation of Fas-L.[112]

LSECs represent a unique population of scavenger APCs which facilitate the passage of molecules from the sinusoidal lumen into the space of Disse through their fenestrae or sieve plates and a lack of a continuous basement membrane. Through increased expression of PD-L1, LSECs can induce antigen-specific tolerance in CD8+ T-cells through cross-presentation of antigens from damaged hepatocytes. LSEC-primed CD8+ T-cells acquire a memory-like phenotype and express the lymphoid adhesion CD62L. Unlike their CD4+ counterparts, the CD8+ cells do not migrate to the gut but return to the secondary lymphoid organs, where they can reactivated by DCs to support anti-infection immunity.[113] In addition, LSECs have shown to actively suppress CD4+ T-cells effector function and cell contact-dependent suppression of Th1 and Th17 responses, but not proliferation.[114] Moreover, compared to other liver cell types, LSECs have shown to be the most efficient in inducing fork-head-winged helix transcription factor (Foxp3+) Tregs in the liver. Treg induction by LESCs occurred in a TGFβ-dependent and antigen-specific manner.[115]

HSCs, located at the perisinusoidal space between LSECs and hepatocytes, also play a critical role in immune regulation and suppression within the liver. It is well understood that HSCs release anti-inflammatory mediators which prevent activation and progression to an inflammatory state. After inflammatory stress, HSCs release IL1Ra, IDO1, and IL-10, promoting tolerance and preventing the immune system from mounting a response.[116] In addition, activated HCSs show a marked increase in expression of PD-L1, which results in the decreased responsiveness of T-cells associated with a high T-cell apoptotic rate.[117] Moreover, in the presence of DCs, HSCs inhibit activation of CD8+ T-cells via a CD54-dependent mechanism and promote differentiation of naïve CD4+ to Tregs.[118119]

DCs also play a crucial role in promoting tolerance within the liver. The reprogramming of CD117+ hematopoietic progenitor cells to differentiate into tolerogenic DCs which suppress T-cell proliferation and induce apoptosis of activated T-cells is promoted by the liver microenvironment.[120] Liver DCs promote tolerance though priming IL-10 producing T-regs, inducing the generation of CD4+CD25+ Tregs and through utilization of PD-L1 to induce and maintain T-cell unresponsiveness.[121] In addition, pDCs show a high expresson of NOD that down-regulates immune response through increasing PD-L1 expression, dampen TLR signaling, and inhibition of pro-inflammatory cytokine secretion (IL-6, IL12p70, TNF-a, and IFN-y).[122] The fine-tuned interaction between hepatic Treg population and DCs is responsible for the maintenance of a tolerogenic environment within the liver.

5.3. Dysregulation and Disruption of Tolerance Pathways. Understanding the mechanisms by which the fine-tuned tolerance pathways are disrupted in a range of other disease states such as infection and inflammation may provide insight into the mechanisms behind the progression of DILI. Over a quarter of most common DILI drugs are indicated as antimicrobials or antibiotics, suggesting patients have recently suffered a microbial infection.[123] One possible concept is that certain bacterial infections or disease states are associated with unique changes in the gut microbiota profile, which may be linked to diffusion of normal tolerance pathways. As the liver interacts with the gut through the hepatic portal and bile secretion systems, gut microbiota as well as bacterial endotoxins (such as LPS) and their regulation pathways could be associated with liver disease. This theory raises the possibility that the concurrent infection and inflammation may render an individual more susceptible to DILI. However, the translocation of endotoxins or the presence of modest inflammation cannot be labeled as the sole trigger disrupting tolerance but a contributing factor. In addition, disruption of liver tolerance through immune checkpoint inhibitors (ICIs) treatment that commonly block B7/CTLA-4 and PD1/PD-L1 pathways can promote the activation and proliferation of effector T-cells, resulting in immune-mediated liver injury. Indeed, the incidence of immune-related acute hepatitis of all grades is estimated to affect 4−9% of patients with monotherapy, and 18% of patients treated with the combination therapy. Treatment of ICIs has led to massive infiltration of lymphocytes, largely CD8+ T-cells, in portal tracts and lobules, resulting in inflammatory liver injury.[124] In an animal model with an impaired immune tolerance, Uetrecht et al. showed that co-treatment of C57BL/6 mice with amodiaquine and anit-CTLA4 resulted in a greater injury than treatment with amodiaquine alone.[125] Together the intrinsic toxicity of the drug, individual differences and external factors, cell population changes as well as pre-existing inflammation may contribute to impair the liver tolerance, resulting in increased liver injury.

6. CONCLUSION

Advanced bioanalysis has enabled characterization of both stable and potentially reactive metabolites that may be involved in DILI. In particular, recent investigations using immunopeptidomics have enabled the identification of drug associated antigens that may be involved in the activation of T-cells. However, the hepatic protein targets and the exact location of neoantigens formed in the liver remain 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.[43,44] Therefore, the critical question remains, what determines switching from tolerance to immunity?

Although considerable progress has been made in understanding the molecular and cellular mechanisms underlying DILI, the lack of *in vitro* and animal models, and definitive prognostic biomarkers, makes the prediction and prevention of immune-mediated DILI extremely challenging. Recent advances in developing a more physiological *in vitro* model, such as liver-derived organoids,[126,127] has made the future prediction of DILI more promising. Specifically, the co-culture of immune cells with target cells, such as hepatocytes, has allowed us to investigate the complex cellular mechanisms involved in killing target cells (Unpublished data). 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. As it is challenging to achieve the same balance between tolerance and immunity occurring in the liver, developing *in vitro* models for investigating the mechanisms of immune-mediated DILI has been problematic. However, recent attempts using HLA transgenic animal

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models with impaired immune tolerance have shown some promising results. Transgenic mice positive for HLA-B*57:01 showed induced toxicity to abacavir, in contrast to those carrying HLA-B*57:03. While abacavir hypersensitivity does not result in liver injury, these models will be particularly valuable for the preclinical screening of the immunogenicity of new drug candidates if they can be applied to general DILI drugs.[125,128,129] Due to the complexity of both innate and adaptive immunity involved in DILI, physiologically relevant *in vitro* models incorporating both immune and liver cells are in great need. In particular, co-culturing hepatic cells derived from human pluripotent stem cells with HLA matched drug-specific immune cells will allow the monitoring of immune-mediated toxicity. Moreover, future study on the patient related risk factors such as disease states and regulatory pathways that determine susceptibility to DILI will certainly provide an opportunity to develop better predictive test systems for the prediction and prevention of DILI.

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Notes

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James C. Waddington obtained a Master of Biological Sciences degree from the University of Liverpool in 2015, with honors in microbiology. Previous research focuses included the implementation of novel techniques to analyze microbial metagenomics signatures in order to identify disease biomarkers, cellular responses to HIV viral infection, and toxin gene regulation in bacterial viruses. In 2015, James joined the MRC Centre for Drug Safety Science at the University of Liverpool as a Ph.D. student in pharmacology. Current research focuses on the characterization of naturally processed MHC peptides that act as T-cell antigens in patients with drug hypersensitivity.

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■ ABBREVIATIONS

AHS, abacavir hypersensitivity syndrome; APAP, acetaminophen; ALF, acute liver failure; ADA, antidrug antibody; TNF, antitumor necrosis factor; APCs, antigen presenting cells; ConA, Concanavalin A; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; Foxp3, fork-head-winged helix transcription factor; HSP, heat shock protein; HSCs, hepatic stellate cells; GSH, glutathione; HMGB1, high mobility group box 1; HLA, human leukocyte antigen; HSA, human serum albumin; iDILI, idiosyncratic drug-induced liver injury; KCs, Kupffer cells; LSECs, liver sinusoidal endothelial cells; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NK, natural killer; NVP, nevaripine; NSAIDs, nonsteroidal anti-inflammatory drugs; PD-1, programmed cell death protein 1; PD-L1, PD-ligand1; RMs, reactive metabolites; SE, suicidal emperipolesis; SMX-NO, sulfamethoxazole nitroso; TCRs, T-cell receptors; TPs, therapeutic proteins; TLRs, toll-like receptors; TNF, tumor necrosis factor

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