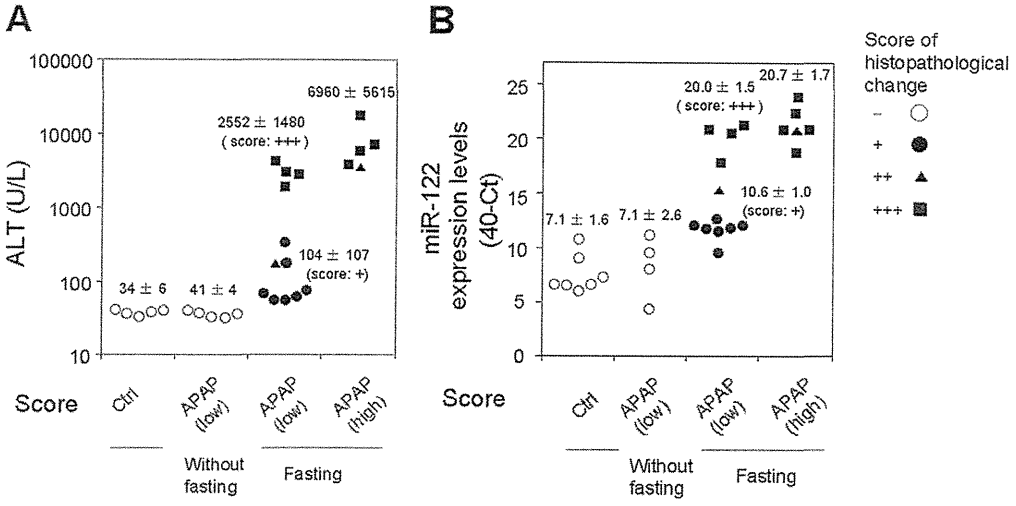

Figure 6. Association of plasma miR-122 level with hepatocellular injury. The extent of hepatocellular necrosis and inflammation was scored + (closed circle), ++ (closed triangle), and +++ (closed square) by histopathological examination, and was compared with the plasma ALT (A) and miR-122 (B) levels in rats administered 1000 mg/kg (high dose) or 500 mg/kg (low dose) of APAP with fasting, low dose of APAP without fasting, and CMC (as a control).
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associated with proteins [32]. It seems that there are three types of miRNAs: miRNAs which are dominantly vesicle-associated, those which are dominantly associated with protein, and those which are equally distributed. In addition, the mechanisms by which miRNAs are taken up by cells are not fully understood. To understand the relationship between the miRNA expression profiles in plasma and those in liver, the complex export and import systems of the miRNAs in various organs should be clarified.

By the comparison of the miRNA expression profiles in rat models of various types of liver injury, we could identify miRNAs that could be specific and sensitive biomarkers of hepatocellular injury, cholestasis, steatosis, steatohepatitis, and fibrosis. It is conceivable that the plasma miRNAs would be a superior noninvasive biomarker in human that could distinguish the different types of liver injury to conventional biomarkers such as ALT and ALP, although the analysis to compare the plasma miRNA expression profiles in patients suffering from various type liver injury remains to be performed. The plasma miRNAs have a potential to be used to know the types of liver injury to decide appropriate therapy, or to know the progress or restoration of liver injury in clinics. In addition, the plasma miRNAs would be useful in drug development, since they could detect liver injury caused by treatment with drug candidates at the early stage, resulting in saving time and resources in nonclinical study.

In conclusion, the present study demonstrated that the expression profiles of plasma miRNAs differed according to the type of liver injury. Although earlier studies reported the changes of some miRNAs in plasma or tissues with disease using a single model, implying the possibility of associations with the development of disease, comparison of the miRNA expression profiles across models would be important for understanding the physiological implications of the miRNAs changes. We could identify miRNAs which could be specific and sensitive biomarkers of each type of liver injury (e.g. acute/chronic liver injury or hepatocellular injury/cholestasis/steatosis/steatohepatitis/fibrosis) using rat models. Further studies are warranted to elucidate whether the miRNAs could be used as biomarkers in patients with various types of liver injury.

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Author Contributions

Conceived and designed the experiments: YY MN ST TY. Performed the experiments: YY ST. Analyzed the data: YY MN ST. Contributed reagents/materials/analysis tools: YY MN ST KT. Wrote the paper: YY MN TF TY.

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Mechanism of Exacerbative Effect of Progesterone on Drug-Induced Liver Injury

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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 generally believed that women exhibit worse outcomes from DILI than men. Recently, we found that pretreatment of mice with estradiol attenuated halothane (HAL)-induced liver injury, whereas pretreatment with progesterone exacerbated it in female mice. To investigate the mechanism of sex difference of DILI, we focused on progesterone in this study. We found the exacerbating effect of progesterone in thioacetamide (TA), α-naphthylisothiocyanate, and dicloxacillin-induced liver injury only in female mice. Higher number of myeloperoxidase-positive mononuclear cells infiltrated into the liver and increased levels of Chemokine (C-X-C motif) ligand 1 and 2 (CXCL1 and CXCL2) and intercellular adhesion molecule-1 in the liver were observed. Interestingly, CXCL1 was slightly increased by progesterone pretreatment alone. Progesterone pretreatment increased the extracellular signal-regulated kinase (ERK) phosphorylation in HAL-induced liver injury. Pretreatment with U0126 (ERK inhibitor) significantly suppressed the exacerbating effect of progesterone and the expression of inflammatory mediators. In addition, pretreatment with gadolinium chloride ($GdCl_3$: inhibitor of Kupffer cells) significantly suppressed the exacerbating effect of progesterone pretreatment and the expression of inflammatory mediators. Moreover, post-treatment of RU486 (progesterone receptor antagonist) 1 h after the HAL or TA administration ameliorated the HAL- or TA-induced liver injury, respectively, in female mice. In conclusion, progesterone exacerbated the immune-mediated hepatotoxic responses in DILI via Kupffer cells and ERK pathway. The inhibition of progesterone receptor and decrease of the immune response may have important therapeutic implications in DILI.

Key Words: **drug-induced liver injury; CXCL1; sex difference; Kupffer cell; progesterone receptor antagonist.**

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. In most cases, the mechanisms of hepatotoxicity are not elucidated, but it is likely to arise from complex interactions among drug properties, daily dose, genetic variations, age, sex, diseases, and environmental factors (Chalasani and Björnsson, 2010; Li, 2002). In general, women are more susceptible to liver injury by therapeutic drugs than men. Seventy-four percent of all acute liver failure cases are women (Miller, 2001). It has been reported that 78% of DILI cases are in women and a significantly greater number of women show DILI than men (Björnsson and Olsson, 2005; DeValle *et al.*, 2006; Ostapowicz *et al.*, 2002). Although some reports described that female sex is not a predisposing factor for DILI, it was also reported that patients with severe DILI who underwent liver transplantation were more frequently women (76%) and that nearly 90% of patients with fulminant liver injury from DILI were women (Andrade *et al.*, 2005; Lucena *et al.*, 2009; Russo *et al.*, 2004). From these lines of study, women appear to be at greater risk of developing severe liver injury, but it is not clear why women exhibit the worst outcomes from liver injury.

It has been reported that women elicit more vigorous cellular and humoral immune reactions and suffer in greater numbers from autoimmune disease than men (Ansar *et al.*, 1985; Ostensen, 1999). Moreover, immune-mediated diseases in women may be exacerbated during the reproductive phase (Ansar *et al.*, 1985; Ostensen, 1999). Circulating levels of estradiol (E2) and progesterone fluctuate as a result of the reproductive phase and pregnancy in females (Barkley *et al.*, 1979; Wood *et al.*, 2007). There is evidence that the immune system is regulated by circulating level of sex steroid hormones, E2, progesterone, and testosterone (Grossman, 1985). It was also reported that E2 decreased and progesterone increased the production of proinflammatory cytokines in oxidative stress-stimulated murine peritoneal macrophage and human mononuclear cells and their receptor antagonists, ICI 182,780 and RU486, blocked these effects, respectively (Huang *et al.*, 2008; Yuan *et al.*, 2008). Recently, there have been many reports that immune reactions may have a critical role in DILI and that hepatic inflammation determines the

extent of liver injury (Adams *et al.*, 2010; Deng *et al.*, 2009; Holt and Ju, 2006). However, there has been little information concerning the involvement of female sex hormones in DILI. There are some reports that E2-attenuated liver injury caused by ischemia-reperfusion, trauma-hemorrhage, and acetaminophen (APAP) (Chandrasekaran *et al.*, 2011; Shimizu *et al.*, 2008; Yokoyama *et al.*, 2005), but there is little information about the effect of progesterone in liver injury.

We recently reported that the progesterone pretreatment exacerbated the immune-mediated hepatotoxic responses in halothane (HAL)-induced liver injury in female mice (Toyoda *et al.*, 2011). In this study, we investigated the underlying mechanism of the progesterone-induced exacerbation of DILI using a mouse model.

MATERIALS AND METHODS

Materials. HAL was purchased from Takeda Yakuhin (Osaka, Japan) and Isoflurane (ISO) was from Abbott Japan (Tokyo, Japan). Progesterone, gadolinium chloride ($GdCl_3$), and dicloxacillin (DCX) were purchased from Sigma-Aldrich (St Louis, MO). Mifepristone (RU486) and α-naphthylisothiocyanate (ANIT) were from Tokyo Kasei (Tokyo, Japan). U0126, SB203580, and thioacetamide (TA) were from Wako Pure Chemical Industries (Osaka, Japan). SP600125 was from Calbiochem (Los Angeles, CA). ICI 182,780 (ICI) was from TOCRIS Bioscience (Ellisville, MO). Fuji Dri-Chem slides of GPT/ALT-PIII and GOT/AST-PIII to measure alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT) and aspartate aminotransferase (AST)/glutamic oxaloacetic transaminase (GOT), respectively, were from Fuji Film Med. Co. (Tokyo, Japan). Rabbit polyclonal antibody against mouse myeloperoxidase (MPO) was from DAKO (Carpinteria, CA). Rat polyclonal antibody against F4/80 was from U.K.-Serotec (Oxford, U.K.). The monoclonal antibodies of anti-Thr202/Tyr204 phosphorylated extracellular signal-regulated kinase (ERK) 1/2, anti-Thr180/Tyr182 phosphorylated p38 mitogen-activated protein (MAP) kinase, and anti-Thr183/Tyr185 phosphorylated c-Jun N-terminal kinase (JNK) 1/2 were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal antibodies against ERK1/2 and JNK1/2 and the polyclonal antibody against p38 MAP kinase were also from Cell Signaling Technology. All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals were of the highest grade commercially available.

Animals. Female BALB/cCrSlc mice (8 weeks old, 20–25 g) were obtained from SLC Japan (Shizuoka, Japan). Animals 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 for a week before use for the experiments. Animal maintenance and treatment were performed 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.

Administration of hepatotoxic compounds in progesterone-pretreated mice. The progesterone pretreatment methods were described previously (Toyoda *et al.*, 2011). In brief, female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by the administration of HAL (15 or 30 mmol/kg, ip), TA (50 mg/kg, ip), ANIT (80 mg/kg, po), DCX (600 mg/kg, ip), or ISO (15 mmol/kg, ip) 1.5 h after the last treatment of progesterone. In the ANIT experiments, the mice were fasted for 15 h prior to the ANIT administration. Six hours after DCX administration and 24 h after HAL, TA, ANIT, or ISO administration, the mice were sacrificed, and the plasma and the liver were collected. The liver was fixed in buffered neutral 10% formalin and used for immunohistochemical staining. The degree of liver injury was assessed by hematoxylin-eosin (H&E) staining, and the plasma AST and ALT levels were determined using Fuji Dri-Chem 4000V (Fuji Film Med. Co.). The mononuclear cells infiltration was assessed by immunostaining for MPO as previously described (Kumada *et al.*, 2004).

Administration of HAL in U0126- or $GdCl_3$-pretreated mice. Mice were pretreated with progesterone for 7 days. In experiments using ERK inhibitor, mice were treated with U0126 (ERK inhibitor, 10 mg/kg, ip) 1 h before the HAL administration (30 mmol/kg, ip). In experiments using an inhibitor of Kupffer cells, mice were treated with $GdCl_3$ (10 mg/kg, iv) 24 and 48 h before the HAL administration (30 mmol/kg, ip). Twenty-four hours after the HAL administration, the mice were sacrificed. It was reported that a 40–61% reduction of the number of Kupffer cells in the mouse liver tissue occurred when treated with $GdCl_3$ in this method (Mosher *et al.*, 2001).

Administration of RU486 and HAL in mice. Mice were pretreated with RU486 (progesterone receptor antagonist, 50 μg/mouse, sc) for 7 days followed by HAL administration (30 mmol/kg, ip) 1.5 h after the last RU486 treatment, according to the method described previously (Toyoda *et al.*, 2011). In the experiments of postadministration of RU486, mice were administered RU486 (1 mg/kg, iv) 1 h after the HAL administration (30 mmol/kg, ip). Twenty-four hours after the HAL administration, the mice were sacrificed.

Real-time reverse transcription PCR analysis. RNA from mouse liver was isolated using RNAiso according to the manufacturer's instructions. Tumor necrosis factor α (TNFα), Chemokine (C-X-C motif) ligand 1 and 2 (CXCL1 and CXCL2), intercellular adhesion molecule-1 (ICAM-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time reverse transcription (RT)PCR. The primer sequences used in this study are shown in Table 1. The RT process and real-time PCR were performed as described previously (Kobayashi *et al.*, 2009).

Enzyme-linked immunosorbent assay. The CXC chemokines, CXCL1 and CXCL2, in plasma were measured by Quantikine Mouse CXCL1/KC ELISA and Quantikine Mouse CXCL2/MIP-2 ELISA (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's instructions.

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli (1970). Whole liver homogenates (50 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membranes were probed with the monoclonal antibodies of anti-ERK1/2, anti-JNK1/2, anti p38 MAP kinase, anti-Thr202/Tyr204 phosphorylated ERK1/2, anti-Thr183/Tyr185 phosphorylated JNK1/2, and anti-Thr180/Tyr182 phosphorylated p38 MAP kinase and the corresponding fluorescent dye–conjugated second antibody. An Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) was used for the detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.).

TABLE 1
Sequence of Primers Used for Real-Time RT-PCR Analyses in This Study

Target	Primer	Sequence
TNFα	FP	5′-TGT CTC AGC CTC TTC TCA TTC C-3′
	RP	5′-TGA GGG TCT GGG CCA TAG AAC-3′
CXCL1	FP	5′-GAT TCA CCT CAA GAA CAT CCA GAG-3′
	RP	5′-GAA GCC AGC GTT CAC CAG AC-3′
CXCL2	FP	5′-AAG TTT GCC TTG ACC CTG AAG-3′
	RP	5′-ATC AGG TAC GAT CCA GGC TTC-3′
ICAM-1	FP	5′-CAA GGA GAT CAC ATT CAC GG-3′
	RP	5′-CTT CCA GGG AGC AAA ACA AC-3′

Note. FP, Forward primer; RP, Reverse primer.

Cell culture and progesterone treatment. The mouse macrophage cell line RAW264.7 was kindly provided by Dr K. Miyamoto (Kanazawa University, Japan). RAW264.7 was maintained in Dulbecco's Modified Eagle's Medium from Nissui Pharmaceutical (Tokyo, Japan) containing 10% fetal bovine serum (BioWhitaker, Walkersville, MD), 3% glutamine, and 16% sodium bicarbonate in a 5% CO_2 atmosphere at 37°C. RAW264.7 were seeded at a density of 1×10^5 cells/well in 24-well plates with the medium containing the indicated concentration of progesterone and then incubated at 37°C. In experiments using MAP kinase inhibitors, cells were treated with 1μM of MAP kinase/ERK (MEK) 1/2 inhibitor U0126, p38 MAP kinase inhibitor SB203580, or JNK1/2 inhibitor SP600125. In other experiments, cells were treated with progesterone receptor antagonist RU486 or E2 antagonist ICI. After incubation, the cells were harvested, and total RNA was prepared using RNAiso according to the manufacturer's protocols.

Statistical analysis. Data are presented as mean ± SD. Comparison of the two groups was made with an unpaired two-tailed Student's *t*-test. Comparison of multiple groups was made with ANOVA followed by Dunnett or Tukey test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Progesterone Pretreatment on the Time-Dependent Changes of Plasma Transaminase Levels in HAL-Induced Liver Injury

To investigate the effects of progesterone pretreatment on the time-dependent changes of plasma transaminase levels in HAL-induced liver injury, mice pretreated with progesterone (0.3 mg/mouse, sc) were administered HAL (15 mmol/kg, ip), which resulted in a significant increase of the ALT and AST levels at 24 and 36 h after the HAL administration in female mice but not in male mice (Fig. 1A and Supplementary fig. 1A).

Effect of Progesterone Pretreatment on the Time-Dependent Changes of CXC Chemokines in HAL-Induced Liver Injury

To investigate whether the changes in liver injury in mice pretreated with progesterone after HAL administration resulted in increases of chemokines, we measured the hepatic messenger RNA (mRNA) expression and serum protein levels of CXCL1 and CXCL2. The hepatic CXCL1 and CXCL2 mRNA levels were markedly increased at 3 and 24 h after HAL administration in progesterone-pretreated mice, respectively (Fig. 1B). Interestingly, progesterone pretreatment alone increased CXCL1 mRNA (4.1-fold) and serum protein (2.3-fold) (0 h point in Figs. 1B and C). CXCL1 expression in response to HAL administration peaked at an earlier time point compared with CXCL2 expression. The time-dependent changes of the mRNA and protein levels were similar in CXCL1 and CXCL2. Thus, changes of mRNA expression were mainly followed in the subsequent experiments.

Among male mice, there was no marked difference in the mRNA expression levels of CXCL1 and CXCL2 after HAL administration in progesterone-pretreated mice compared with vehicle-pretreated mice (Supplementary fig. 1B). As with HAL-induced liver injury, the time-dependent changes of the transaminase levels and mRNA levels of CXCL1 and CXCL2 were similar to those associated with TA-induced liver injury (Supplementary fig. 2).

Effects of Progesterone Pretreatment on Various Hepatotoxic Compound–Induced Liver Injury

To investigate the effects of progesterone pretreatment on various compounds, TA, ANIT, DCX, or ISO were administered to the progesterone-pretreated mice. Female mice pretreated with progesterone showed significantly increased ALT and AST levels after the administration of TA, ANIT, or DCX, but not ISO, compared with vehicle-pretreated mice (Fig. 2A). However, male mice showed no effects on the transaminase levels by the administration of TA or ANIT as well as HAL (Supplementary fig. 3). Histopathological changes demonstrated that progesterone pretreatment enhanced TA- or ANIT-induced hepatocyte degeneration and damage. In addition, immunohistochemical analyses with anti-MPO antibody demonstrated that progesterone pretreatment increased the number of MPO-positive cells infiltrated in liver at 24 h after TA or ANIT administration (Fig. 2B). There was no change in either the histopathology findings or the number of MPO-positive cells in the liver of mice pretreated with progesterone alone. As with HAL-induced liver injury, hepatic mRNA level of CXCL1 and CXCL2 was increased significantly by progesterone pretreatment after TA, ANT, and DCX administration compared with vehicle-pretreated mice (Fig. 2C).

Effects of Progesterone Pretreatment on Activation of MAP Kinase-Signaling Pathway in HAL-Induced Liver Injury

MAP kinases, including ERK1/2, p38 MAP kinase, and JNK1/2, are important components for many intracellular signaling pathways. The phosphorylation of MAP kinases, which are required for the enzyme activity, activates signaling cascades, the downstream effects of which have been linked to the regulation of the inflammatory responses (DeFranco *et al.*, 1998). To clarify the role of the MAP kinase-signaling pathway in the liver after the HAL administration, the phosphorylation of ERK1/2, p38 MAP kinase, and JNK1/2 in liver were assessed by immunoblot analyses. Progesterone pretreatment alone significantly increased the phosphorylation of ERK in female mice but not in male mice (Fig. 3 and Supplementary fig. 4). The phosphorylation of ERK was also significantly increased in female mice by the HAL administration compared with control (Fig. 3). Progesterone pretreatment and HAL administration had no effect of the phosphorylation of p38 MAP kinase and JNK1/2 in female and male mice (data not shown).

Effects of ERK Pathway on Progesterone Pretreatment–Induced Exacerbation of HAL-Induced Liver Injury

To investigate whether the activation of the ERK pathway is involved in the progesterone pretreatment–induced exacerbation of liver injury, mice pretreated with progesterone were treated with an ERK inhibitor U0126, followed by the administration of

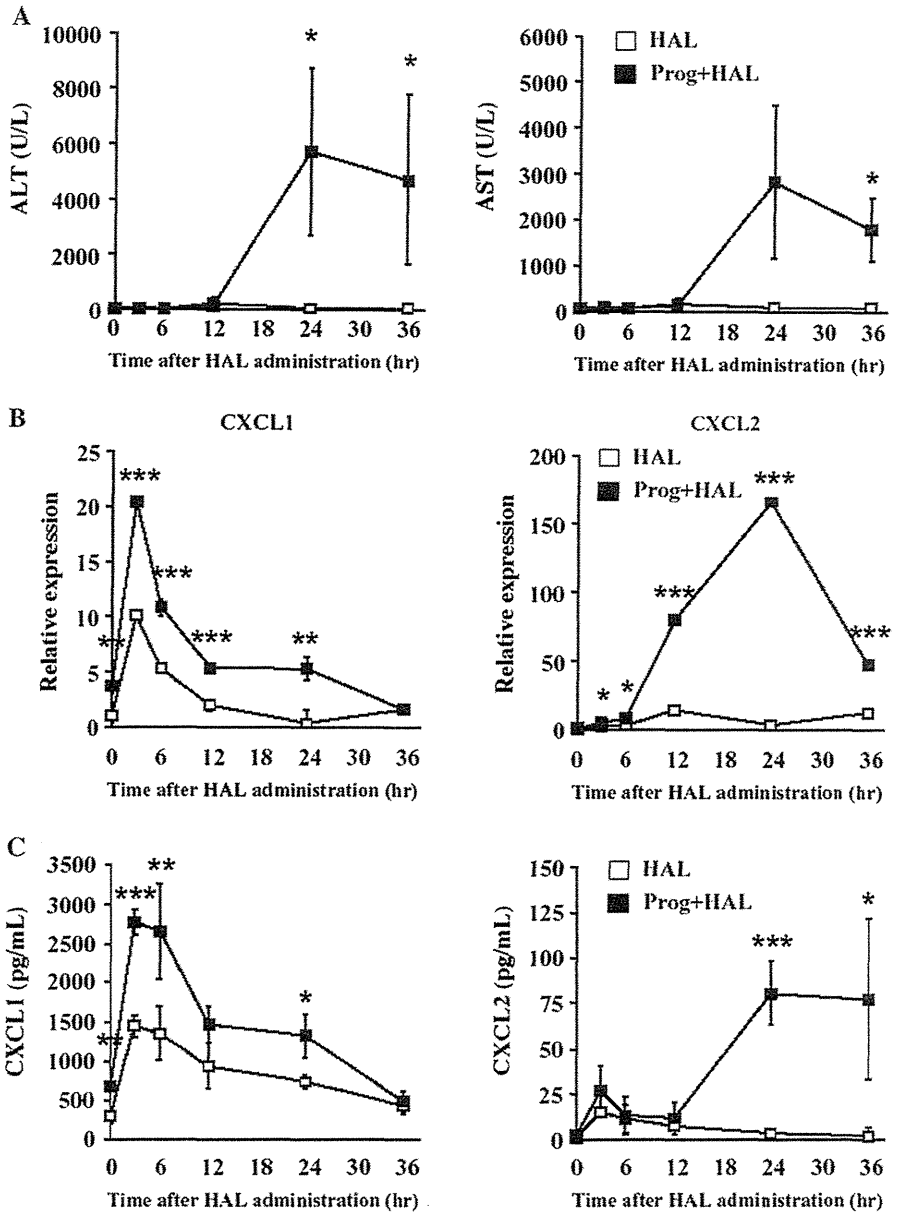

FIG. 1. Time-dependent effects of progesterone pretreatment on HAL-induced liver injury. Female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by HAL (15 mmol/kg, ip) administration 1.5 h after the last treatment of progesterone. Plasma and liver samples were collected 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

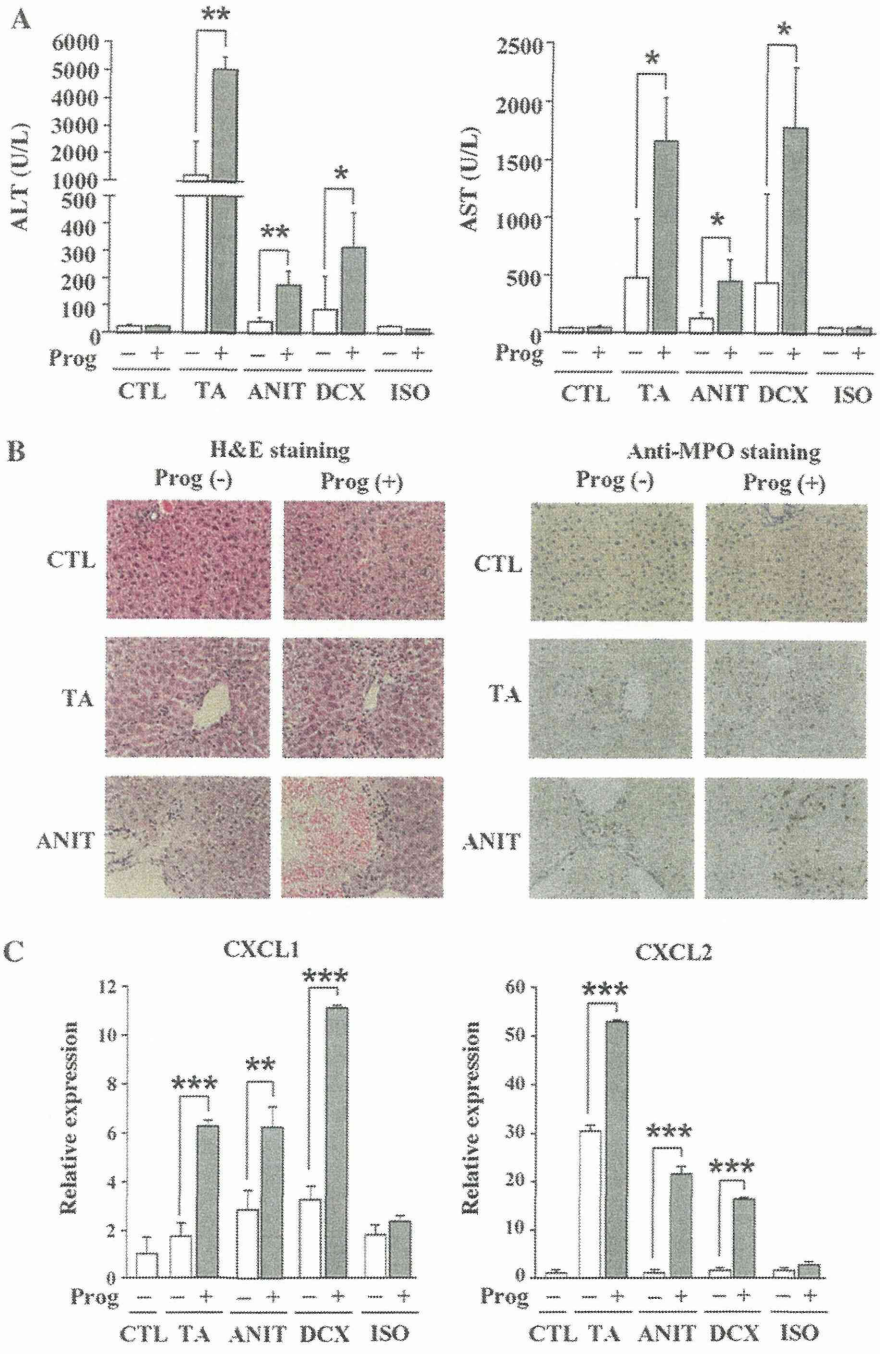

FIG. 2. Effects of progesterone pretreatment on various hepatotoxic compound–induced liver injury. Female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by TA (50 mg/kg, ip), ANIT (80 mg/kg, po), DCX (600 mg/kg, ip), or ISO (15 mmol/kg, ip) administration 1.5 h after the 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

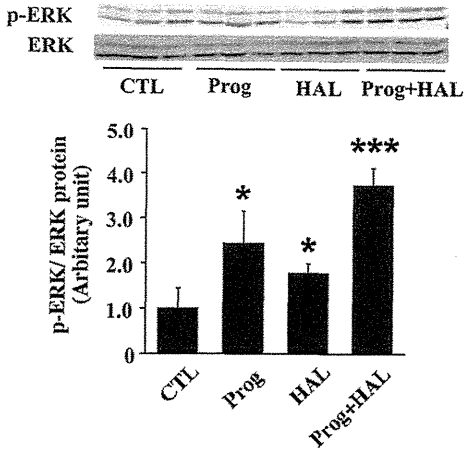

FIG. 3. Activation of ERK signaling pathways in liver after HAL administration in progesterone-pretreated female mice. Experimental conditions for animal treatments were the same as those in Figure 1. Female mice were pretreated with progesterone for 7 days followed by HAL administration 1.5 h after 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.

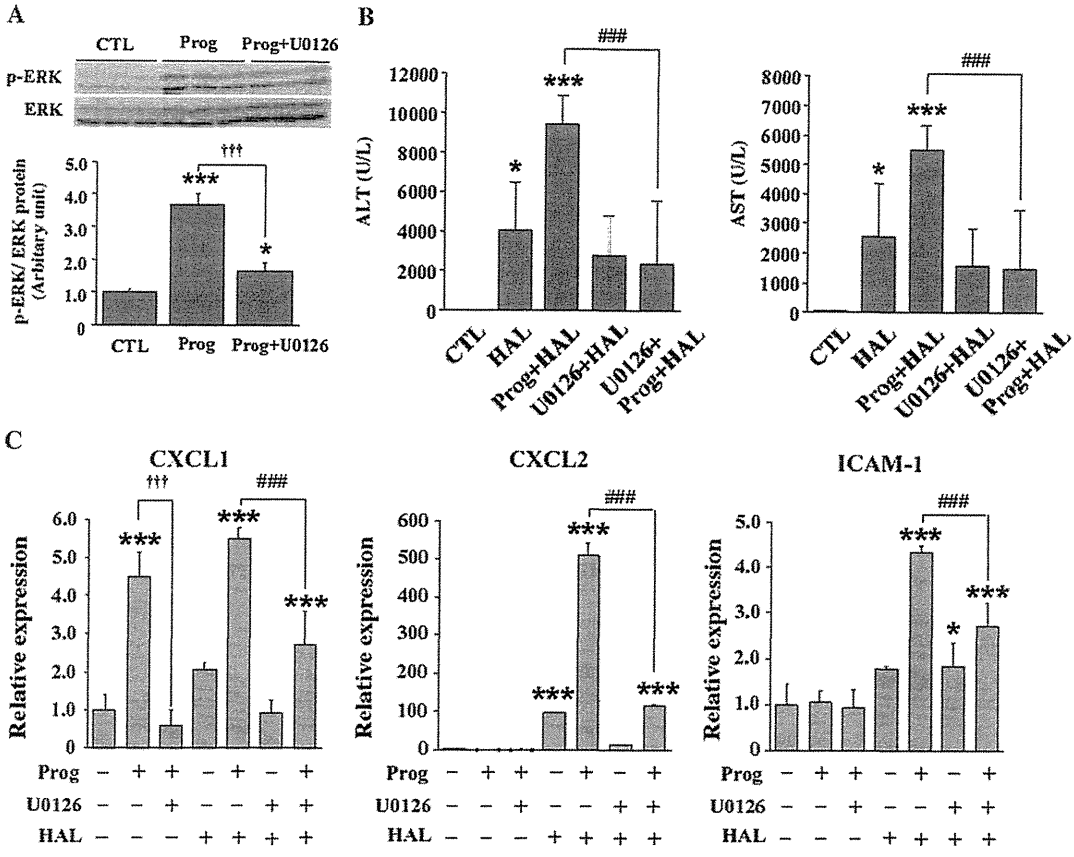

FIG. 4. Role of ERK pathway on the effect of progesterone pretreatment in HAL-induced liver injury. Female mice pretreated with progesterone for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last progesterone treatment, then U0126 (10 mg/kg, ip) was administered 1 h before the HAL administration. 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

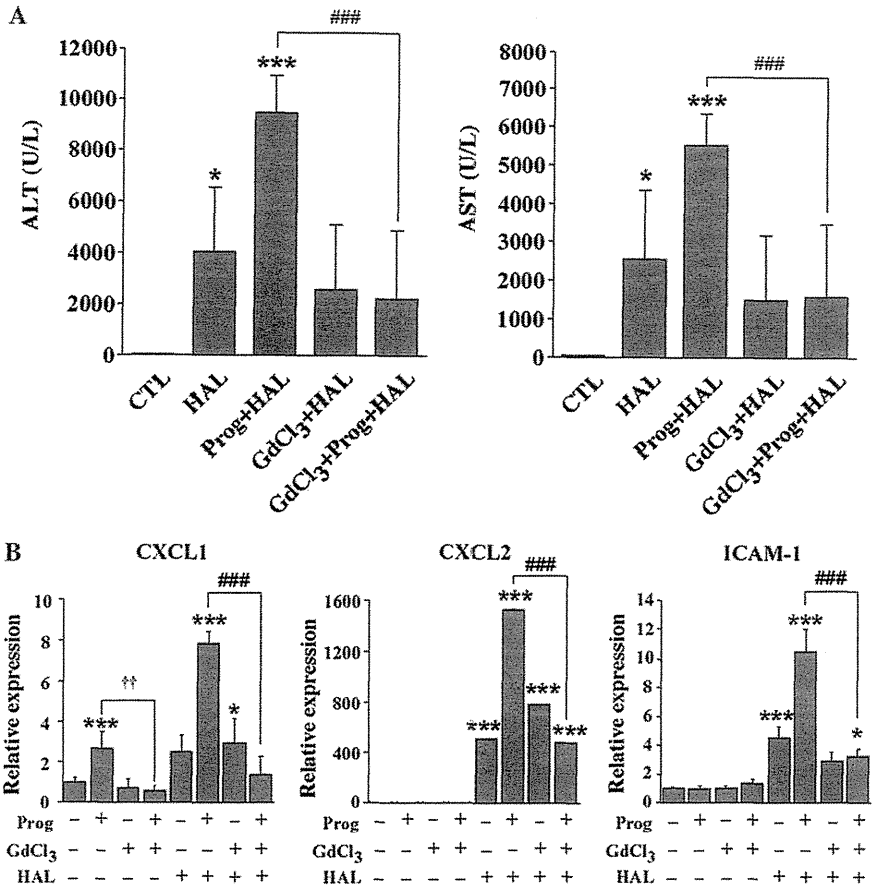

FIG. 5. Role of Kupffer cells in the effect of progesterone pretreatment in HAL-induced liver injury. Female mice pretreated with progesterone for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of progesterone. $GdCl_3$ (10 mg/kg, iv) was administered two times at 24 and 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. $††p < 0.001$, 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

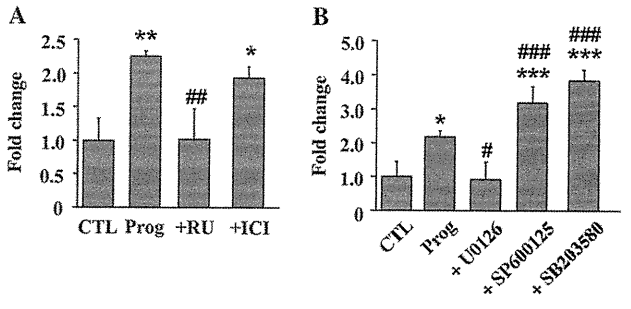

FIG. 6. Changes of the expression levels of CXCL1 mRNA in RAW264.7 cells treated with progesterone. The cells were treated with progesterone (0.1μM), progesterone and estrogen receptor antagonists (RU486 or ICI; 1.0μM) (A), and MAPK inhibitors (U0126, SP600125, SB203580; 1.0μM) (B). After 24-h incubation, 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

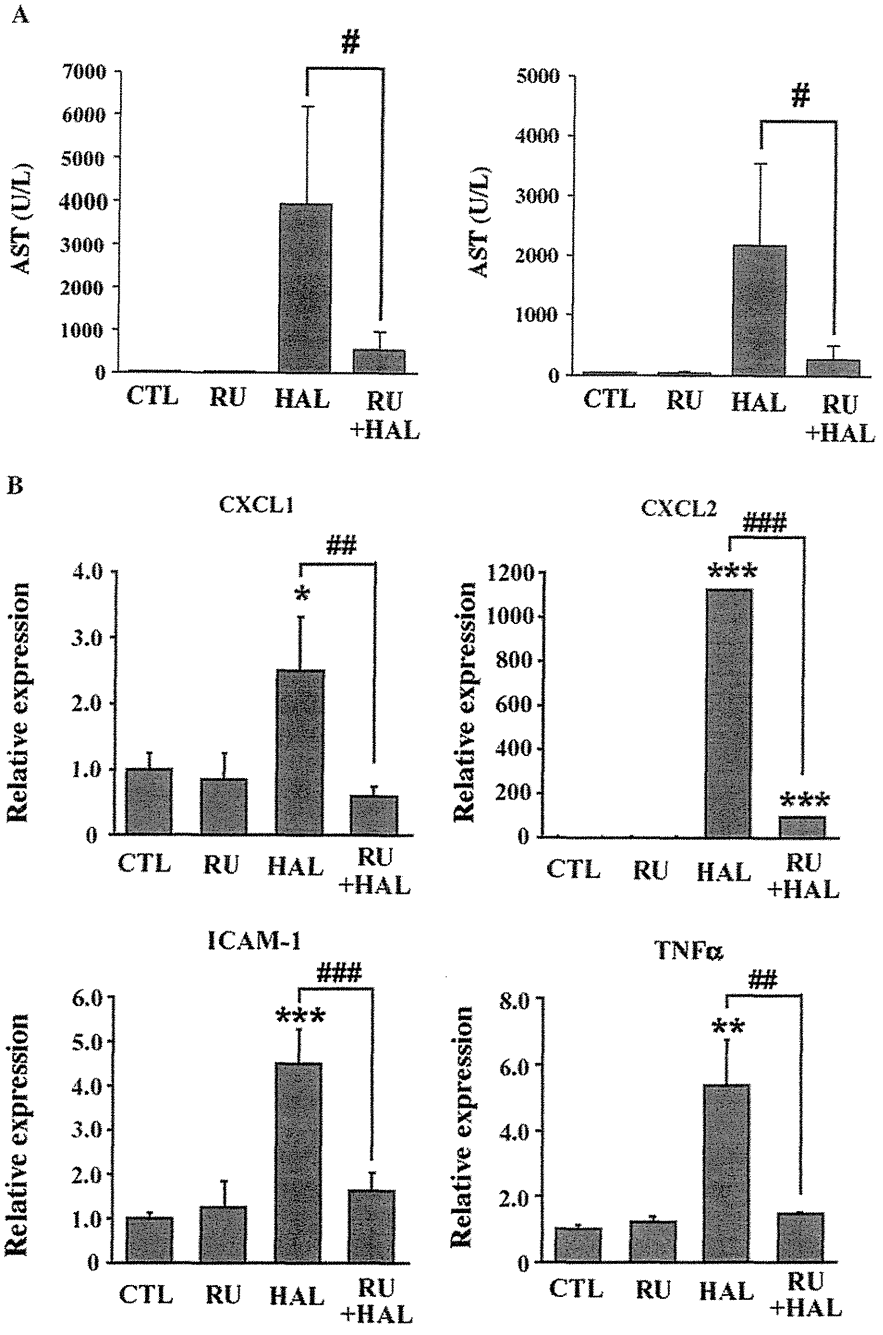

FIG. 7. Effects of RU486 pretreatment on HAL-induced liver injury. Female mice pretreated with RU486 (50 μg/mouse, sc) for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of RU486. Plasma and liver samples were collected for assessment of the transaminase levels (A) and the 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

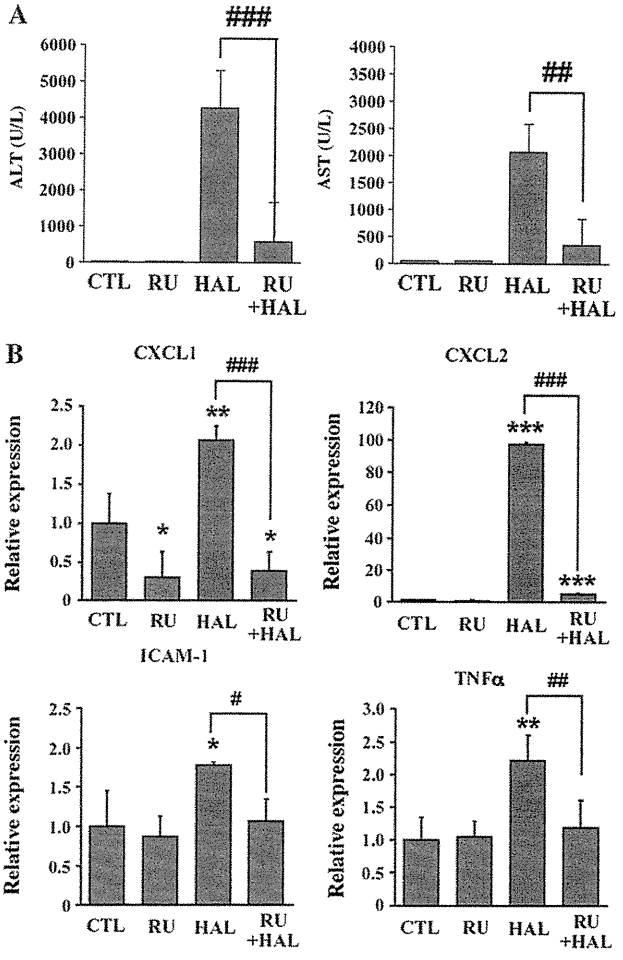

FIG. 8. Effects of RU486 posttreatment on HAL-induced liver injury. Female mice were administered HAL (30 mmol/kg, ip) and administered RU486 (1 mg/kg, iv) 1 h after the HAL administration. Plasma and liver samples were collected for assessment of the transaminase levels (A) and the expression of hepatic 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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Supplementary Material and Methods

Administration of hepatotoxic compounds to Prog-pretreated mice.

Male BALB/c mice were pretreated with Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by the administration of HAL (15 mmol/kg, *i.p.*), TA (50 mg/kg, *i.p.*) or ANIT (80 mg/kg) 1.5 h after the last Prog treatment. In the ANIT experiments, the mice were fasted for 15 h prior to the ANIT administration. Twenty-four hours after the administration of HAL, TA, or ANIT, the mice were sacrificed and the plasma and liver samples were collected.

Administration of TA in Prog-pretreated mice.

Female BALB/c mice were pretreated with Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by the administration of TA (50 mg/kg, *i.p.*) 1.5 h after the last Prog treatment. Mice were sacrificed and plasma and liver samples were collected 0, 3, 6, 12, 24 or 36 h after the TA administration.

Administration of RU486 and TA in mice.

Female BALB/c mice were administered RU486 (1 mg/kg, *i.v.*) 1 h after the TA (100 mg/kg, *i.p.*) administration. Twenty-four hours after the TA administration, the mice were sacrificed.

Supplementary Figure Legends

Supplementary Fig. 1. Time-dependent effects of Prog treatment on HAL-induced liver injury in male mice. Male mice were pretreated with Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by HAL (15 mmol/kg, *i.p.*) administration 1.5 h after the last treatment of Prog. Plasma and liver samples were collected 0, 3, 6, 12, 24 or 36 h after the HAL administration. Plasma transaminase levels were measured (A). Relative expression of hepatic mRNA was measured for CXC chemokines, adhesion molecule and TNFα. Expression of hepatic mRNA was normalized to Gapdh mRNA (B). The data are mean ± SD of 4 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with only HAL-administered mice.

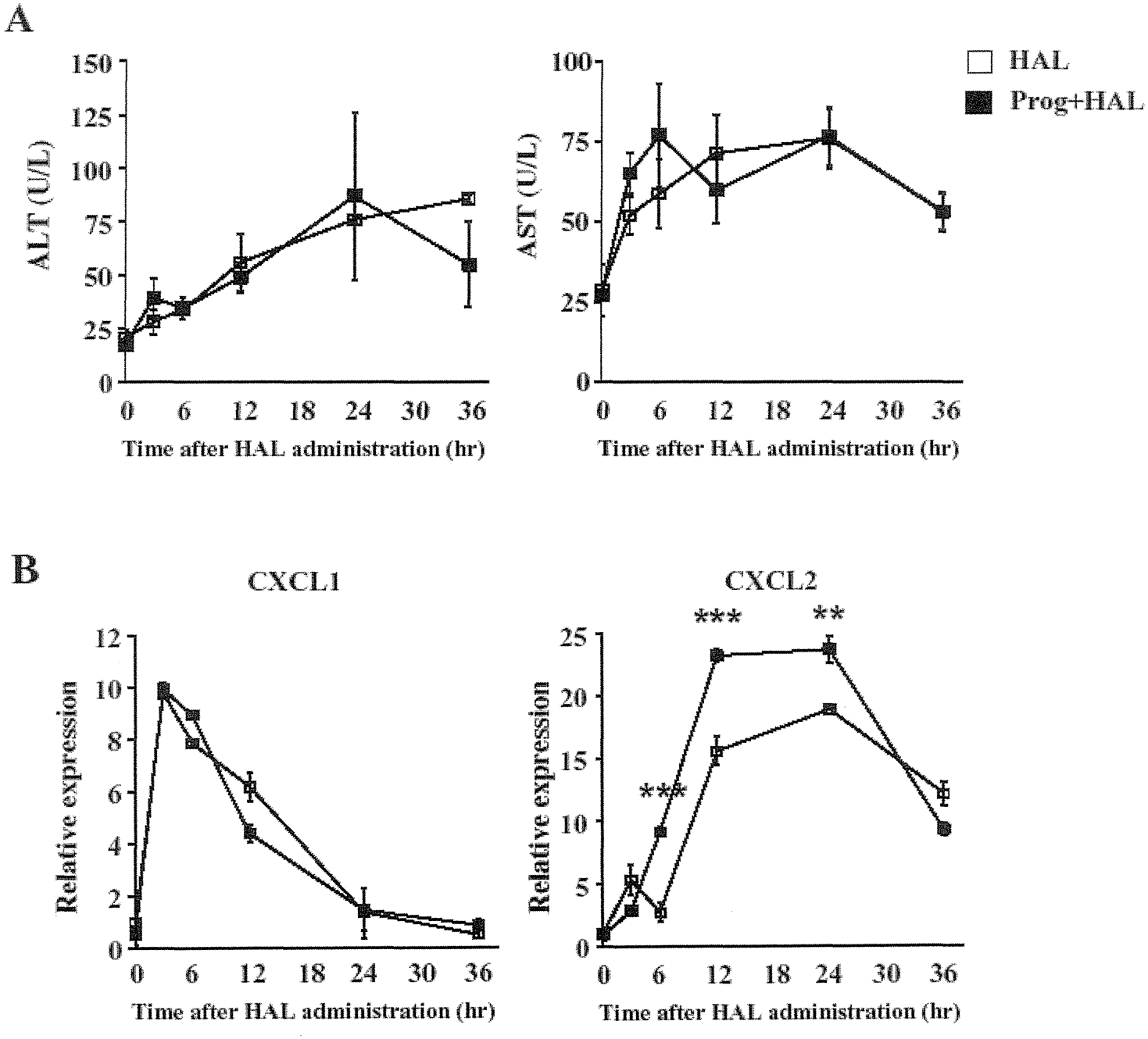
Supplementary Fig. 2. Time-dependent effects of Prog treatment on TA-induced liver injury. Female mice were pretreated with Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by TA (50 mg/kg, *i.p.*) administration 1.5 h after the last treatment of Prog. Plasma and liver samples were collected 0, 3, 6, 12, 24 or 36 h after the HAL administration. Plasma transaminase levels were measured (A). Relative expression of hepatic mRNA was measured for CXC chemokines and adhesion molecule. Expression of hepatic mRNA was normalized to Gapdh mRNA (B). The data are mean ± SD of 4 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with only TA-administered mice.

Supplementary Fig. 3. Effects of Prog pretreatment on various hepatotoxic compounds-induced liver injury. Male mice were pretreated with Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by HAL (15 mmol/kg, *i.p.*), TA (50 mg/kg, *i.p.*) or ANIT (80 mg/kg, *p.o.*) administration 1.5 h after the last treatment of Prog. Twenty-four hours after the administration of HAL, TA or ANIT, plasma was collected for assessment of the transamimase levels. The data are mean ± SD of 4 mice.

Supplementary Fig. 4. Activation of ERK signaling pathways in liver after HAL administration in Prog-pretreated male mice. Experimental conditions for animal treatments were the same as those in Supplementary Fig.1. Liver samples were collected 3 h after the HAL administration. Immunoblot of ERK proteins in whole liver homogenates was performed and quantified according to the same methods in Figure 3. Each lane showed an individual mouse (50 μg/lane). The data are mean ± SD of 3 mice.

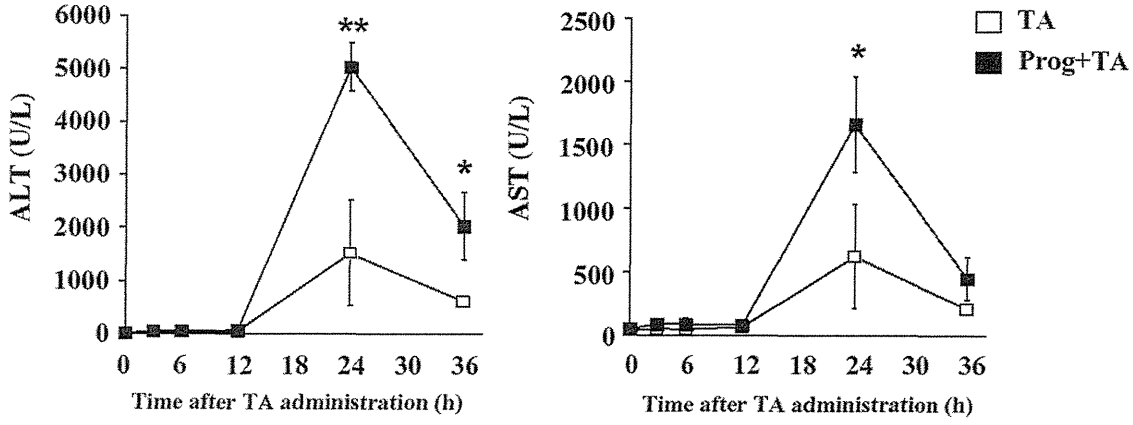
Supplementary Fig. 5. Effects of RU486 on TA-induced liver injury. Female mice were administered TA (100 mg/kg, *i.p.*) and administered RU486 (1 mg/kg, *i.v.*) 1 h after the TA administration. Plasma samples were collected for assessment of the transamimase levels 24 h after the TA administration. The data are mean ± SD of 5 mice. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

Supplementary Figure 1



Supplementary Figure 2

A



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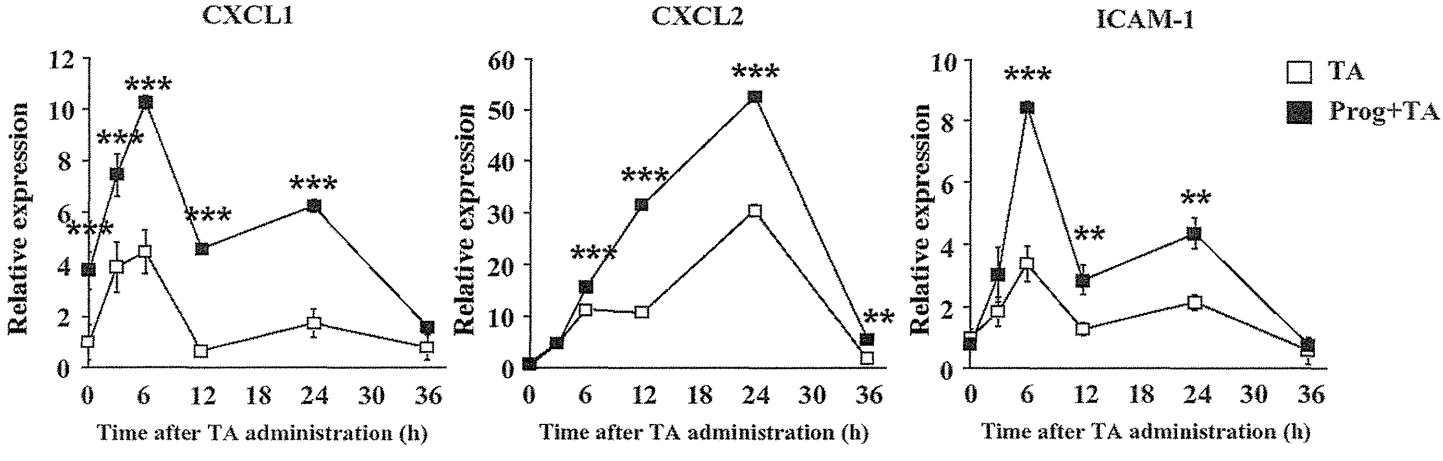

Supplementary Figure 3