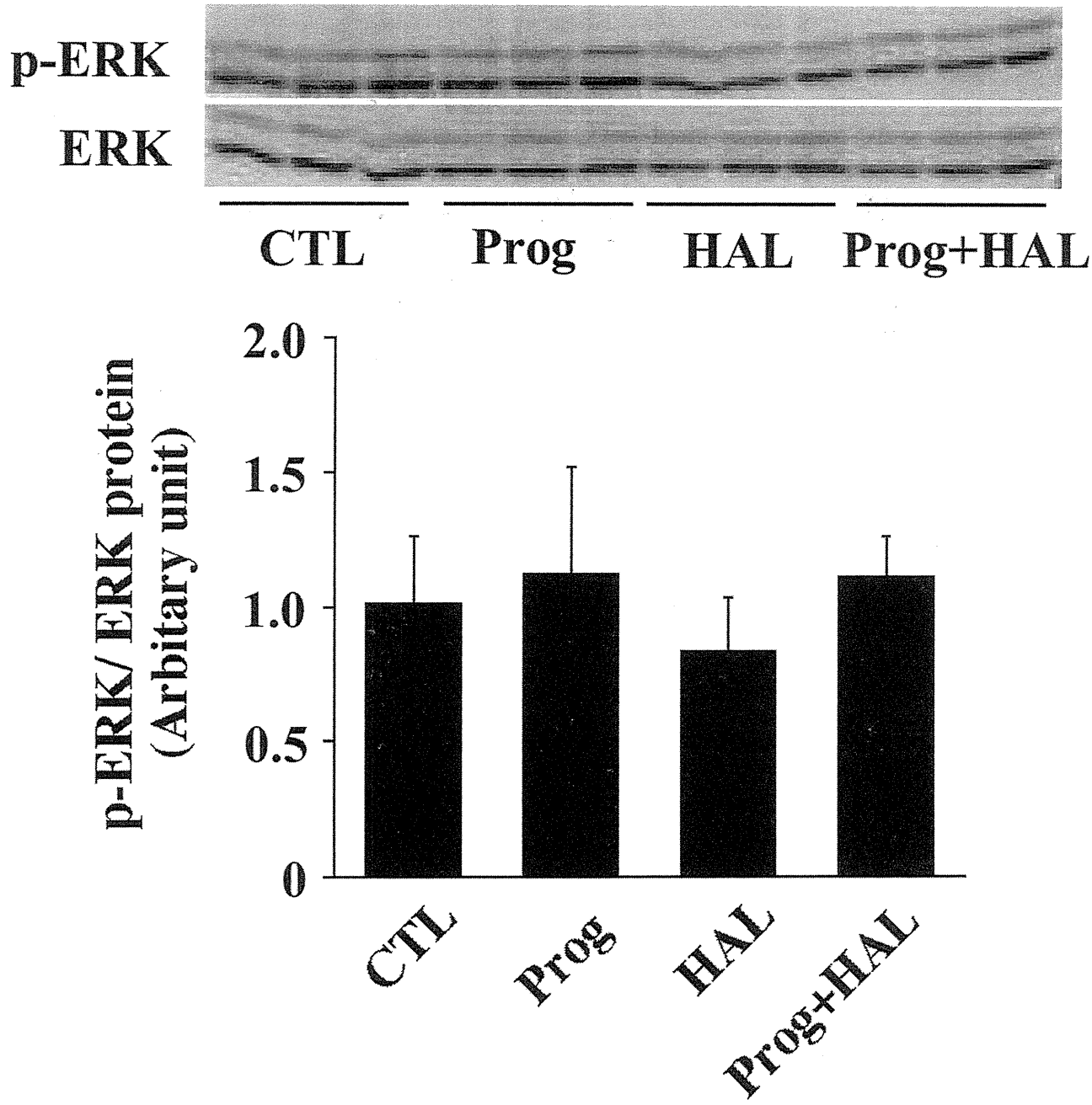
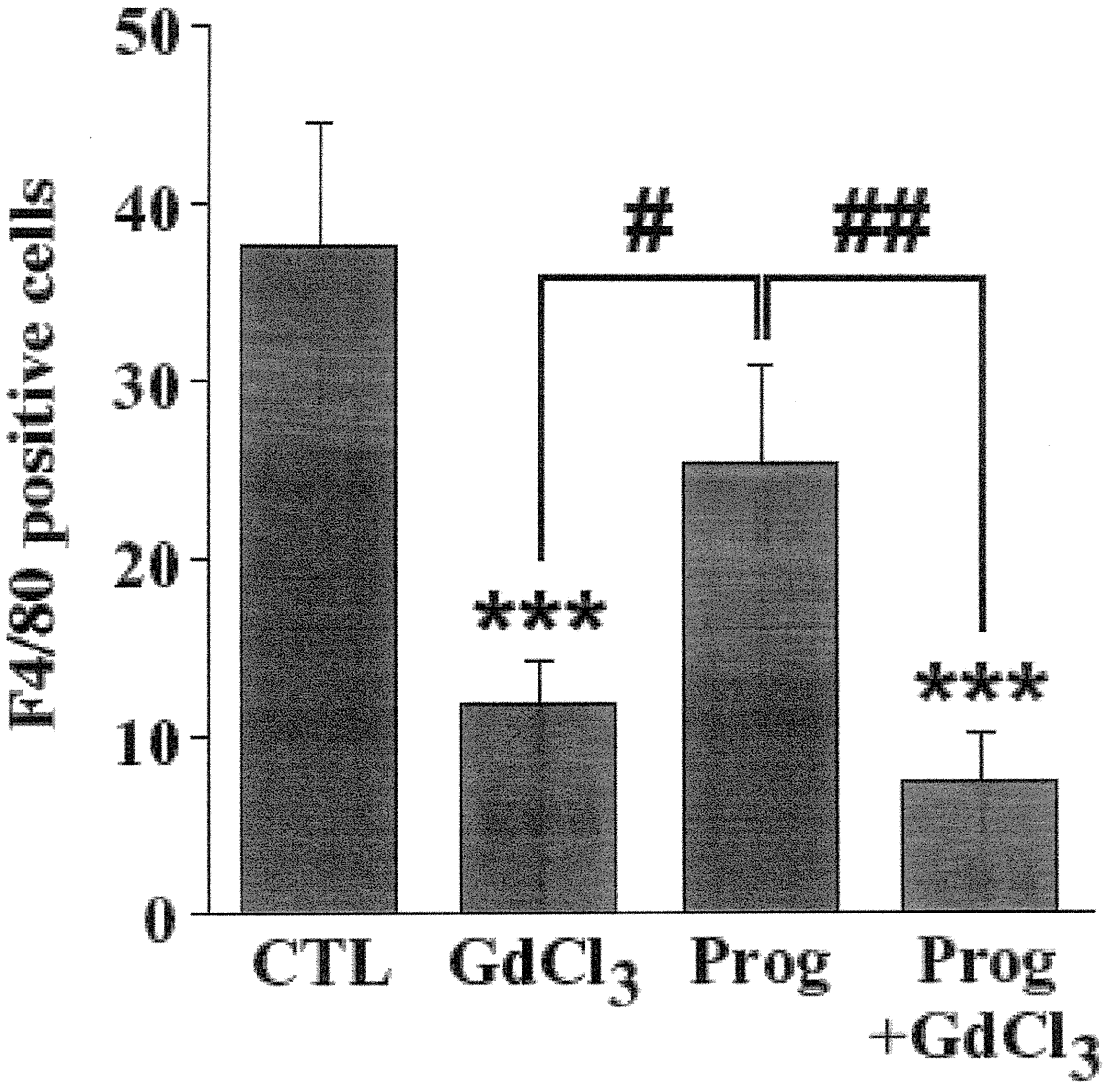
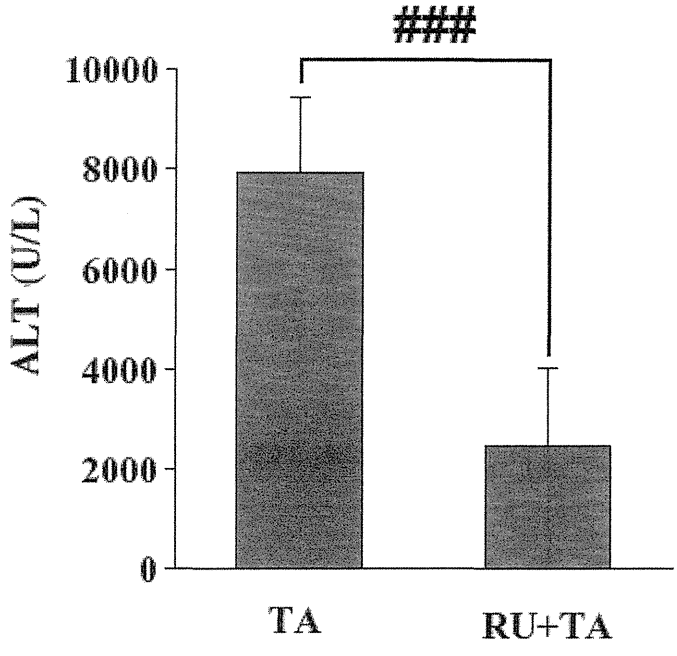
Supplementary Figure 4

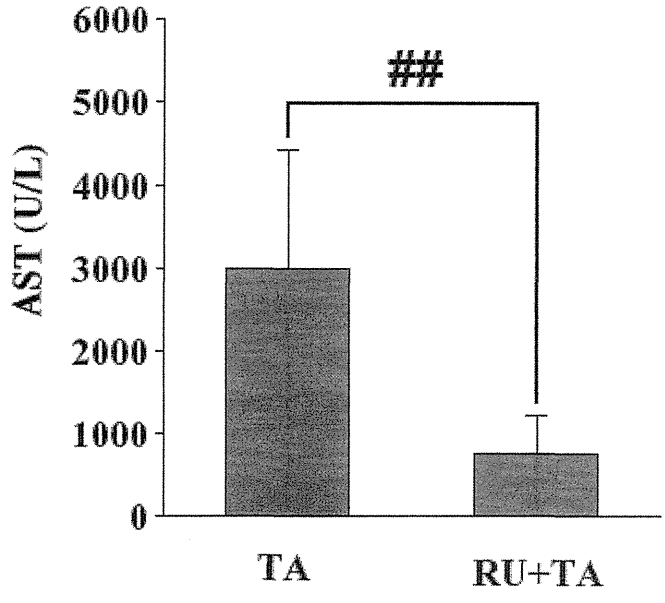


Supplementary Figure 5



Supplementary Figure 6





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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patients because numerous drugs have been linked to hepatotoxicity and it is the most common reason for a drug to be withdrawn.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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($DK\text{-}PGD_2$) exacerbated dicloxacillin-induced liver injury. $DK\text{-}PGD_2$ 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). $DK\text{-}PGD_2$ 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

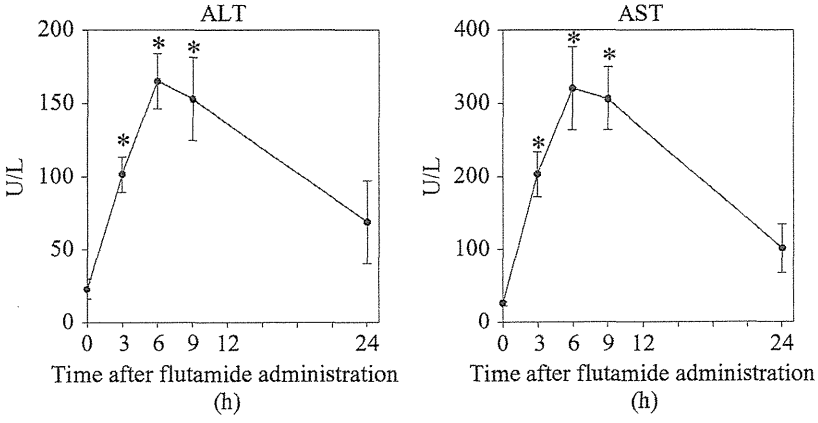

Figure 1. Time-dependent changes of plasma ALT and AST levels in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg in a non-fasting condition. Plasma ALT and AST levels were measured 3, 6, 9 and 24 h after the administration. Data are mean ± SD (n = 4). Significantly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

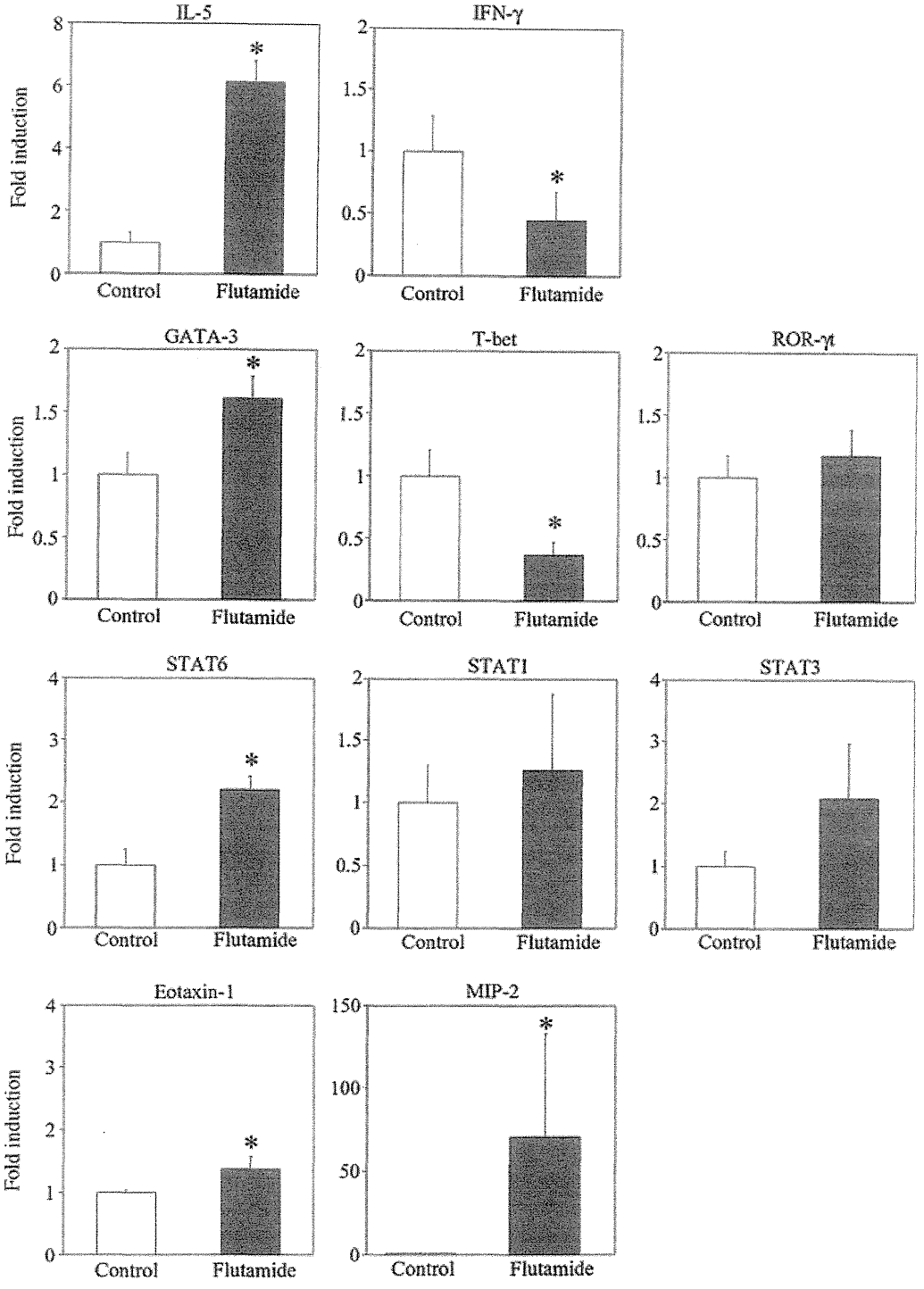

Figure 2. Hepatic mRNA levels of transcriptional factors, cytokines and chemokines in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg and mRNA levels of T-bet, GATA-3, ROR-gt, IFN-g, IL-5, STAT6, STAT1, STAT3, Eotaxin-1, and MIP-2 in the liver were measured by real time RT-PCR 6 h after the administration. Data are mean ± SD (n = 4). Significantly different from control mice (*$p < 0.05$).

involved in the flutamide-induced liver injury, we measured plasma IL-4 in flutamide-administered mice (Fig. 3A). The plasma IL-4 level was significantly increased in flutamide-administered mice compared with control mice. In nontreated control mice, plasma IL-4 was not detected. To further investigate whether IL-4 was involved in the flutamide-induced liver injury, we performed IL-4 neutralization studies (Fig.3B). The i.p. administration of anti-mouse IL-4 antibody significantly reduced the plasma ALT, but rat IgG2a treatment demonstrated no effect on the flutamide-induced liver injury.

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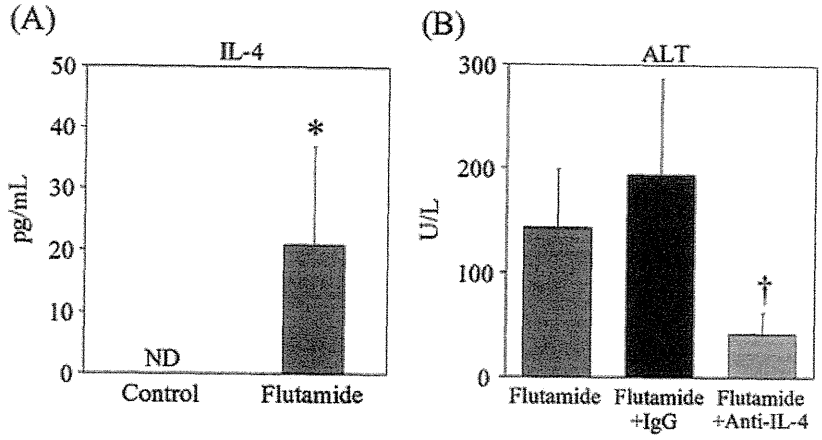

Figure 3. PlasmaIL-4 level and effects of anti-mouse IL-4 antibody administration on plasma ALT in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg, and plasma IL-4 level was measured by ELISA 6 h after the administration (A). Anti-mouse IL-4 antibody (0.1 mg/mouse, i.p.) was administered one hour before the flutamide administration. Plasma ALT levels were measured 6 h after the flutamide administration (B). Data are mean ± SD (n = 4). Significantly different from control mice (*$p < 0.05$); significantly different from flutamide-plus control IgG2a-administered group ($^{\dagger}p < 0.05$).

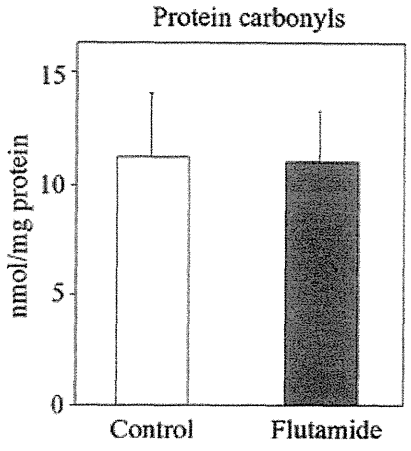

Figure 4. Protein carbonyl level in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg. The plasma protein carbonyl level was measured 6 h after the administration. Data are mean ± SD (n = 4). Significantly different from control mice (*$p < 0.05$).

Protein Carbonyl Level in Flutamide-administered Mice

It has been reported that oxidative stress is involved in flutamide-induced liver injury (Kashimshetty *et al.*, 2009; Yoshikawa *et al.*, 2009b). Then, we measured protein carbonyl, which is a general biomarker for oxidative stress (Davies, 1987). The protein carbonyl level in plasma was not changed in flutamide-administered mice, compared with control mice (Fig. 4). This result suggested that oxidative stress is not likely to be involved in this flutamide-induced liver injury model.

Effects of DK-PGD_2 Treatment on Flutamide-induced Liver Injury

We previously demonstrated the effects of the DK-PGD_2, a selective CRTh2 agonist, on dicloxacillin-induced liver injury. The DK-PGD_2 administration exacerbated dicloxacacllin-induced liver injury (Higuchi *et al.*, 2011). We investigated the effects of DK-PGD_2 on flutamide-induced liver injury. The plasma ALT level was significantly increased in flutamide/DK-PGD_2-cotreated mice, compared with flutamide-administered mice (Fig. 5A). DK-PGD_2 administration alone had no effects on the plasma ALT level. This set of mice was different from that which was used in Figs 1–4. In the histopathological study, a few infiltrating mononuclear cells were observed in flutamide-administered mice, whereas spotty necrosis and mononuclear cells infiltration were observed in flutamide/DK-PGD_2-cotreated mice (Fig. 5B).

Effects of DK-PGD_2 Treatment on Plasma IL-4 Level and Hepatic mRNA Expressions

To investigate the underlying mechanisms responsible for the increased susceptibility of DK-PGD_2 treated mice to flutamide-induced liver injury, the plasma IL-4 level and hepatic mRNA expressions were measured. The plasma IL-4 level was significantly increased in flutamide/DK-PGD_2-cotreated mice compared with flutamide-administered mice. The hepatic mRNA levels of GATA-3 and MIP-2 were significantly increased compared with flutamide-administered mice (Fig. 5C). In particular, MIP-2 mRNA was markedly increased 200-fold compared with nontreated control. In contrast, the expressions of IFN-γ, IL-5, T-bet, ROR-γt, and Eotaxin-1 mRNA were not changed (data not shown). These results suggested that Th2 cytokines are mainly involved in the exacerbation of flutamide-induced hepatotoxicity by DK-PGD_2.

DISCUSSION

Previous studies demonstrated that a high dose of flutamide (500 mg kg^{-1}, daily for 3 days by oral gavage) caused an increase of the liver–body weight ratio, but no increase in the serum ALT level in rat (Coe *et al.*, 2006), and repeated high doses of flutamide (400 mg kg^{-1}, daily for 28 days) did not cause an increase in the plasma ALT level in SV129 mouse (Matsuzaki *et al.*, 2006). Thus, these reports suggested that it is difficult to induce flutamide-induced liver injury at repeated high doses in rodents. After testing many different conditions for flutamide

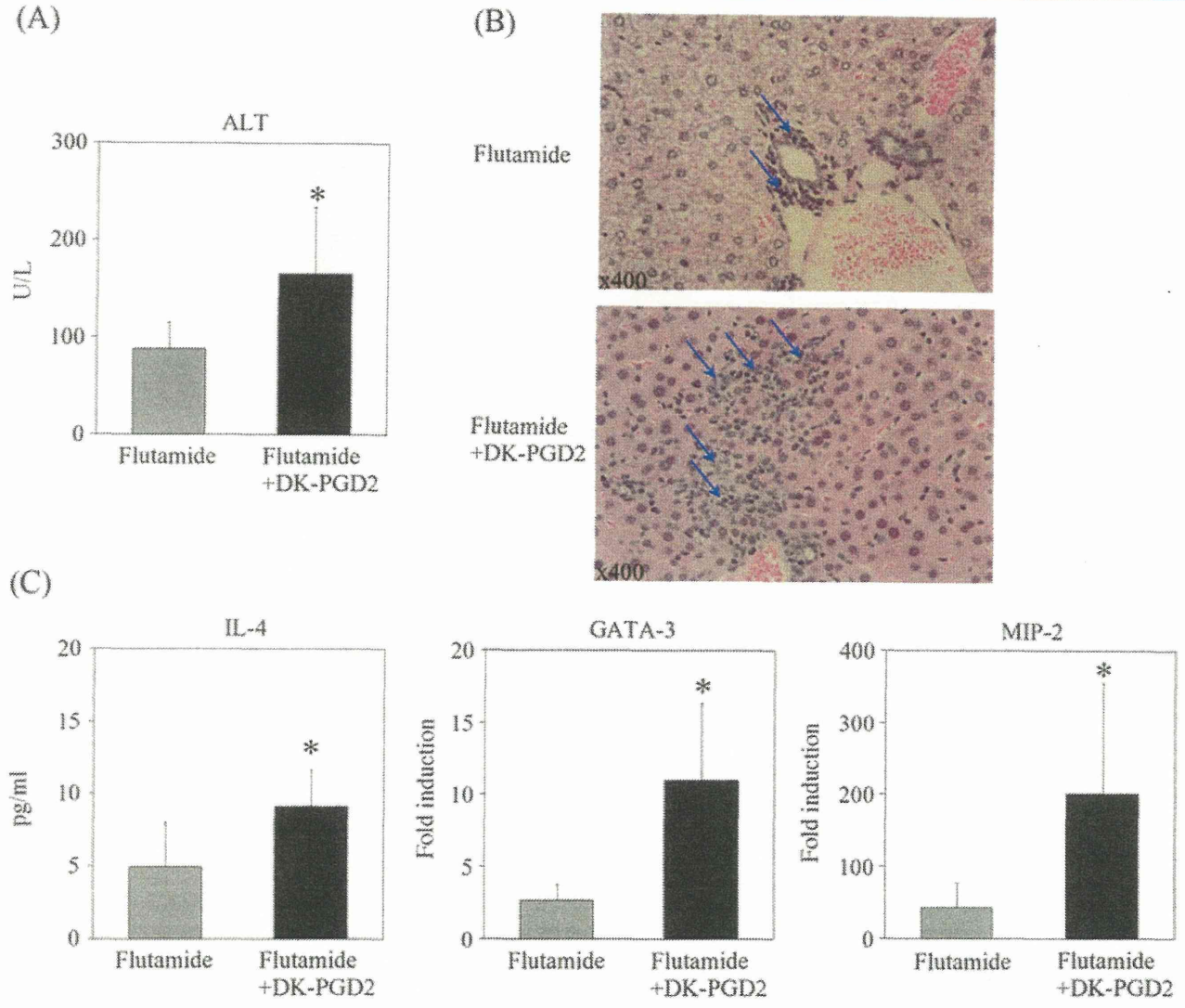

Figure 5. Effects of DK-PGD_2 treatment on flutamide-induced liver injury in mice. Flutamide was orally administered at a dose of 1500 mg/kg. The plasma ALT level was measured 6 h after the administration. DK-PGD_2 (10 μg/mouse, i.p.) was administered 1 h after the flutamide administration (A). Liver specimens were prepared 6 h after the flutamide administration. Liver tissue sections were stained with H&E (B). Arrows indicate necrotic cells with infiltration of neutrophils. Plasma IL-4 level was measured by ELISA and hepatic GATA-3 and MIP-2 mRNA levels were measured by real time RT-PCR (C). Data are mean ± SD (n = 5 for flutamide, 7 for flutamide + DK-PGD_2). Significantly different from flutamide-administered mice (*$p < 0.05$).

administration in mice, finally, we found that administration of flutamide (1500 mg kg^{-1}, single, p.o.) in a nonfasting condition causes flutamide-induced acute hepatotoxicity in mice.

The plasma ALT and AST levels were significantly increased in flutamide-administered mice after 3, 6 and 9 h compared with control mice, and were attenuated by 24 h (Fig. 1). In the present study, we investigated the involvement of immunological factors in flutamide-induced liver injury. The administration of flutamide significantly increased the expression of IL-5, GATA-3, STAT6 and Eotaxin-1. These results suggested that Th2-immune factors would be involved in flutamide-induced liver injury in BALB/c mice, and the plasma IL-4 level was significantly increased in flutamide-administered mice. In addition, neutralization of IL-4 suppressed the hepatotoxicity of flutamide. IL-4 plays a central role in Th2-dominant responses and activates STAT6 and GATA-3, which induce IL-5 and Eotaxin (Agnello *et al.*, 2003; Jaruga *et al.*, 2003), and IL-5 and Eotaxin-1 play roles in development and accumulation of eosinophils and basophils, followed by allergic inflammation (Kay, 2001). In addition, it has been reported that the plasma IL-4 levels were elevated in patients with liver injury of various kinds (Spanakis *et al.*, 2002; Harada *et al.*, 1997). Thus, more investigations are needed into whether IL-4 might be involved the pathogenesis of many DILI. MIP-2 mRNA expression was remarkably increased in flutamide-administered mice. MIP-2 is mainly secreted from macrophages and plays a role in neutrophil accumulation; halothane administration also greatly increases MIP-2 mRNA expression in BALB/c mice (Jaeschke and Hasegawa 2006; Kobayashi *et al.*, 2009). MIP-2, followed by mononuclear cell migration into the liver, might be involved in the flutamide hepatotoxicity.

We demonstrated that IL-4 is involved in flutamide hepatotoxicity by the administration of anti-IL-4 monoclonal antibody

(Fig. 3B). In general, IL-4- or IL-5-deficient mice were suggested to be useful for the investigation of allergic mechanisms for IL-4 or IL-5 (Hogan *et al.*, 1998). However, cytokines markedly change hepatic P450 expressions (Abdel-Razzak *et al.*, 1993). Since flutamide is metabolized by various CYPs (Matsuzaki *et al.*, 2006; Ohbuchi *et al.*, 2009), changes in the expression of drug metabolizing enzymes should be carefully pre-evaluated using IL-4 or IL-5 knockout mouse. Thus, we did not use knockout mouse in the present study.

Next, we investigated the effects of oxidative stress on flutamide-induced liver injury. Flutamide is known as an inhibitor of complex I of mitochondria, and inhibition of complex I leads to increased levels of superoxide, which induce oxidative stress (Fau *et al.*, 1994). In an *in vivo* study, a heterozygous deficiency of superoxide dismutase 2 (SOD2) and the depletion of glutathione, which serves an important function in protecting tissues against oxidative damage, exacerbated flutamide hepatotoxicity in mouse (Kashimshetty *et al.*, 2009) and rat (Morita *et al.*, 2009). These reports suggested that oxidative stress was involved in flutamide-induced liver injury. Therefore, we analyzed the protein carbonyl level, which is widely used as an oxidative stress marker (Davies, 1987). The protein carbonyl level was not changed in flutamide-administered mice. This result suggested that oxidative stress is not likely to be involved in flutamide-induced liver injury in BALB/c mice.

CRTh2, one of the PGD_2 receptors, plays a major role in atopic dermatitis, allergic asthma and airway inflammation, and it was demonstrated that CRTh2 is responsible for PGD_2 chemotaxis of Th2 cells, eosinophils, basophils and monocytes (Kostenis and Ulven, 2006). DK-PGD_2, a CRTh2-selective agonist, enhances Th2-type inflammation (Spik *et al.*, 2005). In a previous study, we demonstrated that DK-PGD_2 administration exacerbated dicloxacillin-induced liver injury in mouse, which involved Th2 immune factors such as IL-4 and Eotaxin (Higuchi *et al.*, 2011). We hypothesized that DK-PGD_2 may exacerbate flutamide-induced liver injury. To examine this hypothesis, BALB/c mice were cotreated with flutamide and DK-PGD_2. The plasma ALT and IL-4 levels were significantly increased in flutamide/DK-PGD_2-cotreated mice compared with flutamide-administered mice, which supported the involvement of Th2 factors. The plasma IL-4 level in flutamide-administered mice was higher in Fig. 3 than in Fig. 5(C), but it was not significant. The experimental conditions were the same. In this study, mice were administered orally flutamide in nonfasting condition, and the stomach content might affect flutamide absorption, resulting in the difference in IL-4 levels.

In the histopathological study, spotty necrosis and mononuclear cell infiltration were observed in flutamide/DK-PGD_2-cotreated mice (Fig. 5B). It has been reported that flutamide activates neutrophil accumulation (Srinivasan *et al.*, 1997), and MIP-2 mRNA expression was remarkably increased in flutamide/DK-PGD_2-cotreated mice (Fig. 5C), and was followed by the infiltration of mononuclear cells into the liver. Compared with our previous dicloxacillin study, the expression levels of Eotaxin-1 and monocyte chemoattractant protein-1 mRNA were not changed in flutamide/DK-PGD_2-cotreated mice, which might be due to differences in the IL-4 secretion level induced by flutamide and dicloxacillin. Th2 immune factors-mediated liver injury was exacerbated by DK-PGD_2, which could be a novel method to detect hepatotoxicity of new compounds in drug development.

In conclusion, we reported for the first time that immunological factors are involved in flutamide-induced liver injury in mice, and that DK-PGD_2 could be useful for the detection of Th2 immune factors that mediate liver injury. The present study may shed light on the mechanisms of DILI.

Acknowledgments

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