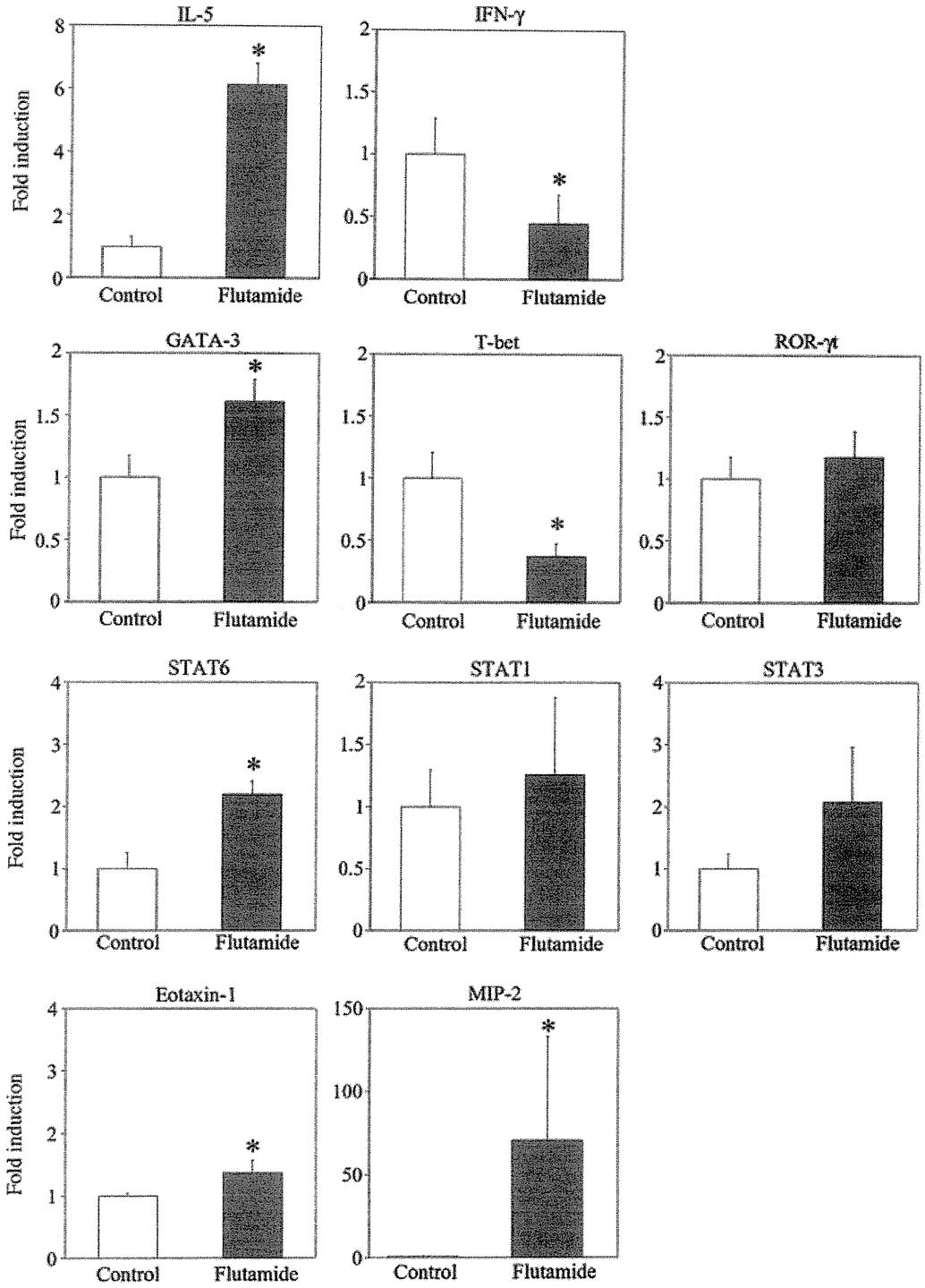

Figure 2. Hepatic mRNA levels of transcriptional factors, cytokines and chemokines in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg and mRNA levels of T-bet, GATA-3, ROR-gt, IFN-g, IL-5, STAT6, STAT1, STAT3, Eotaxin-1, and MIP-2 in the liver were measured by real 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.

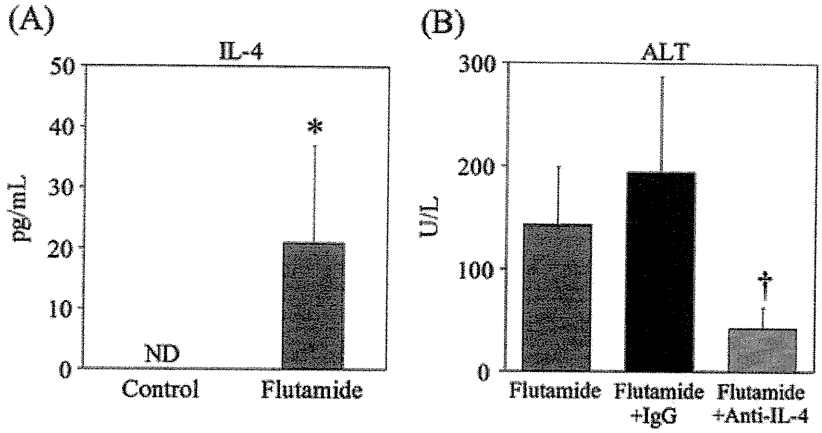

Figure 3. PlasmaIL-4 level and effects of anti-mouse IL-4 antibody administration on plasma ALT in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg, and plasma IL-4 level was measured by ELISA 6 h after the administration (A). Anti-mouse IL-4 antibody (0.1 mg/mouse, 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 (†$p < 0.05$).

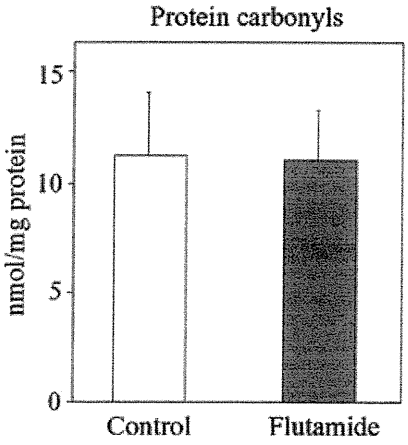

Figure 4. Protein carbonyl level in flutamide-administered mice. Flutamide was orally administered at a dose of 1500 mg/kg. The plasma protein carbonyl level was measured 6 h after the administration. Data are mean ± SD (n = 4). Significantly different from control mice (*$p < 0.05$).

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

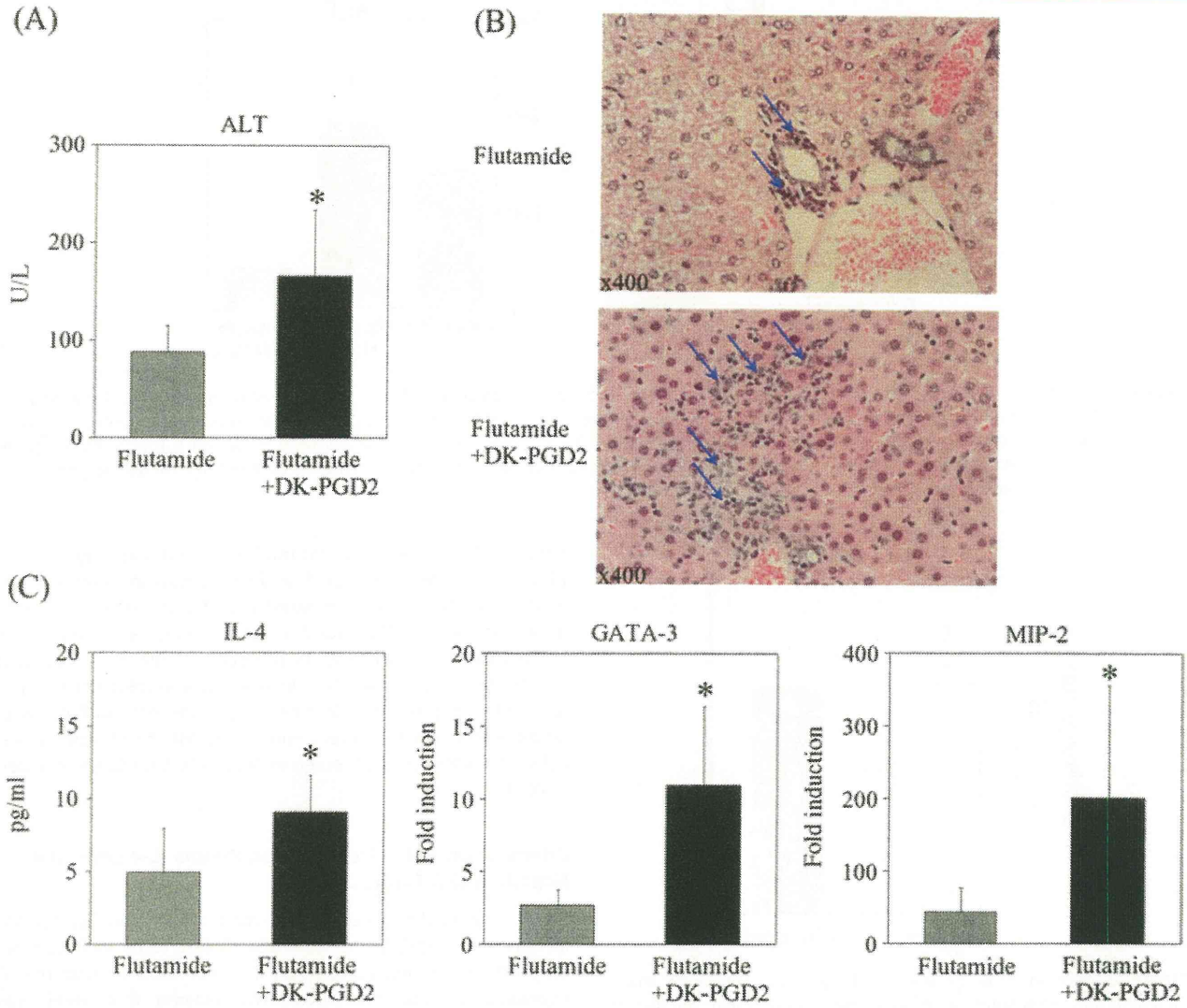

Figure 5. Effects of DK-PGD_2 treatment on flutamide-induced liver injury in mice. Flutamide was orally administered at a dose of 1500 mg/kg. The plasma ALT level was measured 6 h after the administration. DK-PGD_2 (10 μg/mouse, i.p.) was administered 1 h after the flutamide administration (A). Liver 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 (*$p < 0.05$).

adminitration 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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Th2 cytokine-mediated methimazole-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 practice. The pathogenesis of DILI usually involves the participation of the parent drug or metabolites that either affect cellular function or elicit an immune response. However, the mechanisms leading to DILI are unknown in most cases. Methimazole (MTZ) is used as an antithyroid drug and is well known to have induced liver injuries such as cholestatic hepatitis in a small number of human cases. Immune-mediated reactions were also suggested to play a role in MTZ-induced acute liver injury, but the mechanism underlying this process has not been elucidated. To address this issue, we measured plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, hepatic glutathione levels, hepatic expression of $CD4^+$ Th cell-related transcriptional factors, cytokines and chemokines, plasma interleukin (IL)-4 levels and histopathological changes in the liver following MTZ (450 mg kg^{-1}, p.o.) administration in mice. The hepatic expression of mRNA for Th2 cell-related factors, such as GATA-binding protein, macrophage inflammatory protein-2 (MIP-2) and plasma IL-4 levels, as well as plasma AST and ALT levels, was significantly increased in mice treated with MTZ. These changes were markedly enhanced by pre-treatment with L-buthionine sulfoximine (3 mmol kg^{-1}, i.p.) and MTZ (15 mg kg^{-1}, p.o.). Neutralization of IL-4 using a monoclonal anti-mouse IL-4 antibody (100 μg/mouse, single i.p.) suppressed the hepatotoxic effect of MTZ. In conclusion, this report is the first to demonstrate that Th2 cytokine-mediated immune responses are involved in MTZ-induced acute liver injury in mice.

Keywords: drug-induced liver injury; cytokines; IL-4; eotaxin; helper T cells

INTRODUCTION

Drug-induced liver injury (DILI) remains an important cause of acute liver failure and is a major reason for the withdrawal of approved drugs from the market. The pathogenesis of drug-induced liver injury usually involves the participation of the parent drug or metabolites that either directly affect cell functions or elicit an immune response. Some drugs, such as tienilic acid, amodiaquine and halothane, are suggested to induce immune-related DILI (Bugelski, 2005). In most cases, the mechanisms underlying DILI are unknown because predictive *in vitro* screening methods and animal models are lacking.

Methimazole (MTZ), which is widely used to treat hyperthyroidism, is well known to induce DILI, such as acute hepatic necrosis and cholestatic hepatitis, with a low incidence (Luca *et al.*, 2009; Sadoul *et al.*, 1993; Vitug and Goldman, 1985). The metabolic activation of MTZ by either hepatic cytochrome P-450 (P450) or flavin-containing monooxygenases (FMO) has been proposed to produce reactive metabolites that may covalently bind to proteins and inactive P450 (Lee and Neal, 1978; Kedderis and Rickert, 1985). It was reported that the inadequate detoxification of reactive metabolites was responsible for the hepatotoxicity observed in glutathione (GSH) depleted mouse and rat models using L-buthionine sulfoximine (BSO) (Mizutani *et al.*, 1999; Shimizu *et al.*, 2011). However, it is not clear whether immune-mediated factors are involved in MTZ-induced acute liver injury.

Although the pathogenesis of DILI is not fully understood, it can be divided into a two-stage process based on studies of the mechanisms involved in animal models (Laverty *et al.*, 2010). In the first step, chemical stress responses, such as mitochondrial dysfunction, are induced owing to excessive drug accumulation and/or metabolic activation. The second step is the process of adaptation or failure of the response mediated by immune-related cells. In addition, as knowledge concerning the role of immune cells in hepatic pathology accumulates, it is becoming clear that immune responses possibly play an essential role in DILI (Adams *et al.*, 2010). Therefore, it is important to understand the mechanisms by which cells that play a role in the immune system are activated during DILI.

Helper T cells ($CD4^+$ Th cells) are an important regulator 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 that target liver cells and immune cells by activating multiple signaling cascades (Leonard and O'Shea, 1998). Th cells are subdivided into Th1, Th2, regulatory T cells (Treg) and Th17 based on their unique production of cytokines and characteristic transcription factors (Kidd,

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2003; Zhu and Paul, 2008). Th1 cells require 'T-box expressed in T cells' (T-bet) and secrete interferon (IFN)-γ. Th2 cells require GATA-binding domain-3 (GATA-3) and produce IL-4 and IL-5. In addition, Th17 cells secrete IL-17 and IL-22 require 'retinoid-related orphan receptor γt' (ROR-γt) for differentiation (Zhu and Paul, 2008).

Th2 cell-mediated responses influence a wide range of events associated with allergic inflammation via IL-4 and IL-5. IL-4 promotes the development of mast cells. IL-5 is involved in the development of eosinophils (Kay, 2001). In concanavalin (Con) A-mediated hepatitis, a widely accepted mouse model for studying T cell-mediated liver injury, IL-4 stimulates the secretion of eotaxin-1 and enhances IL-5 production, resulting in attraction of neutrophils and eosinophils to the liver, which leads to hepatitis (Jaruga *et al.*, 2003).

We recently reported that IL-17 is involved in halothane- and α-naphthylisothiocyanate-induced acute liver injury in mice (Kobayashi *et al.*, 2009, 2010) and that IL-4 is involved in dicloxacillin-induced acute liver injury in mice (Higuchi *et al.*, 2011). In this study, we found that Th2 cytokine-mediated immune responses play a role in MTZ-induced acute liver injury in mice.

MATERIALS AND METHODS

Materials

MTZ and BSO were purchased from Sigma-Aldrich (St Louis, MO, USA). GSH was obtained from Wako Pure Chemical Industries (Osaka, Japan). β-NADPH and GSH reductase came from Oriental Yeast (Tokyo, Japan). RNAiso came from Nippon Gene (Tokyo, Japan). ReverTra Ace came from Toyobo (Tokyo, Japan). Random hexamers and SYBR Premix Ex Taq were from Takara (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). A rabbit polyclonal antibody against myeloperoxidase (MPO) was obtained from Thermo Fisher Scientific Anatomical Pathology (Cheshire, UK). A monoclonal anti-mouse IL-4 antibody came from U-Cytech Biosciences (Utrecht, Netherland). A monoclonal rat IgG2a isotype, used as a negative control, was purchased from R&D Systems (Abingdon, UK). A Ready-SET-GO! Mouse IL-4 enzyme-linked immunosorbent assay (ELISA) kit was obtained from eBioscience (San Diego, CA, USA). Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII, which were used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, came from Fujifilm (Tokyo, Japan). Other chemicals used were of analytical or the highest grade commercially available.

MTZ Administration

Female BALB/cCrSlc mice (6 weeks old, 15–20 g) were obtained from SLC Japan (Hamamatsu, Japan). The animals were housed in a controlled environment (temperature 25 ± 1 °C, humidity 50 ± 10%, 12 h light/12 h dark cycle) in the institution's animal facility with *ad libitum* access to food and water. The animals were acclimatized before use in the experiments. MTZ was dissolved in saline (45 mg ml^{-1}) and administered orally to the mice at a dose of 450 or 15 mg kg^{-1} following overnight fasting. Two hours after MTZ administration, the mice were again allowed access to food and water *ad libitum*. Animal maintenance and treatments 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.

An MTZ dose of 450 mg kg^{-1} was set at approximately one-half the oral LD_{50} value based on the results of experiments to determine the effects of MTZ doses of 860 (the oral LD_{50} in mice), 450 and 225 mg kg^{-1} as follows. It was difficult to assess the hepatotoxicity of MTZ at a dose of 860 mg kg^{-1} because symptoms of moribundity, such as decreased locomotor activity and tremor, were observed in all mice treated with MTZ at this dose. In addition, plasma AST and ALT values exhibited a high degree of individual variation (AST and ALT values were 3165 ± 7175 and 4087 ± 8063 IU l^{-1}, respectively). There was no difference in plasma AST and ALT values between the MTZ 450 mg kg^{-1} (AST and ALT values were 104 ± 29 and 79 ± 4 IU l^{-1}, respectively) and MTZ 225 mg kg^{-1} groups (AST and ALT values were 74 ± 16 and 70 ± 12 IU l^{-1}, respectively; the *P*-values for AST and ALT were 0.06 and 0.09, respectively). Based on these results, the dose of MTZ was set at 450 mg kg^{-1}.

The dose of MTZ was set at 15 mg kg^{-1} for experiments involving the co-administration of BSO and MTZ because it was reported that MTZ at doses of 11.4, 22.8 and 45.6 mg kg^{-1} resulted in dose-dependent increases (12- to 180-fold of the values in controls) in serum ALT activity (Mizutani *et al.*, 1999).

Administration of BSO

BSO was dissolved in water and administered intraperitoneally at a dose of 3 mmol/20 ml kg^{-1} to mice 1 h before MTZ (15 mg kg^{-1}) administration. The dose of BSO was set at 3 mmol kg^{-1} because it was confirmed in several previous studies that hepatic GSH was decreased after intraperitoneal injection of mice with BSO at a dose of approximately 3 mmol kg^{-1} (Mizutani *et al.*, 1999; Tirmenstein and Nelson, 1991; Shimizu *et al.*, 2009).

Administration of Anti-mouse IL-4 Antibodies

One hour prior to MTZ administration, the mice were administered an anti-mouse IL-4 antibody intraperitoneally (100 µg of anti-mouse IL-4 antibody in 0.5 ml of sterile PBS). As a negative control, rat IgG2a was administered (100 µg of rat IgG2a in 0.5 ml of sterile PBS).

Sample Collection and Histopathology

At 6 h after MTZ administration, the animals were sacrificed, and their plasma and livers were collected because it was reported that serum AST and ALT activities were markedly increased 6 h after MTZ administration and slightly decreased at 24 h (Mizutani *et al.*, 1999). A portion of each excised liver was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a 4 µm thickness and subjected to hematoxylin–eosin (HE) staining and immunohistochemical staining for MPO. The degree of liver injury was confirmed by HE staining. Neutrophil infiltration was assessed by immunostaining for MPO. After deparaffinization, liver sections were microwaved in a target retrieval solution (TRS, pH6.1, Dako) for 15 min to unmask the antigens. Then, the

sections were immersed in an aqueous 3% H_2O_2 solution for 5 min to inactivate endogenous peroxidase following blocking of unspecific proteins with Brock Ace (DS Pharma Biomedical Co., Osaka, Japan) for 1 h at room temperature. Diluted primary antibody (1:150) was applied to the sections, which were then incubated at 4 °C. After overnight treatment, the sections were incubated with Histofine Simple Stain Mouse MAX PO® (Nichirei Corp., Tokyo, Japan) for 30 min according to the manufacturer's instructions.

Quantification of Hepatic MPO-positive Cells

The numbers of MPO-positive cells were counted throughout an entire section from each of three mice and expressed as numbers of cells per square millimeter.

Real-time Reverse Transcription-PCR

RNA from mouse livers was isolated using RNAiso according to the manufacturer's instructions. T-bet, GATA-3, RORγt, FoxP3, interferon-γ (IFN-γ), IL-5, tumor necrosis factor α (TNFα), eotaxin, chemokine (C–X–C motif) ligand 1 (Cxcl-1), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory ptrotein-2 (MIP-2) and Gapdh were quantified using real-time reverse transcription (RT)-PCR. The primer sequences used in this study are shown in Table 1. For the RT step, total RNA (10 μg) and 150 ng random hexamers were mixed and incubated at 70 °C for 10 min. An 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 and 42 °C for 1 h then heated at 98 °C for 10 min to inactivate the enzyme. Real-time RT-PCR was performed using Mx3000P (Stratagene, La Jolla, CA, USA). The PCR mixture contained either 1 or 2 μl of template cDNA, SYBR Premix Ex Taq solution and 8 pmol of forward and reverse primers. The amplified products were monitored directly by measuring the increase in the intensity of the SYBR Green I dye (Molecular Probes, Eugene, OR, USA) binding to the double-stranded DNA amplified by PCR.

GSH Levels

Total GSH (the sum of both the reduced and oxidized form) was measured using reduced GSH as a standard. Mouse liver tissue was homogenized with a glass homogenizer on ice with cold 5% sulfosalicylic acid and centrifuged at 8000 ***g*** at 4 °C for 10 min. The resultant supernatant was diluted with distilled water, and finally, the GSH concentration was measured as described previously (Tietze, 1969). The GSH standard curve (0.035–0.55 μmol g^{-1} protein) showed good linearity ($R > 0.99$).

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

Gene		Sequence
T-bet	FP	5′- CAA GTG GGT GCA GTG TGG AAA G-3′
	RP	5′- TGG AGA GAC TGC AGG ACG ATC-3′
IFNγ	FP	5′- GGC CAT CAG CAA CAT AAG C-3′
	RP	5′- TGG ACC ACT CGG ATG AGC TCA-3′
GATA-3	FP	5′- GGA GGA CTT CCC CAA GAG CA-3′
	RP	5′- CAT GCT GGA AGG GTG GTG A-3′
IL-5	FP	5′- AAA GAG ACC TTG ACA CAG CTG-3′
	RP	5′- AGA GCT CTG TCT AGG TCC TGG-3′
FoxP3	FP	5′- CTA GCA GTC CAC TTC ACC AAG-3′
	RP	5′- GCT GCT GAG ATG TGA GTG TC-3′
RORγt	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′
TNFα	FP	5′- TGT CTC AGC CTC TTC TCA TTC C-3′
	RP	5′- TGA GGG TCT GGG CCA TAG AAC-3′
Eotaxin	FP	5′- TCC ACA GCG CTT CTA TTC CT-3′
	RP	5′- CTA TGG CTT TCA GGG TGC AT-3′
MIP-2	FP	5′- AAG TTT GCC TTG ACC CTG AAG-3′
	RP	5′- ATC AGG TAC GAT CCA GGC TTC-3′
Cxcl-1	FP	5′- GAT TCA CCT CAA GAA CAT CCA GAG-3′
	RP	5′- GAA GCC AGC GTT CAC CAG AC-3′
MCP-1	FP	5′- TGT CAT GCT TCT GGG CCT G-3′
	RP	5′- CCT CTC TCT TGA GCT TGG TG-3′
Gapdh	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; T-bet, T-box expressed in T cells; IFNγ, interferon-γ; GATA-3, GATA-binding domain-3; IL-5, interleukin-5; FoxP3, forkhead box P3; RORγt, retinoid-related orphan receptor γt; TNFα, tumor necrosis factor α; MIP-2, macrophage inflammatory ptrotein-2; Cxcl-1, chemokine (C–X–C motif) ligand 1; MCP-1, monocyte chemotactic protein-1.

Plasma IL-4 Levels

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

Statistical Analysis

Statistical analyses were performed using SAS 9.1.3. Comparison of two groups was carried out with a two-tailed Student's t-test. Comparison of multiple groups was accomplished using the Dunnet test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Changes in Plasma AST, ALT and Hepatic GSH levels, and Histopathological Changes in the Liver in MTZ-treated Mice

Female BALB/c mice were administered MTZ orally at a dose of 450 mg kg^{-1}. A slight but significant increase in the levels of AST (45 ± 5 and 104 ± 29 IU l^{-1} in the control and MTZ 450 mg kg^{-1} group, respectively) and ALT (25 ± 6 and 79 ± 4

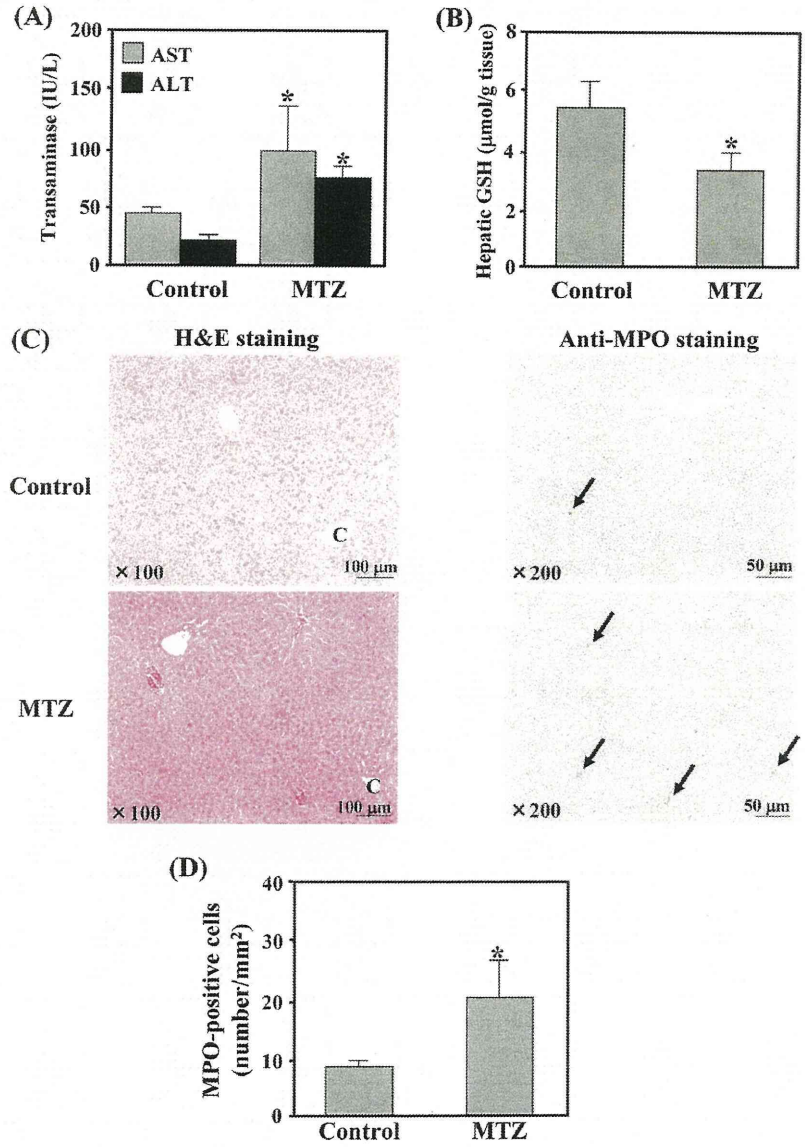

Figure 1. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and hepatic glutathione (GSH) levels; histopathological presentation of liver injury and quantification of hepatic myeloperoxidase (MPO) positive cells in methimazole (MTZ) treated mice. Mice were administered MTZ (450 mg kg^{-1}, p.o.), and plasma and liver tissue were collected 6 h after MTZ administration. Plasma AST and ALT (A) and hepatic GSH levels (B) were measured. Data are shown as the means ± SD of results from five mice. Differences compared with the control group were considered significant at $^*P < 0.05$. Histopathological examination of the liver (C). Liver tissue sections were stained with HE or immunostained with an anti-MPO antibody. Arrows indicate MPO-positive cells. C, central vein. The numbers of MPO-positive cells in three mice were counted throughout the entire section and are expressed as the numbers of cells per square millimeter. The numbers of MPO-positive cells were compared with those in mice administered MTZ (D). Differences compared with the control group were considered significant at $^*P < 0.05$.

IU l^{-1} in the control and MTZ 450 mg kg^{-1} group, respectively) in plasma was observed 6 h after MTZ administration (Fig. 1A). The GSH level in the liver was significantly lower in mice treated with MTZ than in control mice (Fig. 1B). The histopathological changes observed in the mouse livers 6 h after MTZ administration are shown in Fig. 1(C). Based on HE staining, a decrease of glycogen was observed in the hepatocytes of MTZ-treated mice. In the immunohistochemical analysis, a small number of cells reacted to the anti-MPO antibody 6 h after MTZ administration. An increase in the number of MPO-positive cells was observed in the MTZ-treated mice (Fig. 1D).

Expression of mRNA for $CD4^+$ Th Cell-related Transcription Factors and Cytokines in the Livers of MTZ-treated Mice

To investigate the effects of MTZ administration on immunological factors in the mouse liver, the hepatic mRNA expression levels of transcriptional factors for each T helper lineage were measured using real-time RT-PCR (Fig. 2). In previous studies by our group (Kobayashi *et al.*, 2010; Higuchi *et al.*, 2011), we confirmed that the expression levels of mRNA and protein were similar for interleukins and chemokines. Thus, changes in mRNA were mainly followed in the present study, except for IL-4. The hepatic mRNA expression levels of T-bet and IFN-γ, which are a master regulator and a major cytokine of Th1 cells, respectively, in mice treated with MTZ were significantly decreased compared with the levels in control mice (Fig. 2A and B). The hepatic mRNA expression levels of GATA-3 and IL-5, which are a master regulator and a major cytokine of Th2 cells, respectively, in mice treated with MTZ were 3-fold higher than those in control mice (Fig. 2C and D). However, the hepatic mRNA expression levels of FoxP3 and RORγt, which are master regulators of Treg and Th17 cells, respectively, in mice treated with MTZ were not changed compared with control mice (Fig. 2E and F).

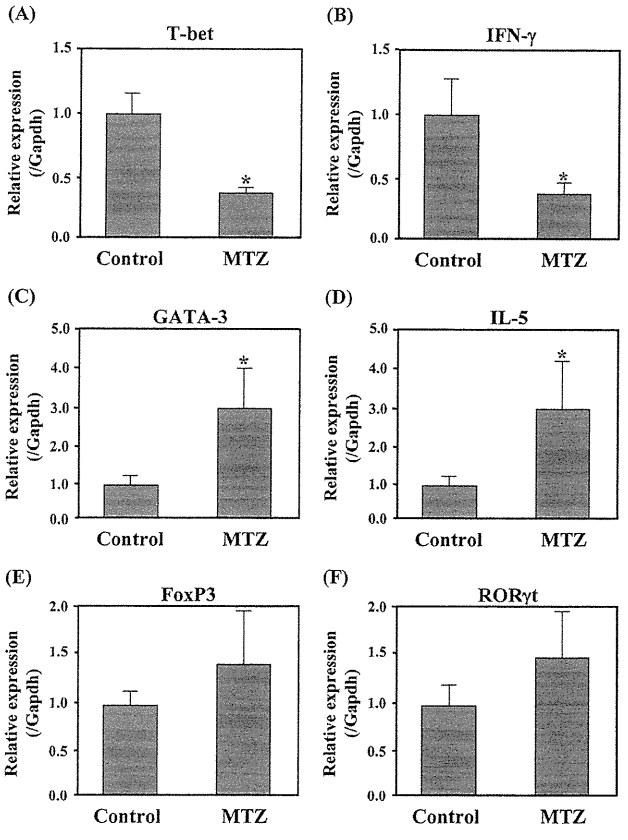

Figure 2. Hepatic mRNA expression levels of $CD4^+$ Th cell-related transcription factors and cytokines 6 h after MTZ administration. The relative hepatic expression levels of T-box expressed in T cells (T-bet, A), interferon-γ (IFN-γ B), GATA-binding domain-3 (GATA-3, C), interleukin-5 (IL-5, D), forkhead box P3 (FoxP3, E) and retinoid-related orphan receptor γt (RORγt, F) mRNA were measured using real-time reverse transcriptase (RT)-PCR and normalized to Gapdh mRNA. Data are shown as the means ± SD of results from five mice. Differences compared with the control group were considered significant at $^*P < 0.05$.

Changes of Proinflammatory Cytokine and Chemokine Genes in the Livers of MTZ-treated Mice

To investigate whether the neutrophil infiltration observed in mice treated with MTZ resulted from increases in the levels of proinflammatory cytokines and chemokines, the hepatic expression levels of TNFα, eotaxin, MIP-2, Cxcl-1 and MCP-1

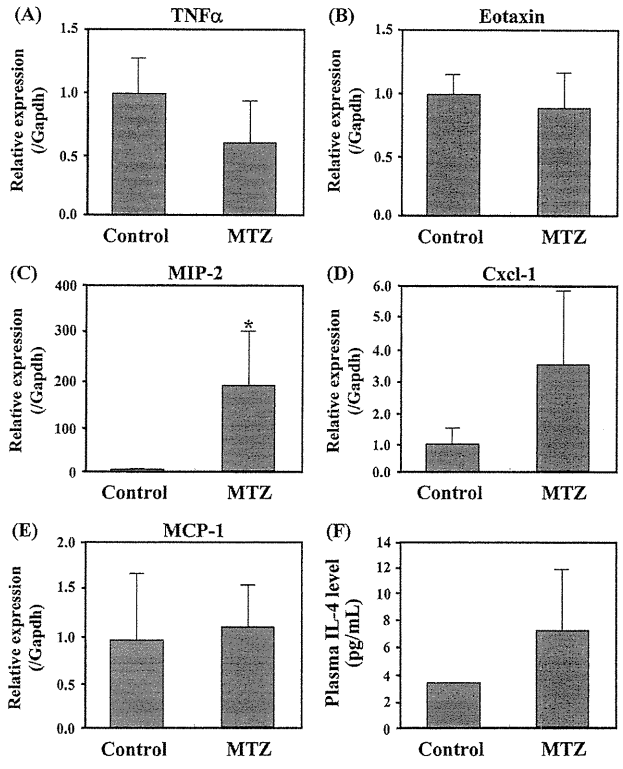

Figure 3. Hepatic mRNA expression levels of proinflammatory cytokines and chemokines and plasma IL-4 levels 6 h after MTZ administration. The relative expression levels of tumor necrosis factor αTNFα A), eotaxin (B), macrophage inflammatory ptrotein-2 (MIP-2, C), chemokine (C–X–C motif; Cxcl-1, D) and monocyte chemotactic protein-1 (MCP-1, E) mRNA were measured using real-time RT-PCR and normalized to Gapdh mRNA. The plasma IL-4 level was measured using ELISA. The limit of detection for plasma IL-4 was 3.9 pg ml^{-1} (F). Data are shown as the means ± SD of results from five mice. Differences compared with the control group were considered significant at $^*P < 0.05$.

mRNA were measured. The expression levels of TNFα, eotaxin and MCP-1 mRNA were not altered by MTZ administration (Fig. 3A, B and E). However, the level of MIP-2 mRNA was markedly increased 6 h after MTZ administration (Fig. 3C).

Changes in Plasma IL-4 Levels

IL-4 is a major cytokine of Th2 cells. We attempted to measure the mRNA expression of hepatic IL-4, but no expression was detected in either MTZ-treated or control samples, as suggested previously (Higuchi *et al.*, 2011). Thus, plasma IL-4 was measured using ELISA. Plasma IL-4 was not detectable in control mice by ELISA but was detected in mice treated with MTZ (Fig. 3F). The detection limit of this assay was 3.9 pg ml^{-1}.

Co-treatment of Mice with BSO and MTZ (15 mg kg^{-1})

A group of mice was subjected to treatment with BSO (3 mmol kg^{-1}, i.p., 1 h before MTZ administration) and MTZ (15 mg kg^{-1}, p.o.). BSO was administered to inhibit the ability to generate GSH upon experiencing a challenge (e.g. reactive metabolites of MTZ or reactive oxygen species). In mice treated with BSO alone, no elevation of ALT and AST levels and no histopathological changes were observed. However, the hepatic GSH level was significantly decreased (Fig. 4A, B, E and F). The activity of γ-glutamylcysteine synthetase (γ-GCS) was measured to validate the effectiveness of BSO as described previously (Hamel *et al.*, 1992). It was confirmed that 1 and 6 h after BSO administration at a dose of 3 mmol kg^{-1}, hepatic γ-GCS activities were 23.6 ± 7.4 and 22.6 ± 1.7%, respectively, of those in the vehicle-treated control, using five mice in each case. The plasma AST (44 ± 5 and 13 184 ± 4500 IU l^{-1} in the MTZ 15 mg kg^{-1} alone and BSO and MTZ 15 mg kg^{-1} groups, respectively) and ALT (21 ± 8 and 20 424 ± 7180 IU l^{-1} in the MTZ 15 mg kg^{-1} alone and BSO and MTZ 15 mg kg^{-1} groups, respectively) levels were markedly increased in mice treated with BSO and MTZ at a dose of 15 mg kg^{-1} compared with mice treated with MTZ alone at 15 mg kg^{-1} (Fig. 4C). The GSH level in the liver was significantly decreased in mice

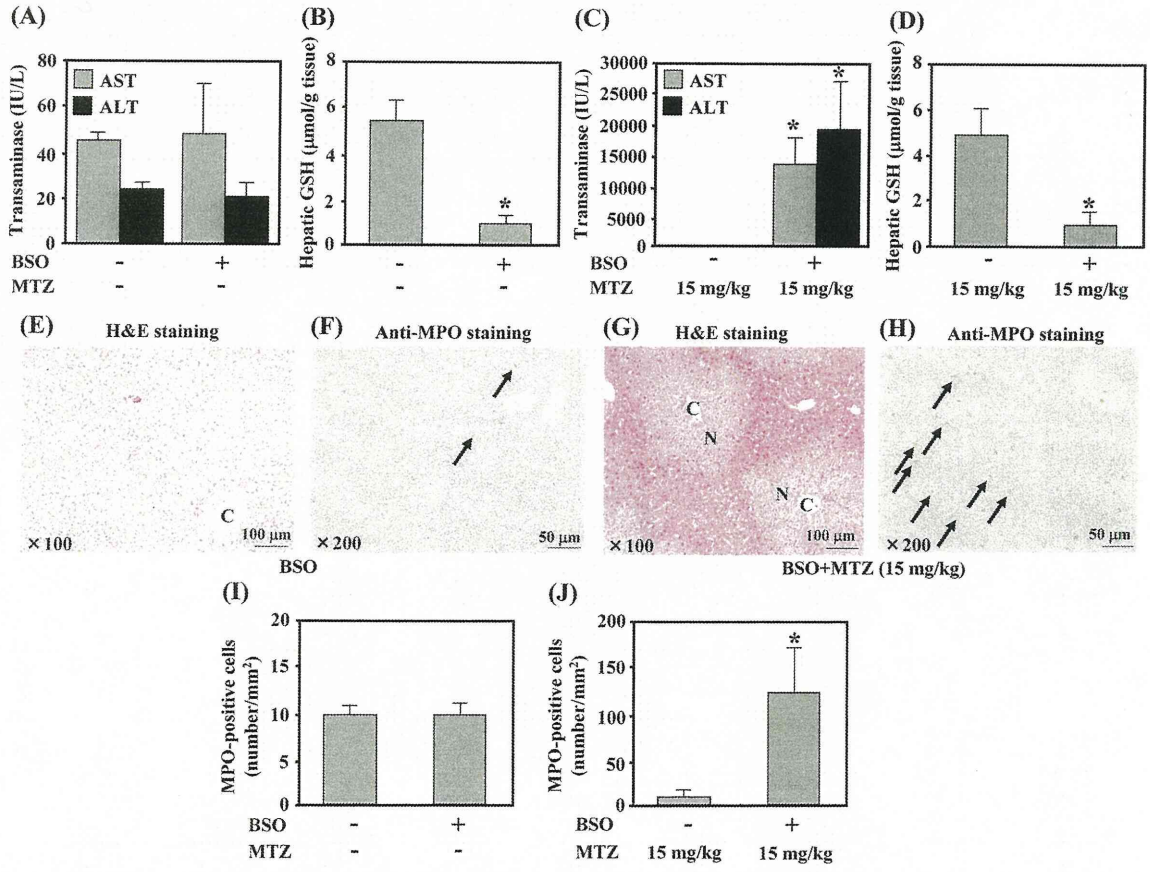

Figure 4. Plasma AST and ALT, hepatic GSH levels, histopathological presentation of liver injury and quantification of hepatic MPO-positive cells in mice treated with L-buthionine sulfoximine (BSO) and MTZ. Mice were administered BSO alone (3 mmol kg^{-1}, i.p.) (A, B), MTZ alone (15 mg kg^{-1}, p.o.) (C, D) or MTZ (15 mg kg^{-1}, p.o.) 1 h after pre-treatment with BSO (C, D), and plasma and liver tissue were collected 6 h after administration. Plasma AST and ALT (A, C) and hepatic GSH levels (B, D) were measured. Data are shown as the means ± SD of results from five mice. Differences compared with the control group or the MTZ alone (15 mg kg^{-1}) group were considered significant at $^*P < 0.05$. Histopathological examination of the liver (E–H). Liver tissue sections from mice treated with BSO alone (E, F) or BSO and MTZ (15 mg kg^{-1}) (G, H) were stained with hematoxylin–eosin (HE) or immunostained with an anti-MPO antibody. Arrows indicate MPO-positive cells. C, central vein; N, necrotic area. The numbers of MPO-positive cells were counted throughout an entire section from each of three mice and are expressed as the numbers of cells per square millimeter. The numbers of MPO-positive cells were compared with mice administered BSO (I). In the BSO and MTZ (15 mg kg^{-1}) treated groups, the numbers of MPO-positive cells were significantly increased compared with the group treated with MTZ at 15 mg kg^{-1} alone ($^*P < 0.05$) (J).

treated with BSO and MTZ at 15 mg kg^{-1} compared with mice treated with MTZ alone at 15 mg kg^{-1} (Fig. 4D). The histopathological changes observed in the mouse liver 6 h after MTZ administration are shown in Fig. 4G and H. Using HE staining, centrilobular degeneration and necrosis were observed in mice treated with BSO and MTZ at 15 mg kg^{-1} (Fig. 4G). However, no histopathological changes were observed in mice treated with MTZ at 15 mg kg^{-1} alone (data not shown). In the immunohistochemical analysis, a large number of cells was observed to react to the anti-MPO antibody 6 h after MTZ administration (Fig. 4H). The number of MPO-positive cells was remarkably increased in mice treated with BSO and MTZ at 15 mg kg^{-1} compared with mice treated with MTZ 15 mg kg^{-1} alone (Fig. 4J). There was no difference in the number of MPO-positive cells between the control and BSO groups (Fig. 4I).

To investigate whether the liver injury detected in mice treated with BSO and MTZ at 15 mg kg^{-1} was related to immunological factors, the changes in the levels of mRNA expression for transcription factors, cytokines and chemokines, hepatic GATA-3, IL-5, TNFα, eotaxin, MIP-2, Cxcl-1 and MCP-1 as well as plasma IL-4 protein were measured (Fig. 5). The levels of IL-5 and TNFα mRNA were not altered following co-administration of BSO and MTZ at 15 mg kg^{-1} (Fig. 5B and C). However, GATA-3, eotaxin and MCP-1 mRNA expression was markedly increased at 6 h following treatment of mice with BSO and MTZ at 15 mg kg^{-1} (Fig. 5A, D and G). The plasma IL-4 level was elevated (Fig. 5H). These effects were not observed in response to the administration of BSO alone (data not shown).

Effects of Anti-IL-4 Antibody Administration in Mice Treated with BSO and MTZ

To investigate whether IL-4 was involved in the liver injury observed in mice treated with BSO and MTZ, we conducted a neutralization study. In the neutralization study, intraperitoneal injection of a monoclonal anti-mouse IL-4 antibody (100 μg per mouse) 1 h prior to MTZ administration significantly reduced the plasma AST and ALT levels detected 6 h after MTZ administration (Fig. 6A). No significant difference was observed in the hepatic GSH levels among the three groups (Fig. 6B). In histopathological analyses, centrilobular degeneration, necrosis and infiltration of neutrophils were found to be attenuated in mice treated with the anti-mouse IL-4 antibody (Fig. 6C) compared with mice treated with rat IgG2a (Fig. 6C). Additionally, the infiltration of cells reacting to the anti-MPO antibody was attenuated in mice treated with the anti-mouse IL-4 antibody (Fig. 6C), and the number of MPO-positive cells was decreased in these mice (Fig. 6D). There were no significant differences in the hepatic mRNA expression levels of TNFα, MIP-2, Cxcl-1 and MCP-1, but the expression of hepatic eotaxin mRNA was significantly decreased in mice treated with the anti-mouse IL-4 antibody compared with mice treated with BSO and MTZ at 15 mg kg^{-1} (Fig. 7A–D). The mRNA expression levels of GATA-3 in mice treated with the anti-mouse IL-4 antibody were decreased compared with those in mice treated with BSO and MTZ at 15 mg kg^{-1}, although this difference was not statistically significant (Fig. 7F, P-value = 0.06). The plasma IL-4 level in mice treated with the anti-mouse IL-4 antibody was significantly decreased compared with that in mice

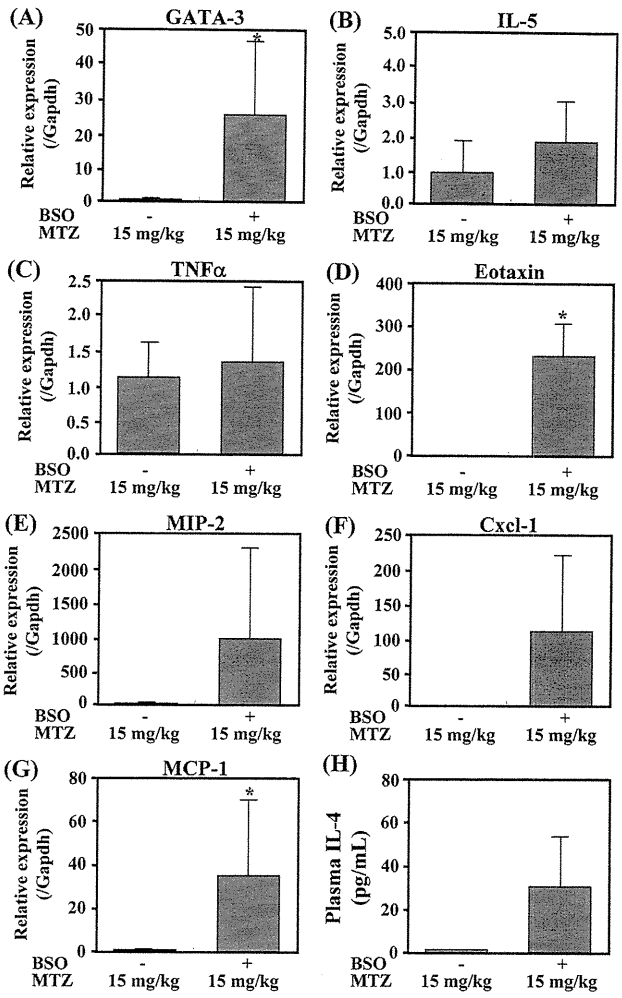

Figure 5. Effect of BSO pre-treatment on the hepatic mRNA expression of a transcription factor, cytokines and chemokines as well as plasma IL-4 protein levels 6 h after MTZ administration. Mice were administered MTZ alone (15 mg kg^{-1}, p.o.) or MTZ (15 mg kg^{-1}, p.o.) 1 h after pre-treatment with BSO, and liver tissue was collected 6 h after administration. The relative expression levels of hepatic mRNA for GATA-3 (A), IL-5 (B), TNFα (C), eotaxin (D), MIP-2 (E), Cxcl-1 (F) and MCP-1 (G) were measured using real-time RT-PCR and normalized to Gapdh mRNA. The fold induction of the mRNA level is shown compared with nontreated control mice. The plasma IL-4 level (H) was measured using ELISA. The limit of detection for plasma IL-4 was 3.9 pg ml^{-1}. Data are shown as the means ± SD of results from five mice. Differences compared with the MTZ alone (15 mg kg^{-1}) group were considered significant at $^{*}P < 0.05$.

treated with BSO and MTZ at 15 mg kg^{-1} (Fig. 7G). These changes were not observed following the administration of rat IgG2a.

DISCUSSION

Reactive metabolite formation followed by covalent binding is believed to be associated with idiosyncratic toxicity, possibly through immune-related mechanisms. Reactive metabolites of MTZ biotransformed by both P450 and FMO are known to bind covalently to proteins and inactive P450 (Lee and Neal, 1978; Kedderis and Rickert, 1985). The

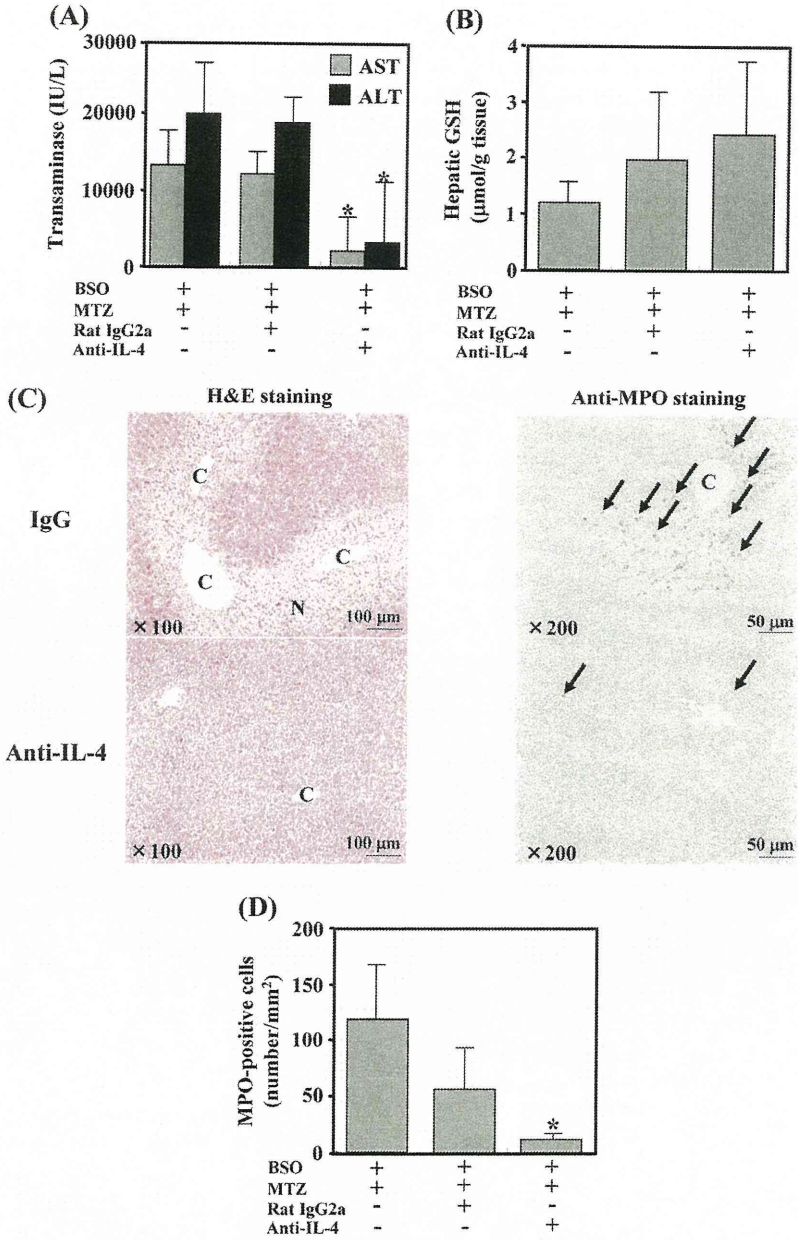

Figure 6. Effect of an anti-mouse IL-4 antibody on plasma AST and ALT, hepatic GSH levels, histopathological presentation of liver injury and quantification of hepatic MPO-positive cells in mice treated with BSO and MTZ. Mice were injected with a monoclonal anti-mouse IL-4 antibody (100 μg per mouse, i.p.) or IgG2a 1 h prior to MTZ administration (15 mg kg^{-1}), and plasma and liver tissue were collected 6 h after MTZ administration. Plasma AST and ALT (A) and hepatic GSH levels (B) were measured. Data are shown as the means ± SD of results from five or six mice. Differences compared with the BSO and MTZ (15 mg kg^{-1}) treated group were considered significant at $^*P < 0.05$. Histopathological examination of the liver (C). Liver tissue sections were stained with HE or immunostained with an anti-MPO antibody. Arrows indicate MPO-positive cells. C, central vein; N, necrotic area around central vein. The numbers of MPO-positive cells were counted throughout an entire section in each of three mice and are expressed as the numbers of cells per square millimeter. The numbers of MPO-positive cells were compared with those in mice administered BSO and MTZ (15 mg kg^{-1}) (D). In the groups administered the anti-mouse IL-4 antibody, the numbers of MPO-positive cells were significantly decreased compared with the BSO and MTZ (15 mg kg^{-1}) treated group ($^*P < 0.05$) (D).

resulting metabolites are likely to be detoxified by GSH (Mizutani *et al.* 1999). It has been reported that glucocorticoids improve MTZ-induced severe cholestatic jaundice in humans (Zhang *et al.*, 2010), suggesting that MTZ-induced liver injury may involve immune-mediated reactions. In the present study, we investigated whether immune-mediated factors play an important role in MTZ-induced acute liver injury.

An acute toxicity analysis demonstrated that the oral LD_{50} value for MTZ in mice is 860 mg kg^{-1} (7.5 mmol kg^{-1}; Brock and Lorenz, 1954). A lethal dose of MTZ appears to cause small fatty and necrotic changes in hepatocytes. In this study,

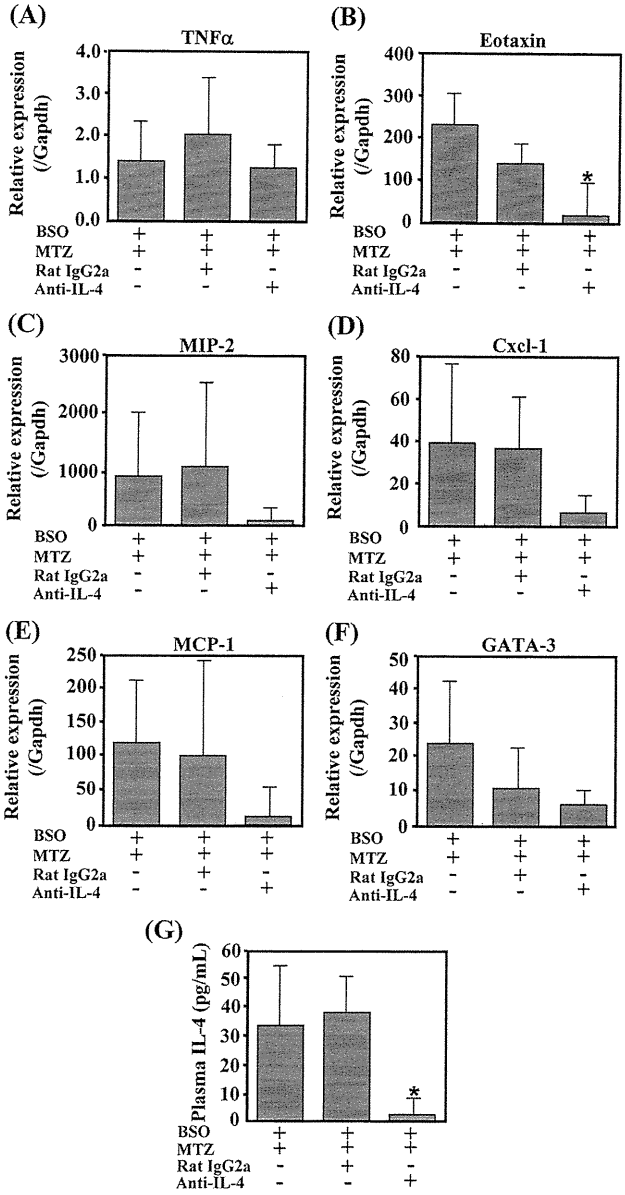

Figure 7. Effect of an anti-mouse IL-4 antibody on the hepatic mRNA expression of a cytokine, chemokines and a transcription factor as well as plasma IL-4 protein levels 6 h after BSO and MTZ administration. Mice were injected with a monoclonal anti-mouse IL-4 antibody (100 μg per mouse, i.p.) or IgG2a 1 h prior to MTZ administration (15 mg kg^{-1}), and liver tissue was collected 6 h after MTZ administration. The relative expression levels of TNFα (A), eotaxin (B), MIP-2 (C), Cxcl-1 (D), MCP-1 (E) and GATA-3 (F) were measured using real-time RT-PCR and normalized to the Gapdh mRNA level. The fold induction of the mRNA level is shown compared with nontreated control mice. The plasma IL-4 level (G) was measured using ELISA. Data are shown as the means ± SD of results from five or six mice. Differences compared with the BSO and MTZ (15 mg kg^{-1}) treated group were considered significant at $^{*}P < 0.05$.

the MTZ dose of 450 mg kg^{-1} was set at approximately one-half the oral LD_{50} value based on the results of experiments to determine the effects of MTZ doses of 860 (the oral LD_{50} in mice), 450 and 225 mg kg^{-1}. Following treatment of mice with an MTZ dose of 450 mg kg^{-1}, small numbers of MPO-positive cells were observed using an anti-MPO antibody (Fig.1), suggesting that neutrophil infiltration had occurred. Furthermore, changes in the hepatic expression of $CD4^{+}$ T cell-related transcription factors and cytokines were measured. The expression of GATA-3, IL-5, IL-4 and MIP-2 was significantly increased in MTZ-treated mice (Figs 2C, D and 3C), suggesting that Th2-related immune factors are activated, even in mice with a mild liver injury.

To establish a more sensitive mouse model for MTZ-induced acute liver injury, we adapted a BSO pre-administration method. Previously, Mizutani *et al.* (1999) reported that serum ALT levels were 76.5 and 3,150 IU l^{-1} in ICR mice treated with MTZ alone at a dose of 228 mg kg^{-1} or with BSO (3 mmol kg^{-1}, i.p.), and MTZ at a dose of 22.8 mg kg^{-1}, respectively. Using the BSO-enhanced mouse model, DILI is expected to be more clearly detectable. In the present study, the dose of MTZ (15 mg kg^{-1}) was reduced to one-thirtieth of the dose without BSO pre-treatment, resulting in greatly increased levels of AST (44 ± 5 and 13 184 ± 4500 IU l^{-1} in the MTZ 15 mg kg^{-1} alone and BSO and MTZ 15 mg kg^{-1} groups, respectively) and ALT (21 ± 8 and 20 424 ± 7180 IU l^{-1} in the MTZ 15 mg kg^{-1} alone and BSO and MTZ 15 mg kg^{-1} groups, respectively). The difference in the degree of enhancement of liver injury following BSO pre-treatment observed in the present study compared with Mizutani's data may be due to the different mouse strains used. Moreover, in the present study, the hepatic GSH level following administration of BSO alone (Fig. 4B) appeared to be lower than that found in other studies (Mizutani *et al.*, 1999; Shimizu *et al.*, 2009). This might be the reason why no further decrease in the hepatic GSH level was observed in mice treated with BSO and MTZ (15 mg kg^{-1}) compared with mice treated with BSO alone.

The levels of Th2-related factors, such as IL-4, GATA-3 and eotaxin, were clearly higher in mice treated with BSO and MTZ (15 mg kg^{-1}) compared with those treated with MTZ alone (450 mg kg^{-1}), and a Th1/Th2 imbalance may be involved in MTZ-induced acute liver injury. IL-4 is a multifunctional Th2 cytokine that is reported to play a pivotal role in concanavalin (Con) A-induced liver injury (Jaruga *et al.*, 2003) and to promote the hapten-induced pro-inflammatory responses associated with trifluoroacetyl chloride-induced liver injury (Njoku *et al.*, 2009). In the present study, we found that the plasma IL-4 level was significantly increased by the administration MTZ alone and by co-treatment with BSO. IL-5 is involved in allergic inflammation (Kay, 2001) and plays a critical role in recruiting eosinophiles (Coffman *et al.*, 1989). In this study, changes in IL-5 expression were not observed to be correlated with the severity of liver injury. Moreover, no eosinophil infiltration of the liver was observed in the BSO-enhanced mouse model, suggesting that IL-5 may not play an essential role in this process.

It is well known that hepatic GSH plays an important role in the detoxification of active metabolites. The activity of hepatic glutathione *S*-transferase (GST) and the hepatic GSH content are 10–20 times and approximately 2 times higher in mice than in humans, respectively (Grover and Sims, 1964; Woodhouse *et al.*, 1983; Watanabe *et al.*, 2003), suggesting that the GSH-depleted animal model could sensitively detect DILI. In the present study, the GSH-depleted mouse model presented significantly increased sensitivity to MTZ-induced DILI at one-thirtieth of the dose

without BSO pre-treatment. Thus, this GSH-depleted mouse model would be useful for testing certain drugs to elucidate the mechanisms of DILI.

It has been reported that the expression of hepatic cytochrome P450s was markedly suppressed in an interleukin-concentration dependent manner (Siewert *et al.*, 2000) and that the detoxification abilities of Phase II enzymes were decreased by interleukins (Romero *et al.*, 2002). Therefore, interleukin-knockout mice should be carefully applied in this kind of study. This is one reason why we performed an IL-4 neutralization study using an anti-mouse IL-4 antibody, instead of using interleukin-knockout mice. In a previous study by our group addressing dicloxacillin-induced acute liver injury (Higuchi *et al.*, 2011), the appropriate dose of the anti-IL-4 antibody was found to be 100 μg per body, and the timing of anti-IL-4 injection was 1 h before drug administration. In this study, neutralization of IL-4 significantly attenuated hepatotoxicity in mice treated with BSO and MTZ (Fig. 6A), suggesting that IL-4 is mainly involved in MTZ-induced acute liver injury.

Eotaxin is a CC chemokine that is known to stimulate the migration of eosinophils by acting on CC chemokine receptor 3 (Forssaman *et al.*, 1997; Rankins *et al.*, 2000) and other leukocytes and lymphocytes via other chemokine receptors (Menzies-Gow *et al.*, 2002). In the present neutralization study, the hepatic mRNA expression level of eotaxin was significantly decreased, and MTZ-induced acute liver injury was attenuated (Fig. 7B). Expression of eotaxin was reported to be induced by IL-4 in primary mouse hepatocytes, sinusoidal cells and HepG2 cells (Jaruga *et al.*, 2003). Depletion of eotaxins markedly attenuated Con A-induced liver injury (Jaruga *et al.*, 2003), which closely resembles the pathology of human autoimmune hepatitis (Tiegs *et al.*, 1992). In addition, the recruitment of tissue leukocytes, which are primarily attracted to tissues during drug-induced liver injury in humans, might depend on eotaxin expression (Pham *et al.*, 2001). Based on these and similar reports, it was proposed that decreased IL-4 levels attenuated DILI via decreasing eotaxin in the neutralization study.

With respect to investigating the mechanisms underlying DILI, lipopolysaccharide (LPS)-treated rodents are known to be sensitive to human hepatotoxic drugs, such as sulindac, chlorpromazine and trovafloxacin (Zou *et al.*, 2009; Shaw *et al.*, 2009). Various cytokines are upregulated after activation of Toll-like receptor 4 by LPS (Shaw *et al.*, 2009). However, the involvement of Th2 factors in DILI in LPS-treated rodents has never been reported. Thus, LPS-treatment methods are probably not appropriate for studying Th2 factor-related DILI.

In the present study, we first demonstrated that IL-4 mediated immune responses are involved in MTZ-induced acute liver injury in mice. Investigation of immune-related factors using a GSH depletion animal model might be useful to elucidate the mechanisms of DILI and to develop drugs by revealing the potential for DILI.

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