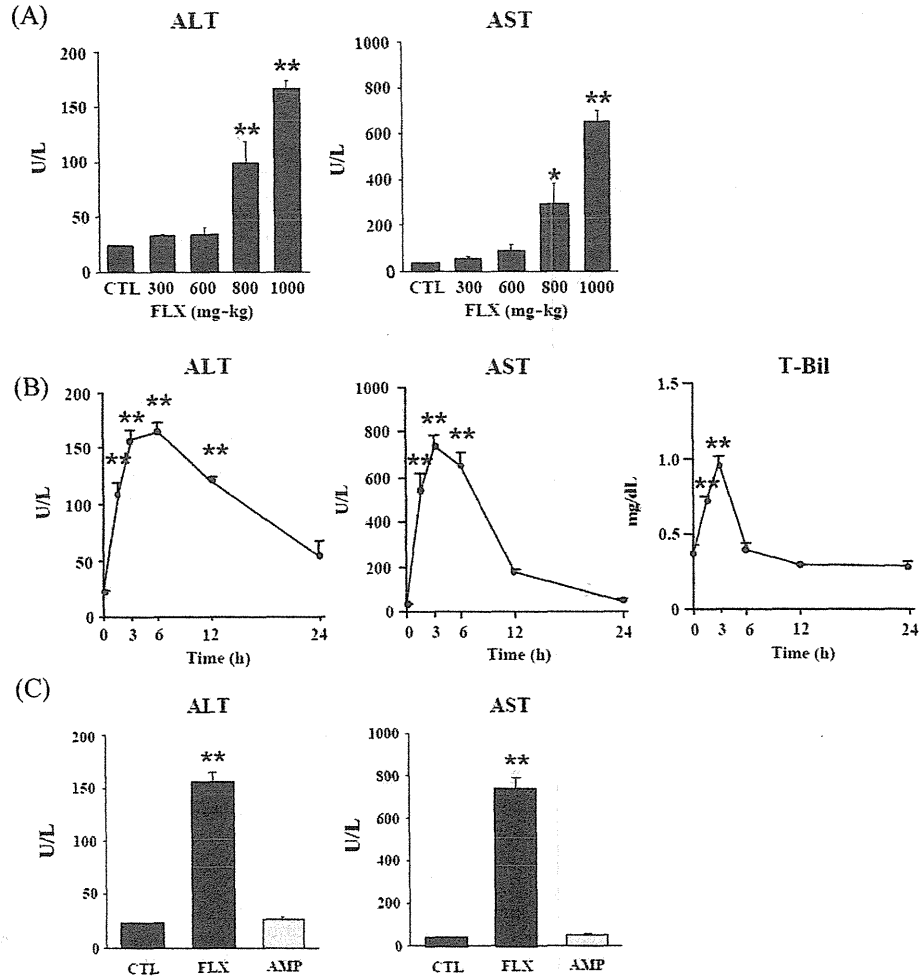

Figure 1. Dose- and time-dependent changes in plasma biomarkers in flucloxacillin (FLX)-administered mice. (A) Mice were administered FLX [300, 600, 800 and 1000 mg kg^{-1}, intraperitoneally (i.p.)]. Six hours after the administration, the plasma, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured. (B) The plasma ALT, AST and total bilirubin (T-Bil) levels were measured 1.5, 3, 6, 12 and 24 hafter administration of FLX (1000 mg kg^{-1}, i.p.). (C) Mice were administered FLX (1000 mg kg^{-1}, i.p.) or ampicillin (AMP) (1000 mg kg^{-1}, i.p.). Three hours after the administration, the plasma ALT and AST levels were measured. The data are shown as the mean ± SEM of the results from four to six mice. The differences compared with the control mice (A), (C), or with the time before the administration (B) were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

increased at 1.5, 3, 6 and 12 h, 1.5, 3 and 6 h, and 1.5 and 3 h, respectively, after FLX administration (Fig. 1B). To investigate the impact of the pharmacological effect on FLX-induced liver injury, we used AMP, which is a β-lactam antibiotic with much less hepatotoxicity and similar pharmacological properties and chemical structure to FLX. The administration of AMP did not result in an increase in the plasma ALT or AST (Fig. 1C).

Expression of TLR4 Ligands in FLX-Induced Liver Injury

To investigate the involvement of TLR4 in FLX-induced hepatotoxicity, the hepatic mRNA levels of S100A8 and S100A9 and the plasma HMGB1 level were measured by real-time RT-PCR and ELISA, respectively. HMGB1 is released from necrotic cells or activated immune cells, and S100A8 and S100A9 are localized in the cytoplasm and/or nucleus of a wide range of cells and are released by phagocytes. HMGB1, S100A8 and S100A9 are ligands of TLR4 expressed in immune-related cells. They activate downstream signaling pathway of TLR4, and then induce inflammatory factors such as IL-1β and TNFα. Thus, the activation of TLR4 by those ligands is necessary to develop inflammation. As the results, the hepatic mRNA levels of S100A8 and S100A9 were significantly increased at 3 h, 1.5 and 3 h, respectively, after the administration of FLX compared with the non-treated controls (Fig. 2A). The plasma HMGB1 level was significantly increased at 1.5 and 3 h (Fig. 2B). These changes suggested the involvement of a TLR4-associated signal cascade.

Time-Dependent Changes in the mRNA Expression of Inflammatory-Related Factors in FLX-Induced Liver Injury

To investigate if inflammatory-related factors are involved in FLX-induced liver injury, the hepatic mRNA levels of IL-1β, TNFα, MCP-1, MIP-2 and CXCL1 were measured by real-time RT-PCR. The hepatic mRNA levels of IL-1β, TNFα, MCP-1, MIP-2 and CXCL1 were significantly increased at 3 and 6 h, 6 h, 3 and 12 h, 1.5 and

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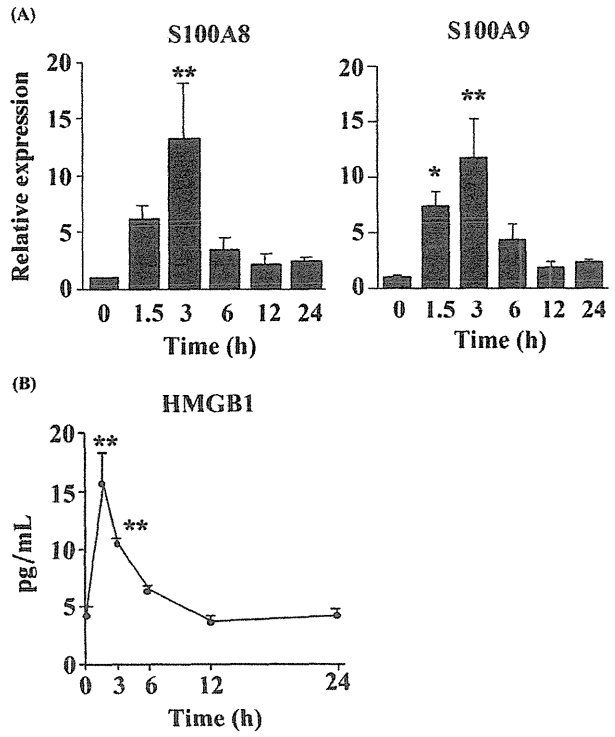

Figure 2. Time-dependent changes in the hepatic expression levels of S100A8 and S100A9 mRNA and the plasma HMGB1 level in flucloxacillin (FLX)-administered mice. (A) The expression levels of hepatic S100A8 and S100A9 mRNA levels were measured by real-time RT-PCR 1.5, 3, 6, 12 or 24 h after administration of FLX [1000 mg kg^{-1}, intraperitoneally (i.p.)]. The expression level of hepatic mRNA was normalized to that of β-actin mRNA. (B) The plasma HMGB1 protein levels were measured by enzyme-linked immunosorbent assay (ELISA). The data are shown as the mean ± SEM of the results from four to six mice. The differences compared with the control mice were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

3 h, 1.5 and 3 h, respectively in the FLX-induced liver injury mice compared with the non-treated controls. This suggests that these inflammatory-related factors might be involved in the FLX-induced liver injury (Fig. 3).

Time-Dependent Changes in the mRNA Expression of Immune-Related Transcriptional Factors and Cytokines in FLX-Induced Liver Injury

To investigate the contribution of immune-related transcriptional factors and cytokines to FLX-induced liver injury, the expression of the hepatic mRNA of T-bet, GATA-3, IFN-γ, IL-5 and IL-6 were measured by real-time RT-PCR. The expression levels of T-bet were significantly decreased at 1.5 to 12 h after the FLX administration compared with the non-treated controls. The expression levels of IFN-γ and GATA-3 were unchanged. The expression levels of ROR-γt, IL-5 and IL-6 were significantly increased at 3 and 12 h, 1.5 h, 1.5 and 3 h, respectively, after the administration of FLX compared with the non-treated controls (Fig. 4). It is known that IFNγ and GATA-3 are the representative factor of Th1 and Th2 cells, respectively. Thus, it was suggested that IFNγ and GATA-3 were not involved in FLX-induced liver injury (Fig. 4). ROR-γt is a transcription factor of NK cells, NKT cells and Th17 cells. IL-6 is a pleiotropic cytokine involved in the enhancing expression and activation of IL-17. The mRNA expression levels of ROR-γt and IL-6 were significantly increased (Fig. 4), therefore we thought that IL-17 is involved in FLX-induced liver injury.

Effect of rIL-17 and anti-IL-17 Antibody Administration in FLX-Induced Liver Injury

To investigate if IL-17 is involved in FLX-induced liver injury, FLX was co-administered with rIL-17 (2 mg/body, i.p.). The plasma ALT was significantly increased, and the plasma AST showed a tendency to increase in the mice co-administered FLX and rIL-17 (Fig. 5A) compared with the FLX-administered mice. In the histopathological examinations of the liver, immune cell infiltration and necrotic cells were observed in the mice administered FLX and rIL-17 by hematoxylin-eosin staining (Fig. 5B). In the immunohistochemical analysis, the numbers of MPO-positive cells were significantly increased in the mice administered FLX and rIL-17 (Fig. 5C) compared with the FLX-administered mice. In a neutralization study, 1 h before the administration of FLX, the mice were administered an anti-IL-17 antibody. At 3 h after the administration of FLX, the plasma ALT and AST levels showed a tendency to decrease compared with the FLX-administered mice (Fig. 5D).

Involvement of GSH and Oxidative Stress in FLX-Induced Liver Injury

The hepatic GSH content was significantly decreased at 1.5 and 3 h in the FLX-administered mice compared with the non-treated mice. The GSSG level was significantly increased at 1.5 and 6 h after the administration of FLX compared with the non-treated controls. The GSH/GSSG ratio, which is used as an oxidative stress marker, exhibited a similar profile to that obtained for the GSH level (Fig. 6A). The protein carbonyl levels were not significantly changed compared with the non-treated controls (Fig. 6B).

Discussion

In this study, we established a mouse model of FLX-induced liver injury and suggested that immune- and inflammatory-related factors are involved in the hepatotoxicity of FLX. Many clinical case reports of FLX-induced liver injury have been published since 1982 (Olsson *et al.*, 1992), but the mechanism of FLX-induced liver injury has not been fully elucidated. This is the first report to clarify the mechanism of FLX-induced liver injury using a mouse model.

The administration of FLX in mice resulted in an increase in the plasma ALT, AST and T-Bil levels. The plasma AST level was more elevated than the plasma ALT level (Fig. 1A and B), which is similar to the results of a clinical case report (Törnhage *et al.*, 2009). The elevation of the plasma T-Bil level in the mouse was similar to that in humans because jaundice was observed in 95% of patients with FLX-induced liver injury (Devereaux *et al.*, 1995). To investigate if the pharmacological effect of FLX has an effect on the liver injury, AMP, which has similar pharmacological properties and chemical structure to those of FLX and has been reported to be much less hepatotoxic than FLX (Andrade & Tulkens, 2011), was administered to mice (Fig. 1C). The results showed that the pharmacological effect was not involved in the FLX-induced liver injury.

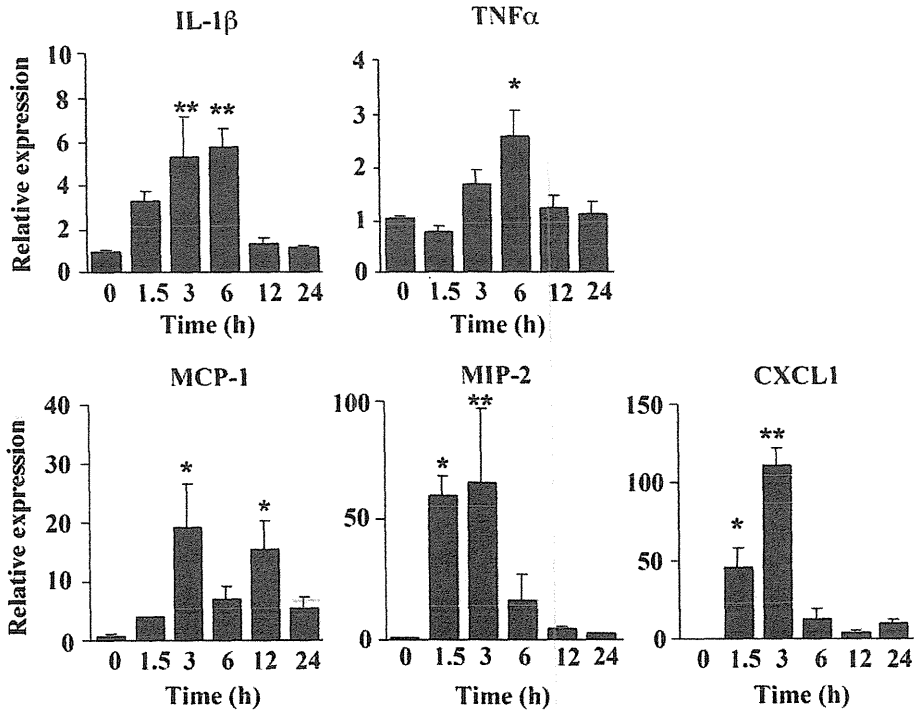

Figure 3. Time-dependent changes in the hepatic mRNA expression levels of inflammatory-related factors in flucloxacillin (FLX)-administered mice. The expression levels of hepatic mRNA were measured by real-time reverse transcription (RT)-PCR 1.5, 3, 6, 12 or 24 h after the FLX administration [1000 mg kg^{-1}, intraperitoneally (i.p.)]. The expression level of hepatic mRNA was normalized to that of β-actin mRNA. The data are shown as the mean ± SEM of the results from four to six mice. The differences compared with the control mice were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

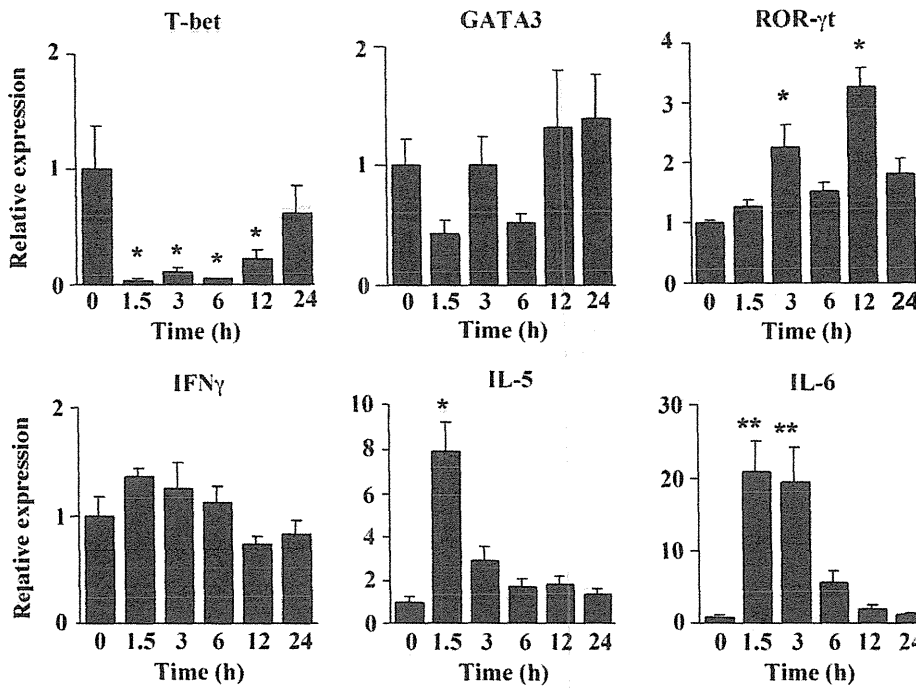

Figure 4. Time-dependent changes in the hepatic expression levels of transcriptional factors and cytokines in flucloxacillin (FLX)-administered mice. The hepatic mRNA levels were measured by real-time reverse transcription (RT)-PCR 1.5, 3, 6, 12 or 24 h after the administration of FLX [1000 mg kg^{-1}, intraperitoneally (i.p.)]. The expression level of hepatic mRNA was normalized to that of β-actin mRNA. The data are shown as the mean ± SEM of the results from four to six mice. The differences compared with the control mice were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

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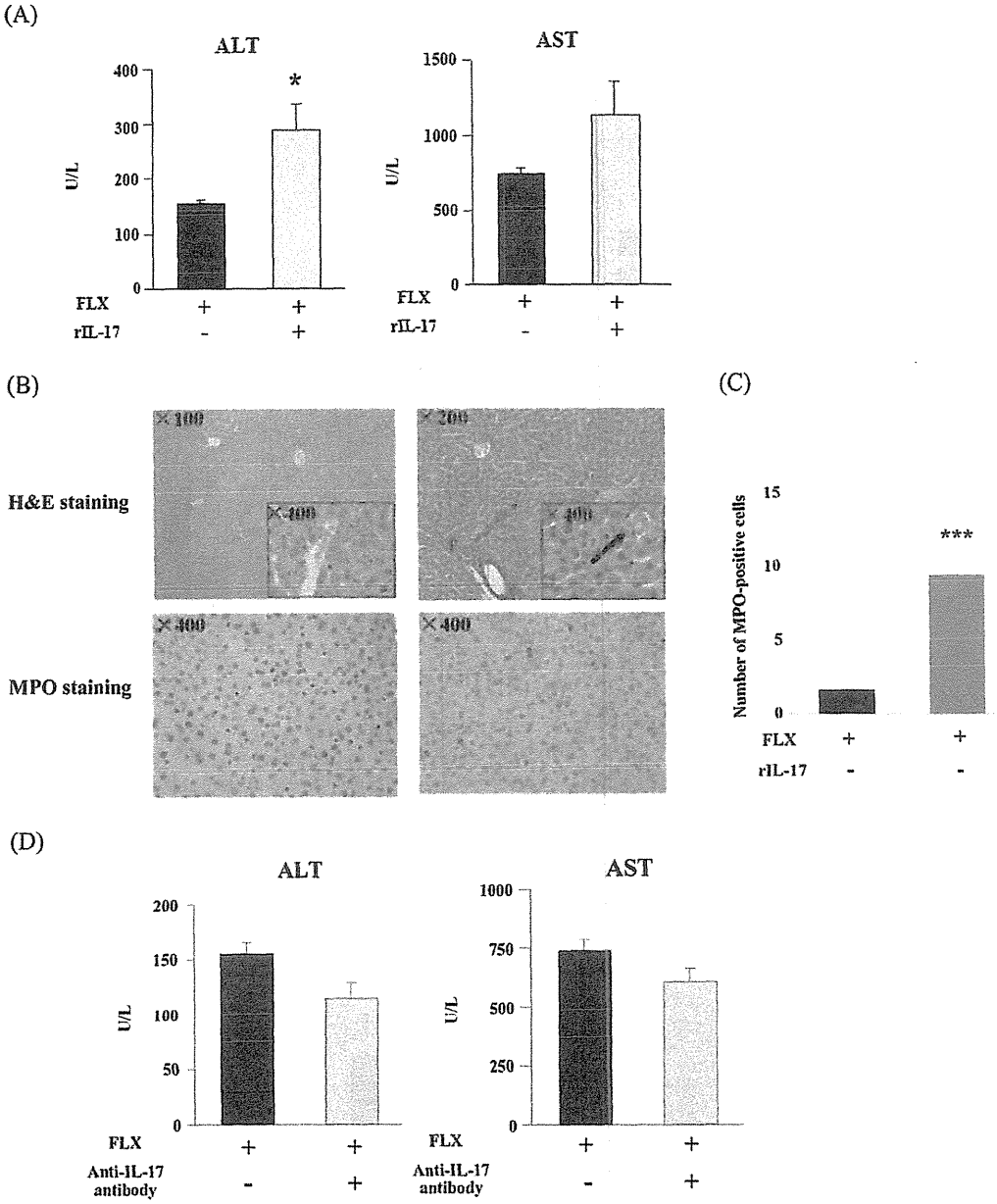

Figure 5. Involvement of interleukin (IL)-17 in flucloxacillin (FLX)-induced liver injury in mice. (A) One hour after the administration of FLX [1000 mg kg^{-1}, intraperitoneally (i.p.)], rIL-17 (2 μg per body weight, i.p.) was administered. Three hours after the administration of FLX, the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured. (B) Liver sections from the FLX (1000 mg kg^{-1}, i.p.)-administered mice were stained with hematoxylin and eosin (H&E) or immunostained with an anti-myeloperoxidase (MPO) antibody. A green arrow indicates an immune cell infiltration, and a black arrow indicates a necrotic cell. (C) The number of MPO-positive cells was compared with those in mice given FLX. (D) One hour before FLX (1000 mg kg^{-1}, i.p.) administration, an anti-IL-17 antibody (100 μg body weight, i.p.) was administered. Three hours after the administration of FLX, the plasma ALT and AST levels were measured. The data are shown as the mean ± SEM of the results from four. The differences compared with the control mice were considered significant at $^{*}P < 0.05$ and $^{***}P < 0.001$.

TLR4 was first recognized as a receptor for host defenses. TLR4 is involved in many diseases, and its endogenous ligands such as S100A8/A9 and HMGB1 have been identified. S100A8 and S100A9 are released from activated phagocytes, and they are associated with inflammation and cancer (Gebhardt *et al.*, 2006; Foell *et al.*, 2007). S100A8 and S100A9 are also increased in the plasma by TLR4 activation (Boyd *et al.*, 2008; Ehrchen *et al.*, 2009). HMGB1 is released from necrotic cells and activated immune cells and binds TLR4 to promote the production of inflammatory cytokines (Sims *et al.*, 2010). It has been reported that HMGB1 is responsible for hepatic ischemia and reperfusion and acetaminophen- and halothane-induced liver injury (Tsung *et al.*, 2005; Antoine *et al.*, 2009; Dugan *et al.*, 2011). We investigated the expression changes of S100A8, S100A9 and HMGB1. The maximum level of HMGB1 was demonstrated 1.5 h after the administration of FLX, which is earlier than that of the plasma ALT, AST, S100A8 and S100A9 (Fig. 2A and B). Considering from these data, we thought that the mechanism is as follows: (1) inflammatory-related cells were activated by FLX or its metabolite(s) in the liver; (2) the released HMGB1

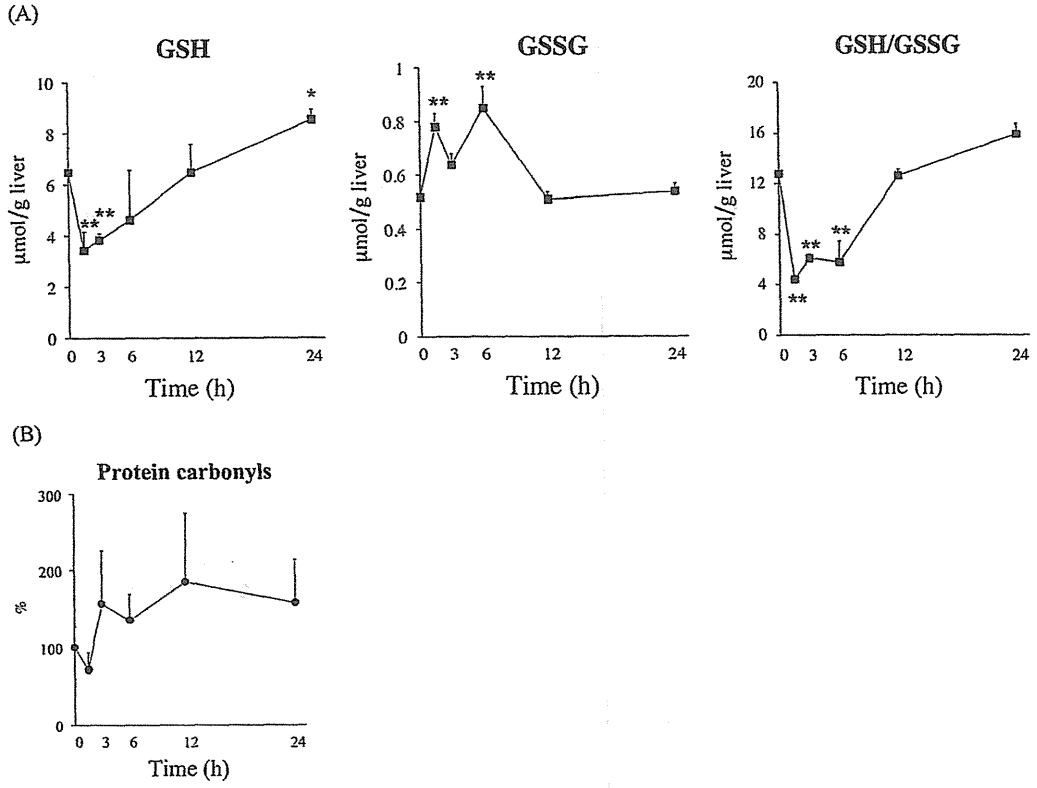

Figure 6. Time-dependent changes in the hepatic GSH and GSSG levels and GSH/GSSG ratio and the levels of protein carbonyls in flucloxacillin (FLX)-administered mice. (A) The content of GSH, GSSG and (B) protein carbonyl were measured in the liver tissue homogenate. The data are shown as the mean ± SEM of the results from four to six mice. The differences compared with the control mice were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

binds to TLR4 in macrophages; and (3) the production of S100A8, S100A9 and inflammatory factors was increased by the activated TLR4.

To further investigate if TLR4 promoted inflammation in FLX-induced liver injury, we measured the expression levels of IL-1β, TNFα, MCP-1, MIP-2 and CXCL1 mRNA produced by the activation of TLR4 (Asehnoune *et al.*, 2004; De Filippo *et al.*, 2008; Li *et al.*, 2011). The expression levels of these proteins were reported to be increased when the mRNAs are elevated (Sass *et al.*, 2002; Roche *et al.*, 2008; Imaeda *et al.*, 2009). IL-1β has roles in inflammation and the activation of immune cells (Dinarello, 2011). TNF-α is associated with cell apoptosis (Malhi *et al.*, 2006). MCP-1, MIP-2 and CXCL1 attract immune cells in tissues (Adams *et al.*, 2010). These proteins are involved in immune cell-associated inflammation and cell death. TLR4 activation may lead to the production of inflammatory factors and immune cell activation in FLX-induced liver injury.

It is known that IFNγ, IL-4 and IL-17 are exacerbation factors in liver injury *via* T cell-related immune reactions. The expression of IL-4 was not detected in the present study (data not shown). The expression level of IL-4 is known to correlate with that of GATA-3, demonstrating no changes as shown in Fig. 4. IFNγ and GATA-3 are the representative factor for Th1 and Th2 cells, respectively (Ranganath *et al.*, 1998), thus, it was suggested that IFNγ and GATA-3 were not involved in FLX-induced liver injury.

The plasma IL-17 level showed a low threshold level in the present study (data not shown). ROR-γt is a transcription factor of NK cells, NKT cells and Th17 cells. IL-6 is a pleiotropic cytokine involved in the enhancing expression and activation of IL-17. The mRNA expression levels of ROR-γt and IL-6 were significantly increased (Fig. 4), therefore we thought that IL-17 might be involved in FLX-induced liver injury.

We investigated the effect of rIL-17 and anti-IL-17 antibodies. The plasma ALT was elevated by the co-administration of rIL-17 and FLX, and liver injury was confirmed by histopathology (Fig. 5A and 5B). Previously, we reported that administration of anti-mouse IL-17 antibody (100 μg per mouse) significantly attenuated α-naphthylisothiocyanate-, carbamazepine-, halothane- and phenytoin-induced liver injury in mice (Kobayashi *et al.*, 2009; Kobayashi *et al.*, 2010; Higuchi *et al.*, 2012; Sasaki *et al.*, 2013). IL-17 had been identified as involved in thee drug-induced liver injury. Therefore, we conducted the same method to neutralize IL-17 in the present study, suggesting the involvement of IL-17 in FLX-induced liver injury (Fig. 5D). Taking these results into consideration, IL-17 would be an exacerbating factor, but its contribution to DILI might not be strong.

GSH is known to have two functions: the antioxidant regent by forming GSSG and the conjugated substrate of reactive metabolites. Therefore, we measured the cellular GSH content to determine which has the major role. We considered that oxidative stress had a limited effect on FLX-induced liver injury, because protein carbonyl, an oxidative biomarker, did not change significantly (Fig. 6B). It has been suggested that the parent compound of FLX has no or much less toxicity, but 5′-OH FLX induced cytotoxicity in culture of human hepatocytes and biliary epithelial cells (Lakehal *et al.*, 2001). However, GSH conjugation of FLX metabolite(s) *in vivo* has never been reported. It has been reported

that penicillin derivatives are metabolized to penicillanic acid and penicilloic acid, which are sulfur-containing compounds and bind to protein (Stachulski *et al.*, 2013). GSH has the ability to bind sulfur-containing groups such as the thiol group. Thus, we thought GSH conjugates the sulfur-containing group of FLX-metabolites. In the present study, GSH and the GSH/GSSG ratio decreased until 6 h after the administration of FLX, and the protein carbonyl levels showed no significant changes (Fig. 6A and B). These results suggest that GSH might conjugate reactive metabolite(s) of FLX, but the contribution of reactive metabolite(s) for DILI might be limited.

In our previous study, DCX-induced liver injury was exacerbated by IL-4 (Higuchi *et al.*, 2011), but it was unclear why the exacerbation factors were different for FLX and DCX. There is only one atom difference of fluorine (F) and chloride (Cl) at position 6 of phenyl group. Olsson *et al.* (1992) reported that the patients who developed FLX-induced liver injury did not have detectable antibodies, but approximately half of the patients who developed DCX-induced liver injury had detectable antibodies. The differences in the atomic radius and electronegativity between F and Cl affect the responses of the immune cells.

An HLA association with the *HLA-B*57:01* allele has been reported in FLX-induced liver injury in humans (Daly *et al.*, 2009; Wuillemin *et al.*, 2013). The involvement of activated T cells in the immune pathomechanism of liver damage has been suggested (Monshi *et al.*, 2013). This study will provide useful information to clarify the detailed immune pathomechanism of FLX-induced liver injury.

This study showed that immune- and inflammatory-related factors are involved in the pathogenesis and exacerbation of FLX-induced liver injury. The TLR4 signaling cascade was suggested to be associated with the etiology, and IL-17 might be an exacerbating factors. This study might provide beneficial information about FLX-induced liver injury and similar drug-induced hepatotoxicities.

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Conflict of Interest

The Authors did not report any conflict of interest.

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Development of a cell-based assay system considering drug metabolism and immune- and inflammatory-related factors for the risk assessment of drug-induced liver injury

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Abbreviations: ALT, alanine aminotransferase; AMP, ampicillin; APAP, acetaminophen; AST, aspartate aminotransferase; CXCL1, chemokine (C-X-C motif) ligand 1; CYP, Cytochrome P450; DAMP, damage-associated molecular pattern molecule; DIC, diclofenac; DILI, Drug-induced liver injury; DIX, dicloxacillin; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FLU, flutamide; GATA-3, GATA-binding domain-3; HAL, halothane; HLM, human liver microsomes; HMGB1, high-mobility group box 1; HSP70, heat shock protein 70; IBU, ibuprofen; IFN-γ, interferon-γ; IL, interleukin; ISO, isoflurane; KC, Kupffer cell; LPS, Lipopolysaccharide; MBZ, mebendazole; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; NALP3, NATCH, LRR, and pyrin domain-containing protein 3; NK cell, natural killer cell; NKT cell, NK cell with T cell receptors; RAGE, receptor for advanced glycation endproducts; ROR-γt, retinoid-related orphan receptor-γt; STAT, signal transducer and activators of transcription factor; T-bet, T-box expressed in T cells; TIM, T cell immunoglobulin and the mucin domain; TLR, toll-like receptor; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule.

Abstract

Drug-induced liver injury (DILI) is a major safety concern in drug development and clinical pharmacotherapy. However, prediction of DILI is difficult because the underlying mechanisms are not fully understood. To establish a novel cell-based screening system to suggest drugs with hepatotoxic potential in preclinical drug development, comprehensive gene expression analyses during *in vivo* DILI are necessary. Using *in vivo* mouse DILI models and 4 sets of hepatotoxic positive and non-hepatotoxic drugs, we found that the hepatic mRNA levels of S100A8; S100A9; "NATCH, LRR, and pyrin domain-containing protein 3" (NALP3); interleukin (IL)-1β; and the receptor for advanced glycation endproducts (RAGE) were commonly increased in hepatotoxic drug-administered mice compared to non-hepatotoxic drug-administered mice. To clarify whether these 5 *in vivo* biomarkers can be applied to a cell-based screening system, we adapted human liver microsomes (HLM) in the presence of NADPH to assess the metabolic activation reaction, and we also adapted human monocytic leukemia cells HL-60, K562, KG-1 and THP-1 to assess the effects on mRNA expression of immune- and inflammatory-related factors. We investigated 30 clinical drugs with different safety profiles with regard to DILI and found that the total sum score of gene expression levels of S100A8, S100A9, RAGE, NALP3 and IL-1β mRNA in HL-60 or K562 cells incubated with HLM, could identify drugs at high risk for hepatotoxicity. We proposed the use of the total sum score of gene expression level for assessing metabolic activation by drug-metabolizing enzymes and immune- and inflammatory-related factors for the risk assessment of DILI in preclinical drug development.

1. Introduction

Drug-induced toxicity is an important human health problem. Although toxic candidate compounds are mostly eliminated during preclinical safety studies in drug development, in some cases, toxicity is detected only in late clinical phases or during postmarketing evaluation. Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of an approved drug from the market and also a major cause of attrition in drug development (Lee, 2003). The drugs nefazodone, troglitazone, and bromfenac were withdrawn from the market because of DILI. For the pharmaceutical industry, it is important that drugs with the potential risk of DILI are screened out in the early phase of discovery and development process. However, in most cases of DILI, the mechanism is largely unknown despite many efforts to clarify them. The low incidence of DILI in humans together with the very large inter- and intra-individual variability of drug metabolism abilities in humans has hampered detailed mechanistic studies of DILI (Evans *et al.*, 2004; Walgren *et al.*, 2005; Takakusa *et al.*, 2008).

Although the mechanisms of DILI are not fully understood, it is generally believed that one of the triggers of the pathogenesis of DILI is that the drug and/or its metabolites are chemically reactive. Cytochrome P450 (CYP) enzymes play an important role in metabolic activation and may generate reactive metabolites. Because a high proportion of drugs are capable of generating reactive metabolites, which covalently bind to various target macromolecules through nucleophilic substitution, it is thought that metabolic activation of a drug might be a necessary first step in DILI in many cases (Uetrecht, 1999; Walgren *et al.*, 2005).

In addition, there is some evidence that immune- and inflammatory-related factors are involved in the pathogenesis of DILI (Ramaiah and Jaeschke, 2007). The liver is selectively enriched in Kupffer cells (KCs), natural killer (NK) cells, and NK cells with T cell receptors (NKT cells), which are key components of the innate immune system that can develop intracellular networks mediated by cytokine and chemokine signaling (Racanelli and

Rehermann, 2006). Several mediators have been suggested to induce hepatotoxicity, including tumor necrosis factor (TNF) α, interleukin (IL)-1β, high-mobility group box (HMGB) 1, and IL-17 (Ishida *et al.*, 2004; Tukov *et al.*, 2007; Antoine *et al.*, 2010; Kobayashi *et al.*, 2009). These mediators can induce hepatocyte cell death by causing an excessive inflammatory response. Thus, comprehensive analyses of gene expression are necessary to establish a new *in vitro* screening system to identify drugs with hepatotoxic potential.

We recently reported the development of mouse *in vivo* DILI models of dicloxacillin (DIX), flutamide (FLU), halothane (HAL), and diclofenac (DIC) using wild type Balb/c mice (Higuchi *et al.*, 2011; Higuchi *et al.*, 2012a; Kobayashi *et al.*, 2009, Yano *et al.*, 2012). FLU- and HAL-induced liver injury were extensively studied in mice and rats (Deng *et al.*, 2006 and 2008; Dugan *et al.*, 2010 and 2011; Cheng *et al.*, 2010; You *et al.*, 2006). In the present study, we conducted analyses of hepatic mRNA expression profiles in the hepatotoxic drugs-induced *in vivo* mouse model, using non-hepatotoxic drugs as negative controls, to determine the common risk biomarkers of DILI. The pharmacological properties and chemical structures of the controls are similar to those of the hepatotoxic drugs. Next, we used 17 representative hepatotoxic and 13 non-hepatotoxic drugs to investigate the application of the risk biomarkers to an *in vitro* cell-based assay system to determine the risk of DILI for each of these drugs. Taking drug metabolism reactions and immune- and inflammatory-related reactions in human into consideration, we proposed the calculation of a "total sum score of gene expression level" for risk assessment of DILI.

2. Materials and Methods

2.1. Materials.

Caffeine, DIC, DIX, lipopolysaccharide (LPS), mebendazole (MBZ), procainamide, valproic acid, warfarin, and zomepirac were purchased from Sigma-Aldrich (St. Louis, MO). HAL and isoflurane (ISO) were kindly provided from Takeda Yakuhin (Osaka, Japan) and Abbott Japan (Tokyo, Japan), respectively. Ampicillin (AMP), APAP, ibuprofen (IBU), FLU, aminopyrine, carbamazepine, ticlopidine, clopidogrel, erythromycin, furosemide, indomethacin, phenytoin, sulfamethoxazole, tacrine, acetylsalicylic acid, dexamethasone, losartan, pravastatine, and theophylline were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Bicalutamide (BIC) was obtained from Enzo Life Sciences (Farmingdale, NY). Rosiglitazone was obtained from LKT Labs (St. Paul, MN). Olanzapine, pioglitazone and valsartan were purchased from Toronto Research Chemicals (Toronto, Canada). Levofloxacin was purchased from BioChemika (Buchs, Switzerland). β-NADPH and glutathione reductase were from Oriental Yeast (Tokyo, Japan). Troglitazone and eritoran were kindly provided by Daiichi–Sankyo (Tokyo, Japan) and Eisai (Tokyo, Japan), respectively. The Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were purchased from Fujifilm (Tokyo, Japan). RNAiso, random hexamer, and SYBR Premix Ex Taq were purchased from Takara (Ohtsu, Japan). ReverTra Ace was purchased from Toyobo (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Monoclonal anti-mouse/rat RAGE antibody and rat IgG2a isotype (used as a control) were obtained from R&D systems (Abingdon, UK). The HMGB1 enzyme-linked immunosorbent assay (ELISA) kit II was purchased from Sino-Test Corporation (Tokyo, Japan). Pooled HLMs (n = 50, testosterone 6β-hydroxylase activity of 5,700 pmol/mg/min) were purchased from BD Gentest (Woburn, MA). All other chemicals were either analytical grade or the highest commercially available grade.

2.2. Drug-induced liver injury mouse models.

Female BALB/cCrSlc mice (8 weeks old) were obtained from SLC Japan (Hamamatsu, Japan). Mice were housed in a controlled environment (temperature 25 ± 1°C, humidity 50 ± 10%, and 12 h light/12 h dark cycle) in the institutional animal facility with access to food and water *ad libitum*. Animals were acclimatized before the experiments. HAL or ISO (30 mmol/kg in 2 μL olive oil, *i.p.*), DIC or IBU (150 mg/kg in saline, *i.p.*), FLU or BIC (1500 mg/kg in 0.5% carboxymethylcellulose (CMC), *p.o.*), and DIX (600 mg/kg in saline, *i.p.*) or AMP (1000 mg/kg in saline, *i.p.*) were administered to mice in a non-fasting condition. APAP (300 mg/kg in saline, *i.p.*) was administered to mice in an overnight fasting condition. Blood samples from the inferior vena cava and the largest lobe of the liver were collected 1, 3, 6 and 24 h after drug administration under diethyl-ether anesthesia ($n = 5$ for each drug-treated group). As previously reported by our group (Higuchi *et al.*, 2011; Higuchi *et al.*, 2012a; Kobayashi *et al.*, 2009, Yano *et al.*, 2012), the liver damage was confirmed by histopathology in APAP, HAL, DIC, FLU, and DIX-administered mice. Animals were treated and maintained in accordance with the Japan National Institutes of Health Guide for Animal Welfare, and the animal protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan (AP-#111985).

2.3. Real-time reverse transcription (RT)-PCR.

RNA from the mouse liver or human monocytic leukemia cells were isolated using RNAiso according to the manufacturer's instructions. The expression levels of mouse chemokine (C-X-C motif) ligand (CXCL) 1, Fas ligand (FasL), GATA-binding domain (GATA)-3, granzyme B, HMGB1, heat shock protein (HSP) 70, interferon (IFN)-γ, IL-1β, IL-6, IL-12p35, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-2, "NATCH, LRR, and pyrin domain-containing protein 3" (NALP3), perforin, receptor for advanced glycation endproducts (RAGE), retinoid-related orphan receptor

(ROR)-γt, signal transducer and activators of transcription factor (STAT) 1, STAT3, STAT6, S100A8, S100A9, T-box expressed in T cells (T-bet), T cell immunoglobulin and the mucin domain (TIM) family (TIM1, TIM2, TIM3, TIM4), TNFα toll-like receptor (TLR) 4, vascular cell adhesion molecule (VCAM) 1, and GAPDH were quantified by real-time RT-PCR. The proinflammatory calgranulins S100A8 and S100A9 are cytoplasmic proteins that are expressed by neutrophils, monocytes and activated macrophages under physiological conditions. The primer sequences are shown in Table 1. The expression levels of human NALP3, RAGE, S100A8, S100A9, IL-1β,and GAPDH were also quantified by real-time RT-PCR. The primer sequences are shown in Table 2. For the RT step, total RNA (10 μg) and 150 ng of random hexamer were mixed and incubated at 70°C for 10 min. The RNA solution was added to a reaction mixture containing 100 units of ReverTra Ace, reaction buffer and 0.5 mM dNTPs in a final volume of 40 μL. The resulting reaction mixture was incubated at 30°C for 10 min and 42°C for 1 h and then heated at 98°C for 10 min to inactivate the enzyme. Real-time RT-PCR was performed using an Mx3000 P instrument (Stratagene, La Jolla, CA). The PCR mixture contained 1 μL of template cDNA, SYBR Premix Ex Taq solution and 8 pmol of forward and reverse primers. The amplified products were monitored directly by measuring the increase of the SYBR Green I (Molecular Probes, Eugene, OR) dye intensity.

2.4 Administration of a TLR4 antagonist and an anti-mouse RAGE antibody.

Mice were intravenously treated with eritoran, a TLR4 antagonist (50 μg/mouse in 0.2 mL sterile saline), or an anti-mouse/rat RAGE antibody (100 μg/mouse in 0.2 mL sterile PBS) simultaneously with the drug administration, as previously described (Higuchi *et al.*, 2012b). Rat IgG2a was administered as a control for the RAGE neutralization study (100 μg rat IgG2a in 0.2 mL sterile saline). Blood was collected from the inferior vena cava at 6 h after DIC and DIX administration, and 24 h after the HAL administration.

2.5. Cell culture.

Human monocytic leukemia THP-1 cells were obtained from Riken Gene Bank (Tsukuba, Japan). Human promyelocytic leukemia HL-60 cells and human acute myeloid leukemia K562 cells and KG-1 cells were obtained from American Type Culture Collection (Manassas, VA). THP-1 and K562 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). HL-60 and KG-1 cells were cultured in RPMI 1640 medium supplemented with 20% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2. In the experiment using differentiated THP-1 cells, THP-1 cells were treated with PMA (phorbol 12-myristate 13-acetate) at 20 ng/mL for 48 h to trigger THP-1 cells to undergo differentiation into macrophages.

2.6. Drug treatment of human monocytic leukemia cells and the effects of HLM-dependent drug metabolism.

In LPS or MBZ exposure experiments, human monocytic leukemia cells, THP-1, HL-60, K562 and KG-1 cells were seeded at a density of 1×10^6 cells/well in 24-well plates with culture medium containing 10 μg/mL LPS or 10 μM MBZ and then incubated at 37°C for 3 and 6 h. In the experiments conducted to investigate the effect of the HLM-dependent drug metabolism of 30 drugs, HL-60 or K562 cells were seeded at a density of 1×10^6 cells/well in 24-well plates with medium containing the indicated concentration of the drugs (shown in Table 4) and 5% FBS, 1 mM β-NADPH and 1 mg/mL HLM. The cells were then incubated at 37°C for 6 h. Heat-inactivated (treated at 56°C for 30 min) HLMs were used as a control. The final concentration of dimethyl sulfoxide (DMSO) in the culture medium was 0.1%. We confirmed that cell viability was above 80% under the condition for any drug treatment.

2.7. Statistical analysis.

The data are shown as the means ± SEM. Comparison of multiple groups was made with Kruskal-Wallis ANOVA followed by Dunn's test. Comparison of two groups was made using Welch's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Time-dependent changes of plasma transaminase levels in hepatotoxic drug-administered mice.

To conduct a comprehensive analyses of the time-dependent changes of hepatic gene expression in mice with drug-induced hepatotoxicity, drugs known to cause DILI, including APAP (300 mg/kg, *i.p.*), HAL (30 mmol/kg, *i.p.*), DIC (150 mg/kg, *i.p.*), FLU (1500 mg/kg, *p.o.*), and DIX (600 mg/kg, *i.p.*) were administered to female BALB/c mice. Plasma ALT and AST levels were significantly increased in hepatotoxic drug-administered mice (Fig. 1). Slight increases in the plasma ALT and AST levels were observed 1 h after these drugs were administered, and increases in the ALT and AST levels were observed 6 h after DIC, FLU, and DIX administration. Increased levels were also observed 24 h after APAP and HAL administration. ISO, IBU, BIC, and AMP were used as the non-hepatotoxic control drugs; these control drugs have pharmacological properties and chemical structures that are similar to HAL, DIC, FLU, and DIX, respectively. The control drugs showed no hepatotoxic or much less hepatotoxic effects when administered with the same dosing program as the hepatotoxic drugs.

3.2. Time-dependent mRNA expression profiles in mouse models of drug-induced liver injury.

Time-dependent expression profiles of hepatic mRNAs in the mouse models of drug-induced liver injury were investigated by real-time RT-PCR analysis focusing on inflammation- and immune-related factors (Table 3). The hepatic mRNA expression levels of Th cell stimulation-related factors (T-bet, GATA-3, ROR-γt, STAT1, STAT3, STAT6), cytokines (TNFα, IL-1β, IL-6, IFN-γ, IL-12), chemokines (MIP-2, MCP-1, CXCL1, VCAM1), damage-associated molecular pattern molecules (DAMPs: S100A8, S100A9, HSP70, HMGB1), receptors (TLR4, NALP3, RAGE), CD8-positive cell-related factors

(granzyme B, perforin, FasL), and the TIM family members (TIM1, TIM2, TIM3, TIM4) were measured (Table 3, Figs. 2A and 2B). In our previous studies (Higuchi *et al.*, 2011; Kobayashi *et al.*, 2009), we confirmed that the expression profiles of mRNA and protein were similar for the interleukins, chemokines and receptors listed in Table 3, except in the case of HMGB1. Thus, changes in the mRNA expression levels were mainly investigated in the present study. Time-dependent changes in the expression levels of the hepatic mRNAs were shown in the hepatotoxic or non-hepatotoxic drug-administered groups compared with the vehicle-administered control groups (Fig. 2A). For example, the expression level of RAGE was increased after APAP, DIC, FLU, and DIX were administered to mice compared with the levels in mice who received vehicle. Increased MIP-2 mRNA expression was also observed after APAP, HAL, DIC, IBU, FLU, DIX, and AMP administration (Figs. 2A). In particular, 6 h after HAL administration, S100A8 and S100A9 expression were markedly increased, whereas the plasma ALT levels were not markedly increased (Fig. 3). In addition, the increase of S100A8 and S100A9 expression is likely specific for the hepatotoxic drug-administered groups, suggesting that those genes might be useful biomarkers for hepatotoxicity induced by drugs. However, S100A8 and S100A9 are not suitable biomarkers for FLU-induced hepatotoxicity, suggesting that several factors are necessary for developing a panel of biomarkers to determine the risk for drug hepatotoxicity. In Fig. 2B, the time-dependent changes of the expression levels of the S100A8, S100A9, RAGE, NALP3, and IL-1β were demonstrated in detail. Taking those expression profiles into consideration, changes in the mRNA expression of these 5 factors were relatively specific in the groups that received hepatotoxic drugs compared with those that received non-hepatotoxic drugs (Fig. 2A and 2B). Therefore, we focused on these 5 factors as candidate biomarkers to determine the risk of hepatotoxicity for the drugs in the *in vitro* studies. The change of the expression level of MIP-2 was the highest among the immune- and inflammatory-related factors in all the DILI-positive drugs. However, the results of MIP-2 showed high variability among the *in vivo*

experiments, which considerably affect the changes total sum score of gene expression level. To avoid low reproducibility, MIP-2 was not selected.

3.3. Role of TLR4 and RAGE in the pathogenesis of drug-induced liver injury

S100A8 and S100A9 are ligands of TLR4 and RAGE; HMGB1 is also a well-known ligand. HMGB1 is secreted from activated immune cells and is also passively released from necrotic cells (Wang *et al.*, 2004). It has been demonstrated that the release of HMGB1 is not correlated with changes in hepatic HMGB1 mRNA expression (Higuchi *et al.*, 2012b). Thus, the plasma HMGB1 level was measured using ELISA, which demonstrated a significant increase in expression in the hepatotoxic drug-administered groups compared with the non-hepatotoxic drug-administered groups (Fig. 4A). To investigate whether the TLR4 and RAGE signaling pathways are involved in DILI, eritoran, a TLR4 antagonist, or a monoclonal anti-RAGE antibody was administered to mice according to our previously described methods (Higuchi *et al.*, 2012b). Eritoran treatment significantly suppressed the plasma ALT levels in HAL-, DIC-, and DIX-administered mice (Fig. 4B), and anti-RAGE antibody administration also ameliorated DIX-induced liver injury (Fig. 4C). These results suggested that the TLR4 and RAGE signaling pathways might be involved in DILI.

3.4. Effect of immunostimulatory drugs on the mRNA level of the 5 biomarkers in human monocytic cell lines

To evaluate the 5 biomarkers for their utility in an *in vitro* assay system to distinguish the risk of the hepatotoxicity of drugs, we investigated the changes of the expression levels of NALP3, RAGE, S100A8, and S100A9 mRNA in human monocytic leukemia cells, such as THP-1, K562, KG-1, and HL-60, and differentiated THP-1 cells. Before considering the metabolic activation reaction in the cell-based assay, the effect of a parent drug (before being metabolized) on the expression of 4 biomarkers should be considered. The hepatotoxic drugs