

RESEARCH ARTICLE

Bee Venom Phospholipase A2 Protects against Acetaminophen-Induced Acute Liver Injury by Modulating Regulatory T Cells and IL-10 in Mice

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Abstract

The aim of this study was to investigate the protective effects of phospholipase A2 (PLA2) from bee venom against acetaminophen-induced hepatotoxicity through CD4⁺CD25⁺Foxp3⁺ T cells (Treg) in mice. Acetaminophen (APAP) is a widely used antipyretic and analgesic, but an acute or cumulative overdose of acetaminophen can cause severe hepatic failure. Tregs have been reported to possess protective effects in various liver diseases and kidney toxicity. We previously found that bee venom strongly increased the Treg population in splenocytes and subsequently suppressed immune disorders. More recently, we found that the effective component of bee venom is PLA2. Thus, we hypothesized that PLA2 could protect against liver injury induced by acetaminophen. To evaluate the hepatoprotective effects of PLA2, C57BL/6 mice or interleukin-10-deficient (IL-10^{-/-}) mice were injected with PLA2 once a day for five days and sacrificed 24 h (h) after acetaminophen injection. The blood sera were collected 0, 6, and 24 h after acetaminophen injection for the analysis of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). PLA2-injected mice showed reduced levels of serum AST, ALT, proinflammatory cytokines, and nitric oxide (NO) compared with the PBS-injected control mice. However, IL-10 was significantly increased in the PLA2-injected mice. These hepatic protective effects were abolished in Treg-depleted mice by antibody treatment and in IL-10^{-/-} mice. Based on these findings, it can be concluded that the protective effects of PLA2 against acetaminophen-induced hepatotoxicity can be mediated by modulating the Treg and IL-10 production.

Introduction

Acetaminophen is an effective antipyretic and analgesic drug that is commonly used. It is considered safe at its therapeutic dose, but it can cause severe hepatic necrosis, nephrotoxicity, additional hepatic lesions, and even death in experimental mice and humans when taken in high doses [1, 2]. Many researchers have attempted to demonstrate the mechanism underlying acetaminophen-induced acute injury, particularly the signaling pathways leading to tissue damage and toxicity in the liver [3, 4, 5, 6].

Tregs have been known to play a pivotal role in the maintenance of tolerance in the immune system, and Treg deficiency can be a cause of autoimmune disease [7]. Tregs also have various functions in the control of transplantation tolerance, tumor immunity, allergy, and infection [8, 9, 10].

Previous studies demonstrated that Tregs mediate therapeutic potential against immune-mediated hepatic injury [11, 12, 13]. The expression of anti-inflammatory factors, such as IL-10, has been found to be increased in the normal response to drug-induced liver injury [14]. The increased susceptibility to acetaminophen-induced hepatic injury appeared to be correlated with an elevated expression of proinflammatory cytokines, such as TNF and IL-6 [15].

PLA2 is known to be a major component of snake venoms and hydrolyzes the fatty acids in membrane phospholipids [16]. PLA2 from bee venom is a prototypic group III enzyme that hydrolyzes fatty acids, and it has been reported that melittin in bee venom enhances the activity of PLA2 [17, 18]. In addition, it has been demonstrated that this bee PLA2 prevents neuronal cell death and spinal cord injury [19, 20]. In this study, we demonstrate that PLA2 protects against hepatic dysfunction and induces antiinflammatory cytokine production in acetaminophen-injected mice by upregulation of the Treg population. Therefore, PLA2 may have therapeutic potential in preventing acetaminophen-induced hepatotoxicity.

Materials and Methods

Mouse

Male C57BL/6 mice (seven to eight weeks old, Charles River Korea, Seungnam, Korea), weighing 20–21 g each, were used in most of the experiments. Male $\text{Foxp3}^{\text{EGFP}}$ C57BL/6 mice (C. Cg-*Foxp3*^{tm2Tch}/J, six weeks old) and IL-10^{-/-} mice (B10.129P2(B6)-*Il10*^{tm1Cgn}/J, 7–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained under specific pathogen-free conditions with an air conditioning system and a 12-h light/12-h dark cycle. The mice had free access to food and water during the experiments. This research was approved by the Animal Care and Use Committee of Kyung Hee University (KHUASP (SE)-11-041). Mice were sacrificed by CO₂ asphyxiation.

Chemicals and treatment

PLA2 from honey bee venom and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Before the acetaminophen injection, the mice were intraperitoneally injected with PLA2 at a concentration of 0.2 mg/kg body weight once a day for five days. The control group received an equal volume of PBS. Acetaminophen was dissolved in PBS at a concentration of 20 mg/ml. Two days after the last administration of PLA2 or PBS, all of the mice received a single intraperitoneal (i.p.) injection of acetaminophen (500 mg/kg). The mice were sacrificed by CO₂ asphyxiation 24 h after the acetaminophen injection. Blood, spleen, and liver samples were obtained for further analysis.

Flow cytometry analysis

Splenocytes were isolated from Foxp3^{EGFP} mice for analysis of the Treg population change by PLA2 treatment. The cells were treated with PBS or PLA2 (0.01, 0.1, 1 and 10 µg/ml) and cultured in complete RPMI 1640 media containing 2.5 µg/ml anti-mouse CD3 antibody and 2 µg/ml anti-mouse CD28 antibody for 72 h. The cells were incubated with fluorescently tagged Abs for CD4 and CD25 staining (eBioscience, San Diego, CA, USA). The FACS data were acquired with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed by Cell Quest Pro (BD Biosciences, San Jose, CA, USA).

Assessment of serum AST, ALT and IL-10

Blood samples were collected 0, 6, and 24 h after the acetaminophen injection for the measurement of hepatic dysfunction by quantification of AST and ALT. The blood samples were maintained at room temperature for 1 h and then centrifuged for 10 min at 1,000 g to separate the serum. The AST and ALT levels were measured using a Fuji Dri-Chem 3500i instrument (Fuji Photo Film Ltd., Tokyo, Japan). The serum IL-10 level was measured by ELISA (BD Biosciences, San Jose, CA, USA).

H&E staining

The separated livers were fixed in 4% paraformaldehyde (PFA) for 1 day and then embedded in paraffin. The paraffin samples were sliced into 5-µm-thick slices and then deparaffinized. To observe the tissues, we stained the samples in hematoxylin for 90 s and dipped then slowly three times in eosin. After washing for 10 min in running water, the samples were covered with a cover glass. The portal and periportal areas in the liver were captured by microscopy.

Injection of anti-CD25 antibody for Treg depletion

Anti-mouse CD25 rat IgG1 (anti-CD25; clone PC61) antibody was generated from hybridomas collected from ATCC (Manassas, VA, USA). To deplete Tregs,

an anti-CD25 antibody (0.1 mg/mouse) was injected i.p. each day before the PLA2 and acetaminophen injections. The depletion of Tregs was confirmed by flow cytometry analysis using PE-anti-mouse CD25 and FITC-anti-mouse CD4 antibodies.

Assessment of proinflammatory cytokines and nitrite in the liver

Separated livers were maintained in a deep freezer (-70°C) to measure liver tissue inflammation after acetaminophen injection. Frozen liver tissues were homogenized in a protein extraction solution (PRO-PREP; Intron biotechnology, Sungnam, Korea), incubated for 30 min on ice and then centrifuged at 13,000 rpm (4°C) for 10 min. The TNF and IL-6 protein levels in the liver were measured by an enzyme linked immunosorbent assay (ELISA; BD Biosciences, San Jose, CA, USA). To measure the nitrite levels, the samples were incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The protein concentrations of the samples were measured by a BCATH Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The final concentrations were calculated with the total amount of protein, and the results are expressed as pg/mg or pmol/mg.

Statistical analysis

All of the results are expressed as the means \pm S.E.M. The data were analyzed using two-tailed *t* test or one-way ANOVA with Tukey's test. Differences were considered to be significant at $p < 0.05$.

Results

Upregulation of the Treg population in splenocytes by PLA2

To evaluate the immune-modulating effect of PLA2 in splenocytes, the splenocytes from Foxp3^{EGFP} mice were treated with PLA2 or PBS for three days. The Treg population was dose-dependently increased in the PLA2-treated group compared with the PBS-treated group ([Fig. 1](#)).

Protective effects of PLA2 on acetaminophen-induced hepatotoxicity

The mice were injected with a high dose of acetaminophen (500 mg/kg) after PLA2 pre-treatment. Blood samples were collected at 0, 6, and 24 h to measure the AST and ALT levels, and the liver tissues were separated 24 h after the acetaminophen injection for H&E staining. The hepatic cell death in the periportal area was protected by PLA2 injection. In addition, AST and ALT were significantly increased upon acetaminophen administration, and the PLA2 injection significantly suppressed the observed increases in AST and ALT ([Fig. 2](#)).

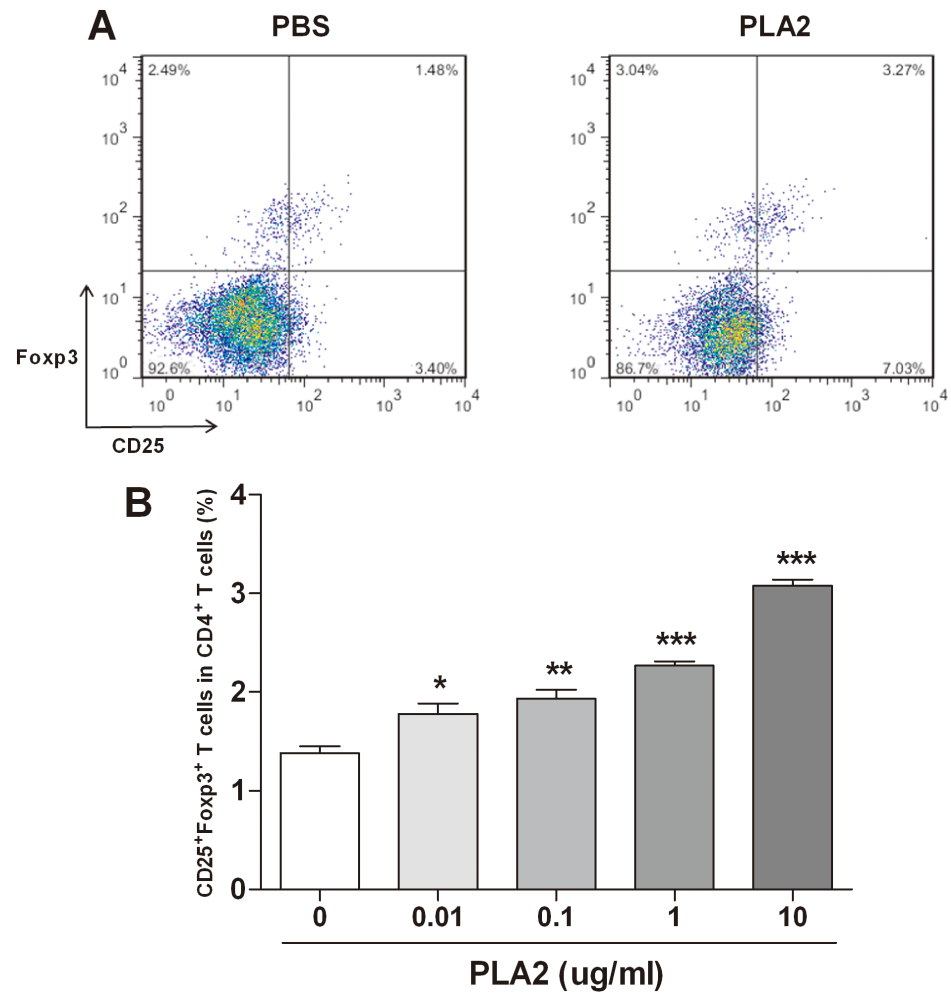


Figure 1. Increase of the Treg population in splenocytes by PLA2. Splenocytes from Foxp3^{EGFP} mice were treated with various concentrations of PLA2 and PBS for three days. The flow cytometry data showed a population of Tregs in the groups of PBS-treated and PLA2 (10 μ g/ml)-treated CD4⁺ T cells (A). The populations of CD25⁺Foxp3⁺ T cells treated with various concentrations of PLA2 are depicted as percentages of the total CD4⁺ T cells (B). The values shown indicate the means \pm S.E.M. *P<0.05 vs. PBS, **P<0.01 vs. PBS, ***P<0.01 vs. PBS.

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Hepatoprotective effects of PLA2 in Treg-depleted mice

To verify whether the effect of PLA2 on acetaminophen-induced hepatotoxicity is mediated by Tregs, we tested the PLA2 effects in a CD4⁺CD25⁺ T cell depletion model by administering mice with an anti-CD25 antibody (0.1 mg/mouse, i.p.). We measured the levels of AST and ALT in the serum. PLA2 treatment had no effect on liver histopathology, AST and ALT level in Treg-depleted mice. The hepatoprotective effects of PLA2 were diminished in Treg-depleted mice. These results demonstrated that Treg depletion eliminated the hepatotoxic protective effects of PLA2 and strongly suggest that the hepatoprotective effects of PLA2 were Treg-dependent (Fig. 3).

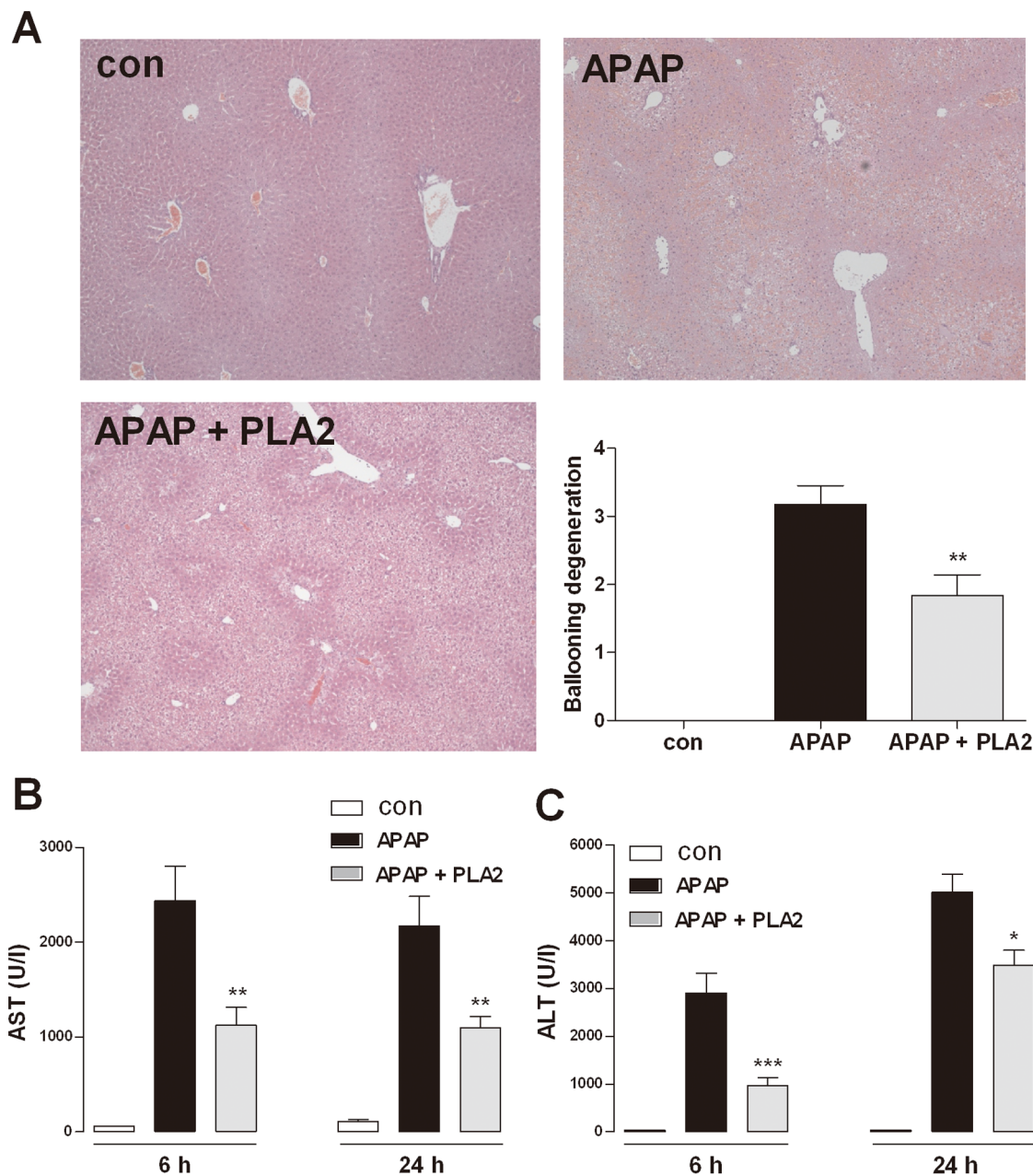


Figure 2. Protective effects of PLA2 on acetaminophen-induced hepatotoxicity. Mice were administered PLA2 (0.2 mg/kg) once a day for five days. The control group received the same volume of PBS. After the fifth administration of PLA2 or PBS, all of the mice received a single injection of acetaminophen (500 mg/kg). Blood samples were collected 0, 6 and at 24 h, and the mice were sacrificed under ether anesthesia 24 h after acetaminophen injection (n=10). Hepatic dysfunction was confirmed based on the AST (B) and ALT levels (C) and H&E staining (A). The values shown indicate the means \pm S.E.M. *P<0.05 vs. APAP, **P<0.01 vs. APAP, ***P<0.001 vs. APAP.

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Proinflammatory cytokines in the liver

To evaluate the antiinflammatory responses of PLA2 treatment in acetaminophen-induced hepatic injury, the levels of TNF, IL-6 and NO in the liver tissue were measured 24 h after acetaminophen injection. Acetaminophen-treated mice

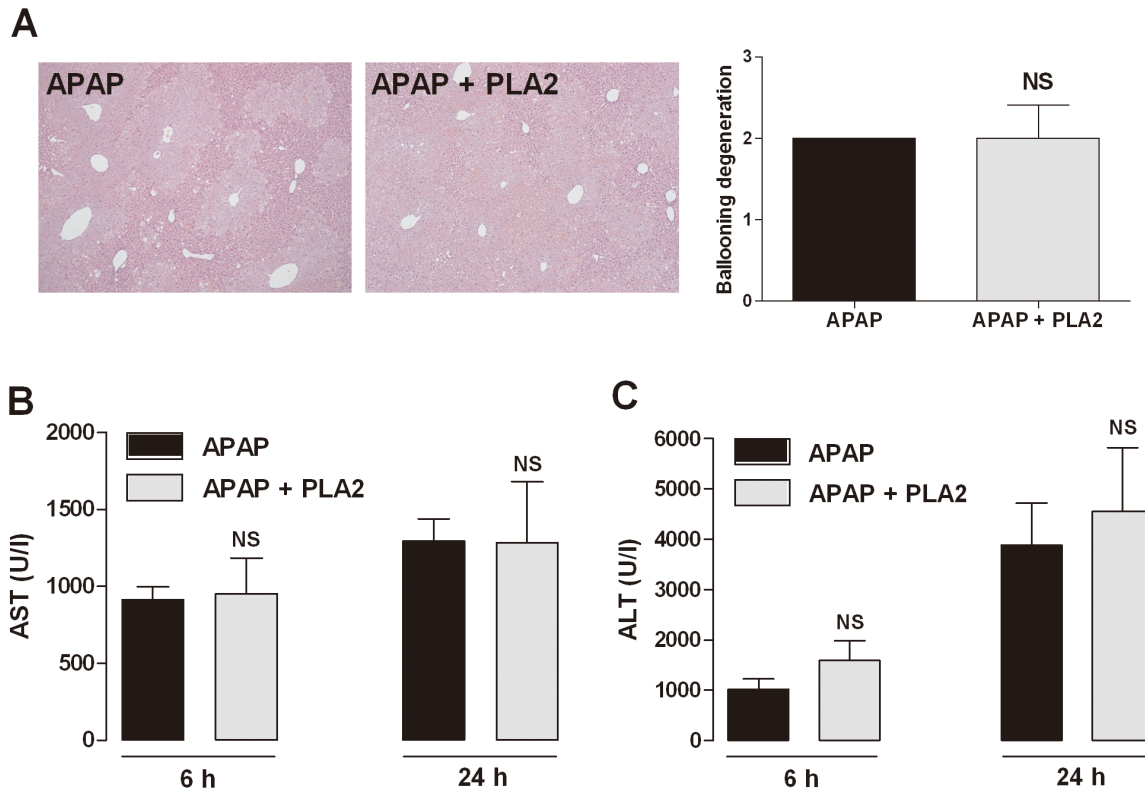


Figure 3. Effect of PLA2 in CD25-depleted mice. To deplete Tregs, an anti-CD25 antibody (0.1 mg/mouse) was injected i.p. each day before the PLA2 and acetaminophen injections. Blood was collected 6 and 24 h after the acetaminophen injection for the measurement of the AST (A) and ALT levels (B) (n=6). There was no difference between the PBS-treated (APAP) and PLA2-treated (APAP + PLA2) CD25-depleted mice.

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exhibited increased levels of TNF, IL-6 and NO. However, the PLA2-treated mice showed significantly lower levels of these inflammatory responses than the control mice, and Treg depletion incapacitated the protective effects of PLA2 (Fig. 4).

Hepatoprotective effects of PLA2 were mediated by IL-10 production

It has known that IL-10 protects against acetaminophen-induced liver injury and lethality [21]. PLA2 treatment significantly increased IL-10 production in acetaminophen-treated mice (Fig. 5A). To examine whether the hepatoprotective effect of PLA2 is dependent on IL-10, we used IL-10-deficient mice. We measured the levels of AST and ALT in the serum 24 h after acetaminophen injection. The results showed that the PLA2 effects on acetaminophen-induced hepatotoxicity were abolished in IL-10-deficient mice, suggesting that IL-10 is essential in the PLA2-mediated protective effects in hepatotoxicity (Fig. 5).

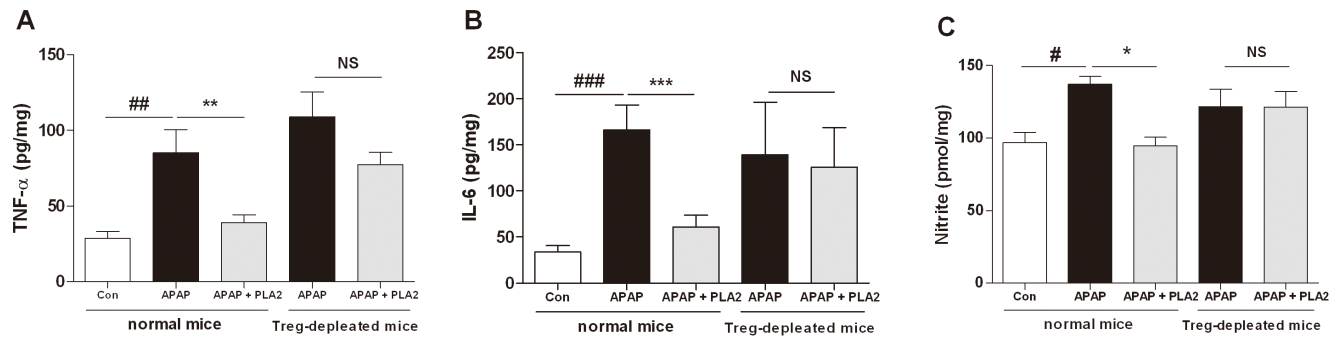


Figure 4. Proinflammatory cytokines in the liver. The proinflammatory cytokines and NO in the liver tissue from normal and CD25-depleted mice were measured by ELISA and the Griess method, respectively. The hepatic TNF (A), IL-6 (B) and NO levels (C) were decreased in the PLA2-treated group (APAP + PLA2) compared with the PBS-treated group (APAP). However, there was no difference between the PBS-treated and PLA2-treated mice in the anti-CD25 model. The values shown indicate the means \pm S.E.M. * $P < 0.05$ vs. APAP, ** $P < 0.01$ vs. APAP, *** $P < 0.001$ vs. APAP, # $P < 0.05$ vs. Con, ## $P < 0.01$ vs. Con, ### $P < 0.001$ vs. Con, NS; $P > 0.05$ vs. APAP in anti-CD25 mice ($n = 6-8$).

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Discussion

The liver is the main organ in the detoxification of drugs and toxins [22]. There are various mechanisms [23, 24] through which drugs may damage the liver [15, 25, 26]. Above all, an overdose of the analgesic drug acetaminophen often causes severe acute hepatotoxicity in experimental animals and humans [27, 28, 29].

In a previous study, Tregs attenuated inflammation in liver injury [30, 31]. The adoptive transfer of Tregs into mice successfully inhibits acute liver injury, whereas the depletion of Tregs aggravated the hepatic toxicity [14]. In our research, we demonstrated that bee venom attenuates inflammatory immune diseases through Treg regulation [32, 33, 34]. In particular, PLA2 from bee venom

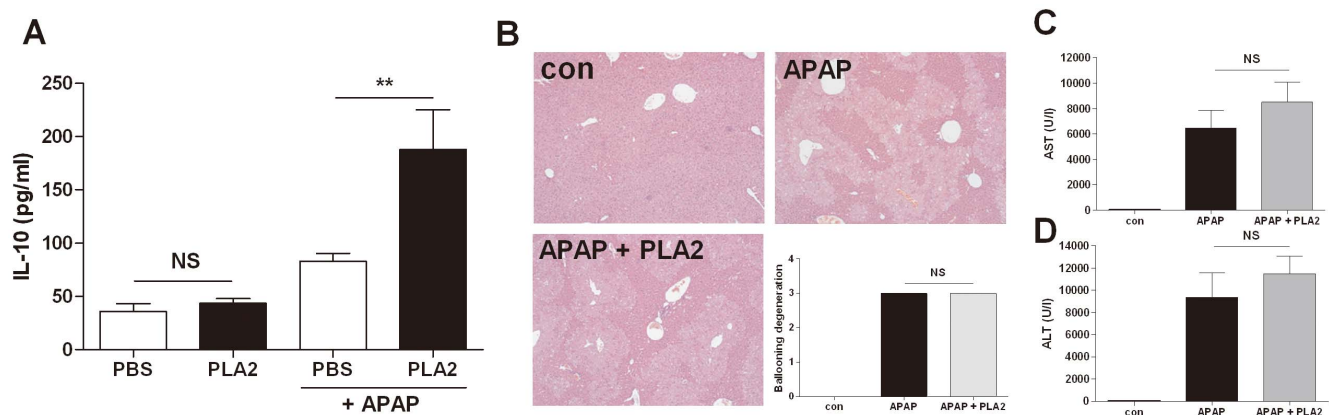


Figure 5. Hepatoprotective effects of PLA2 were mediated by IL-10 production. Mice were injected with acetaminophen (500 mg/kg, i.p.) after PBS or PLA2 injection. Blood samples were collected 6 h after acetaminophen injection ($n = 10$). IL-10 production in the serum was measured by ELISA (A). IL-10-deficient mice were injected with acetaminophen (500 mg/kg, i.p.) after PBS or PLA2 injection. Blood samples were collected 24 h after acetaminophen injection (B~D, $n = 4$). Hepatic dysfunction was reflected by the levels of AST (C) and ALT (D). There was no difference between the PBS-treated (APAP) and PLA2-treated (APAP + PLA2) IL-10^{-/-} mice (B~D).

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distinctly increased the Treg population in splenocytes. Based on these results, we hypothesized that PLA2 could have protective effects on acetaminophen-induced hepatotoxicity through Treg modulation.

Fifteen distinct groups of PLA2 have been discovered, and these are categorized into four groups: secreted sPLA2s, cytosolic cPLA2s, calcium-independent iPLA2s, and platelet-activating factor acetyl hydrolase/oxidized lipid lipoprotein-associated PLA2s [35]. Bee venom PLA2 (group III), Indian cobra (group IA) PLA2 and the new world rattlesnake PLA2 (group II) belong to secreted sPLA2. PLA2 is the second most abundant component of bee venom after melittin, and there are many therapeutic effects of bee venom called apitherapy. Although there is a previous report on the hepatoprotective effect of bee venom [36], it did not show which component was responsible for this effect. Other researchers have previously demonstrated the ability of bee venom PLA2 to activate T helper Type 2 cells and the importance of enzymatic activity to this effect [37, 38]. Palm et al., showed that bee venom PLA2 induces the Th2 response through the cleavage of membrane phospholipids and production of lysophospholipids, such as lysophosphatidylcholine [38]. It should be elucidated whether the enzymatic activity of PLA2 is critical for Treg differentiation as a further study.

AST and ALT are secreted into the blood stream in acute liver injury and are important indicators of hepatotoxicity [39, 40, 41]. PLA2-injected mice showed lower levels of AST and ALT in acetaminophen-induced hepatotoxicity compared with PBS-injected mice (Fig. 2). An increased ALT level is associated with hepatic expression of inducible NO synthase (iNOS). The high levels of NO within the liver by iNOS may also promote damage via interference with mitochondrial respiration [42]. NO is an important mediator of acetaminophen-induced hepatotoxicity [43, 44, 45]. PLA2-injected mice exhibited a lower level of NO compared with PBS- treated mice (Fig. 3).

To analyze whether the effect of PLA2 is dependent on Tregs, an anti-CD25 antibody was used to deplete Tregs, and the hepatoprotective effect of PLA2 disappeared as a result. This finding indicates that the hepatoprotective effect of PLA2 is mediated through Tregs. Tregs are associated with the secretion of IL-10 in inflammatory responses [46, 47] and inhibit the secretion of proinflammatory cytokines, such as TNF and IL-6, in liver injury [21, 48]. It has been reported that IL-10 is crucial for tolerance induction in hepatitis and is mainly expressed by Tregs and Kupffer cells. Treg adoptive transfer prevented liver injury, and the depletion of Tregs resulted in reduced plasma IL-10 levels. These findings suggested that Tregs are crucial for primary IL-10 production and augmentation in tolerized mice [14]. It has also been reported that IL-10 protects against acetaminophen-induced liver injury and lethality [21]. Moreover, Louis et al. demonstrated that the administration of recombinant IL-10 protects against hepatotoxicity in a galactosamine and lipopolysaccharide mouse model [49].

Thus, we used IL-10-deficient mice to confirm whether the hepatoprotective effect of PLA2 is dependent on IL-10. The results show that the protective effect of PLA2 was abolished in IL-10-deficient mice (Fig. 5). These results suggest that the protective effect of PLA2 is mediated by IL-10 secretion via Tregs.

The hepatotoxicity of APAP has been attributed to the formation of a highly reactive metabolite N-acetyl p-benzoquinonimine (NAPQI) by the hepatic cytochrome P-450. Substantial amounts of NAPQI are secreted by conjugation with glutathione (GSH). However, in the case of over-dose of APAP, the sulfonation reaction becomes saturated and the over-production of NAPQI depletes GSH in the liver, causing further accumulation of NAPQI. Unconjugated NAPQI binds to proteins and induces cell death that can lead to liver injury [50]. N-acetyl-cysteine (NAC) which is known to stimulate the production of GSH is used as an antidote for overdose of APAP. PLA2 might not have any effects on the APAP metabolism unlike NAC, because protective effects of PLA2 was disappeared in IL-10^{-/-} (Fig 5B~D) or Treg depleted (Fig 3 and 4) APAP mice suggesting that the hepatoprotective effects of PLA2 were not directly associated with APAP metabolites. It is proposed that PLA2 could be used as an alternative drug for NAC or simultaneously treated with NAC to reduce liver injury by acetaminophen.

In conclusion, PLA2 induces the secretion of IL-10 through Treg modulation and inhibits acute injury in the liver. We suggest that PLA2 has hepatoprotective effects in acetaminophen-induced acute toxicity through modulation of Tregs and IL-10 in mice.

Author Contributions

Conceived and designed the experiments: HK HSC HB. Performed the experiments: HK DJK JWK. Analyzed the data: HK HSC. Contributed reagents/materials/analysis tools: HSC JWK. Wrote the paper: HK HSC HB.

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