T Cells Infiltrate the Liver and Kill Hepatocytes in HLA-B*57:01-Associated Floxacillin-Induced Liver Injury

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Drug-induced liver injury is a major safety issue. It can cause severe disease and is a common cause of the withdrawal of drugs from the pharmaceutical market. Recent studies have identified the HLA-B*57:01 allele as a risk factor for floxacillin (FLUX)-induced liver injury and have suggested a role for cytotoxic CD8+ T cells in the pathomechanism of liver injury caused by FLUX. This study aimed to confirm the importance of FLUX-reacting cytotoxic lymphocytes in the pathomechanism of liver injury and to dissect the involved mechanisms of cytotoxicity. IHC staining of a liver biopsy from a patient with FLUX-induced liver injury revealed periportal inflammation and the infiltration of cytotoxic CD3+CD8+ lymphocytes into the liver. The infiltration of cytotoxic lymphocytes into the liver of a patient with FLUX-induced liver injury demonstrates the importance of FLUX-reacting T cells in the underlying pathomechanism. Cytotoxicity of FLUX-reacting T cells from 10 HLA-B*57:01 healthy donors toward autologous target cells and HLA-B*57:01-transduced hepatocytes was analyzed in vitro. Cytotoxicity of FLUX-reacting T cells was concentration dependent and required concentrations in the range of peak serum levels after FLUX administration. Killing of target cells was mediated by different cytotoxic mechanisms. Our findings emphasize the role of the adaptive immune system and especially of activated drug-reacting T cells in human leukocyte antigen-associated, drug-induced liver injury. (Am J Pathol 2014, 184: 1677–1682; http://dx.doi.org/10.1016/j.ajpath.2014.02.018)

Drug-induced liver injury (DILI) is one of the most frequent causes of acute liver failure and the need for liver transplantation in the US.1 Nonetheless, the exact mechanism underlying DILI remains largely unknown. Direct or intrinsic hepatotoxicity by the drug or its metabolites can be distinguished from unpredictable DILI—often called idiosyncratic as it is due to an individual predisposition. Among idiosyncratic DILI, one further discriminates between immune-mediated and non-immune-mediated reactions. Immunological hepatotoxicity is the least understood form of DILI, and the exact contribution of the immune system remains to be clarified. Despite the detection of drug-specific antibodies and T cells, it has been difficult to directly prove the pathogenic role of the adaptive immune system in DILI, in part because of the lack of available liver biopsies from patients.2 In the past decade, associations between drug-hypersensitivity reactions and human leukocyte antigen (HLA) alleles have been discovered as, for example, liver injury caused by amoxicillin-clavulanic acid, which has been associated with HLA-A*02:01, HLA-DRB1*15:01, HLA-DRB5*01:01, and HLA-DQB1*06:02,4–5 and floxacillin-induced liver injury (FLUX-DILI), which has been associated with HLA-B*57:01.6 As HLA molecules are involved in antigen presentation to T cells, HLA associations with DILI represent a strong indication for the involvement of the adaptive immune system, and in particular activated T cells, in the pathomechanism of these types of DILI.

FLUX-DILI is a rare but a highly relevant form of DILI that occurs with an 80-fold increased probability in patients with the HLA-B*57:01 allele.7,8 Other studies have suggested a role for activated T cells in DILI.9–10 To further investigate the role of the adaptive immune system, we performed this study to confirm the importance of FLUX-reacting cytotoxic lymphocytes in the pathomechanism of liver injury caused by FLUX and to dissect the different mechanisms of cytotoxicity.
HLA-B*57:01+ individuals. Consequently, the HLA-B*57:01 allele has been suggested to play a mechanistically important role. Recent studies by us and others have reported activation of peripheral blood mononuclear cells in patients with FLUX-DILI as well as in HLA-B*57:01+ FLUX-naïve individuals on FLUX exposure. This led to the speculation that FLUX-reacting cytotoxic and inflammatory CD8+ T cells infiltrate the liver and cause tissue damage in vivo.

Cytotoxic T lymphocytes (CTLs) have been reported to play an essential role in maculopapular exanthema and bullous skin diseases. Fulminant liver failure described in drug-hypersensitivity reactions is probably also caused by massive infiltration of activated drug-reacting cytotoxic T cells. A recent study by Mennicke et al revealed infiltration of granulocyte B+CD3+ T lymphocytes in close proximity to apoptotic hepatocytes in a patient with fulminant liver failure after vancomycin intake. A similar mechanism can be presumed for FLUX-DILI. Indeed, Monshi et al reported that FLUX-reacting T cells from patients as well as from HLA-B*57:01+ healthy donors (HDs) expressed the chemokine receptors CCR2, CCR4, and CCR9, which are thought to be involved in the migration and accumulation of immune cells in the liver.

CTLs can kill target cells via different pathways involving granule exocytosis, activation of death receptors, and/or release of cytokines. The perforin/granzyme mechanism relies on granule exocytosis on cell contact and specific activation of CTLs. Other proteins that are found in human CTL granules are granulysin and T cell intracytoplasmic antigen (TIA)-1. TIA-1 induces DNA fragmentation in target cells and regulates alternative splicing of the death receptor Fas (CD95), thereby promoting the proapoptotic membrane-bound form at the expense of the anti-apoptotic soluble Fas molecule. Induction of apoptosis may also occur by the surface expression of Fas ligand (FasL), which binds to Fas on target cells. FasL-mediated killing not only can induce specific lysis of antigen-presenting target cells but also has been reported to be responsible for bystander killing, meaning the destruction of cells not presenting the activating antigen. Such a mechanism could also be possible in the case of immune-mediated DILI.

Here we investigated the impact and mechanism of FLUX-mediated T-cell cytotoxicity toward hepatocytes. We report the infiltration of cytotoxic CD3+CD8+ T cells into the liver of a FLUX-DILI patient. In vitro data confirmed that FLUX-reacting T cells kill FLUX-presenting autologous Epstein-Barr virus-B lymphoblastoid cell lines (EBV-BLCLs) and HLA-B*57:01-transduced human lymphoblastoid cell lines. FLUX-mediated killing of target cells was concentration dependent and involved various cytoxic mechanisms.

Materials and Methods

Healthy Donors

Ten FLUX-naïve HLA-B*57:01+ HDs were selected from the Regional Blood Transfusion Service (Swiss Red Cross, Bern, Switzerland) according to their HLA-B*57:01 status and were enrolled in the study. All HDs gave written informed consent, and the study protocol was approved by the local ethics committee.

IHC Staining

Immunohistochemical (IHC) analysis was performed on liver biopsy sections from formalin-fixed tissue using an avidin-biotin complex method (Vectastain ABC detection kit; Vector Laboratories, Inc., Burlingame, CA). Briefly, following pressure cooker-mediated antigen retrieval in 0.001-mol/L EDTA (pH 8.0), the sections were incubated with 10% normal goat serum (Dako North America, Inc., Carpinteria, CA) for 20 minutes. Endogenous peroxidase activity was blocked using 0.5% H2O2. Slides were then incubated with CD8 and CD3 antibodies (Dako North America, Inc.). Staining for TIA-1 was performed using the modified streptavidin-biotin complex/alkaline phosphatase method as previously described, using the anti-TIA-1 antibody (Abcam plc, Cambridge, UK).

Primary Induction with FLUX and Cytotoxicity Assay

Primary induction with FLUX was performed as described earlier. Primary release cytotoxicity assays were performed as described previously. Briefly, target cells were labeled with 50-μCi sodium chromate solution (PerkinElmer, Schwerzenbach, Switzerland) for 60 minutes at 37°C. Unless otherwise stated, the effector/target ratio was 10:1 and autologous EBV-BLCLs served as target cells. Specific lysis was calculated as:

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100 \times \left[ \frac{(\text{experimental release with drug} - \text{experimental release without drug})}{(\text{maximal release} - \text{spontaneous release})} \right]
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To block perforin/granzyme B-mediated cytotoxicity or FasL-mediated cytotoxicity, T cells were preincubated with 100 nmol/L/mL concanamycin A (CMA; SAFC Buchs, Buchs, Switzerland) or 10-μg/mL brefeldin A (SAFC Buchs, respectively, for 2 hours at 37°C. Because previous studies revealed that FasL-mediated killing did not occur at a significant level before 8 hours, specific lysis of target cells was determined after 4 and 18 hours of co-incubation with FLUX-reacting T cells.

Viral Transduction of Huh7 and SKHep1 Hepatocytes

HLA-B*57:01 murine stem cell virus/puro construct (vector from Clontech Laboratories, Inc., Mountain View, CA) was generated as described previously for 721.221 cells. The HLA-B*57:01+ hepatocyte cell lines Huh7 and SKHep1 [kind gift from Thomas Kaufmann (University of Bern, Bern, Switzerland)] were infected with the generated constructs, and transduced hepatocytes were selected under antibiotic pressure (0.5 to 1μg/mL puromycin; Grogg Chemie AG, Stettlen, Switzerland). Successful transduction was confirmed by RT-PCR with 5'-GTCTCACATCATCCAGGT-3' and...
5′-CGCCTCCCACTTGCGCTGGG-3′ as forward and reverse primers, respectively (Microsynth AG, Balgach, Switzerland).

FasL-Mediated Bystander Killing of Nontransduced Hepatocytes

To assess bystander killing of nontransduced parental hepatocytes, 2500 hepatocytes labeled for 60 minutes with 50 μCi sodium chromate solution were co-incubated together with 2500 unlabeled autologous EBV-BLCLs and T cells at various effector/target ratios. Specific lysis after 18 hours was calculated as described above.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA). Experiments were performed in triplicate, and each experiment was repeated at least twice. Results are expressed as means ± SD. Comparisons were drawn using an unpaired t-test. P < 0.05 were regarded as statistically significant.

Results

T Cells Infiltrate the Liver

Recent studies by us and others7,8 reported the in vitro induction of FLUX-reacting T cells from FLUX-DILI patients as well as from HLA-B*57:01+ FLUX-naïve individuals. Those studies hypothesized that the FLUX-reacting cytotoxic and inflammatory CD8+ T cells infiltrate the liver and damage liver cells in vivo. To test these assumptions, we analyzed infiltration of lymphocytes into the liver by IHC examination of a biopsy from a FLUX-DILI patient. The biopsy showed periportal inflammation with infiltrates of TIA-1+ CD3+ CD8+ lymphocytes (Figure 1).

FLUX-Mediated Killing Is Concentration-Dependent and Involves Various Cytotoxic Mechanisms

We previously showed primary induction of FLUX-reacting CD8+ T cells in 100% of HLA-B*57:01+ FLUX-naïve individuals.7 To determine whether these FLUX-reacting T cells were cytotoxic, 51Cr-release cytotoxicity assays were performed. Titration of the FLUX concentration revealed that cytotoxicity was concentration dependent. Specific lysis of target cells was detected at concentrations as low as 50 μg/mL, and a plateau was reached at 250 μg/mL (Figure 2A).

To discriminate between perforin/granzyme B-mediated killing and FasL-mediated cytotoxicity, we performed51Cr-release assays in the presence of brefeldin A and/or CMA. CMA is a potent inhibitor that blocks perforin-mediated killing, whereas brefeldin A selectively inhibits FasL-mediated cytotoxicity.24 Cytotoxicity in the 4 hours assay (Figure 2B) was dominated by perforin/granzyme B-mediated killing, whereas full cytotoxicity was observed in the presence of CMA in the 18-hour assay (Figure 2C),...
indicating the importance of FasL-mediated killing. The simultaneous addition of both inhibitors completely abrogated cytotoxicity (Figure 2, B and C). Our data indicate that the two killing pathways appear to coexist.

**FLUX-Reacting T Cells Kill HLA-B*57:01-Transduced Hepatocytes**

In the liver, the targets of FLUX-reacting T cells are thought to be hepatocytes. Therefore, the cytotoxic potential of HLA-B*57:01 FLUX-reacting CTLs toward hepatocytes was assessed in vitro. To this end, SKHep1 and Huh7 hepatocyte cell lines were transduced with HLA-B*57:01, and successful transduction was confirmed by PCR (Figure 3A). HLA-B*57:01 FLUX-reacting T cell lines specifically killed HLA-B*57:01-transduced hepatocytes, but not the parental nontransduced hepatocyte lines (Figure 3, B and C).

**FLUX-Reacting T Cells Can Kill via FasL-Mediated Bystander Killing**

CTLs have been reported to mainly kill antigen-bearing cells through perforin/granzyme B secretion.28 FasL has been reported to be responsible for bystander killing, meaning the destruction of cells that are not presenting the activating antigen. In hepatitis C virus infection, bystander killing of noninfected cells aggravates liver damage. To investigate whether FLUX-reacting T cells could also kill non-FLUX-presenting hepatocytes, we performed bystander killing assays with nontransduced hepatocytes. These do not express HLA-B*57:01 and therefore cannot directly activate HLA-B*57:01-restricted FLUX-reacting T cells. Although some killing of nontransduced hepatocytes was observed in the absence of FLUX, the presence of FLUX significantly increased specific lysis (Figure 3D).

**Discussion**

We report the infiltration of cytotoxic CD3+ CD8+ lymphocytes into the liver in FLUX-DILI. This finding supports a role for drug-reacting T cells in the pathogenesis of HLA-associated DILI. In vitro analysis of FLUX-reacting T cells from HLA-B*57:01 HDs revealed that killing of autologous target cells depended on the available FLUX concentration and involved various cytotoxic mediators.

We observed an overlap of the different killing pathways. FLUX-reacting CTLs were able to kill target cells in a perforin/granzyme B-dependent manner, which relies on the rapid exocytosis of granules containing cytotoxic molecules. Importantly, CTL-induced cytotoxicity can also be contact independent. In this case, target cells are killed by the FasL pathway. Indeed, by analyzing cytotoxicity toward hepatocytes, we found that HLA-B*57:01 FLUX-reacting CTLs also kill nontransduced hepatocytes, which are not able to present the activating antigen (FLUX) in an HLA-B*57:01-restricted manner. This bystander killing could lead to exacerbation of the liver injury caused by FLUX. Hepatocytes appear to be extremely susceptible to Fas-mediated killing, as the injection of Fas-activating anti-Fas antibodies induces massive injury to the liver and not elsewhere.29 The striking susceptibility might be due to a high expression of Fas in liver tissue, and high sensitivity of the liver cells to FasL-mediated cell death.30 Therefore, the bystander killing caused by FasL might explain why the liver is predominantly involved in FLUX-hypersensitivity reactions.

Recently, Yun et al31 reported that, in contrast to the widely held dogma that idiosyncratic adverse reactions are dose independent, dose is crucial for both immunogenicity and reactivity of allopurinol- and oxypurinol-reacting T cells. We have shown that activation of FLUX-reacting T cells was concentration dependent and required a high concentration of soluble antigen. Peak plasma concentration was in the range of 190 µg/mL after i.v. administration of 1000-mg FLUX32 and around 12 µg/mL after oral ingestion of 500-mg

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**Figure 3** FLUX-reacting T cells kill hepatocytes. SKHep1 and Huh7 hepatocyte cell lines were transduced with HLA-B*57:01. A: mRNA from nontransduced and transduced hepatocytes was reverse-transcribed and analyzed for the expression of HLA-B*57:01. 721.221 HLA-B*57:01 cells served as positive control. B and C: Killing of HLA-B*57:01-transduced SKHep1 (B) and Huh7 (C) hepatocytes was analyzed in a 4-hour 51Cr-release assay. One representative experiment of five is shown. D: Bystander killing of nontransduced SKHep1 hepatocyte cells was analyzed in an 18-hour cytotoxicity assay. One representative experiment of three is shown. Experiment was performed in triplicate and data were analyzed by unpaired t-test. Data are expressed as means ± SD (B and C). **P < 0.01 (D). APC, antigen presenting cell; EBV-BLCL, Epstein-Barr virus-B lymphoblastoid cell lines; E/T, effector/target; HD, healthy donors; TCL, T-cell lines.
FLUX. Typical doses in patients with Staphylococcus infections are in the range of 2 g every 6 hours i.v., certainly exceeding the threshold concentration of 50 μg/mL required to activate FLUX-reacting CTLs. After oral ingestion, maximal plasma levels are lower, but local concentrations in the liver may be higher due to high portal concentrations of FLUX during the absorption phase. Interestingly, FLUX-DILI is primarily located in the periportal region, where the highest FLUX concentrations can be expected. Furthermore, the fenestrated endothelium of the sinusoids favors the passage of drugs such as FLUX into the space of Disse, allowing an intensive contact between drugs and hepatocytes. FLUX-DILI has been reported to be more common in female patients, in elderly patients, and in patients with long-term treatment courses and high dosages. All of these factors are associated with an increased hepatic exposure to the drug. The liver may be a predominant target of FLUX-DILI because the required threshold concentration can be exceeded and the contact with target cells is intensive.

In a previous study, analysis of the T-cell reactivity pattern revealed that the HLA-B*57:01 molecule is crucial for stimulation according to the pharmacological interaction with immune receptor concept. On the contrary, in HLA-B*57:01 individuals, the presentation followed the hapten concept. Although the particular FLUX binding to the HLA-B*57:01 molecules might explain the enhanced pathogenicity of FLUX in HLA-B*57:01 individuals, the predominant liver manifestation remains intriguing. In some patients, DILI might also be accompanied by other manifestations, such as rash or eosinophilia. Unfortunately, most descriptions do not clarify the presence of extrahepatic symptoms in FLUX-DILI patients. In addition, FLUX can cause other hypersensitivity reactions such as nephritis or exanthema, but potential HLA-B*57:01 associations of these hypersensitivity reactions have not been investigated to date. An animal model with a humanized immune system carrying the HLA-B*57:01 allele could help to identify FLUX-DILI etiopathology.

To conclude, our findings suggest a role of the adaptive immune system and especially of activated drug-reacting T cells in HLA-associated DILI. IHC staining revealed lymphocyte infiltration and tissue damage, and in vitro T-cell culture showed a cytotoxic potential toward hepatocytes in the presence of FLUX. Thus, FLUX-activated T cells seem to play a key role in the pathomechanism of FLUX-DILI.

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References

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