

Protectin D1 reduces concanavalin A-induced liver injury by inhibiting NF- κ B-mediated CX3CL1/CX3CR1 axis and NLR family, pyrin domain containing 3 inflammasome activation

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Abstract. Protectin D1 (PD1) is a bioactive product generated from docosahexaenoic acid, which may exert anti-inflammatory effects in various inflammatory diseases. However, the underlying molecular mechanism of its anti-inflammatory activity on concanavalin A (Con A)-induced hepatitis remains unknown. The aim of the present study was to investigate the protective effects of PD1 against Con A-induced liver injury and the underlying mechanisms via intravenous injection of PD1 prior to Con A administration. C57BL/6 mice were randomly divided into four experimental groups as follows: Control group, Con A group (30 mg/kg), 20 μ g/kg PD1 + Con A (30 mg/kg) group and 10 μ g/kg PD1 + Con A (30 mg/kg) group. PD1 pretreatment was demonstrated to significantly inhibit elevated plasma aminotransferase levels, high mobility group box 1 and liver necrosis, which were observed in Con A-induced hepatitis. Furthermore, compared with the Con A group, PD1 pretreatment prevented the production of pro-inflammatory cytokines, including tumor necrosis factor- α , interferon- γ and interleukin-2, -1 β and -6. In addition, pretreatment with PD1 markedly downregulated cluster of differentiation (CD)4⁺, CD8⁺ and natural killer T (NKT) cell infiltration in the liver. PD1 pretreatment was observed to suppress the messenger RNA and protein expression levels of NLR family, pyrin domain containing 3 and Toll-like receptor (TLR) 4 in liver tissue samples. Further data indicated that PD1 pretreatment inhibited the activation of the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) signaling pathway and chemokine (C-X3-C motif) ligand 1

(CX3CL1)/chemokine (C-X3-C motif) receptor 1 (CX3CR1) axis by preventing phosphorylation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α and NF- κ B in Con A-induced liver injury. Therefore, these results suggest that PD1 administration protects mice against Con A-induced liver injury via inhibition of various inflammatory cytokines and, in part, by suppressing CD4⁺, CD8⁺ and NKT cell infiltration in the liver and the NF- κ B-activated CX3CL1/CX3CR1 signaling pathway. The beneficial effect of PD1 may be associated with the inhibition of TLR4 expression and the downregulation of NF- κ B activation. In conclusion, PD1 appears to be a potential natural bioproduct, and provide a promising strategy, for the prevention of hepatic injury in patients with chronic or acute liver disease.

Introduction

Hepatitis is an inflammation of the liver without a specific cause and presents a major threat to human health worldwide (1). Hepatitis is a medical condition defined by inflammation of the liver and characterized by the presence of inflammatory cells in the organ tissue. Previous studies have indicated the main causes as follows: i) Various disorders, including a viral or bacterial infection of the liver (1,2); ii) the intake of toxic substances (1,3); iii) interruption of the normal blood supply to the liver; and iv) an autoimmune disorder (4). Notably, the hepatitis viruses, designated hepatitis A, B and C, and autoimmune hepatitis (AIH), result in the greatest liver injury. During investigations of AIH, concanavalin A (Con A)-induced hepatitis, a well-established mouse model of immune-mediated liver injury, has been recognized as the best experimental model for AIH research (5-7). In addition, previous studies suggest that pro-inflammatory cytokines and T lymphocytes are important in the pathogenesis of AIH (6,8). Furthermore, a single intravenous (i.v.) injection of Con A has been demonstrated to induce fulminant, T cell-dependent hepatitis and major inflammatory cytokine production, including tumor necrosis factor (TNF)- α , interferon (IFN)- γ and interleukin (IL)-6 (8). Overexpression of pro-inflammatory cytokines is associated with recruitment and activation of immune cell infiltration, which results in aberrant expression

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of inflammatory genes, ultimately resulting in hepatitis or other diseases (such as lung injury, lung cancer, gastritis and neurogenic inflammation) (9).

Fractalkine (FKN), also termed chemokine (C-X3-C motif) ligand 1 (CX3CL1) is important in the recruitment of intraepithelial lymphocytes and the adhesion of inflammatory cells (10,11). Chemokine (C-X3-C motif) receptor 1 (CX3CR1) is a specific CX3CL1 receptor, which has been implicated in the pathogenesis of liver injury in humans (11). Wollberg *et al* (12) and Pirvulescu *et al* (13) demonstrated the underlying mechanisms of CX3CR1 in cellular adhesion, migration, metastasis, and CX3CL1/CX3CR1-induced migration and recruitment of inflammatory cells. Furthermore, CX3CL1 expression in endothelial and vascular smooth muscle cells, and CX3CL1-mediated leukocyte adhesion markedly promote the development of inflammatory diseases (14). However, the role of CX3CL1 in Con A-induced liver injury remains to be elucidated. Previous studies have demonstrated that the CX3CL1/CX3CR1 axis was markedly upregulated via activation of the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) signaling pathway (14,15). Notably, the generation of inflammatory cytokines, resulting from CX3CL1/CX3CR1 acceleration, promotes increased production of inflammatory cytokines and exacerbation of the inflammatory response (16).

Protectin D1 (PD1) is a bioactive product generated from docosahexaenoic acid (DHA), which has been reported to exert anti-inflammatory effects in various disorders, including acute kidney injury, neurodegenerative diseases and acute lung injury (17-20). Previous studies suggest PD1 attenuates inflammatory action by inhibiting inflammatory signaling pathways, such as NF- κ B and p38 mitogen-activated protein kinases (20). Furthermore, Yan *et al* (20) demonstrated that ω -3 fatty acids (including DHA and eicosapentaenoic acid) potentially have the ability to inhibit caspase-1 expression, IL-1 β secretion and NLR family, pyrin domain containing 3 (NLRP3) inflammasome formation. Hence, the present study hypothesized that PD1, as a bioactive product generated from DHA, may suppress NLRP3 expression. Furthermore, Tsutsui *et al* (21) indicated that Toll-like receptor (TLR) 4, NF- κ B and NLRP3-mediated pro-inflammatory cytokine and chemokine expression is a major factor in the development of liver injury. Therefore, the current study hypothesizes that PD1 may be key in the alleviation of Con A-induced hepatitis. The present study identified that PD1 pretreatment suppressed systemic inflammation, in part, via inhibition of NF- κ B activation, CX3CL1 expression and NLRP3 inflammasome formation. These findings suggest that PD1 may be considered a promising agent for the treatment of liver-related diseases.

Materials and methods

Animals and administration of Con A and PD1. A total of 60 male C57BL/6 mice (age, 8 weeks; weight, 20-25 g) were purchased from the Animal Experimentation Center of the Second Military Medical University (Shanghai, China). Mice were acclimatized to their environment for one week. They were housed in a pathogen-free, temperature and

humidity-controlled environment (25 \pm 2 $^{\circ}$ C; 50 \pm 5% humidity) with a standard 12-h light/dark cycle, and allowed free access to food and water. All procedures were performed in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China (<http://www.most.gov.cn>). The Institutional Animal Care and Use Committee at Jilin University (Changchun, China) approved the animal study protocols. PD1 was obtained from the Cayman Chemical Company (Ann Arbor, MI, USA) and prepared in Hank's buffer (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PD1 solution was prepared 10 min prior to use and placed in cold storage upon reaching a peak concentration of 200 μ g/ml. Con A (cat. no. 11028-71-0; purity \geq 98%) was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA) and dissolved in Hank's balanced salt solution buffer (Gibco; Thermo Fisher Scientific, Inc.) at a concentration of 25 mg/ml. The mice were randomly divided into four groups as follows: i) Control group (a tail vein injection with 0.5 ml Hank's buffer); ii) Con A group (30 mg/kg); iii) 20 μ g/kg PD1 pretreatment group (HPD1; high dose of PD1); iv) 10 μ g/kg PD1 pretreatment group (LPD1; low dose of PD1). Mice were deprived of food for 24 h, but given free access to water. The mice were pretreated with 20 or 10 μ g/kg PD1 via i.v. injection for 4 h. They were subsequently injected (i.v.) with Con A (30 mg/kg) for 24 h, at different time-points (4, 12 and 24 h), blood samples (1.5 ml) were collected by cardiac puncture following sevoflurane anesthesia.

Serum transaminase activity assays and pro-inflammatory cytokine levels. Serum was obtained following centrifugation at 650 x g for 15 min. Alanine transaminase (ALT) and aspartate transaminase (AST) serum levels were analyzed spectrophotometrically using an Olympus AU1000 automated chemistry analyzer (Olympus Corporation, Tokyo, Japan). The TNF- α (DY410), IFN- γ (DY485), IL-6 (DY406) and CX3CL1 (DY472) levels in the serum (markers of Con A-induced liver injury) were measured in the mice using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocols. The high mobility group protein B1 (HMGB1) was quantified using sandwich immunoassays from Bio-Gene Technology, Ltd. (Shanghai, China). The remaining serum was stored at -80 $^{\circ}$ C.

Histopathological examination of the liver. Liver tissue samples were obtained 24 h after Con A administration, fixed in 10% neutral buffered formalin and embedded in paraffin (both purchased from Nanjing Jiancheng Biology Engineering Institute, Nanjing, China). For morphometric analysis, the sections were stained with hematoxylin and eosin (Thermo Fisher Scientific, Inc.) to monitor histological changes. Following dehydration, thin sections (4-6 μ m) were evaluated by light microscopy (OLYMPUS CX23; Olympus Corporation) according to the previously described method (22).

Preparation of liver mononuclear cells (MNCs). Liver samples were collected 24 h after Con A administration. The liver MNCs were prepared according to the method by Tsutsui *et al* (21) with certain modifications. To avoid CD3 $^{+}$ interference, blood was collected from the eyeball rather than

by conducting liver perfusion. The liver tissue samples were infiltrated with collagenase type II (Invitrogen; Thermo Fisher Scientific, Inc.) for 40 min. Liver samples were washed with cold Hank's buffer three times, the tissues were compressed and passed through a stainless steel mesh (pores, size 60; Sigma-Aldrich, St. Louis, MO, USA), and suspended in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). The cell suspensions were centrifuged at 75 x g for 5 min to remove debris and impurities prior to being filtered through a nylon mesh (Jilin Futian Bioproduct Corporation), which had been presoaked in Hank's buffer. The supernatants containing hepatic MNCs were harvested and washed once with Hank's buffer, and the cells were re-suspended in 40% Percoll® (Sigma-Aldrich). All cell suspensions were placed over 70% Percoll® and centrifuged for 30 min at 350 x g. MNCs were enriched at interphase, and washed twice in Hank's buffer.

Flow cytometric analysis. Single-cell suspensions of liver tissue were collected 24 h following Con A injection (30 mg/kg). Cells were immediately stained with fluorescent-labeled antibodies, anti-CD4 allophycocyanin (APC) (dilution, 1:1,000; cat. no. 17-0041-82), anti-CD8 phycoerythrin (PE) (dilution, 1:1,000; cat. no. 11-0081-85), anti-killer cell lectin-like receptor subfamily B, member 1 (NKT1.1) PE (dilution, 1:1,000; cat. no. 12-5941) and anti-CD3 APC (dilution, 1:1,000; cat. no. 17-0031), which were obtained from eBioscience, Inc., San Diego, CA, USA). The number of CD4⁺, CD8⁺ and NKT cells infiltrating the mouse livers was analyzed by flow cytometry (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Cell viability and cell cycle analysis. A rapid method for isolating mice lymphocytes was used to evaluate the inhibitory effect of PD1 on T cell proliferation and pro-inflammatory responses. T lymphocytes of mouse livers (that had not been administered with therapeutic agents) were separated and prepared in Hank's buffer. The isolation was based on the preparation of liver MNCs. All the cells were washed using Hank's buffer (containing 0.1% bovine serum albumin; BSA; Sigma-Aldrich China, Inc., Shanghai, China) three times and resuspended in the RPMI-1640 culture medium. The cells (1x10⁷ cells/ml) were seeded in 24- or 96-well plates containing RPMI-1640 with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich China, Inc.). The cells were either untreated or treated with 20 µg/ml Con A in the presence of 0, 2.5, 5, 10 or 20 nM PD1 for 48 h. The cells were maintained in an atmosphere of 5% CO₂ at 37°C. The MTT assay was used to analyze cell viability. All of the medium was removed and 5 µl MTT solution (10 mg/ml; Sigma-Aldrich China, Inc.) was added to 100 µl phenol red-free growth medium (Sigma-Aldrich China, Inc.), and the plates were incubated at 37°C in 5% CO₂ for 4 h. Subsequently, a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the absorbance of each well at a wavelength of 540 nm. For cell cycle analysis, cells were plated at 1x10⁷ cells/well in 24-well plates and left untreated or treated with 20 µg/ml Con A in the presence of 20 nM PD1 for 18 h. Subsequently, the cells were incubated with 20 µg/ml RNase A, followed by 25 µg/ml propidium iodide (PI; Sigma-Aldrich China, Inc.). A flow cytometer

(MACSQuant; Miltenyi Biotec GmbH.) with an argon laser and 570-nm Bandpass filter (Sigma-Aldrich China, Inc.) was used to measure the intensity of PI fluorescence to determine the phase of the cell cycle.

Western blot analysis. The liver tissues and cells were homogenized in 10% (w/v) hypotonic buffer [25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 µg/ml leupeptin, 1 mM Pefabloc® SC, 50 µg/ml aprotinin, 5 µg/ml soybean trypsin inhibitor and 4 mM benzamide (Shanghai Bogoo Biotech, Co., Ltd., Shanghai, China)] to yield a homogenate. The final supernatants were obtained by centrifugation at 12,000 rpm for 20 min. Protein concentration was determined using an ASSAYMicro BCA protein assay kit (Thermo Fisher Scientific, Inc.) with BSA serving as a standard. The total protein extract (5 µg) was used for western blot analysis. Equal quantities of total protein from the liver tissues samples were subjected to 10 or 12% SDS-PAGE (Sigma-Aldrich China, Inc.; 150 V for 1 h). Immunoblotting was conducted using the following primary polyclonal rabbit antibodies: Rabbit anti-GAPDH (dilution, 1:1,000; cat. no. 5174), NF-κB (dilution, 1:1,000; cat. no. 8242), phosphorylated-NF-κB (dilution, 1:1,000; cat. no. 4887), TLR4 (dilution, 1:1,000; cat. no. 14358), nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) (dilution, 1:1,000; cat. no. 4814), inhibitor of nuclear factor κ-B kinase subunit beta (IKKβ) (dilution, 1:1,000; cat. no. 8943), myeloid differentiation primary response gene 88 (MyD88) (dilution, 1:2,000; cat. no. 4283), NLRP3 (dilution, 1:1,000; cat. no. 13158), IL-1β (dilution, 1:1,000; cat. no. 12426), caspase-1 (dilution, 1:1,000; cat. no. 3866) (Cell Signaling Technology, Inc., Danvers, MA, USA), CX3CR1 (cat. no. ab8021) and CX3CL1 (dilution, 1:1,000; cat. no. ab25088) (Abcam, Cambridge, MA, USA). Immunoreactive bands were visualized by a Pierce enhanced chemiluminescence immunoblot detection system (Thermo Fisher Scientific, Inc.) and exposed to Kodak X-ray film (Kodak, Rochester, NY, USA). Expression levels of each protein were defined as grey values (ImageJ software, version 1.4.2b; ImageJ, National Institutes of Health, Bethesda, MD, USA), standardized to the housekeeping gene, GAPDH and expressed as a fold of the control.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissue and cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and 1 µg total RNA was reverse transcribed using the M-MLV-RT system (Promega Corporation, Madison, WI, USA). This was performed at 42°C for 1 h and terminated by deactivation of the enzyme at 70°C for 10 min. qPCR was conducted using SYBR® Green (Bio-Rad Laboratories, Inc.) in an ABI PRISM 7900HT detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All the primers for GAPDH, TNF-α, NF-κB p65, IL-6, IL-1β, IFN-γ, IκBα, IKKβ, caspase-1, NLRP3, HMGB1, CX3CL1 and CX3CR1 were produced by Thermo Fisher Scientific, Inc. and the sequences are presented in Table I. Amplification of pre-denatured products was conducted at 94°C for 60 sec; followed by 45 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec; followed by 95°C for 10 sec, 65°C for 45 sec, and 40°C for 60 sec. Fold induction values were calculated according to 2^{-ΔΔC_q} expression (15),

where ΔC_q represents the differences in cycle threshold number between the target gene and GAPDH, and $\Delta\Delta C_q$ represents the relative change in the differences between the control and treatment groups.

Immunoprecipitation (IP). IP was performed using a Pierce Classic IP kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Lysates from cell cultures were obtained by homogenizing the cells in 10% (w/v) hypotonic lysis buffer (Shanghai Bogoo Biotech, Co., Ltd., Shanghai, China). Then, 100 μ l bead slurry (Thermo Fisher Scientific, Inc.) was added to the lysate and incubated for 10 to 30 min at 4°C with gentle agitation. To increase the yield, the beads were washed 1 or 2 additional times in lysis buffer, and the supernatants were collected. The cell lysate was put on ice and, in a microcentrifuge tube, 100 μ l culture supernatant (Thermo Fisher Scientific, Inc.) was added to 10-50 μ g cell lysate. The samples were then incubated with the following primary polyclonal antibodies overnight at 4°C: rabbit anti-NF- κ B (dilution, 1:1000, cat. no. 8242), TLR4 (dilution, 1:1000; cat. no. 14358), I κ B (dilution, 1:1000; cat. no. 4814), MyD88 (dilution, 1:2000; cat. no. 4283) (all purchased from Cell Signaling Technologies, Inc.), CX3CL1 (dilution, 1:1000; cat. no. ab25088) and CX3CR1 (dilution, 1:1000; cat. no. ab8021) (both purchased from Abcam). The samples were then incubated with horseradish peroxidase-conjugated rabbit anti-biotin (dilution, 1:15,000; cat. no. 5571; Cell Signaling Technologies, Inc.) for 1.5 h at 37°C. The beads were then washed with washing buffer or lysis buffer three times to remove non-specific binding. Samples were then centrifuged at 1,000 x g and western blot analysis was performed using 30 μ g protein samples. Protein expression levels were defined as grey value and analyzed using Image J software (version 1.4.2b, National Institutes of Health, Bethesda, MA, USA), and standardized to GAPDH and expressed as a fold of the control.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean. Treated tissue samples and the corresponding controls were compared using GraphPad PRISM software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA), and one-way analysis of variance with Dunn's least significant difference tests or Student's t-tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of PD1 on serum transaminase activity and pro-inflammatory cytokine secretion in Con A-induced hepatitis. Serum ALT and AST levels significantly increased following intravenous Con A administration. By contrast, pretreatment with HPD1 and LPD1 markedly suppressed the serum concentration of AST and ALT when compared with that of the Con A group (Fig. 1A and B), indicating that PD1 has a protective effect on Con A-induced hepatitis. The association between pro-inflammatory cytokine secretion and the inhibitory effect of PD1 was also investigated. Following Con A injection, ELISA kits were used to measure the key pro-inflammatory cytokines and analyze the inhibitory effect of PD1 on IL-1 β , TNF- α , IFN- γ , IL-6, CX3CL1 and HMGB1 expression levels. As expected, the

Table I. Primer sequences used in the present study.

Gene	Primer sequence (5'-3')
TNF- α	F: CAGGCGGTGCCTATGTCTC R: CGATCACCCCGAAGTTCAGTAG
IL-2	F: TGAGCAGGATGGAGAATTACAGG R: GTCCAAGTTCATCTTCTAGGCAC
IL-6	F: CTGCAAGAGACTTCCATCCAG R: AGTGGTATAGACAGGTCTGTTGG
HMGB1	F: GCATCCTGGCTTATCCATTGG R: GGCTGCTTGTTCATCTGCTG
NF- κ B	F: ATGGCAGACGATGATCCCTAC R: CGGATCGAAATCCCCTCTGTT
I κ B α	F: AAGATGTCGTTCAAGGAGGTGCG R: ATCCTCTGAGATTTGACGCTTTG
IKK β	F: GGTGTGAAATTGAGACAATTGAAAAC R: GTTTCCTGTCAGTACCAAGGTTGA
Caspase-1	F: ACGCCTTGCCCTCATAAT R: TCTAATACATCTGGGACTTCTT
CX3CL1	F: GCTAGCATGGCTCCCTCGCCGCTCGCG R: ATGCAGGACCACGGTCACACTTGCGCA
IL-1 β	F: CCAGGATGAGGACCCAAGCA R: TCCCGACCATTGCTGTTTCC
IFN- γ	F: CCTCAAACCTGGCAATACTCA R: CTCAAGTGGCATAGATGTGGA
CX3CR1	F: TCATCCAGACGCTGTTTCC R: AGGCATTTCCCATACAGGTG
NLRP3	F: CTTCTCTGATGAGGCCCAAG R: GCAGCAAACCTGGAAAGGAAG
GAPDH	F: TTCACCACCATGGAGAAGGC R: GGCATGGACTGTGGTCATGA

TNF- α , tumor necrosis factor- α ; IL, interleukin; HMGB1, high mobility group B1; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; I κ B α , nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α ; IKK β , inhibitor of nuclear factor κ -B kinase subunit β ; CX3CL1, chemokine (C-X3-C motif) ligand 1; CX3CR1, chemokine (C-X3-C motif) receptor 1; NLRP3, NLR family, pyrin domain containing 3.

results demonstrated that PD1 downregulated the release and production of inflammatory cytokines in a dose-dependent manner (Fig. 1C-H). These results indicate that PD1 inhibits the hepatitis induced by Con A i.v. injection.

PD1 attenuates Con A-induced liver injury in mice. To further confirm the protective effect of PD1 on liver damage, hematoxylin and eosin staining of the liver was performed to evaluate whether PD1 inhibits pathological development. Following i.v. Con A administration, marked hepatocyte necrosis was observed in the Con A group. Four pathologists blinded to the experimental conditions evaluated the sections, indicating that the Con A group induced marked bridging necrosis, which was observed under a light microscope (Fig. 2A). Furthermore, the pathological scores in the PD1 pretreatment groups were lower than that of the Con A group (Fig. 2B).

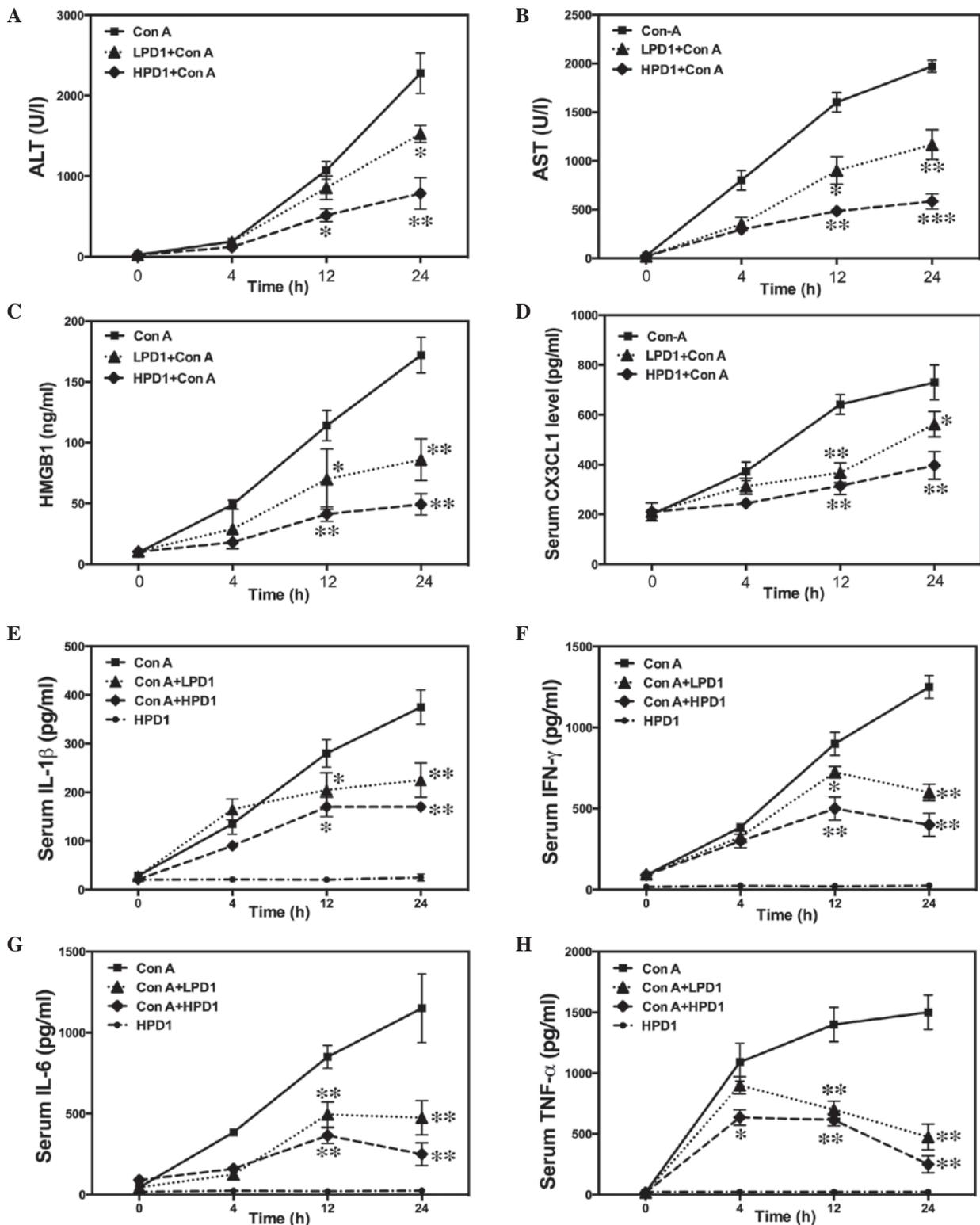


Figure 1. PD1 inhibits Con A-induced liver injury in mice. Following Con A administration (30 mg/kg) for 24 h, the serum samples were obtained by cardiac puncture at different time-points. (A) ALT and (B) AST serum levels were analyzed using an Olympus AU1000 automated chemistry analyzer. (C) HMGB1 was quantified via sandwich immunoassay. ELISA kits were used to examine levels of (D) CX3CL1, (E) IL-1 β , (F) IFN- γ , (G) IL-6 and (H) TNF- α in the serum. The bars indicate the mean \pm standard error of the mean (n=9). *P<0.05, **P<0.01, ***P<0.001 vs. Con A (30 mg/kg). PD1, protectin D1; Con A, concanavalin A; ALT, alanine transaminase; AST, aspartate transaminase; HMGB1, high mobility group B1; CX3CL1, chemokine (C-X3-C motif) ligand 1; IL, interleukin; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; HPD1, 20 μ g/kg PD1 pretreatment group; LPD1, 10 μ g/kg PD1 pretreatment group.

Effect of PD1 on inflammation-associated gene expression in Con A-induced hepatitis. Pro-inflammatory cytokines and inflammatory signaling pathways are important in

Con A-induced hepatitis. Results from the present study demonstrated that in PD1 pretreatment groups, including LPD1 and HPD1, the upregulated messenger (m)RNA expression

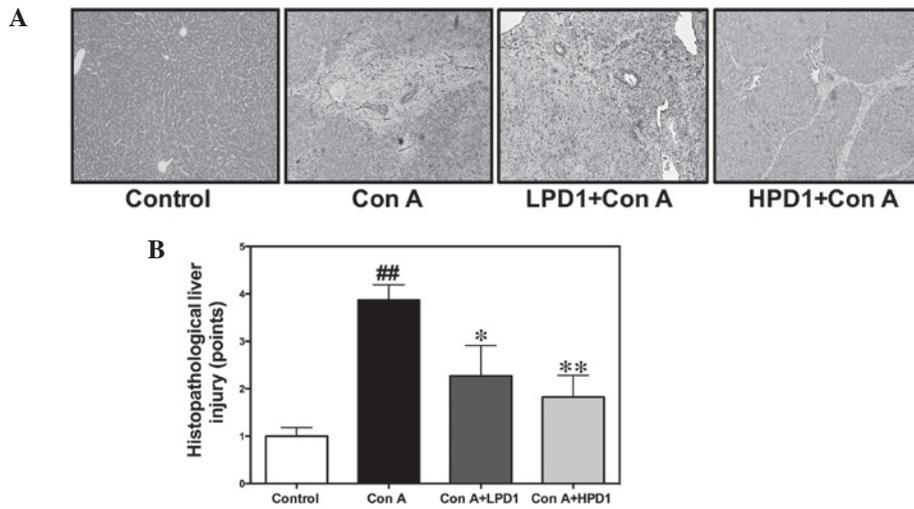


Figure 2. Hematoxylin and eosin staining of liver sections evaluated via light microscopy. (A) Four pathologists blinded to the experimental conditions judged each sample and indicated that Con A (30 mg/kg) induced marked bridging necrosis, which was apparent under the light microscope. Compared with Con A, PD1 markedly protected against hepatocyte necrosis in a dose-dependent manner. Magnification x22. (B) Pathological scores in the PD1 pretreatment groups were lower than that in the Con A group. Bars indicate the mean \pm standard error of the mean. [#]P<0.05 vs. control group. ^{*}P<0.05, ^{**}P<0.01 vs. Con A (30 mg/kg). Con A, concanavalin A; PD1, protectin D1; HPD1, 20 μ g/kg PD1 pretreatment group; LPD1, 10 μ g/kg PD1 pretreatment group.

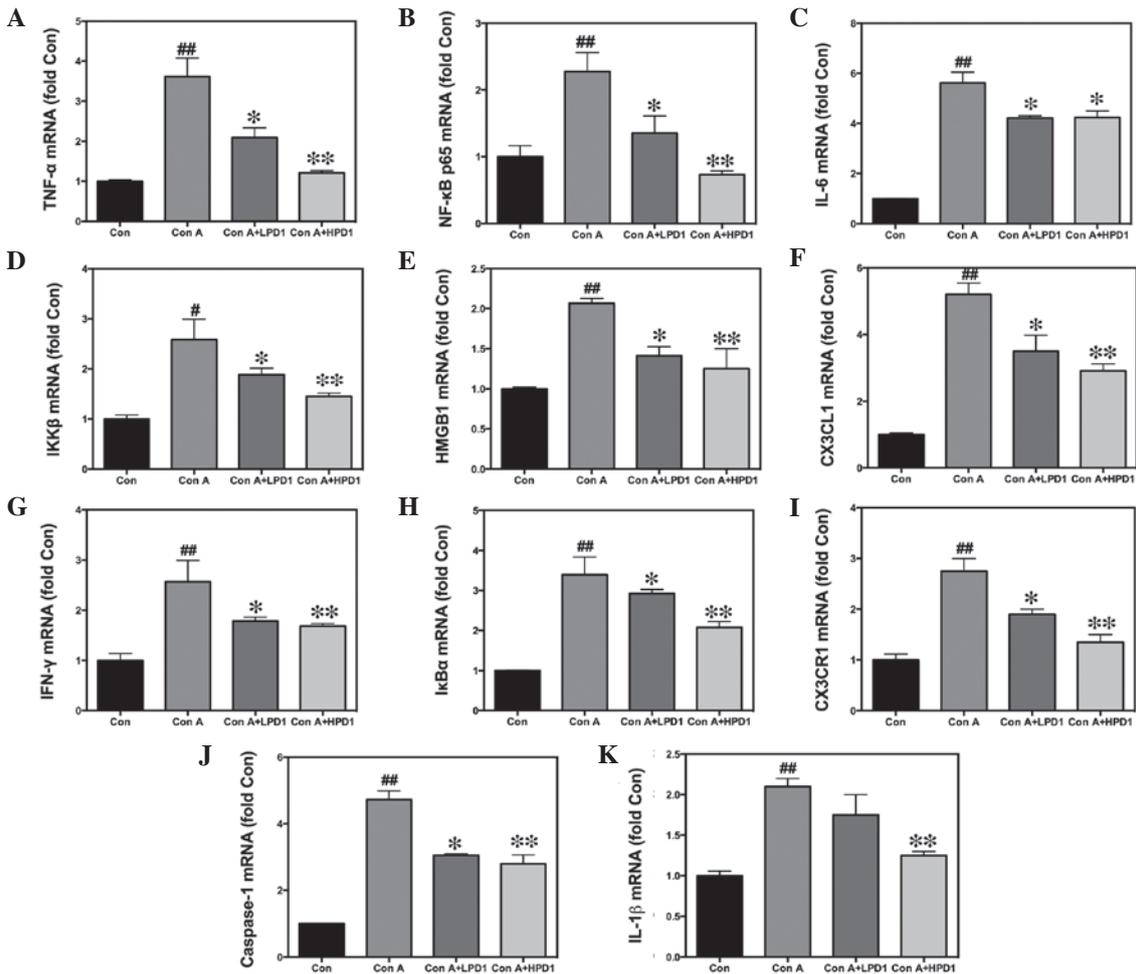


Figure 3. Effects of PD1 on inflammatory gene expression in Con A-induced hepatitis. Livers were removed and RNA was extracted using TRIzol. Quantification of mRNA expression levels of (A) TNF- α , (B) NF- κ B p65, (C) IL-6, (D) IKK β , (E) HMGB1, (F) CX3CL1, (G) IFN- γ , (H) I κ B α , (I) CX3CR1, (J) caspase-1 and (K) IL-1 β in Con A-induced liver tissue. The bars indicate the mean \pm standard error of the mean (n=10). [#]P<0.05, ^{##}P<0.01 vs. control group; ^{*}P<0.05, ^{**}P<0.01 vs. Con A (30 mg/kg). PD1, protectin D1; Con A, concanavalin A; HPD1, 20 μ g/kg PD1 pretreatment group; LPD1, 10 μ g/kg PD1 pretreatment group; TNF- α , tumor necrosis factor- α ; NF- κ B p65, nuclear factor κ -light-chain-enhancer of activated B cells p65 subunit; IL, interleukin; IKK β , inhibitor of nuclear factor κ -B kinase subunit β ; HMGB1, high mobility group B1; CX3CL1, chemokine (C-X3-C motif) ligand 1; IFN- γ , interferon- γ ; I κ B α , nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α ; CX3CR1, chemokine (C-X3-C motif) receptor 1; mRNA, messenger RNA.

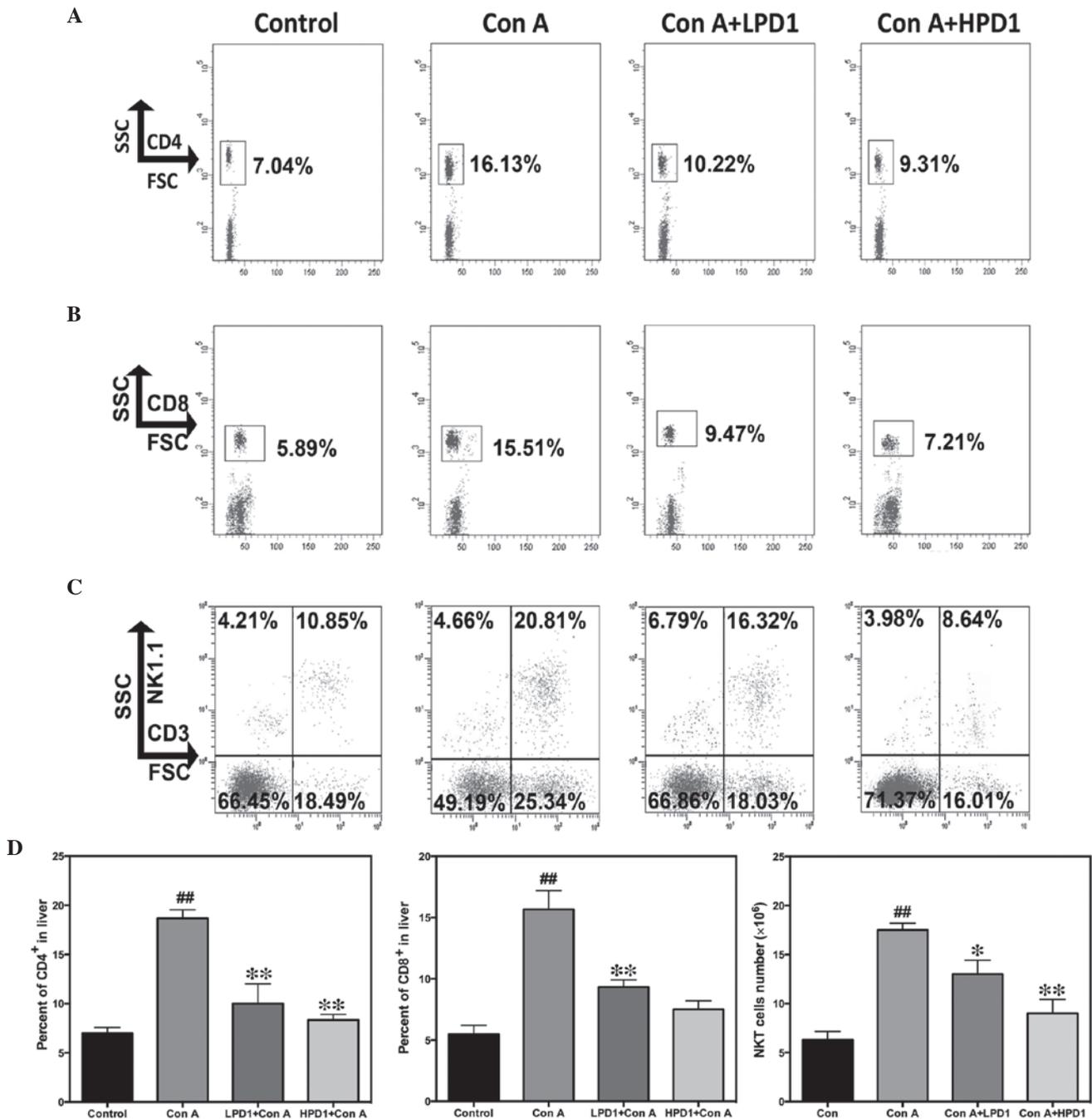


Figure 4. Pretreatment of PD1 inhibited CD4⁺, CD8⁺ and NKT cells infiltrating the liver, as demonstrated by flow cytometric analysis of hepatic lymphocytes following 24 h Con A (30 mg/kg) administration. The T cells were stained with allophycocyanin- or phycoerythrin-conjugated monoclonal antibodies. Flow cytometric analysis of (A) CD4⁺ T cells, (B) CD8⁺ T cells and (C) NKT cells. (D) The percentages of CD4⁺ and CD8⁺ T cells were counted and the absolute number of infiltrated NKT cells was calculated by multiplying the total number of hepatic mononuclear cells with the percentage of NKT cells. The bars indicate the mean ± standard error of the mean (n=10). ##P<0.01 vs. control group; *P<0.05, **P<0.01 vs. Con A. CD, cluster of differentiation; NKT, natural killer T cells; Con A, concanavalin A; PD1, protectin D1; HPD1, 20 μg/kg PD1 pretreatment group; LPD1, 10 μg/kg PD1 pretreatment group; SSC, side scatter; FSC, forward scatter; NK1.1, killer cell lectin-like receptor subfamily B, member 1.

levels of TNF-α, NF-κB p65, IL-6, IL-1β, IFN-γ, IκBα, IKKβ, HMGB1, CX3CL1 and CX3CR1 in response to Con A were attenuated by pretreatment with PD1 (Fig. 3). Hence, these results suggest that the NF-κB-mediated CX3CL1/CX3CR1 pathway was associated (directly or indirectly) with the development of inflammation in Con A-induced liver injury.

Pretreatment with PD1 inhibited CD4⁺, CD8⁺ and NKT cell infiltration in mouse livers. In the present study, the

percentage of CD4⁺ and CD8⁺ T cells in the liver was assessed by flow cytometry. As expected, compared with the Con A group, the percentage of infiltrating CD4⁺ and CD8⁺ T cells was significantly decreased in the liver tissue samples (Fig. 4A and B). The percentage of NKT cells in the liver tissue samples was also analyzed and the data demonstrated that the absolute quantities of infiltrating NKT cells in the liver were significantly upregulated in the Con A group, compared with mice in the PD1 treatment groups (Fig. 4C). These results

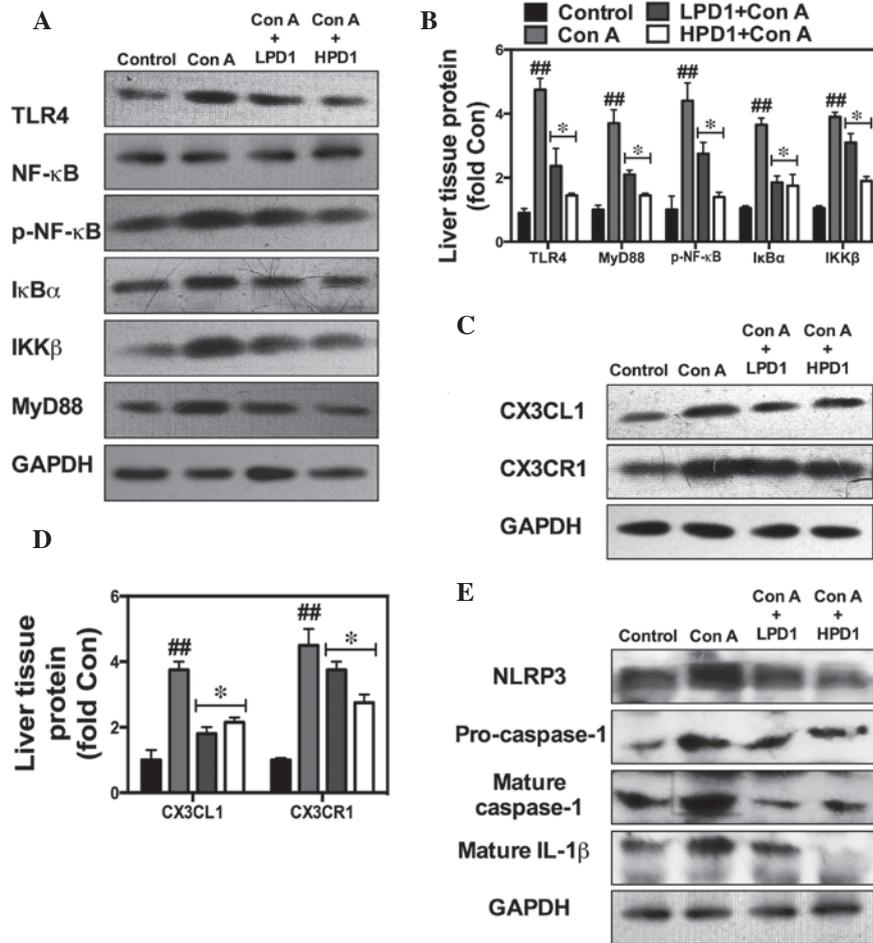


Figure 5. PD1 inhibits NF-κB-stimulated CX3CL1/CX3CR1-dependent inflammatory pathway activation and generation of the inflammasome. Western blot analysis for NF-κB and CX3CL1/CX3CR1 activity was used to demonstrate the effect of PD1 on Con A-induced liver injury and the underlying mechanisms. (A and B) Bands and quantification of TLR4, p-NF-κB, IκBα, IKKβ and MyD88 protein. (C and D) Bands and quantification of CX3CL1 and CX3CR1 protein. (E) Bands and quantification of NLRP3 and caspase-1 protein. The bars indicate means ± standard error of the mean (n=10). ##P<0.01 vs. control group; *P<0.05 vs. Con A (30 mg/kg). PD1, protectin D1; Con A, concanavalin A; HPD1, 20 μg/kg PD1 pretreatment group; LPD1, 10 μg/kg PD1 pretreatment group; TLR4, Toll-like receptor 4; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; p-NF-κB, phosphorylated NF-κB; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α; IKKβ, inhibitor of nuclear factor κ-B kinase subunit β; MyD88, myeloid differentiation primary response gene 88; CX3CL1, chemokine (C-X3-C motif) ligand 1; CX3CR1, chemokine (C-X3-C motif) receptor 1; NLRP3, NLR family, pyrin domain containing 3; IL-1β, interleukin-1β.

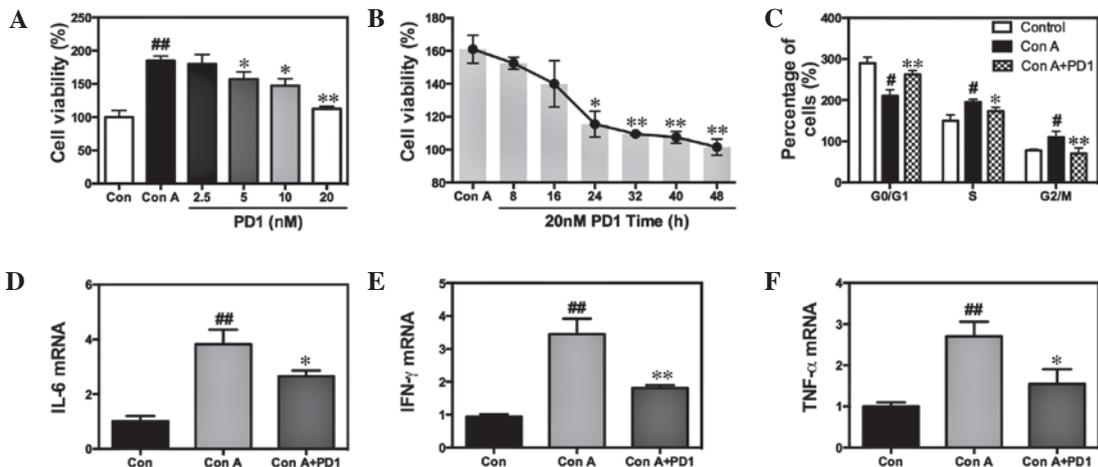


Figure 6. Effects of PD1 on the proliferation of Con A-induced T lymphocytes. (A) T lymphocytes were treated with or without 20 μg/ml Con A in the presence of 0, 2.5, 5, 10 and 20 nM PD1 for 48 h. After the PD1 administration, cell viability was investigated using MTT assay to evaluate the influence of PD1 on proliferation. (B) MTT assay examining cell viability in the condition of Con A+20 nM PD1 treatment. (C) Cell cycles were examined by flow cytometry. Reverse quantitative transcription-polymerase chain reaction demonstrated the mRNA expression levels of (D) IL-6, (E) IFN-γ and (F) TNF-α. The bars indicate the mean ± standard error of the mean (n=10). #P<0.05, ##P<0.01 vs. control group. *P<0.05, **P<0.01 vs. Con A. PD1, protectin D1; Con A, concanavalin A; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; mRNA, messenger RNA.

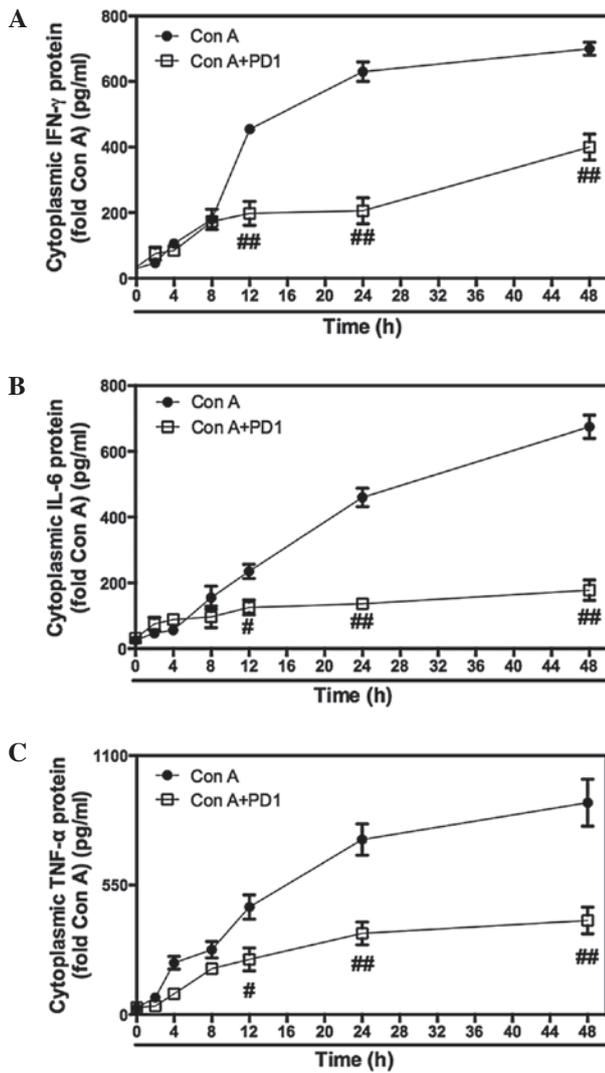


Figure 7. Effects of PD1 (20 nM) on inflammatory cytokine production of Con A (20 μ g/ml)-induced T lymphocytes. ELISA was used to examine the cytoplasmic expression of (A) IFN- γ , (B) IL-6 and (C) TNF- α at different time-points. The bars indicate the mean \pm standard error of the mean (n=10). #P<0.05, ##P<0.01 vs. Con A in t-tests. PD1, protectin D1; Con A, concanavalin A; IFN- γ , interferon- γ ; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α .

suggest that PD1 markedly suppressed the recruitment of CD4⁺, CD8⁺ and NKT cells into the liver in Con A-induced liver injury.

PD1 suppresses inflammatory signaling pathways. The NF- κ B-mediated CX3CL1/CX3CR1 signaling pathway has been reported to be important in hepatitis and associated with inflammatory responses. Hence, the present study investigated whether PD1 inhibits the CX3CL1/CX3CR1 and the TLR4/MyD88/NF- κ B signaling pathways using RT-qPCR. PD1 significantly downregulated the expression of CX3CL1 and CX3CR1 proteins, and NF- κ B phosphorylation. In addition, the IL-1 β , NLRP3 and HMGB1 protein expression levels were suppressed by PD1 treatment, which indicates that NLRP3 and HMGB1 are involved in the development of liver injury (Fig. 5).

PD1 inhibited T lymphocyte proliferation and inflammatory signaling pathways. Various concentrations of PD1 (0, 2.5, 5, 10 and 20 nM) were investigated to assess the

inhibition of proliferation in Con A-stimulated T lymphocytes. As presented in Fig. 6A, PD1 pretreatment markedly inhibited cell proliferation compared with the Con A group. Furthermore, it was observed that 20 nM PD1 affects cell viability in a time-dependent manner (Fig. 6B). Evaluation of cell cycle progression indicated that Con A improved cell cycle progression in T cells, as fewer cells were observed in the G₀/G₁ phase and increasing cells were arrested in the S and G₂/M phase; this effect was partially suppressed by pretreatment with PD1 (Fig. 6C). Subsequently, the inhibitory effect of PD1 on inflammatory cytokines was investigated and RT-qPCR indicated that treatment of PD1 significantly downregulated cytokine expression levels, including TNF- α , IFN- γ and IL-6 (Fig. 6D-F and Fig. 7). Furthermore, PD1 treatment was observed to inhibit NF- κ B/CX3CL1/CX3CR1 activation; IP results indicated that PD1 decreased the formation of NF- κ B/I κ B α , CX3CL1/CX3CR1 and TLR4/MyD88 complexes (Fig. 8). Finally, the inhibitory effect of PD1 on the NLRP3 signaling pathway was investigated. The data indicated that treatment with PD1 significantly suppressed IL-1 β , caspase-1 and NLRP3 mRNA and protein expression levels (Fig. 9).

Discussion

Hepatitis is a serious global health problem associated with high mortality and infection rates (1,2). Therapeutic strategies that target the underlying mechanisms of liver injury are required, particularly in viral and autoimmune hepatitis (2,3). Despite advances in medical science and research regarding the pathogenesis of hepatitis, the search for effective therapeutic strategies remains a major challenge. Hence, elucidation of the physiological and genetic mechanisms of liver damage may aid with the development of effective therapeutic agents. Thus, in the current study, the protective effect of PD1 on Con A-induced hepatitis was investigated. Previous studies have indicated that PD1 exerts a marked anti-inflammatory effect in various disorders, such as acute kidney injury, neurodegenerative diseases and acute lung injury (18,19). In the present study, pretreatment with PD1 demonstrated a potential protective effect against Con A-induced hepatitis as levels of ALT and AST were downregulated, as was the severity of hepatic necrosis. Notably, the underlying mechanisms of PD1 may be associated with the suppression of inflammatory signaling pathways and lymphocyte infiltration by regulating the cell cycle and cycle-related protein expression. Previous studies in a mouse model of Con A-induced hepatitis demonstrated that TNF- α , IL-6 and IFN- γ were significant. Kato *et al* (23) demonstrated that TNF- α , IFN- γ and IL-6 are, directly or indirectly, involved in the regulation of inflammatory cytokine release, as IFN- γ ^{-/-} and TNF- α ^{-/-} mice were free from liver injury following administration of Con A. Therefore, the current study investigated the expression of major inflammatory cytokines, such as TNF- α , IFN- γ , IL-6 and HMGB1 in Con A-induced hepatitis. PD1 pretreatment suppressed the release of pro-inflammatory cytokines, including TNF- α , IFN- γ , IL-6 and HMGB1 production, as well as their mRNA expression in the liver and T cells. In addition, CD4⁺ and CD8⁺ infiltrating T lymphocytes are involved in the development of Con A-induced hepatitis (18-20). In the present study, PD1 treatment markedly inhibited CD4⁺, CD8⁺ and NKT cell infiltration in the liver as

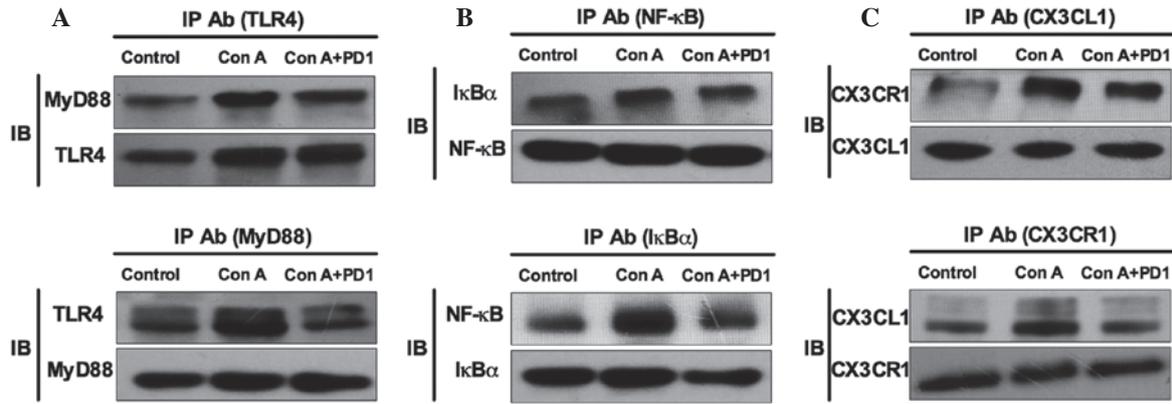


Figure 8. Effects of Con A (20 $\mu\text{g}/\text{ml}$) and PD1 (20 nM) on NF- κB activated CX3CL1/CX3CR1 signaling pathway. IP was used to investigate (A) TLR4/MyD88, (B) NF- κB /I $\kappa\text{B}\alpha$ and (C) CX3CL1/CX3CR1 complex formation. The bars indicate the mean \pm standard error of the mean. (n=10). IP, immunoprecipitation; Ab, antibody; PD1, protectin D1; Con A, concanavalin A; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88; NF- κB , nuclear factor κ -light-chain-enhancer of activated B cells; I $\kappa\text{B}\alpha$, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor; CX3CL1, chemokine (C-X3-C motif) ligand 1; CX3CR1, chemokine (C-X3-C motif) receptor 1.

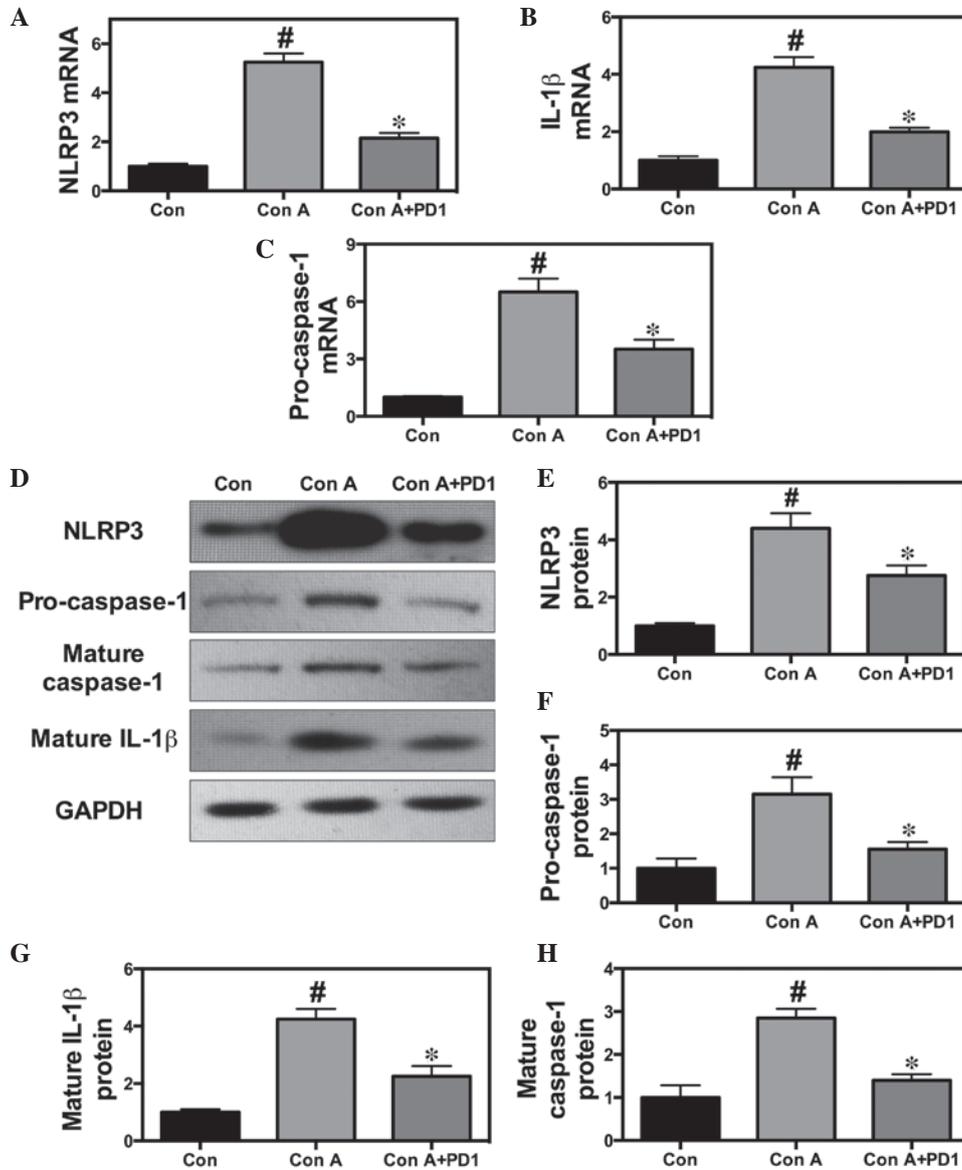


Figure 9. Inhibitory effect of Con A (20 $\mu\text{g}/\text{ml}$) PD1 (20 nM) on the NLRP3 signaling pathway. Total RNA extraction was conducted using TRIzol reagent (48 h). Quantification of mRNA expression levels of (A) NLRP3, (B) IL-1 β and (C) pro-caspase-1 in Con A-induced lymphocytes. (D) Western blot and (E-H) quantification of NLRP3, pro-/mature caspase-1 and mature IL-1 β protein. The bars indicate the mean \pm standard error of the mean (n=10). #P<0.05 vs. control group. *P<0.05 vs. Con A. PD1, protectin D1; NLRP3, NLR family, pyrin domain containing 3; Con A, concanavalin A; IL-1 β , interleukin-1 β ; mRNA, messenger RNA.

compared with the Con A group. These results indicated that PD1 exerted a protective effect, partly dependent on the inhibition of CD4⁺, CD8⁺ and NKT cells, against liver injury. Furthermore, NK cells are a class of lymphocyte distinct from T and B cells, and are the predominant cells involved in autoimmune disorders or viral infections and may result in liver damage by destroying foreign cells (for example, neoplastic cells) and contribute to the progression of inflammation (20,21,24). In the present study, *in vitro* studies with isolates of T lymphocytes were performed to assess whether treatment with PD1 inhibits Con A-induced T lymphocyte proliferation in inflammation and to determine the underlying mechanisms by which it may restore inflammatory responses. The results indicated that different concentrations of PD1 (particularly 20 nM) significantly suppressed lymphocyte proliferation in a time-dependent manner (0–48 h). Notably, PD1 may be important in cell cycle regulation.

CX3CL1 (also referred to as FKN) is a major CX3C chemokine, which binds to its receptor, CX3CR1 and has been associated with the development of inflammation (15). There are two different forms of CX3CL1, the membrane-anchored form and the soluble form, and CX3CL1 may be induced by pro-inflammatory cytokines, TNF- α and IFN- γ . TNF- α and IFN- γ upregulate the expression of CX3CL1 and CX3CR1 in various different types of cells via NF- κ B activation. In addition, the soluble form of CX3CL1, which is released by TNF- α or IFN- γ changing enzyme, is a potent chemoattractant for recruiting monocytes/macrophages, T cells or NK cells. The present study demonstrated that CX3CL1 is involved (directly or indirectly) in the development of inflammation and liver injury in Con A-induced hepatitis. Furthermore, CX3CL1 is involved in the embryonic development of the central nervous system and is key in T cell development and activation (15). Previous studies have demonstrated that the NF- κ B signaling pathway, and TNF- α and IFN- γ cytokines are involved in the development of Con A-induced liver injury (20,21). Thus, the current study investigated whether NF- κ B induced CX3CL1 expression via TNF- α - or IFN- γ -stimulated inflammatory responses. To determine the role of the CX3CL1/CX3CR1 axis in Con A-induced hepatitis, Con A was used to simulate AIH in a mouse model. The results indicated that CX3CL1 expression and NF- κ B activation were significantly inhibited at high and low doses of PD1. Furthermore, the ELISA results demonstrated that soluble CX3CL1 in the serum was downregulated by PD1 administration. In addition, the present study investigated the inhibitory effect of PD1 on Con A-induced T lymphocyte inflammatory responses *in vitro*. Experimental data demonstrated that PD1 markedly inhibited Con A-induced inflammatory cytokine expression via suppression of NF- κ B activation and CX3CL1/CX3CR1 pathways at different time-points. The results of the IP analysis indicated that the CX3CL1/CX3CR1 axis was involved in the progression of Con A-induced inflammation, and that the formation of CX3CL1/CX3CR1 complexes was markedly inhibited by PD1. These results were consistent with previous studies, which demonstrated that PD1 blocks the Con A-induced liver damage by inhibiting NF- κ B-stimulated CX3CL1/CX3CR1-dependent inflammatory signals (10–13).

NLRP3 is a major component of the macromolecular complex that triggers caspase-1-dependent maturation of

IL-1 β and IL-18 cytokine precursors. It has been indicated that the NLRP3 inflammasome is activated by various cellular signals and directly controls collagen synthesis, resulting in increased deposition of collagens in the tissues, such as the lungs, liver, heart and skin (22). Notably, Con A-activated NF- κ B promotes synthesis of pro-IL-1 β , IL-18 and NLRP3 proteins to further promote the inflammatory response (24). Therefore, the role of NLRP3 in the pathological process of Con A-induced liver damage was investigated. Data from the present study demonstrated that PD1 downregulated NLRP3 expression in the Con A-induced damaged liver. In addition, the protein and mRNA expression levels of caspase-1 increased. Thus, pretreatment with PD1 suppresses Con A-induced hepatitis and may indirectly contribute to the inhibition of NLRP3 expression, as well as trigger caspase-1.

In conclusion, the current study demonstrates that PD1 suppressed hepatitis by directly or indirectly inhibiting TLR4/NF- κ B-stimulated CX3CL1/CX3CR1-dependent inflammatory signals and the NLRP3 inflammasome. TLR4 was implicated in the inflammatory response to Con A exposure, in which CX3CL1/CX3CR1-activation is important in the sustained production of pro-inflammatory mediators by regulating nuclear NF- κ B-dependent transcription. These results provide novel insight into the molecular mechanisms that link viral and autoimmune hepatitis to inflammation in liver injury that is caused by various factors. PD1 is a bioactive product generated from DHA and has been demonstrated to suppress Con A-induced hepatitis and inflammatory responses by inhibiting, in part, the NF- κ B-stimulated CX3CL1/CX3CR1 pathway, the NLRP3 inflammasome, lymphocyte proliferation and infiltration of CD4⁺, CD8⁺ and NKT cells to the liver. Therefore, inhibition of inflammation by PD1 may provide a potential therapeutic strategy to recover Con A-induced liver injury.

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