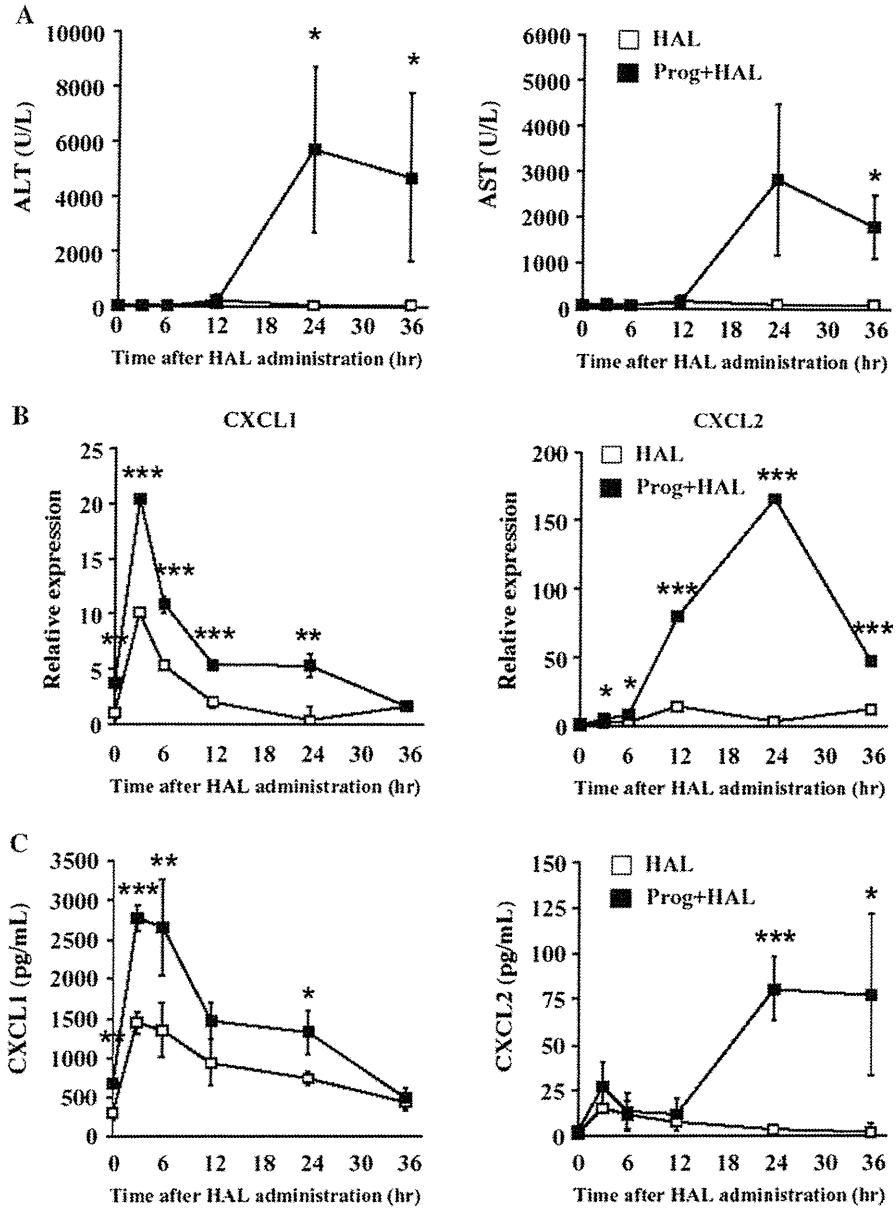

FIG. 1. Time-dependent effects of progesterone pretreatment on HAL-induced liver injury. Female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by HAL (15 mmol/kg, ip) administration 1.5 h after the last treatment of progesterone. Plasma and liver samples were collected 0, 3, 6, 12, 24, or 36 h after the HAL administration. Plasma ALT and AST (A) and relative expression of hepatic mRNA were measured for CXC chemokines (B). Expression of hepatic mRNA was normalized to GAPDH mRNA. Plasma protein levels of CXC chemokines were assessed by ELISA (C). The data are mean ± SD of four mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with only HAL-administered mice.

HAL. U0126 pretreatment decreased of the progesterone-induced phosphorylation of ERK by 50% (Fig. 4A). Although U0126 pretreatment alone did not affect the liver injury induced by HAL alone, U0126 pretreatment significantly decreased the progesterone-induced exacerbation of the HAL-induced liver injury (Fig. 4B). Notably, the CXCL1 mRNA levels increased by progesterone pretreatment alone were significantly decreased by U0126 treatment. Furthermore, U0126 pretreatment significantly decreased the CXCL1, CXCL2, and ICAM-1 mRNA levels increased by progesterone pretreatment after HAL administration (Fig. 4C).

Involvement of Kupffer Cells on Progesterone Pretreatment–Induced Exacerbation of HAL-Induced Liver Injury in Female Mice

Kupffer cells act as a major source of proinflammatory cytokines and CXC chemokines. To determine whether Kupffer

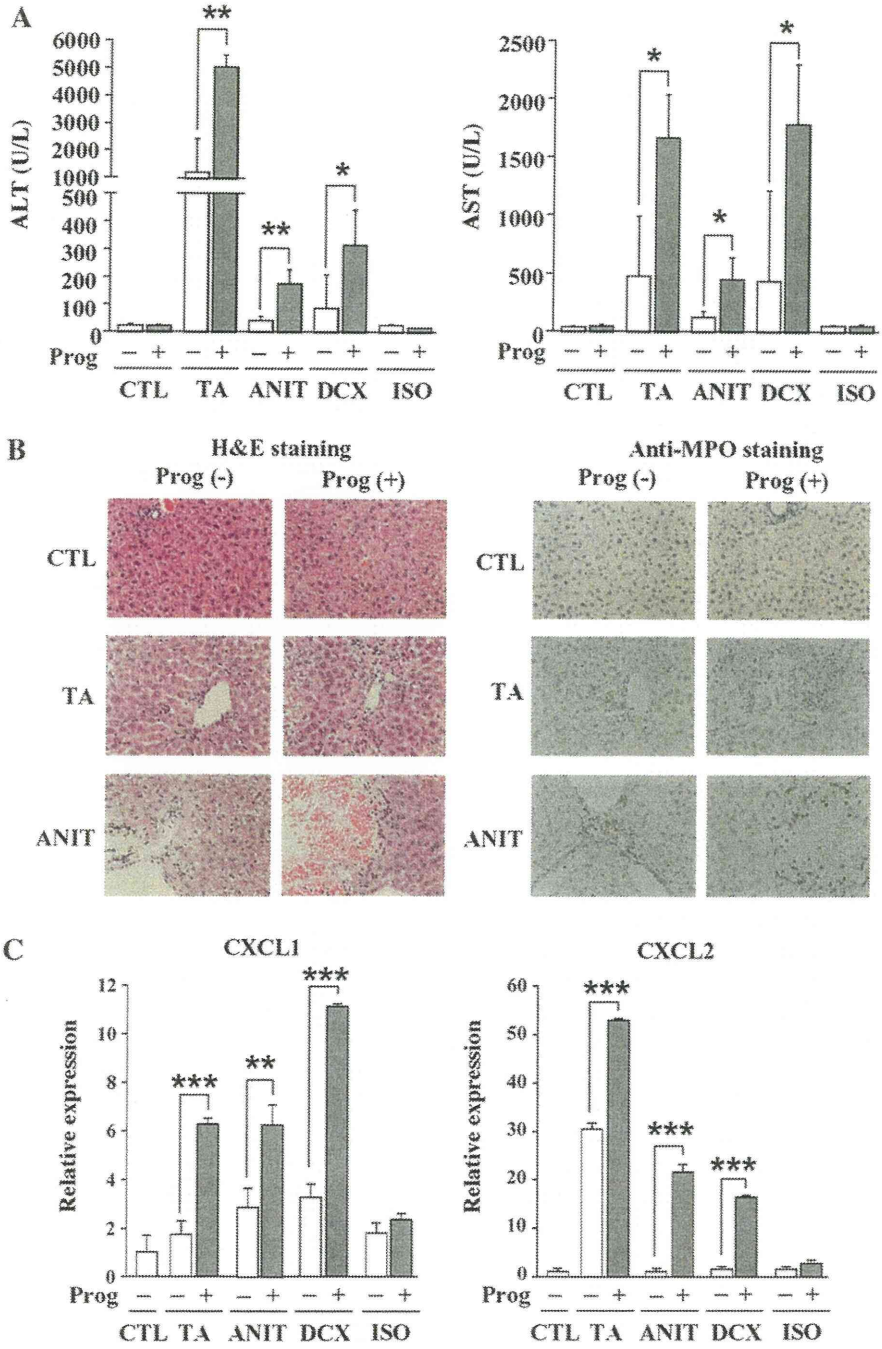

FIG. 2. Effects of progesterone pretreatment on various hepatotoxic compound–induced liver injury. Female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by TA (50 mg/kg, ip), ANIT (80 mg/kg, po), DCX (600 mg/kg, ip), or ISO (15 mmol/kg, ip) administration 1.5 h after the last treatment of progesterone. Six hours after the administration of DCX and 24 h after the administration of TA, ANIT, or ISO, plasma and liver samples were collected for assessment of the transaminase levels (A). Liver tissue sections were stained with H&E or immunostained with anti-MPO antibody (B). Relative expression of hepatic mRNA was measured for CXCL1 and CXCL2 and was normalized to GAPDH mRNA (C). The data are mean ± SD of four mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with CTL (control).

cells are involved in the exacerbation of liver injury, mice pretreated with progesterone were treated with $GdCl_3$, an inhibitor of the Kupffer cell function, followed by the administration of HAL. Although $GdCl_3$ treatment did not affect the liver injury induced by HAL alone, $GdCl_3$ pretreatment significantly decreased the progesterone-induced exacerbation of the

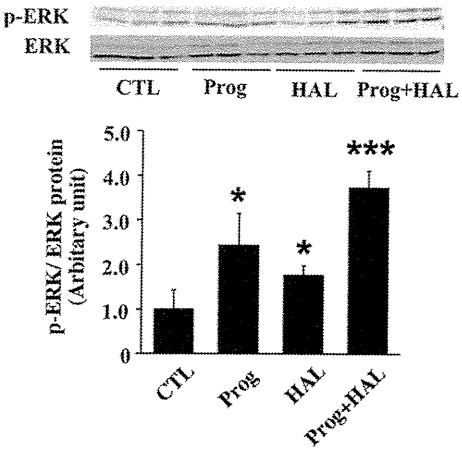

FIG. 3. Activation of ERK signaling pathways in liver after HAL administration in progesterone-pretreated female mice. Experimental conditions for animal treatments were the same as those in Figure 1. Female mice were pretreated with progesterone for 7 days followed by HAL administration 1.5 h after the last pretreatment of progesterone. Liver samples were collected 3 h after HAL administration. Immunoblot of ERK proteins in whole liver homogenates were performed using the monoclonal antibodies against ERK1/2 or against Thr202/Tyr204 phosphorylated ERK1/2 (p-ERK). Each lane of the immunoblot showed an individual mouse (50 μg/lane). The data are mean ± SD of three mice. $*p < 0.05$ and $***p < 0.001$, compared with CTL (control).

HAL-induced liver injury (Fig. 5A). The $GdCl_3$ pretreatment alone did not affect the mRNA levels of CXCL1, CXCL2, and ICAM-1. The increased levels of CXCL1 mRNA by progesterone pretreatment alone were significantly decreased by $GdCl_3$ treatment. Furthermore, $GdCl_3$ pretreatment significantly decreased the CXCL1, CXCL2, and ICAM-1 mRNA levels in mouse liver pretreated with progesterone after HAL administration (Fig. 5B).

To confirm the effect of $GdCl_3$ pretreatment, the number of Kupffer cells was evaluated using F4/80 antibody-staining method and counted number of the F4/80-positive cells microscopically. As shown in Supplementary fig. 5, the numbers of F4/80-positive cells were significantly decreased by $GdCl_3$ pretreatment to 31 and 29% in vehicle-pretreated control mice and in progesterone-pretreated mice, respectively.

Effects of Progesterone on RAW264.7 Cells

To determine whether Kupffer cells are involved in the production of CXCL1 by progesterone treatment, mouse macrophage cell line RAW264.7 cells were treated with progesterone and measured for the expression of CXCL1 mRNA levels. The CXCL1 mRNA levels were significantly increased by progesterone exposure. The increased expression of CXCL1 mRNA was inhibited by cotreatment of RU486 (Fig. 6A). In addition, the increased expression of CXCL1 mRNA was inhibited by cotreatment of U0126 with progesterone but not by ICI, SP600125, or SB203580 (Fig. 6B).

Pretreatment of RU486 Ameliorates HAL-Induced Liver Injury in Female Mice

Since progesterone pretreatment exacerbated the liver injury mediated the activation of immune response, we hypothesized that progesterone receptor antagonist would ameliorate liver injury. To investigate the effect of progesterone receptor antagonist against DILI, mice pretreated with RU486 for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of RU486. RU486 pretreatment alone had no effect on the transaminase levels, but RU486 pretreatment significantly decreased the transaminase levels and mRNA levels of CXCL1, CXCL2, ICAM-1, and TNFα (Fig. 7).

Post-Administration of RU486 Ameliorates HAL-Induced Liver Injury

From a therapeutic point of view, a more clinically relevant approach is to treat RU486 after HAL administration. Therefore, mice were administered RU486 after the HAL administration (30 mmol/kg, ip). As with the pretreatment experiments, posttreatment of RU486 significantly decreased the transaminase levels and mRNA levels of CXCL1, CXCL2, ICAM-1, and TNFα in female mice (Fig. 8). Similarly, posttreatment of RU486 decreased the transaminase levels after TA administration in female mice (Supplementary fig. 6).

DISCUSSION

Progesterone, one of the female sex hormones, plays an important role in the female reproductive function. There is also evidence that the immune system is regulated by the circulating levels of sex hormones (Grossman, 1985). Our previous report demonstrated that progesterone pretreatment exacerbated HAL-induced liver injury, whereas E2 pretreatment resulted in the opposite effect in female mice (Toyoda *et al.*, 2011). It was also demonstrated that HAL-induced liver injury was exacerbated in female mice in estrus, during which the plasma concentration of progesterone is elevated, and ovariectomized mice showed significantly suppressed HAL-induced liver injury (Dugan *et al.*, 2011). These reports suggested that progesterone has an important effect in DILI, therefore, we put the focus on the mechanism of the progesterone-induced exacerbating effect of liver injury in this study.

To investigate the effect of progesterone in DILI, female BALB/c mice pretreated with progesterone were administered with hepatotoxicant. In this study, the plasma progesterone level was 80.4 ± 33.3 ng/ml in mice 24 h after the last progesterone pretreatment and 29.2 ± 14.8 ng/ml in mice pretreated with vehicle. In general, progesterone secretion increased to the maximum plasma progesterone level of 60–120 ng/ml during late pregnancy (Barkley *et al.*, 1979). Thus, the serum progesterone levels of mice pretreated with progesterone in the present study was almost the same as during late pregnancy.

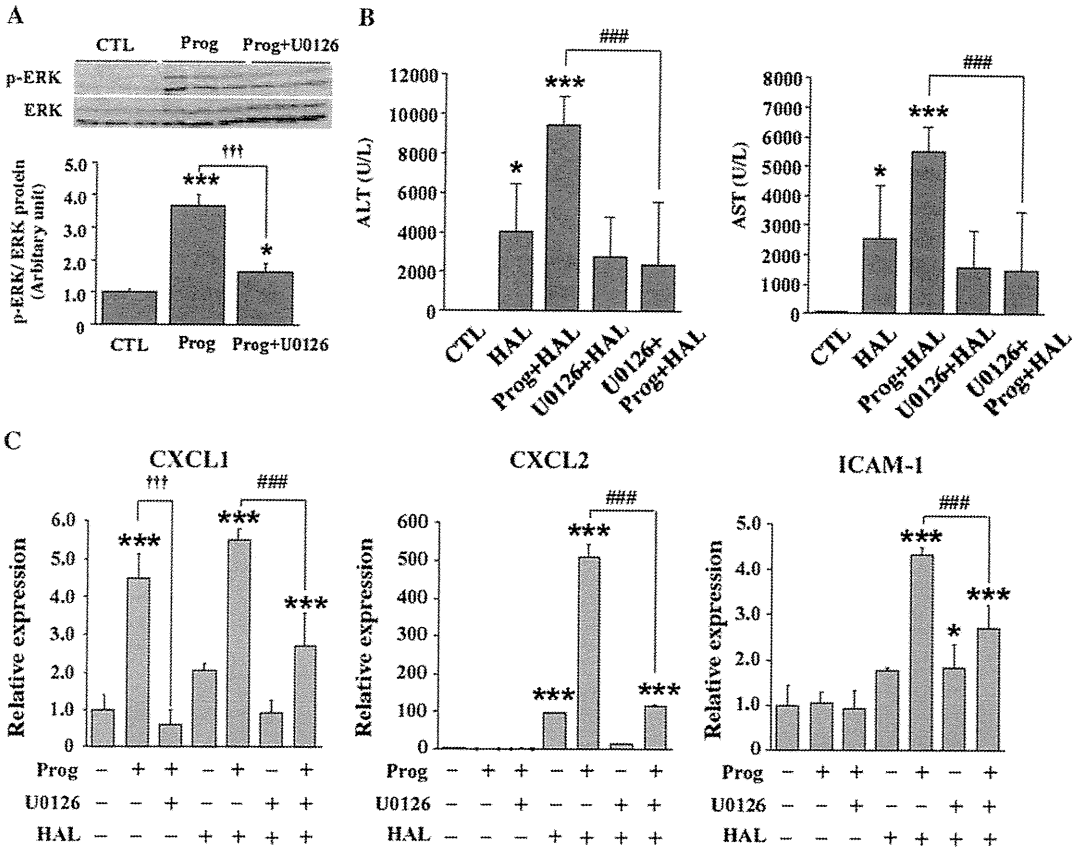

FIG. 4. Role of ERK pathway on the effect of progesterone pretreatment in HAL-induced liver injury. Female mice pretreated with progesterone for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last progesterone treatment, then U0126 (10 mg/kg, ip) was administered 1 h before the HAL administration. Whole liver homogenate was collected 3 h (A) and 24 h (B and C) after the HAL administration. Immunoblot of ERK proteins in whole liver homogenates collected 3 h after the U0126 administration was performed and quantified (A). Each lane shows an individual mouse (50 μg/lane). Plasma and liver samples were collected 24 h after the HAL administration to assess the transaminase levels (B) and expression of hepatic mRNA levels of chemokines and ICAM-1 (C). Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 5–8 mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with CTL. $\dagger\dagger\dagger p < 0.001$, compared with progesterone pretreatment alone. $\#\#\#p < 0.001$, compared with HAL-administered mice pretreated with progesterone.

The transaminase levels, hepatic tissue damage, and mononuclear cells infiltration in HAL-, TA-, ANIT- and DCX-induced liver injury were exacerbated by progesterone pretreatment (Figs. 1 and 2). Progesterone pretreatment alone did not increase the transaminase levels in nontreated mice and did not affect the transaminase levels in ISO-administered mice. ISO is structurally and pharmacology similar to HAL but less hepatotoxic, indicating that the progesterone pretreatment exacerbated the severity of liver injury in female mice. Higher numbers of mononuclear cells infiltrated in the liver of mice pretreated with progesterone after hepatotoxic compounds administration (Fig. 2B). In addition, it was demonstrated that the mRNA levels of CXCL1, CXCL2, and ICAM-1 were correlated with the infiltration and accumulation of MPO-positive cells. Most of MPO-positive cells were considered as neutrophils because of their nuclear morphology in the liver histopathology in this study. Neutrophils have an important role in various types of liver injury (Ramaiah and Jaeschke, 2007). CXC chemokines are considered to attract predominantly neutrophils to the liver under stress conditions and the neutrophils undergo adhesion to hepatocytes via hepatocyte ICAM-1. In this study, CXC chemokines, CXCL1 and CXCL2, were markedly increased after HAL administration in progesterone-pretreated female mice but not in male mice (Fig. 1 and Supplementary fig. 1). Interestingly, progesterone pretreatment alone increased CXCL1 and CXCL1 was quickly and significantly increased after the subsequent administration of hepatotoxic compounds. It was demonstrated that liver injury after carbon tetrachloride administration was exacerbated by injection of recombinant CXCL1, but injection of recombinant CXCL1 did not affect it in normal mice (Stefanovic *et al.*, 2005). In accordance with this report, increased expression of CXCL1 by the progesterone pretreatment demonstrated no hepatotoxic effect in normal mice, but progesterone pretreatment exacerbated liver injury

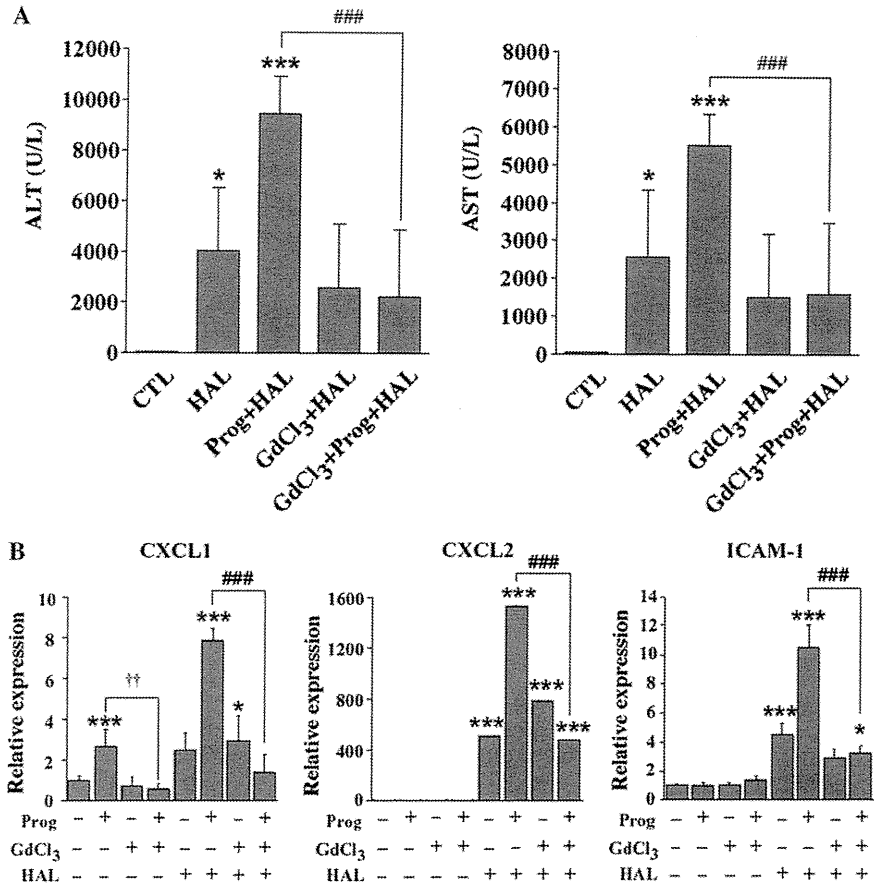

FIG. 5. Role of Kupffer cells in the effect of progesterone pretreatment in HAL-induced liver injury. Female mice pretreated with progesterone for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of progesterone. $GdCl_3$ (10 mg/kg, iv) was administered two times at 24 and 48 h before the HAL administration. Plasma and liver samples were collected for assessment of the transaminase levels (A) and expression of hepatic mRNA levels of chemokines and ICAM-1 (B) 24 h after the HAL administration. Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 5–6 mice. $*p < 0.05$ and $***p < 0.001$, compared with CTL. $\dagger\dagger p < 0.001$, compared with progesterone pretreatment alone. $\#\#\#p < 0.001$, compared with HAL-administered mice pretreated with progesterone.

in mice after hepatotoxicants administration, but not ISO administration, indicating that the increased expression of CXCL1 mediated the activation of immune responses after the hepatotoxicant administration.

Progesterone pretreatment method used in this study did not increase mRNA levels of CXCL1 and subsequent increase of transaminase levels after hepatotoxicants administration in male mice (Supplementary figs. 1 and 3). In human, autoimmune diseases, such as multiple sclerosis, were much higher incidence in women. Some reports indicated that male sex hormones, testosterone, have immunosuppressive effects, which may partly account for the sex difference of autoimmune diseases (Fijak *et al.*, 2011; Gold and Voskuhl, 2009). In mice, serum testosterone levels was 6.9 ± 2.3 ng/ml in male mice and < 0.1 ng/ml in female mice (Bösl *et al.*, 2001). It is thought that testosterone may partly suppress immune activation such as increased expression of CXCL1 by progesterone pretreatment in male mice. It was also reported that liver injury after carbon tetrachloride administration was exacerbated by injection of recombinant CXCL1 in male mice (Stefanovic *et al.*, 2005). Therefore, if CXCL1 is upregulated by modifying the progesterone pretreatment method, the liver injury might be exacerbated in male mice. However, further study of the precise mechanism by which male mice are not responsive to progesterone is needed.

It is suggested that the human serum level of Glo-α, the homolog of CXCL1, is correlated with female sex hormones (Kanda *et al.*, 1997). It is well known that women have greater susceptibility to alcoholic liver injury than men. In alcoholic liver injury, Glo-α was significantly increased in human (Maltby *et al.*, 1996). In addition, female rats fed an ethanol diet showed significantly increased ALT- and cytokine-induced neutrophil chemoattractant (CINC)-1 mRNA, the homolog of CXCL1, after lipopolysaccharide injection (Yamada *et al.*, 1999). This report also demonstrated that gonadectomy totally abolished the sex difference of the CINC-1 mRNA expression. From these

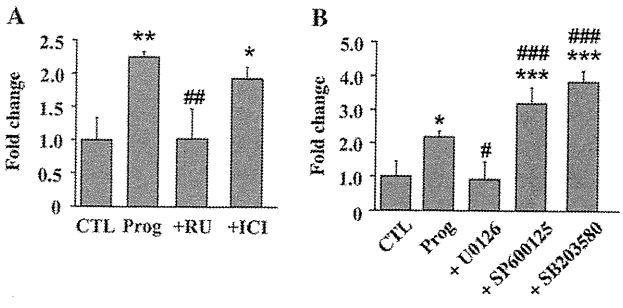

FIG. 6. Changes of the expression levels of CXCL1 mRNA in RAW264.7 cells treated with progesterone. The cells were treated with progesterone (0.1μM), progesterone and estrogen receptor antagonists (RU486 or ICI; 1.0μM) (A), and MAPK inhibitors (U0126, SP600125, SB203580; 1.0μM) (B). After 24-h incubation, the mRNA expression levels of CXCL1 were measured. The expression of mRNA was normalized to GAPDH mRNA. The data are mean ± SD of triplicate determines. $*p < 0.05$ and $***p < 0.001$, compared with CTL. $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$, compared with only progesterone treated groups.

lines of evidence, the sex difference in the ability to produce CXCL1 in the liver is an important factor, which may partly account for the sex difference in the severity of liver injury.

The activation of MAP kinases such as ERK1/2, p38 MAP kinase, and JNK1/2 is important in mediating many functions of macrophages, including the activation of various transcription factors and the production of proinflammatory cytokines and chemokines (DeFranco *et al.*, 1998; Payne *et al.*, 1991). In this study, progesterone activated the ERK signaling pathway in the liver in female mice but not in male mice (Fig. 3 and Supplementary fig. 4). To determine the involvement of ERK in progesterone-induced exacerbation of liver injury, mice were treated with U0126, ERK inhibitor, which did not affect the liver injury and immune response induced by HAL alone, but inhibition of ERK significantly decreased the progesterone-induced exacerbation of HAL-induced liver injury and immune responses. The mRNA levels of CXCL1, CXCL2, and ICAM-1 were suppressed by pretreatment with U0126 after HAL administration, compared with mice untreated with U0126. Importantly, U0126 pretreatment suppressed the increased expression of CXCL1 by progesterone pretreatment alone and progesterone-induced exacerbating effect in transaminase levels (Fig. 4). It has been reported that inhibition of ERK suppressed Fas-induced CXCL1 in murine epithelial cell line (Farnand *et al.*, 2011). It has been also reported that inhibition of the ERK pathway attenuated the inflammatory response and improved the outcome after traumatic injuries and cisplatin-induced renal injury (Hsu *et al.*, 2009; Jo *et al.*, 2005). Thus, the progesterone-induced exacerbation of liver injury is partly mediated via an ERK pathway and subsequent increase of the production of CXC chemokines.

It was also reported that the ERK pathway in Kupffer cells has a critical role in the production of the immune response and liver injury during chronic ethanol feeding (Thakur *et al.*, 2006). In the present study, U0126 treatment decreased the CXCL1 mRNA expression in a progesterone-pretreated mouse macrophage cell line, RAW264.7 (Fig. 6). Thus, the ERK pathway in Kupffer cells may have an important role in the exacerbation of liver DILI by progesterone. Kupffer cells act as a major source of proinflammatory cytokines and CXC chemokines under severe stress and various types of liver injury (Adams *et al.*, 2010; Kaplowitz, 2005; Laskin, 1990; Mosher *et al.*, 2001). Progesterone increased the production of proinflammatory cytokines in monocyte and macrophages via progesterone receptor (Huang *et al.*, 2008; Yuan *et al.*, 2008). In the present study, the inhibition of Kupffer cells by $GdCl_3$ did not affect the liver injury and immune response induced by HAL alone but significantly decreased the progesterone-induced exacerbation of HAL-induced liver injury and immune responses (Fig. 5). Moreover, the inhibition of Kupffer cells significantly decreased the CXCL1 mRNA expression increased by progesterone alone. Although it was reported that Kupffer cells do not contribute to HAL-induced liver injury, cotreatment with poly (I:C), ligand of TLR3, exacerbated the HAL-induced liver injury by activation of Kupffer cells (Cheng *et al.*, 2009, 2010; Dugan *et al.*, 2011). Considering these findings, progesterone led exacerbation of HAL-induced liver injury by activation of Kupffer cells and increased expression of CXCL1 in this study. In addition, GdCl3 attenuated progesterone-induced exacerbation of liver injury and expression of CXCL1.

In the present study, both pretreatment and posttreatment RU486, a potent progesterone receptor antagonist, significantly suppressed HAL- or TA-induced liver injury and immune responses (Figs. 7 and 8 and Supplementary fig. 6). Because progesterone affected the immune responses mediated by Kupffer cells, it is conceivable that RU486 also affects the immune responses mediated by Kupffer cells. The progesterone receptor is expressed on immune cells, natural killer cells, leukocytes as well as Kupffer cells (Gilliver, 2010). Therefore, the mechanism for the suppression of liver injury by RU486 may be due to the effect on immune cells. Moreover, RU486 is also a glucocorticoids (GCs) receptor antagonist. Recently, it was reported that GCs play a role in DILI and pretreatment with RU486 attenuated HAL- and APAP-induced liver injury (Masson *et al.*, 2010). This report also showed that RU486 effects were diminished in adrenalectomized male mice. Thus, they concluded that RU486 play a pathologic role mediated via GC receptor. In general, GCs are thought to have a salutary effect on immune-mediated disease due to their immunosuppressive effects (Prais *et al.*, 2006). Masson *et al.* (2010) also indicated that pretreatment with RU486 exacerbated carbon tetrachloride- and concanavalin A-induced liver injury in male mice. Recent report indicated that estrus cycle and female sex hormones affected HAL-induced liver injury (Dugan *et al.*, 2011; Toyoda *et al.*, 2011). Therefore, RU486 may affect the liver injury via progesterone receptor, but we could not determine whether GCs may be involved in the RU486 effect in this study. Further studies are necessary to investigate the precise mechanism of the RU486, but the inhibition of

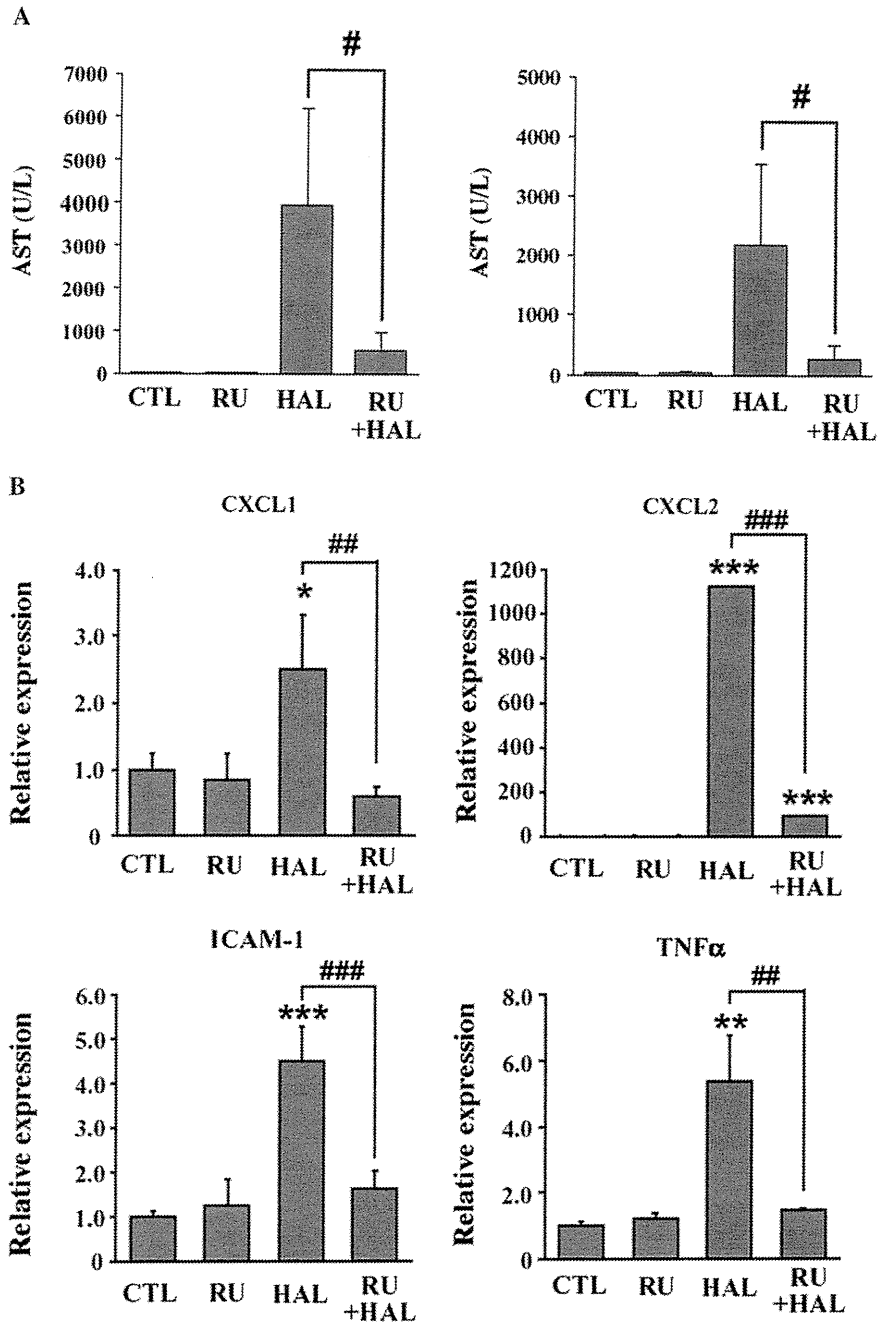

FIG. 7. Effects of RU486 pretreatment on HAL-induced liver injury. Female mice pretreated with RU486 (50 μg/mouse, sc) for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of RU486. Plasma and liver samples were collected for assessment of the transaminase levels (A) and the expression of hepatic mRNA levels (B) 24 h after the HAL administration. Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 3–5 mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with CTL. $\#\#p < 0.01$ and $\#\#\#p < 0.001$, compared with only HAL administered mice.

progesterone receptor and decrease of the immune response may have important therapeutic implications in severe liver injury.

In summary, we demonstrated that progesterone exacerbates the severity of liver injury mediated the activation of immune responses after the administration of hepatotoxicant. The mechanism of the exacerbation by progesterone appears to involve immune responses such as the production of the CXC chemokines and neutrophils infiltration via the activation of ERK pathway and Kupffer cells. Moreover, progesterone receptor antagonist administration suppressed the severity of

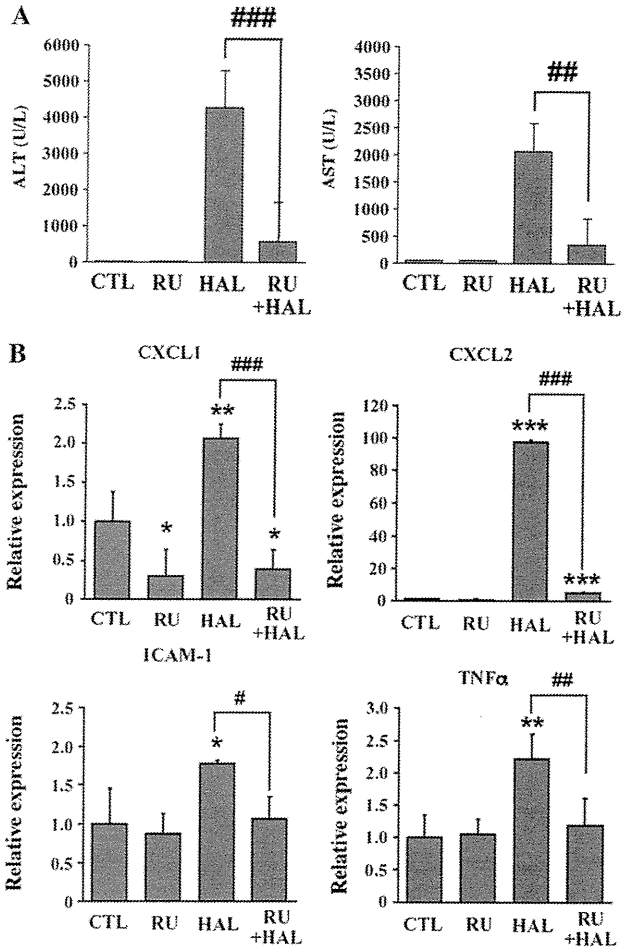

FIG. 8. Effects of RU486 posttreatment on HAL-induced liver injury. Female mice were administered HAL (30 mmol/kg, ip) and administered RU486 (1 mg/kg, iv) 1 h after the HAL administration. Plasma and liver samples were collected for assessment of the transaminase levels (A) and the expression of hepatic mRNA levels (B) 24 h after the HAL administration. Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 5–6 mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with CTL. $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$.

DILI, which suggests the potential clinical application of progesterone receptor antagonist in immune-mediated responses in DILI.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Involvement of immune-related factors in diclofenac-induced acute liver injury in mice

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ABSTRACT

Drug-induced liver injury (DILI) is a major safety concern in drug development and clinical drug therapy. However, the underlying mechanism of DILI is little known. It is difficult to predict DILI in humans due to the lack of experimental animal models. Diclofenac, a non-steroidal anti-inflammatory drug rarely causes severe liver injury in human, but there is some evidence for immunoallergic idiosyncratic reactions. In this study, the mechanism of diclofenac-induced liver injury in mice was investigated. First, we established the dosing condition for liver injury in normal mice. Plasma ALT and AST levels were significantly increased in diclofenac-administered (80 mg/kg, *i.p.*) mice in a dose- and time-dependent manner. Among several interleukins (ILs) and chemokines, mRNA expression of helper T (Th) 17 cell-mediated factors, such as retinoid orphan receptor (ROR)-γt, and signal transducers and activators of transcription factor (STAT) 3 in the liver, and the plasma IL-17 level were significantly increased. Neutralization of IL-17 tended to suppress the hepatotoxicity of diclofenac, suggesting that IL-17 was partly involved. Gadolinium chloride ($GdCl_3$) administration demonstrated that Kupffer cells are not likely to be involved in diclofenac hepatotoxicity. Hepatic expressions of IL-1β mRNA and plasma IL-1β were significantly increased soon after the diclofenac administration. Then, the results of an *in vivo* neutralization study of IL-1β suggested that IL-1β was involved early in the time of pathogenesis of the diclofenac-induced liver injury. In conclusion, we firstly developed a diclofenac-induced acute liver injury model in normal mice, and the involvement of IL-17 and IL-1β was clarified.

1. Introduction

Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of a drug from the market and cessation of new drug development in pharmaceutical companies. Due to their association with significant patient morbidity and mortality, several drugs have been removed from the pharmaceutical market, including bromfenac, ebrotidine, and troglitazone (Holt and Ju, 2006). In most cases, the mechanisms of the hepatotoxicity are unknown and predictive experimental animal models are still lacking.

Diclofenac, a nonsteroidal anti-inflammatory drug, causes an asymptomatic increase of plasma transaminase in approximately 15% of patients, and life-threatening fluminant hepatitis is induced in a very small percentage of patients (Bhogaraju et al., 1999). The molecular mechanisms of liver injury are largely unknown, but the involvement of a toxic metabolite has been suggested (Purcell et al., 1991). Diclofenac is metabolized to oxidative metabolites catalyzed by CYP2C9 and CYP3A4 (Tang et al., 1999), and these metabolites have the potential to be further oxidized to quinoneimine intermediates, which might be involved in the hepatocellular stress caused by diclofenac. On the other hand, there is clinical and experimental evidence that, in some cases, immune-related reactions are involved in diclofenac-induced liver injury, and the histopathological picture suggests hypersensitivity reactions (Boelsterli, 2003). Furthermore, one study reported that pretreatment of lipopolysaccharide (LPS) exacerbates diclofenac-hepatotoxicity in rats (Deng et al., 2006), suggesting that diclofenac-induced liver injury involves immune responses. However, as of now, the involvement of immune reactions underlying the severe liver injury induced by diclofenac remains to be clarified.

Helper T (Th) cell-mediated immune responses play pivotal roles in the pathogenesis of a variety of human liver disorders (Kita et al., 2001). The action of Th cells in the liver is mediated through the release of a variety of cytokines, which target liver cells and/or immune cells by activating multiple signaling cascades, including the signal transducers and activators of transcription factor

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(STAT) family members (Leonard and O'Shea, 1998). Th cells are subdivided into Th1, Th2, and Th17 subsets by their unique production of cytokines and characteristic transcription factors. Th1 cells require "T-box expressed in T cells" (T-bet) and secrete interferon (IFN)-γ. Th2 cells require GATA-binding domain (GATA)-3 and produce interleukin (IL)-4 and IL-5. Retinoid-related orphan receptor (ROR)-γt is indispensable for the differentiation of Th17 cells, which mainly secret IL-17 (Kidd, 2003; Steinman, 2007; McGeachy and Cua, 2008). We previously reported that IL-17 is involved in halothane (Kobayashi et al., 2009) and α-naphthylisothiocyanate (ANIT)-induced liver injury (Kobayashi et al., 2010), and the Th2-mediated cytokine, IL-4, is involved in dicloxacillin-induced liver injury (Higuchi et al., 2011), suggesting that the pathogenesis of DILI involves an altered balance of the Th cells. However, there are a few reports of mechanistic investigations of immune-mediated DILI.

The release of a variety of inflammatory mediators would occur in DILI. The liver is selectively enriched in Kupffer cells (KCs), natural killer (NK) cells, and NK cells with T cell receptors (NKT), which are key components of the innate immune system and develop intracellular networks mediated by cytokine and chemokine signaling (Racanelli and Rehermann, 2006). Among them, KCs have been shown to participate in several types of well-known liver injury, including bile duct ligation (Souto et al., 2001) and acute alcoholic liver damage (Enomoto et al., 2000). However, the protoxicant versus protective role of KCs during acetaminophen (APAP)-induced hepatotoxicity has been widely debated (Laskin et al., 1995). In addition, previous report demonstrated that KC depletion did not affect the severity of liver injury caused by halothane (Cheng et al., 2010). Therefore, the contribution of KC in DILI is still controversial.

In this study, we first induced diclofenac-induced acute liver injury in wild-type normal mice, and demonstrated that Th17-related immunological factors are mainly involved. Furthermore, the early onset of diclofenac-induced hepatic injury was investigated.

2. Methods

2.1. Chemicals

Diclofenac sodium salt, gadolinium (III) chloride hexahydrate ($GdCl_3$) and concanavalin A (Con A) were purchased from Sigma–Aldrich (St. Louis, MO). Ibuprofen was purchased from Wako Pure Chemicals (Osaka, Japan). Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were from Fujifilm (Tokyo, Japan). RNAiso was from Nippon Gene (Tokyo, Japan). ReverTra Ace was from Toyobo (Tokyo, Japan). Random hexamer and SYBR Premix Ex Taq were from Takara (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Rabbit polyclonal antibody against myeloperoxidase (MPO) was purchased from DAKO (Carpinteria, CA). Monoclonal anti-mouse IL-17 antibody, monoclonal anti-mouse IL-1β antibody, rat IgG2a isotype (control for the IL-17 experiment) and rat IgG1 isotype (control for the IL-1β experiment) were from R&D systems (Abingdon, UK). A Ready-SET-GO! Mouse Interleukin-17A (IL-17A) enzyme-linked immunosorbent assay (ELISA) kit and a Ready-SET-GO! Mouse Interleukin-1β (IL-1β) ELISA kit were from eBioscience (San Diego, CA). Other chemicals were of analytical or the highest grade commercially available.

2.2. Diclofenac administration

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 use for the experiments. Diclofenac (dissolved in corn oil, *i.p.*) was administered to mice in a non-fasting condition. At the indicated time after diclofenac administration, the animals were sacrificed, and the blood was collected from the inferior vena cava and the liver from the biggest lobe. A portion of each excised liver was fixed in 10% formalin neutral buffer solution and used for immunohistochemical staining. The degree of liver injury was assessed by hematoxylin–eosin (H&E) staining. Infiltration of mononuclear cells was assessed by immunostaining for myeloperoxidase (MPO). Rabbit polyclonal antibody against MPO was used for immunohistochemical staining of the liver as previously described (Kumada et al., 2004). Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan (AP-111985).

Table 1
Sequences of primers used for real-time RT-PCR analyses.

Target		Sequence
mFasL	FP	5′-AGA AGG AAC TGG CAG AAC TC-3′
	RP	5′-GCG GTT CCA TAT GTG TCT TC-3′
mGATA-3	FP	5′-GGA GGA CTT CCC CAA GAG CA-3′
	RP	5′-CAT GCT GGA AGG GTG GTG A-3′
mIFN-γ	FP	5′-GGC CAT CAG CAA CAT AAG C-3′
	RP	5′-TGG ACC ACT CGG ATG AGC TCA-3′
mIL-1β	FP	5′-GTT GAC GGA CCC CAA AAG AT-3′
	RP	5′-CAC ACA CCA GCA GGT TAT CA-3′
mMCP-1	FP	5′-TGT CAT GCT TCT GGG CCT G-3′
	RP	5′-CCT CTC TCT TGA GCT TGG TG-3′
mMIP-2	FP	5′-AAG TTT GCC TTG ACC CTG AAG-3′
	RP	5′-ATC AGG TAC GAT CCA GGC TTC-3′
mROR-γt	FP	5′-ACC TCC ACT GCC AGC TGT GTG CTG TC-3′
	RP	5′-TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3′
mSTAT1	FP	5′-GTT TCA GCT CTG CTC CAT AC-3′
	RP	5′-CTG CTG AAG CTC GAA CCA C-3′
mSTAT3	FP	5′-TGC AGA GCA GGT ATC TTG AG-3′
	RP	5′-TGC TGC TTC TCT GTC ACT AC-3′
mSTAT6	FP	5′-ATC TTC AAC GAC AAC AGC CTC A-3′
	RP	5′-GGA GAA GGC TAG TGA CAT ATT G-3′
mT-bet	FP	5′-CAA GTG GGT GCA GTG TGG AAA G-3′
	RP	5′-TGG AGA GAC TGC AGG ACG ATC-3′
mTNFα	FP	5′-TGT CTC AGC CTC TTC TCA TTC C-3′
	RP	5′-TGA GGG TCT GGG CCA TAG AAC-3′
mGapdh	FP	5′-AAA TGG GGT GAG GCC GGT-3′
	RP	5′-ATT GCT GAC AAT CTT GAG TGA-3′

FP, forward primer; RP, reverse primer.

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

RNA from the mouse liver was isolated using RNAiso according to the manufacturer's instructions. The expressions of T-bet, ROR-γt, GATA-3, IFN-γ, IL-1β, Fas ligand (FasL), STAT1, STAT3, STAT6, tumor necrosis factor (TNF) α, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein (MIP)-2 were quantified by real-time RT-PCR. The primer sequences used in this study are shown in Table 1. For the RT-process, total RNA (10 μg) and 150 ng random hexamer were mixed and incubated at 70 °C for 10 min. 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 reaction mixture was incubated at 30 °C for 10 min, 42 °C for 1 h, and heated at 98 °C for 10 min to inactivate the enzyme. The real-time RT-PCR was performed using the 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. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR).

2.4. Administration of anti-mouse IL-17 antibody or anti-mouse IL-1β antibody

Anti-mouse IL-17 antibody or rat IgG2a isotype control (0.1 mg per mouse in PBS, i.v.) was administered to mice 6 h after the diclofenac administration (80 mg/kg, *i.p.*) in a non-fasting condition. Anti-mouse IL-1β antibody or rat IgG1 isotype control (0.1 mg per mouse in PBS, *i.p.*) was administered to mice 1 h prior to the diclofenac administration (80 mg/kg, *i.p.*) to mice in a nonfasting condition. Twenty-four hours after the diclofenac administration, the plasma ALT level was measured.

2.5. Measurement of plasma IL-17 and IL-1β levels

The plasma IL-17 and IL-1β levels were measured by ELISA using a kit according to the manufacturer's instructions.

2.6. Kupffer cells depletion

To deplete hepatic KCs, $GdCl_3$ (20 mg/kg, *i.v.*) was administered to mice 24 h prior to diclofenac (80 mg/kg, *i.p.*) or Con A administration (25 mg/kg in saline, *i.v.*). Twenty-four hours after diclofenac administration, the plasma ALT level was measured. As a positive control, Con A was administered to mice, and the plasma ALT level was measured 12 h after the Con A administration.

2.7. Statistical analysis

Data are presented as mean ± SD. Statistical analyses between multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Comparisons between two groups were carried out using two-tailed Student *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Dose- and time-dependent hepatotoxic effects of diclofenac in mice

Diclofenac was administered intraperitoneally at a dose of 50, 80, or 120 mg/kg to female BALB/c mice. Ibuprofen (120 mg/kg) was used as a negative control. Plasma ALT and AST levels were significantly increased in mice administered doses of 80 and 120 mg/kg compared with vehicle-administered control mice (Fig. 1A). No hepatotoxic effect was observed in ibuprofen-administered mice. A dose-dependent increase of ALT and AST was demonstrated at a dose of 80 mg/kg, thus we adapted a dose of 80 mg/kg in the subsequent experiments. The time-dependent hepatotoxic effect of diclofenac was investigated at a dose of 80 mg/kg (Fig. 1B). A slight increase of plasma ALT and AST levels 1 h after the administration, and a marked increase of the ALT and AST levels 24 h after the administration were observed, which then decreased after 24 h. Histopathological examination of liver tissues 24 h after diclofenac administration (80 mg/kg) indicated necrosis and apoptosis (Fig. 1C). In the anti-MPO antibody staining, infiltration of mononuclear cells into the hepatocytes was observed in the diclofenac-administered mice, but not in the vehicle-administered mice.

3.2. Time-dependent changes of the mRNA expression of immune-related transcriptional factors and immune-related factors in diclofenac-administered mouse liver

To investigate the involvement of immune-related factors in the diclofenac-induced hepatotoxicity, the hepatic mRNA expression of immune-related transcriptional factors of Th cells (T-bet, GATA-3, ROR-γt, STAT1, STAT3, STAT6) and inflammatory mediators (IL-1β, TNFα, IFN-γ, MIP-2, MCP-1, FasL) was measured by real-time RT-PCR (Figs. 2 and 3). In our previous studies (Higuchi et al., 2011; Kobayashi et al., 2010), we confirmed that the expression levels of mRNA and protein were similar in ILs and chemokines. Thus, changes of mRNA expression were mainly followed in the present study, except those of some ILs. The hepatic mRNA expressions of Th17 cell-related factors, such as ROR-γt and STAT3, were significantly increased in diclofenac-administered mice compared with the control mice, whereas the expressions of Th1 cell-related factors of STAT1 and T-bet were significantly decreased, while Th2 cell-related factors (GATA3 and STAT6) were not changed (Fig. 2). As shown in Fig. 3, expressions of IL-1β, TNFα, MIP-2, MCP-1, and FasL mRNA expressions were significantly increased in diclofenac-administered mice compared with the control mice, whereas IFN-γ expression was significantly decreased 24 h after diclofenac administration. In particular, IL-1β expression was significantly increased from 3 to 12 h after the administration and peaked at 3 h, suggesting that IL-1β might be related to the early onset of diclofenac-induced liver injury.

3.3. Involvement of IL-17 in diclofenac-induced liver injury

IL-17 is a cytokine, mainly produced by Th17 cells (Nakae et al., 2003), which can induce inflammatory cytokines and chemokines (Yao et al., 1995). Plasma IL-17, measured by ELISA, was detected in mice 24 and 36 h after the diclofenac administration (Fig. 4A), but not in the control mice. The IL-17 neutralization study demonstrated that the administration of anti-mouse IL-17 antibody (0.1 mg/mouse, *i.v.*) 6 h after diclofenac administration resulted in lower levels of the plasma ALT compared with that of IgG2a-treated control mice (Fig. 4B). Plasma IL-17 level in anti-mouse IL-17 antibody-treated mice was significantly decreased compared with mice administered diclofenac alone (Fig. 4C).

3.4. Role of KCs in diclofenac-induced liver injury

KCs are known as one of the sources of cytokines and chemokines in the liver, which suggests that KCs would be involved in the pathogenesis of diclofenac-induced hepatotoxicity. KCs were depleted by $GdCl_3$ (20 mg/kg, *i.v.*) treatment 24 h prior to Con A or diclofenac administration. The dosing condition in this study enabled us to deplete approximately 75% of hepatic KCs (Xu et al., 2010), which attenuated Con A-induced hepatitis (Hatano et al., 2008). In this study, attenuation of the Con A-induced hepatic injury using $GdCl_3$ was demonstrated, but there was no effect on the diclofenac-induced hepatic injury (Fig. 5), suggesting that KCs are not likely involved in the pathogenesis of diclofenac-induced liver injury.

3.5. Involvement of IL-1β in the pathogenesis of diclofenac-induced liver injury

IL-1β is known as an inflammatory cytokine. In the present study, among the many ILs and chemokines, IL-1β mRNA increased early after diclofenac administration, suggesting the involvement of IL-1β in the early onset of hepatic injury. Plasma IL-1β was detected by ELISA from 6 to 36 h after the administration, and a marked increase was observed 24 h after the administration (Fig. 6A). To further investigate whether IL-1β was involved in the diclofenac-induced liver injury, we performed IL-1β neutralization studies. The administration of anti-mouse IL-1β antibody (100 μg/mouse, *i.p.*) 1 h before diclofenac administration significantly reduced the plasma ALT level at 24 h after diclofenac administration compared with IgG1-treated control mice (Fig. 6B). Plasma IL-1β level in anti-mouse IL-1β antibody-treated mice was significantly decreased compared with mice administered diclofenac alone (Fig. 6C).

4. Discussion

Diclofenac is known to cause rare but sometime serious hepatotoxicity in humans (Bhogaraju et al., 1999), but the mechanism of diclofenac-induced liver injury remained to be clarified. There is some evidence suggesting an immune-mediated reaction in diclofenac-induced hepatic injury in human (Greaves et al., 2001; Kretz-Rommel and Boelsterli, 1995). However, there was no animal model that reproduced the diclofenac-induced liver injury. These lines of background prompted us to investigate the immune-mediated mechanisms in diclofenac-induced liver injury.

First, we established the dosing condition for diclofenac-induced acute liver injury in mice. In general, the route of drug administration in clinical practice is an ideal pathway to develop an animal model. However, diclofenac is known to cause gastrointestinal toxicity (Novartis Pharma Co, 2005). Therefore, we adapted *i.p.* administration of diclofenac to avoid gastrointestinal toxicity, as possible. Previous studies demonstrated that diclofenac administration (100 mg/kg dissolved in saline, *i.p.*) caused only a slight increase in the serum ALT level in rat (Deng et al., 2006). Acute toxicity study demonstrated that the LD_{50} value of single *i.p.* administration of diclofenac in female mice was 250 mg/kg (Novartis Pharma Co, 2005). Therefore, in the present study, single *i.p.* administration of diclofenac at dose of 50, 80, and 120 mg/kg,

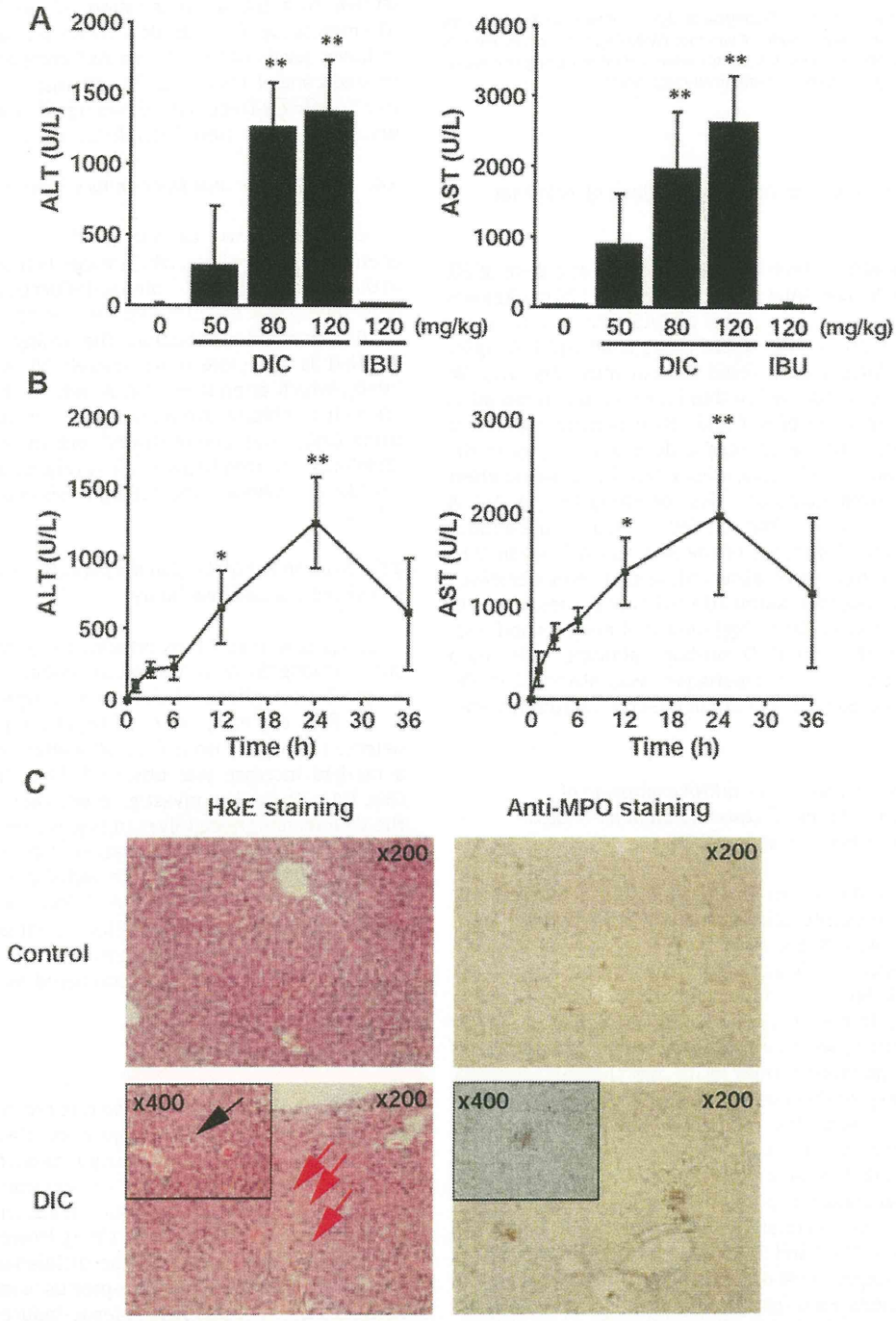

Fig. 1. Dose- and time-dependent changes of plasma ALT and AST and histopathological examination in the liver of diclofenac-administered mice. (A) Diclofenac (DIC: 50, 80, or 120 mg/kg in corn oil, *i.p.*) or ibuprofen (IBU: 120 mg/kg in corn oil, *i.p.*) was administered to mice, and plasma samples were collected for the assessment of ALT and AST 24 h after the administration. (B) Diclofenac (80 mg/kg, *i.p.*) was administered to mice and plasma samples were collected 1, 3, 6, 12, 24 and 36 h after the administration. Data are mean ± SD ($n = 4–5$). Significantly different from vehicle-administered control mice ($^*p < 0.05$, $^{**}p < 0.01$). (C) Histopathological examination of the livers from diclofenac-administered mice. Liver specimens were examined 24 h after the diclofenac administration (80 mg/kg). The liver sections were stained with H&E or immunostained with anti-MPO antibody. Black arrow indicates apoptotic cells and red arrows indicate necrotic cells.

which is less than a half of the *i.p.* LD_{50}, was adapted. After investigating many different conditions, we found that administration of diclofenac 80 mg/kg dissolved in corn oil (*i.p.*) without fasting condition, reproducibly caused diclofenac-induced acute liver injury in mice. Ibuprofen, having similar pharmacological properties as diclofenac, is known to be less hepatotoxic than diclofenac (Rainsford, 2009). Ibuprofen showed no hepatotoxic effect in the same dosing condition as diclofenac, suggesting that the pharmacological effects may not contribute to the diclofenac hepatotoxicity in this model.

In this study, a relationship between diclofenac-induced liver injury and immune-related factors was demonstrated. The administration of diclofenac significantly increased the expression of hepatic ROR-γt and STAT3 mRNA, as well as the plasma IL-17 level (Figs. 2 and 4A). ROR-γt is a master regulator in Th17 cells (Kidd, 2003; Steinman, 2007). STAT3 is also required for the development

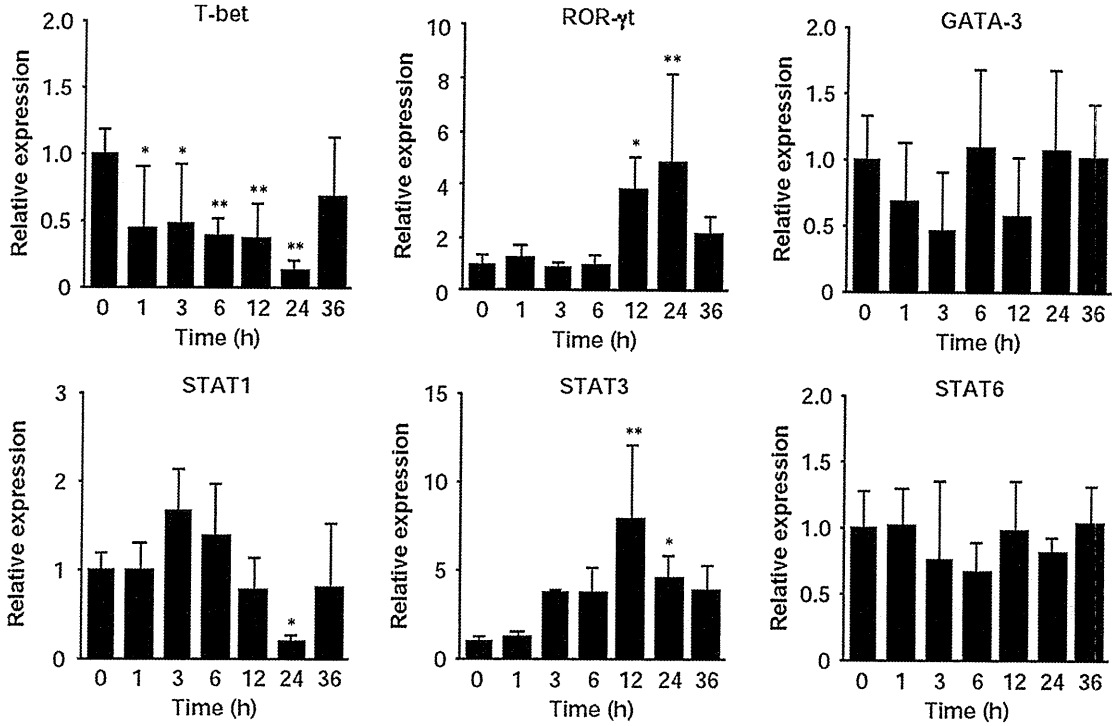

Fig. 2. Time-dependent changes in hepatic mRNA expression of $CD4^+$ Th cells-related transcriptional factors in diclofenac-administered mice. Diclofenac (80 mg/kg, *i.p.*) was administered to mice. After 1, 3, 6, 12, 24, and 36 h, the expressions of T-bet, ROR-γt, GATA-3, STAT1, STAT3, and STAT6 mRNA in the liver were measured by real-time RT-PCR. The expression of hepatic mRNA was normalized to the expression of Gapdh mRNA. Data are mean ± SD (n = 4–5). Significantly different from control (0 h) mice ($^*p < 0.05$, $^{**}p < 0.01$).

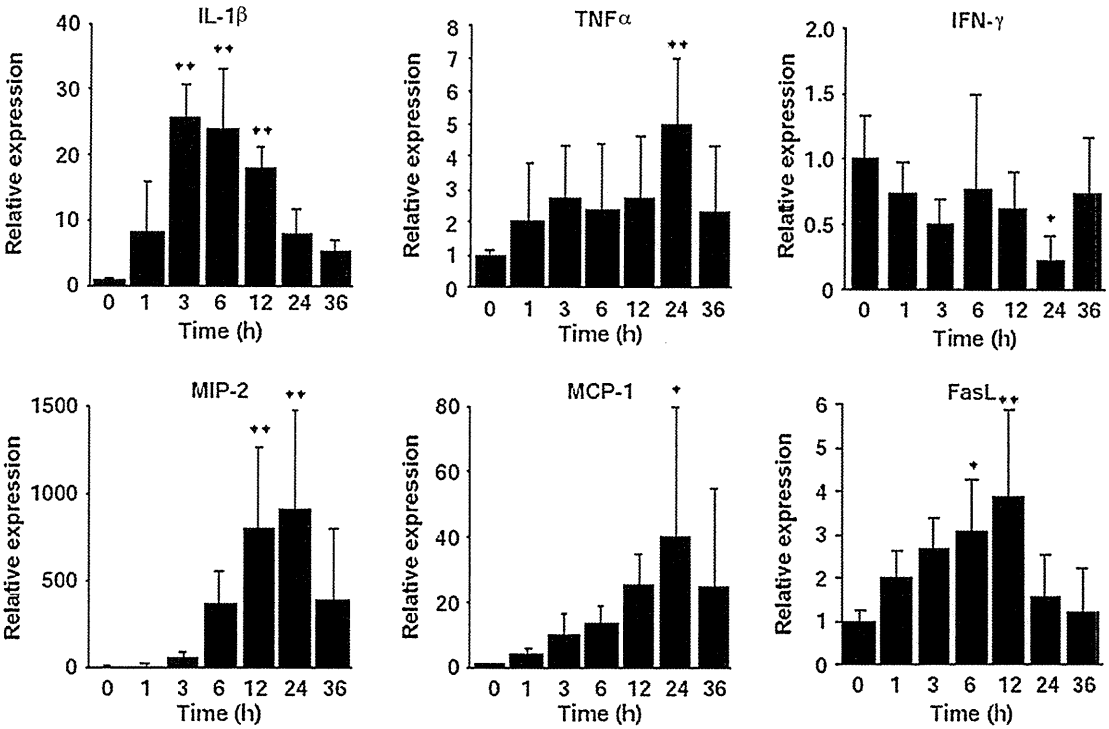

Fig. 3. Time-dependent changes in hepatic mRNA expression levels of cytokines and chemokines in diclofenac-administered mice. Diclofenac (80 mg/kg, *i.p.*) were administered to mice. After 1, 3, 6, 12, 24, and 36 h, the hepatic expressions of IL-1β, TNFα, INF-γ, MIP-2, MCP-1, and FasL mRNA in the liver were measured by real-time RT-PCR. The expression of hepatic mRNA was normalized to the expression of Gapdh mRNA. Data are mean ± SD (n = 4–5). Significantly different from control (0 h) mice ($^*p < 0.05$, $^{**}p < 0.01$).

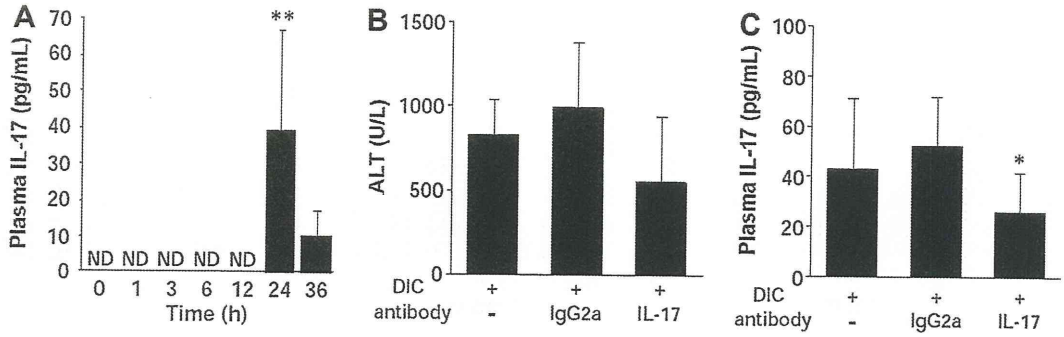

Fig. 4. Time-dependent changes of the plasma IL-17 level and effect of anti-IL-17 antibody administration to diclofenac-administered mice. (A) The plasma IL-17 level was measured by ELISA 1, 3, 6, 12, 24, and 36 h after diclofenac administration (80 mg/kg, *i.p.*). Data are mean ± SD (n = 4–5). (B) Six hours after diclofenac (DIC) administration (80 mg/kg, *i.p.*), IgG2a (control) or anti-mouse IL-17 antibody (0.1 mg/mouse, *i.v.*) was administered. The plasma ALT level was measured 24 h after diclofenac administration. Data are mean ± SD (n = 8–10). (C) The plasma IL-17 level after administered of anti-mouse IL-17 specific antibody or IgG2a was measured by ELISA 24 h after diclofenac administration (80 mg/kg, *i.p.*). Data are mean ± SD (n = 8–10). Significantly different from control (0 h) mice (*p < 0.05, **p < 0.01).

of Th17 cells, and STAT3 deficiency impairs ROR-γt expression (Harris et al., 2007; Yang et al., 2007). It has been suggested that Th17 cell-mediated factors are partly involved in diclofenac-induced hepatotoxicity. It has been reported that the plasma IL-17 levels were elevated in 60% of patients with DILI and occasionally in patients with viral hepatitis (Li et al., 2009), suggesting IL-17 might have a role in liver injury in human.

In our previous study using a halothane-induced liver injury mouse model (Kobayashi et al., 2009), we found that the appropriate dose of anti-IL-17 antibody to suppress the hepatotoxic effect is 100 μg/body. Neutralization of IL-17 tended to inhibit the increase of the plasma ALT level (Fig. 4B), suggesting that IL-17 was partly involved in the diclofenac-induced hepatic injury.

Cytokines and chemokines such as TNFα, IL-1β, MIP-2, and MCP-1 were significantly increased in the diclofenac-administered mice (Fig. 3). TNFα is a pleiotropic pro-inflammatory cytokines produced by a variety of cell types including macrophages, T cells, and mast cells (Tracey, 1994). MCP-1 is increased in APAP-induced liver injury (Dambach et al., 2006), and MIP-2 induces neutrophil recruitment and is markedly increased in halothane-induced hepatotoxicity (Kobayashi et al., 2009). From these lines of data into consideration, it is known that two death receptor ligands, TNFα or FasL, bind to their receptors and induce apoptosis (Nagata, 1997). The expression of hepatic TNFα and FasL mRNA was significantly increased, and hepatocellular apoptosis were observed (Figs. 1C and 3), suggesting that these mediators may cause hepatocellular apoptosis in diclofenac-induced liver injury. Thus, the exacervation of diclofenac-induced liver injury could involve these mediators.

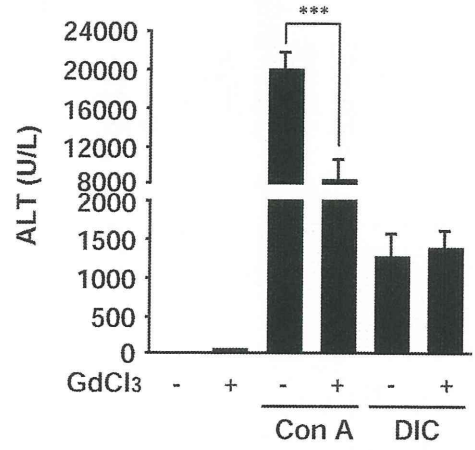

Fig. 5. Role of KCs in diclofenac-induced liver injury. $GdCl_3$ (20 mg/kg, *i.v.*) were administered to mice 24 h prior to diclofenac (80 mg/kg, *i.p.*) or Con A (25 mg/kg, *i.v.*) administration. The Plasma ALT level was measured 24 h after diclofenac administration or 12 h after Con A administration. Data are mean ± SD (n = 4–5). Significantly different from control (without $GdCl_3$ administration) mice (***p < 0.001).

KCs serve important regulatory functions in pathophysiological states of the liver and have the ability to produce a range of inflammatory mediators, including TNFα and IL-1β. It is also reported that KCs play a dispensable role in the development of halothane hepatotoxicity (Cheng et al., 2010). The dosing condition of $GdCl_3$ in this study was determined according to Xu et al.

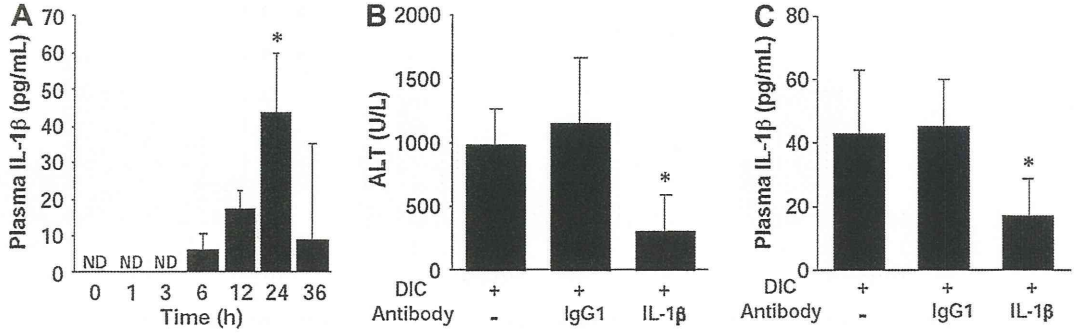

Fig. 6. Time-dependent change of plasma IL-1β level and effect of anti-IL-1β antibody administration to diclofenac-administered mice. (A) The plasma IL-1β level was measured by ELISA 1, 3, 6, 12, 24, and 36 h after diclofenac (80 mg/kg, *i.p.*) administration. (B) One hour before the diclofenac (80 mg/kg, *i.p.*) administration, IgG1 (control) or anti-mouse IL-1β antibody (0.1 mg/mouse, *i.p.*) was administered. The plasma ALT level was measured 24 h after the diclofenac administration. (C) The plasma IL-1β level after administered anti-mouse IL-1β specific antibody or IgG1 was measured by ELISA 24 h after diclofenac administration (80 mg/kg, *i.p.*). Data are mean ± SD (n = 4–5). Significantly different from control mice (A) or IgG1-administered mice (B and C) (*p < 0.05). ND: not detected.

(2010). To confirm whether $GdCl_3$ administration resulted in the depletion of KCs, we revealed the attenuation of Con A-induced hepatitis by $GdCl_3$ (Fig. 5), and the attenuation of plasma ALT was also observed 24 h after Con A administration (data not shown). In this study, we demonstrated that KCs are not likely to be involved in the pathogenesis of diclofenac hepatotoxicity.

In the present study, the increase of hepatic IL-1β mRNA expression and plasma IL-1β level was observed soon after the diclofenac administration (Figs. 3 and 6A). IL-1β is a very potent pro-inflammatory cytokine and the primary sources of IL-1β are blood monocytes and dendritic cells. Hepatocytes and neutrophils also produce IL-1β (Arend et al., 2008). IL-1β acts mainly as a pro-inflammatory mediator activating and recruiting leukocytes, especially neutrophils, into the liver (Bajt et al., 2001). It is well known that activated neutrophils release protease such as elastase that can cause tissue injury. Within the liver, activated neutrophils act as effector cells through cytotoxicity, leading hepatocyte necrosis. In a number of experimental animal models, accumulated neutrophils in the liver were reported to contribute to the progression and severity of a number of experimental animal models such as ischemia-reperfusion injury (Jaeschke et al., 1990; Ramaiah and Jaeschke, 2007), and APAP- and halothane-induced hepatotoxicity (Liu et al., 2006; You et al., 2006). In the present study, neutrophil infiltration occurred in the diclofenac-administered mouse liver (Fig. 1C), and neutralization of IL-1β attenuated diclofenac-induced hepatotoxicity (Fig. 6), suggesting that IL-1β is involved in the early onset of diclofenac-induced liver injury.

A previous study demonstrated a significant increase of reactive oxygen species (ROS) in hepatocytes treated with diclofenac (Gómez-Lechón et al., 2003). Increased ROS levels can trigger "NACHT, LRR, and pyrin domain-containing protein 3" (NALP3) inflammasome activation, which is a multimetimeric protein complex that mediates the processing of the pro-inflammatory caspases and cytokines, such as IL-1β (Agostini et al., 2004). The IL-1β levels are known to be increased during APAP hepatotoxicity (Cover et al., 2006) and halothane-induced liver injury (Toyoda et al., 2011). Therefore, it is conceivable that IL-1β would be released into the extracellular environment by oxidative stress such as ROS and activates immune cells.

In conclusion, we developed a diclofenac-induced acute liver injury model in mice, and demonstrated that Th17-related immunological factors are significantly increased. Furthermore, IL-1β appeared to be involved in the early onset of diclofenac-induced hepatic injury. These findings may shed light on the mechanisms of DILI.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Involvement of Th2 cytokines in the mouse model of flutamide-induced acute liver injury

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ABSTRACT: Drug-induced liver injury is a growing concern for pharmaceutical companies and patients because numerous drugs have been linked to hepatotoxicity and it is the most common reason for a drug to be withdrawn. Flutamide rarely causes liver dysfunction in humans, and immune allergic reactions have been suggested in some cases. In this study, we investigated the mechanisms of flutamide-induced liver injury in BALB/c mice. Plasma alanine aminotransferase and aspartate aminotransferase levels were significantly increased 3, 6 and 9 h after flutamide (1500 mg kg^{-1}, p.o.) administration. The biomarker for oxidative stress was not changed, but Th2-dominant immune-related factors, such as interleukin (IL)-4, IL-5, STAT6 and GATA-binding protein (GATA)-3, were induced in flutamide-administered mice. The pre-administration of monoclonal-IL-4 antibody suppressed the hepatotoxicity of flutamide. In addition, we investigated the effect of 13, 14-dihydro-15-keto-PGD$_2$ (DK-PGD$_2$; 10 μg per mouse, i.p.) administration on flutamide-induced acute liver injury. Coadministration of DK-PGD$_2$ and flutamide resulted in a significant increase in alanine aminotransferase and a remarkable increase of macrophage inflammatory protein-2. In conclusion, we demonstrated that flutamide-induced acute liver injury is mediated by Th2-dominant immune responses in mice. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: cytokines; DILI; MIP-2; IL-4; prostaglandin D$_2$

INTRODUCTION

Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of an approved drug from the market and for failures in drug development in pharmaceutical companies. Several drugs have been withdrawn because of significant adverse drug reactions associated with hepatotoxicity (Holt and Ju, 2006). In most cases, the mechanisms of DILI are unknown and predictive experimental animal models are lacking.

Flutamide is a widely used nonsteroidal anti-androgen for the treatment of prostate cancer, and it is also used for the treatment of hirsutism. Flutamide-therapy is reported to rarely induce severe liver dysfunction in patients, and hepatotoxicity was not recognized in preclinical studies using experimental animals. Approximately 3 in 10 000 users of flutamide were estimated to have developed severe liver injury (Wysowski and Fourcroy, 1996). The hepatic effects can present as either cholestasis or hepatocellular injury (Moller *et al.*, 1990; Wysowski *et al.*, 1993; Dourakis *et al.*, 1994). Oxidative stress, glutathione depletion and protein adducts from *N*-hydroxylation of a flutamide metabolite that is generated by arylacetamide deacetylase (Watanabe *et al.*, 2010) have been suggested to be involved in flutamide-induced liver injury (Kashimshetty *et al.*, 2009; Morita *et al.*, 2009; Ohbuchi *et al.*, 2009). In addition, flutamide-induced liver injury is associated with the infiltration of neutrophils, and lymphocytes have been observed in necrotic areas (Gomez *et al.*, 1992; Dourakis *et al.*, 1994). Although the mechanism of flutamide-induced liver injury is not fully understood, some cases have been associated with peripheral eosinophilia, suggesting the involvement of the immune system (Hart and Stricker 1989; Wysowski *et al.*, 1993). Based on these considerations, we hypothesized that not only oxidative stress but also immunological factors are involved in flutamide-induced liver injury.

T cell-mediated immune responses play pivotal roles in the pathogenesis of a variety of human liver disorders (Kita *et al.*, 2001; Heneghan and McFarlane, 2002). The action of T cells in the liver is mediated through the release of a variety of cytokines, which target liver cells and immune cells by activating multiple signaling cascades (Leonard and O'Shea, 1998). Th cells are subdivided into Th1, Th2 and Th17 subsets by their unique production of cytokines and characteristic transcription factors. Th1 cells require T-box expressed in T cells (T-bet). In contrast, Th2 cells require GATA-binding domain (GATA)-3, and produce IL-4, IL-5 and IL-13. Retinoid-related orphan receptor γt (ROR-γt) is indispensable for the differentiation of Th17 cells, which mainly secret IL-17 and IL-22 (Kidd, 2003; Steinman, 2007).

We previously reported that Th2 responses were involved in dicloxacillin-induced liver injury (Higuchi *et al.*, 2011). Th2 responses are mainly mediated by IL-4 and IL-5, which influence a wide range of events associated with allergic inflammation. We also demonstrated that 13,14-dihydro-15-keto-Prostaglandin D$_2$

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(DK-PGD_2) exacerbated dicloxacillin-induced liver injury. DK-PGD_2 is a metabolite of PGD_2 and a selective agonist of chemoattractant receptor homologous-molecule expressed on T-helper-type-2 cells (CRTh2), which is expressed in eosinophils, basophils and Th2 cells, but not in hepatocytes or endothelial cells (Kostenis and Ulven, 2006). CRTh2 activation plays a significant role in Th2-dependent neutrophil inflammation (Takeshita *et al.*, 2004). In this study, we intended to elucidate the mechanisms of flutamide-induced liver injury.

MATERIALS AND METHODS

Materials

Flutamide was purchased from Wako Pure Chemical Industries (Osaka, Japan). RNAiso was from Nippon Gene (Tokyo, Japan). Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII to measure aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were from FujiFilm (Tokyo, Japan). ReverTra Ace was from Toyobo (Tokyo, Japan). Random hexamer and SYBR Premix Ex Taq were from Takara (Osaka, Japan). DK-PGD_2 was purchased from Cayman Chemical (Denver, CO, USA). Oxiselect Protein Carbonyl enzyme-linked immunosorbent assay (ELISA) kit was from Cell Biolabs (Tokyo, Japan). A Ready-SET-GO! Mouse Interleukin-4 (IL-4) ELISA kit was from eBioscience (San Diego, CA, USA). Monoclonal anti-mouse IL-4 antibody was from U-Cytech Bioscienses (Utrecht, Netherland). Monoclonal rat IgG2a isotype, used as a control, was from R&D Systems (Abingdon, UK). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were of analytical or the highest grade commercially available.

Mouse Models of Flutamide-induced Acute Liver Injury

Female BALB/cCrSlc mice (6 weeks old) were obtained from SLC Japan (Hamamatsu, Japan). Mice were housed in a controlled environment (temperature 23 ± 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 use for the experiments. Mice were administered flutamide orally at a dose of 1500 mg kg^{-1} suspended in 0.5% carboxymethylcellulose in a nonfasting condition. At 3, 6, 9 and 24 h after flutamide administration, the animals were sacrificed and the blood and the liver were collected. A portion of each excised liver was fixed in 10% formalin neutral buffer solution and used for immunohistochemical staining. The degree of liver injury was assessed by hematoxylin–eosin (H&E) staining, and AST and ALT levels were measured by Dri-Chem (FujiFilm) according to the protocol. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan.

Real-time RT-PCR

Total hepatic RNA was isolated using RNAiso according to the manufacturer's instructions. T-bet, GATA-3, ROR-γt, IFN-γ, IL-5, signal transducer and activator of transcription 1 (STAT1), STAT3, STAT6, Eotaxin-1 and macrophage inflammatory ptrotein-2 (MIP-2) were quantified by real-time reverse-transcription polymerase chain reaction (RT-PCR). The primer sequences used in this study are shown in Table 1. For the RT process, total RNA (10 μg) and 150 ng random hexamer were mixed and incubated at 70 °C for

Table 1. Sequence of primers used for real-time RT-PCR analyses

Gene		Sequence
mIFN-γ	FP	5′-GGC CAT CAG CAA CAT AAG C-3′
	RP	5′-TGG ACC ACT CGG ATG AGC TCA-3′
mIL-5	FP	5′-AAA GAG ACC TTG ACA CAG CTG-3′
	RP	5′-CCA CGG ACA GTT TGA TTC TTC-3′
mT-bet	FP	5′-CAA GTG GGT GCA GTG TGG AAA G-3′
	RP	5′-TGG AGA GAC TGC AGG ACG ATC-3′
mGATA-3	FP	5′-GGA GGA CTT CCC CAA GAG CA-3′
	RP	5′-CAT GCT GGA AGG GTG GTG A-3′
mROR-γt	FP	5′-ACC TCC ACT GCC AGC TGT GTG CTG TC-3′
	RP	5′-TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3′
mSTAT-1	FP	5′-GTT TCA GCT CTG CTC CAT AC-3′
	RP	5′-CTG CTG AAG CTC GAA CCA C-3′
mSTAT-3	FP	5′-TGC AGA GCA GGT ATC TTG AG-3′
	RP	5′-TGC TGC TTC TCT GTC ACT AC-3′
mSTAT-6	FP	5′-ATC TTC AAC GAC AAC AGC CTC A-3′
	RP	5′-GGA GAA GGC TAG TGA CAT ATT G-3′
mEotaxin-1	FP	5′-TCC ACA GCG CTT CTA TTC CT-3′
	RP	5′-CTA TGG CTT TCA GGG TGC AT-3′
mMIP-2	FP	5′-AAG TTT GCC TTG ACC CTG AAG-3′
	RP	5′-ATC AGG TAC GAT CCA GGC TTC-3′
mGAPDH	FP	5′-AAA TGG GGT GAG GCC GGT-3′
	RP	5′-ATT GCT GAC AAT CTT GAG TGA-3′

FP, forward primer; RP, reverse primer.

10 min. 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 reaction mixture was incubated at 30 °C for 10 min, 42 °C for 1 h, and heated at 98 °C for 10 min to inactivate the enzyme. The real-time RT-PCR was performed using the Mx3000P (Stratagene, La Jolla, CA). The PCR mixture contained 1 or 2 µl of template cDNA, SYBR Premix Ex Taq solution and 8 pmol of forward and reverse primers. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR, USA) that binds to the double-strand DNA amplified by PCR.

Determination of Protein Carbonyl Content

Increased protein carbonyls are stable indicators of oxidative stress. Plasma protein carbonyl content was measured as described previously (Yoshikawa *et al.*, 2009a).

Administration of Anti-mouse IL-4 Antibody

In the neutralization study, mice were administered anti-mouse IL-4 antibody intraperitoneally (100 µg of anti-mouse IL-4 antibody in 0.5 ml of sterile PBS), 1 h before flutamide administration. As a control, rat IgG2a was administered (100 µg of rat IgG2a in 0.5 ml of sterile PBS).

Treatment of DK-PGD_2

One hour after flutamide administration, mice were treated with DK-PGD_2 intraperitoneally (10 µg per mouse, dissolved in 200 µl of PBS) in a nonfasting condition.

Measurement of Plasma IL-4 Level

The plasma IL-4 level was measured by enzyme-linked immunosorbent assay (ELISA) using a Ready-SET-GO! Mouse Interleukin-4 (IL-4) kit from eBioscience according to the manufacturer's instructions.

Statistical Analysis

Data are presented as means ± SD. Statistical analyses between multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Comparisons between two groups were carried out using two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Increase of Plasma ALT and AST Levels in Flutamide-administrated Mice

Female BALB/c mice were administered flutamide at a dose of 1500 mg kg^{-1} in a nonfasting condition. Plasma ALT and AST levels were significantly increased 3, 6 and 9 h after flutamide administration compared with control mice (Fig. 1), and were attenuated in 24 h. The plasma and liver samples were used for the experiments in Figs 2–4. In addition, plasma ALT level was about 100 U l^{-1} at a dose of 1000 mg kg^{-1} flutamide 6 h after the administration (data not shown).

Expression of mRNA of Transcription Factors, Cytokines and Chemokines in Flutamide-administered Mouse Liver

To investigate the involvement of immunological factors in flutamide hepatotoxicity, the hepatic mRNA levels of IL-5, IFN-γ, GATA-3, T-bet, ROR-γt, STAT6, STAT1, STAT3, Eotaxin-1 and MIP-2 in 6 h after flutamide administration to mice were measured by real-time RT-PCR. In flutamide-administered mice, IL-5, GATA-3, STAT6, Eotaxin-1 and MIP-2 expressions were significantly increased, whereas T-bet expression was significantly decreased and ROR-γt, IFN-γ, STAT1 and STAT3 expressions were not changed (Fig. 2). These results suggested that Th2-related factors, such as IL-5, GATA-3, STAT6 and Eotaxin-1, were involved in the flutamide-induced liver injury in BALB/c mice. In addition, the CXC chemokine MIP-2, which is involved in netrophils recruitment (Biedermann *et al.*, 2000), was significantly increased in flutamide-administered mice. We could not detect the hepatic mRNA expression level of IL-4 owing to very low expression, as reported by Montgomery and Dallman (1991).

Involvement of IL-4 on Flutamide-induced hepatotoxicity

IL-4 plays a central role in the differentiation of Th2 cells and responses (Agnello *et al.*, 2003). To investigate whether IL-4 was

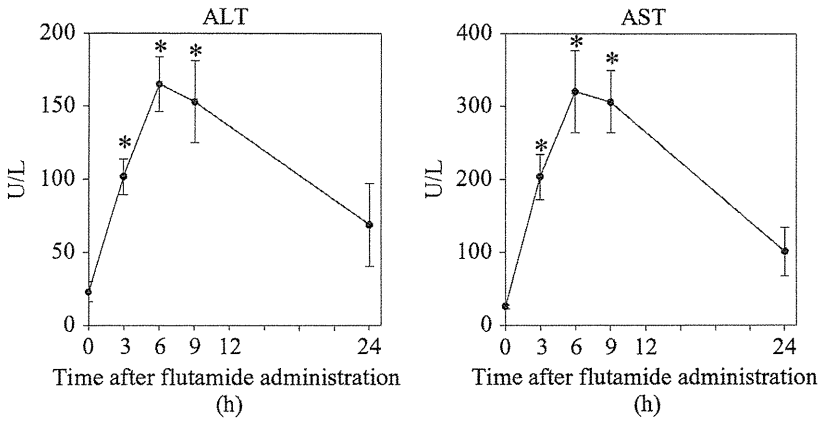

Figure 1. Time-dependent changes of plasma ALT and AST levels in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg in a non-fasting condition. Plasma ALT and AST levels were measured 3, 6, 9 and 24 h after the administration. Data are mean ± SD (n = 4). Significantly different from 0 h mice (*$p < 0.05$).