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1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) reduces hepatic injury in concanavalin A-treated mice

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Abstract

1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), a chemically synthesized mono-acetyldiaglyceride, is one of the constituents in Sika deer antlers and has been known traditionally as having immunomodulatory effects. However, the mechanism by which PLAG controls neutrophil migration, which evokes liver injury in the hepatitis animal model, remains largely unknown. This study was designed to evaluate the immunomodulatory effects of PLAG on cytokine secretion and neutrophil migration in vivo and in vitro. Concanavalin A (Con A) induced leukocyte infiltration in the liver and increased plasma cytokine levels. Pretreatment with PLAG reduced the levels of interleukin (IL)-4, IL-6, IL-10, and CXCL2, but maintained interferon (IFN)-γ levels and modulated neutrophil recruitment toward the liver. Furthermore, the mRNA and protein levels of IL-4 and CXCL2 in liver tissue were also decreased in the Con A-treated mice. Liver histology analyses showed that PLAG reduced Con A-induced hepatic necrosis, which was accompanied by leukocyte infiltration. The in vitro studies revealed that PLAG reduced IL-4 secretion in Con A stimulated T cell and blocked signal transducer and activator of transcription 6 (STAT6) Con A induced hepatocyte. PLAG attenuated IL-4 induced activation of atypical protein kinase C (PKC)/STAT6 in hepatocytes and inhibited neutrophil migration toward the liver tissue through suppression of IL-8/vascular cell adhesion molecule (VCAM) expression. These results suggest that PLAG could mitigate excess neutrophil migration into liver tissue and potentially have a therapeutic effect on immune-mediated liver injury.

KEYWORDS
atypical PKC, concanavalin A-induced hepatitis, neutrophil, PLAG, STAT6

1 | INTRODUCTION

The liver contains large resident and migratory populations of leukocytes that can be rapidly expanded in response to infection or injury by recruiting leukocytes from the circulation into the tissue.[1] Of those leukocytes, neutrophils play a particularly critical role in damage to the liver. Some studies have suggested that CD4 ± T cells[2,3] and NKT cell.[4,5]

are critical in concanavalin a (Con A) induced liver damage and these cells secrete Th1 and Th2 type cytokine including IFN-γ and IL-4. Neutrophils, which express interleukin (IL)-4 receptor, are key initiators of lymphocyte recruitment[1] and play a novel accessory role in Con A induced hepatitis.[6] IL-4 can exhibit both pro- and anti-inflammatory functions in neutrophils.[7] Furthermore, IL-4 directly induces the release of IL-8 from human bronchial epithelial cells[8] and human neutrophils.[9] IL-8 is a potent chemoattractant and activation of this cytokine is required for neutrophil recruitment and activation during inflammation.

The administration of Con A is an established model for studying T cell-mediated hepatitis that closely resembles the pathology of human autoimmune hepatitis. T cell-mediated immune responses play a role in the pathogenesis of a variety of human liver disorders, including autoimmune liver disease, viral hepatitis, and alcoholic liver disease. These conditions induce the expression of a variety of cytokines including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-4 by T cells and macrophages,[10–12] which target liver cells and immune cells via activation of multiple signaling cascades, including members of the signal transducer and activator of transcription (STAT1, 2, 3, 4, 5, and 6) protein family.[3,13–15] Among the various cytokines, increased levels of IFN-γ, and IL-4 are produced in response to Con A in vivo, which plays a critical role in the development of Con A-induced hepatitis.[16,4] Recently, observations have suggested that the activation of STAT6 by IL-4 is essential for the development of liver injury in Con A-induced hepatitis via recruitment of leukocytes into the liver.[17] Furthermore, inhibition of protein kinase C (PKC) isoforms, including an atypical and novel PKC, elicits a reduction in Janus kinase (JAK)/STAT6, and STAT3 in the liver in Con A-induced hepatitis.[18–20] This indicates that PKC interacts with and phosphorylates JAK/STAT. Therefore, an inhibitor of these PKC isoforms might be a promising therapeutic target for hepatitis.

1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) exists in natural resources such as seed oils,[19,21,22] the bovine udder,[22] milk fat,[19] and the antler of Sika deer.[23] We chemically synthesized PLAG from glycerol, palmitic acid, and linoleic acid[23] and confirmed that the synthetic PLAG was chemically identical to its naturally isolated form.[24] Recently, evidence has indicated that PLAG from deer antlers possesses pharmacological activities, such as anti-tumor,[25] immunomodulatory,[17,26] anti-allergic,[27] anti-metastatic,[25] and hematopoiesis[24] effects in in vitro and in vivo experiments. However, the effect of PLAG on liver injury, including leukocyte infiltration caused by Con A, has not been carefully examined. Therefore, in this study, we examined the effects of PLAG on Con A-induced hepatitis in mice and further explored the underlying mechanisms in vitro.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

PLAG and PLG (1-palmitoyl-2-linoleoyl-rac-glycerol) were provided by Enzychem Lifesciences Co. (Daejeon, Republic of Korea). PLAG and PLG were prepared fresh prior to each in vitro and in vivo treatment. Con A (Sigma-Aldrich, Sr. Louis, MO) was purchase.

2.2 | Cell lines and culture

EL-4, Hep3B, HepG2, and HL-60 cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD). Hep3B, HepG2, and HL-60 cells were cultured in RPMI1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Welgene, Gyeongsangbuk-do, Republic of Korea). EL-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO). The cells were cultured at 37°C in a 5% CO_2 humidified atmosphere.

2.3 | Mice and concanavalin A treatment

Balb/c male mice (age 10 weeks and weighing approximately 25 g) were purchased from Koatech Co. (Pyeongtaek, Republic of Korea). The mice were housed under specific pathogen-free conditions and were provided a standard laboratory chow and water ad libitum 1 week before the experiment. All of the experiments were approved by the Korea Research Institute of Bioscience and Biotechnology Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals and with the Korean national laws for animal welfare. The mice were randomly divided into three groups including the control group ($n = 8$), the Con A group ($n = 10$), and the PLAG pretreatment group ($n = 10$). The Con A and PLAG were both dissolved in phosphate buffered saline (PBS). The mice were administered PBS or PLAG orally according to group designations, followed by an intravenous dose of 20 mg/kg body weight (BW) Con A in the Con A group and PLAG pretreatment group 2 h later, respectively. PLAG was administered orally to mice at a dose of 100 mg/kg BW. Blood and liver tissue were harvested 18 h after Con A administration.

2.4 | Cytokine assay

Blood samples were obtained by cardiac puncture 18 h after the Con A injection and centrifuged at 3000 rpm for 10 min. For the measurement of cytokines in liver tissue homogenates, the animals were euthanized and the liver was collected and rinsed with PBS. The liver was then homogenized and filtered through a 70 μm mesh cell strainer (BD Falcon cell strainer, BD Biosciences, Bedford, MA) in PBS. The supernatant was collected via centrifugation at 2000 rpm for

5 min. The cell pellet in the bottom layer was resuspended in PBS and used in the complete blood count (CBC) analysis. The plasma samples and the tissue homogenate supernatants were kept at −20°C until the cytokine measurements. EL-4, Hep3B and HL-60 cells were treated with PLAG for 2 h, and then activated with Con A (12.5, 30 μg/mL, respectively). The dose of Con A has been used to cell activation[28] or hepatitis condition.[29] Twenty four hours later, the supernatant from the treated cells were collected. The concentrations of IL-6, IL-10, IL-4, chemokine (C-X-C motif) ligand 2 (CXCL2), and IFN-γ in the plasma, tissue or cell supernatants were detected by an ELISA as specified by the manufacturer's instructions (BD Bioscience).

2.5 | Complete blood counts

Whole blood and liver samples were collected 18 h after the Con A injection. The liver was excised from the mouse of each group and homogenized in the same manner as described above. The CBC analysis included the quantification of total white blood cells, neutrophils, and lymphocyte fractions of total white blood cells and was performed using the Mindray BC 5300 hematology analyzer (Shenzhen Mindray Bio-medical Electronics, China).

2.6 | Histological analysis

Hepatic sections were obtained from the mice 8 h after the Con A injection. The sections were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm thick sections. After deparaffinization and rehydration, the slices were stained with hematoxylin and eosin (H&E). For immunohistochemical staining, the deparaffinized tissues were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by blocking with 1% BSA. For the identification of neutrophil infiltration, the sections were incubated in the NIMP-R14 neutrophil marker antibody (1:100, Thermo Fisher Scientific, Waltham, MA) at 4°-C overnight. The slides were then incubated with HRP-conjugated goat anti-rat IgG (1:250, Santa Cruz Biotechnology, Dallas, TX) at room temperature for 15 min followed by visualization with the 3-amino-9-ethylcarbazole (AEC) substrate (Dako, Glostrup, Denmark). The tissues were counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted using Crystal Mount™ medium (Sigma-Aldrich). A solution of an irrelevant goat IgG of the same isotype and antibody dilution served as negative control. The images were obtained under light microscopy (Olympus, Tokyo, Japan). All of the specimens were histologically evaluated under a light microscope. The digital images were acquired at a magnification of 100×. The percent of necrotic area and neutrophil infiltration were quantified with NIH IMAGE (Image-J 1.50i) software. The necrosis and neutrophil rate of the hepatocytes in a randomly selected section were calculated according to the necrotic area divided by the liver area in the image.

2.7 | Western blotting

Hep3B cells were seeded in a 12-well plate (2.5×10^5 cells in each well), treated with PLAG after 2 h, and stimulated with Con A or IL-4 for 3 and 1 h, respectively. The total protein extracts were immunoblotted with phospho-STAT6 (p-STAT6), STAT6, phospho-JAK1 (p-JAK1), and phospho-PKCζ/λ (p-PKCζ/λ) antibodies (Cell Signaling, Beverly, MA). HL-60 cells were seeded in a 12-well plate (1×10^6 cells in each well) and treated as above. The total protein extracts were immunoblotted with phospho-PKC (p-PKC) isoforms (δ, θ, ζ/λ, and α/β; Cell Signaling), phospho-ERK 1/2, phospho-p38, phospho-JNK (Cell Signaling), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology Inc.). Detection was conducted using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA).

2.8 | RNA isolation and RT-PCR analysis

EL-4 cells were seeded in a 12-well plate (2×10^6 cells in each well) and treated PLAG after 2 h, and stimulated with Con A for 6 h. HL-60 cells were seeded in a 12-well plate (1×10^6 cells in each well) and treated as described above. Total RNA was isolated from the liver homogenates using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The cDNAs were synthesized from total cellular RNA using oligo-dT primers (Promega, Madison, WI) and M-MLV reverse transcriptase (Promega) following the manufacturer's instructions. The PCR primers were designed using Primer3 program and were purchased from Bioneer (Daejeon, Republic of Korea). The primer sequences were shown in Table 1. The quantity of amplified cDNA in the reaction samples was confirmed by 2% agarose gel (Roche Korea Co. Ltd, Seoul, Republic of Korea) electrophoresis.

2.9 | Luciferase reporter gene assay

The HepG2 and Hep3B cells were transfected with a reporter luciferase plasmid containing four tandem copies of the STAT6 binding site (p4xSTAT6- Luc2P; Addgene, Cambridge, MA). The reporter vector was then transfected into the cell lines using the Attractene transfection reagent (QIAGEN Korea Ltd., Seoul, Republic of Korea) according to the manufacturer's instructions. To activate the STAT6 reporter vectors, IL-4 (10 ng/ml) was used to treat the cells for 6 h and the resulting luciferase activity was measured using a luciferase assay system (Promega) and a luminometer (Micro Lumate Plus, Perkin Elmer Inc., Wellesley, MA). The luciferase activity of each sample was normalized to that of the corresponding sample transfected with pGL4.

TABLE 1 The sequences of primers

Gene	Forward (5′-3′)	Reverse (5′-3′)	Product size
Mouse			
IL-4	TGACGGCACAGAGCTATTGA	TGTTCTTCGTTGCTGTGAGG	203 bp
MIP-2(CXCL2)	AGTGAACTGCGCTGTCAATG	CTTTGGTTCTTCCGTTGAGG	259 bp
CXCL12	GAGCCAACGTCAAGCATCTG	CGGGTCAATGCACACTTGTC	101 bp
CXCR4	GGGGACATCAGTCAGG	GTGGAAGAAGGCGAGGG	360 bp
VCAM	CCTCACTTGCAGCACTACGGGCT	TTTTCCAATATCCTCAATGACGGG	442 bp
ICAM-1	TGCGTTTTGGAGCTAGCGGACCA	CGAGGACCATACAGCACGTGCAG	326 bp
Human			
IL-8(CXCL8)	GCAGGGAATTCACCTCAAGA	CTTCAGGAACAGCCACCAAT	219 bp
VCAM	AGTTGAAGGATGCGGGAGTA	TCTCCAGTTGAACATATCAAGCA	253 bp
GAPDH	CCATCACCATCTTCCAGGAG	ACAGTCTTCTGGGTGGCAGT	346 bp

2.10 | Neutrophil adhesion assay

To determine the effect of PLAG on neutrophil adhesion to fibronectin, 24-well tissue culture plates were coated with fibronectin (Corning Life Sciences, NY) as previously described.[30] The plates were then incubated with 25 μg/mL fibronectin for 2 h at 37°C. The unbound proteins were removed by washing, the neutrophils were pretreated with PLAG 2 h prior to the experiment, and neutrophil activation was evaluated using ConA or IL-4 for 4 h. The non-adherent neutrophils were then eluted by aspiration and washed twice with PBS. The adhered neutrophils were then determined by counting the number of cells in the bottom of each well using a hemocytometer. The remaining adherent cells were left to incubate for 18 h, at which time they were exposed to the WST-1 cell proliferation reagent (Roche Korea Co. Ltd) for the spectrophotometric assessment of cell numbers.

2.11 | Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using SPSS20.0. The data were analyzed using one-way analysis of variance (ANOVA) when comparing among groups (in vivo) or by an independent Student's *t*-test when comparing two experimental groups (in vitro). Statistical significance was assumed if $P < 0.05$.

3 | RESULTS

3.1 | PLAG reduces hepatic injury in Con A-treated mice

The ameliorative effect of PLAG on liver injury was further examined using H&E staining. As shown in Figure 1, histological examination of the liver sections showed that intravenous administration of Con A resulted in massive hepatocyte necrosis, the disorder of the hepatic sinusoids structure, and infiltration of neutrophils into the liver within 8 h. Con A stimulation affected about 40% of necrosis area. However, the necrotic area decreased to about 6% in PLAG treated mice ($P < 0.001$) as shown Figure 1D. Moreover, lower doses of PLAG (10, 50 mg/kg) also attenuated liver injury at the same experimental protocol as described in the section 2. (Supplementary Data 2A). These results indicate that PLAG reduces Con A-induced hepatitis.

3.2 | The effects of PLAG on leukocyte migration into the liver in Con A-injected mice

The numbers of circulating leukocytes were slightly downregulated in the Con A-stimulated mice and the number of circulating neutrophils was significantly increased in the group that received PLAG when compared to other groups as shown in Figure 2A ($P < 0.01$). Meanwhile, the number leukocytes residing in the tissue were increased in the Con A-stimulated mice and the number of neutrophils in the tissue was notably decreased in the PLAG-treated group and the values were similar to those control group, as shown in Figure 2B ($P < 0.05$). These data suggest that leukocyte migration (extravasation) from the blood to the liver was accelerated in the Con A-stimulated mice and that neutrophil transmigration was restored in the PLAG-treated group. In the liver, Con A administration induced the infiltration of a large number of leukocytes, which could subsequently affect tissue destruction. However, PLAG could potentially reduce neutrophil migration into the liver. Overall, the number of neutrophils was estimated by immunohistochemical staining with an anti-neutrophil antibody as shown in Figure 2C. Con A stimulation induced about 6% of neutrophils in liver and infiltrated neutrophil reduced to about 2% in PLAG treated mice ($P < 0.05$).

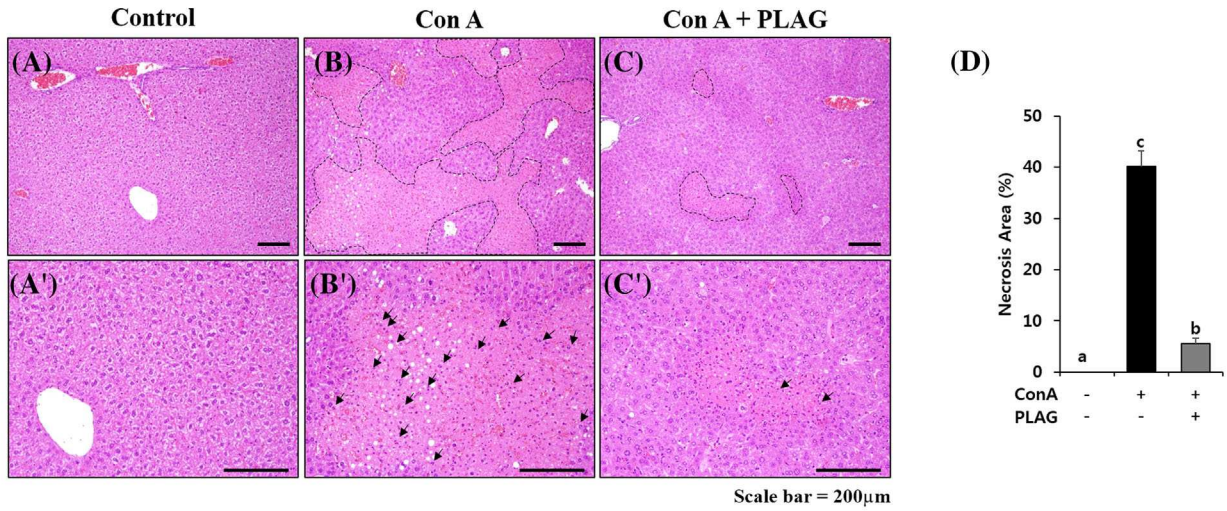

FIGURE 1 Liver damage was evaluated by histological observation in PLAG-treated mouse livers. Liver tissue was collected 8 h after the Con A injection. The liver sections were stained with H&E. The magnified (200×) liver sections from control (A), Con A-induced mice (B), and PLAG-treated mice (C) were observed by light microscopy. The H&E-stained sections were examined for the detection of necrosis (outlined areas) 8 h after Con A injection. (D) The percentage necrotic area in liver was quantified using ImageJ software, and data are expressed as the mean ± SEM. Bars = 100 μm. The arrows represent recruited neutrophils. In the figure above, significant differences among groups at $P < 0.05$ were marked with different letters (a, b, c) and groups that share the same letter are not significantly different

3.3 | PLAG reduced IL-4, IL-6, IL-10, and CXCL2 in the plasma of Con A-injected mice

The concentrations of IL-4, IL-6, IL-10, and CXCL2 in the plasma of Con A-injected mice were significantly higher than those in the control mice. As shown in Figure 3, the levels of IL-4 ($P < 0.001$), IL-6 ($P < 0.001$), IL-10 ($P < 0.01$), and CXCL2 ($P < 0.001$) that were increased by stimulation with Con A and treatment with PLAG at a concentration of 100 mg/ kg significantly reduced, but the increased IFN-γ levels in the Con A-induced group were not significantly reduced by PLAG treatment. In addition, lower doses of PLAG (10, 50 mg/kg) also dose dependently decreased IL-4 and CXCL2 in the plasma but not IFN-γ (Supplementary Data 2B). These results indicate that PLAG could reduce the secretion of cytokines associated with hepatitis-related pathogenesis induced by Con A treatment.

3.4 | PLAG modulates the expression of cytokines, and receptor, and adhesion molecules, in liver tissue of Con A-injected mice

As shown in Figure 4, higher protein and mRNA levels of hepatic IL-4 and CXCL2 were observed in the liver tissue of Con A-injected mice. The enhanced expression of these cytokines in the Con A-injected group was clearly decreased in the PLAG-treated group. In addition, significant increases in the protein level of IL-4 and CXCL2 were reduced in PLAG treated group compared to Con A injected group ($P < 0.01$). These results were consistent with our observation that PLAG regulated the levels of cytokines, such as IL-4 and CXCL2, in the plasma as shown in Figure 3. Modulation of the chemokine receptor CXCR4 mRNA and the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and VCAM following PLAG treatment were also evaluated in the Con A-injected liver tissue. RT-PCR results revealed that PLAG downregulated CXCR4 mRNA expression. However, the adhesion molecules, including ICAM-1 and VCAM, were not affected by PLAG. These results indicate that PLAG could reduce the secretion of the cytokines or chemokines involved in leukocyte infiltration resulting from hepatitis-related pathogenesis induced by Con A treatment.

3.5 | PLAG reduced the secretion and expression of IL-4 via blocking STAT6 phosphorylation

To determine whether PLAG modulate Con A induced signals such as STATs and MAPKs that led to liver injury, we investigated the effect of PLAG on signaling events in Con A induced hepatocyte through western blot. The result showed that phosphorylation of STATs (STAT1, 3, 6) and MAPKs (ERK 1/2, JNK, p38) was induced by Con A stimulation, and phosphorylation of STAT6 was attenuated by PLAG treatment. However, PLAG had no effect on the STAT1, STAT3, and MAPKs signal.

As shown Figures 5B and C, the expression of IL-4 associated with the STAT6 was further analyzed in Con A induced T cell. Con A stimulation of EL-4 cells resulted in an

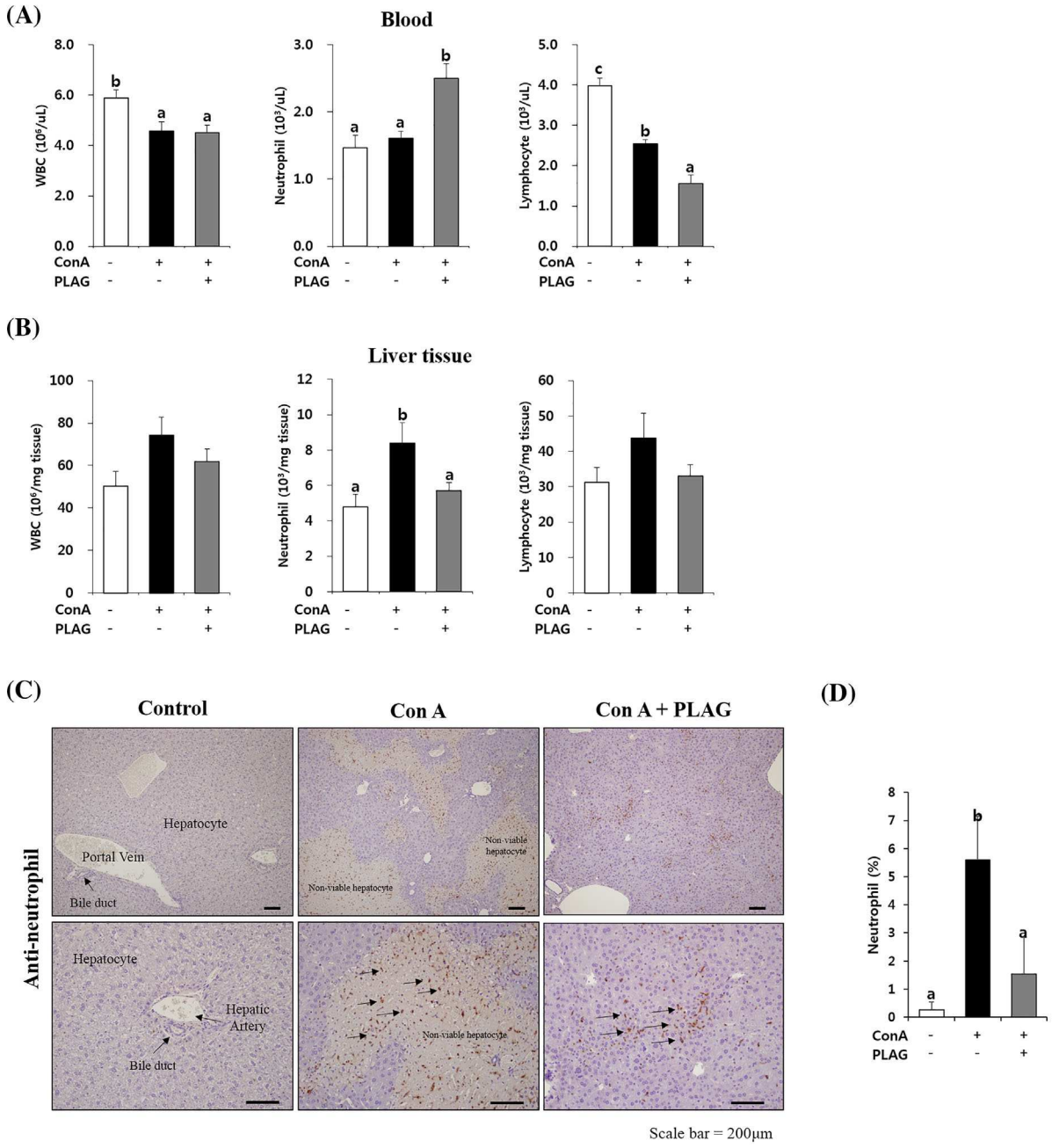

FIGURE 2 Effects of PLAG on leukocyte migration to liver tissue hepatocytes in Balb/c mice with Con A-induced hepatitis. Mice were treated with or without PLAG for 2 h before Con A injection. After 18 h, whole blood (A) and liver tissue (B) was collected and absolute WBC, neutrophil, and lymphocyte counts were determined using a Mindray BC-5500 hematology analyzer. (C) Liver tissue was collected 8 h after Con A injection. Immunohistochemistry was used to detect neutrophil levels in liver sections. (D) Neutrophil infiltration in liver was quantified using ImageJ software, and data are expressed as the mean ± SEM. The mice were randomly divided into three groups: control ($n = 8$), Con A ($n = 10$), and PLAG pretreatment group ($n = 10$). In the figure above, significant differences among groups at $P < 0.05$ were marked with different letters (a, b, c) and groups that share the same letter are not significantly different

approximately sixfold increase in levels of IL-4, and the suppressive effect of IL-4 by PLAG was revealed ($P < 0.05$). STAT6 inhibitor (AS1517499) also showed IL-4 inhibitory effect ($P < 0.001$) when compared to PLAG treatment. These results indicate that PLAG inhibit IL-4 through blocking STAT6 signaling.

3.6 | PLAG attenuates PKC-ζ/STAT6 signal in hepatocytes

The intracellular mechanism underlying attenuation of liver injury by PLAG was further verified using an in vitro model. HepG2 and Hep3B liver cells and HL-60 neutrophil-like cells

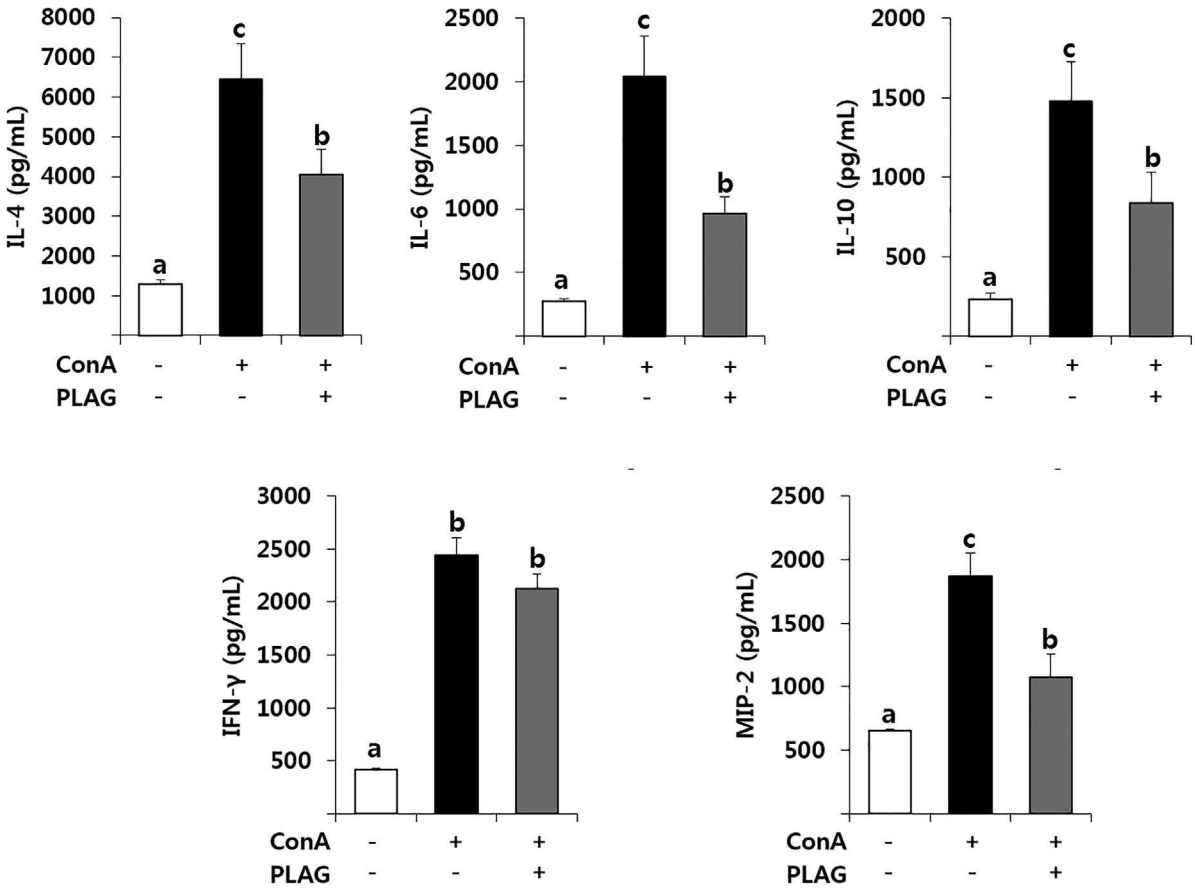

FIGURE 3 PLAG modulates plasma cytokine levels in Con A-induced hepatitis. Balb/c mice were treated with or without PLAG for 2 h before Con A injection. After 18 h, whole blood was collected by retro-orbital bleeding and cytokine levels were determined. Data are expressed as the mean ± SEM. Mice were randomly divided into three groups: control ($n = 8$), Con A ($n = 10$), and PLAG pretreatment group ($n = 10$). In the figure above, significant differences among groups at $P < 0.05$ were marked with different letters (a, b, c) and groups that share the same letter are not significantly different

were used for in vitro assays. The cytotoxicity of EC-18 in hepatocytes and leukocytes was evaluated using a WST assay (Supplementary data 1). The viability of Hep3B and HL-60 cells was not affected during the 24 h incubation with PLAG at concentrations of up to 1000 μg/mL. Based on the results of WST assay, 100, 10, and 1 μg/mL of PLAG were used in the cells.

PKC-ζ, JAK1, and STAT6 activation play an important role in Con A-induced hepatitis[17,18] and the activity of these kinases is increased by IL-4. Western blot analysis showed that phosphorylation of PKC and STAT6 was induced by IL-4 stimulation, and that phosphorylation of these kinases was significantly attenuated by PLAG treatment in a dose-dependent manner as shown in Figure 6A. These results suggest that PLAG effectively controlled IL-4-induced hepatitis by decreasing the activation of PKC-ζ/λ. The transcriptional activity of STAT6 associated with the expression of neutrophil migration genes was further analyzed using a luciferase reporter assay. The assay revealed that the transcriptional activity of STAT6 was effectively inhibited by treatment with 10 and 100 μg/mL of PLAG using 20 and 200 nM of STAT6 inhibitor (AS1517499) as positive control (Figure 6B). These data suggest that EC-18 has an effect on the attenuation of PKC-ζ/λ activation and sequential dephosphorylation of JAK1, which results in the dephosphorylation of STAT6 and the deactivation of PKCζ/JAK1/STAT6 cascade.

3.7 | In vitro assay for the analysis of PLAG function on neutrophil activation and adhesion

Neutrophil adhesion assays were conducted to examine neutrophil trans-endothelial migration activity. We examined that the modification of HL-60 activity by Con A or IL-4 was calculated using the WST-1 assay and cell counting. Con A induced HL60 cell adhesion and PLAG treatment significantly reduced adhesion as shown Figures 7A and 7B

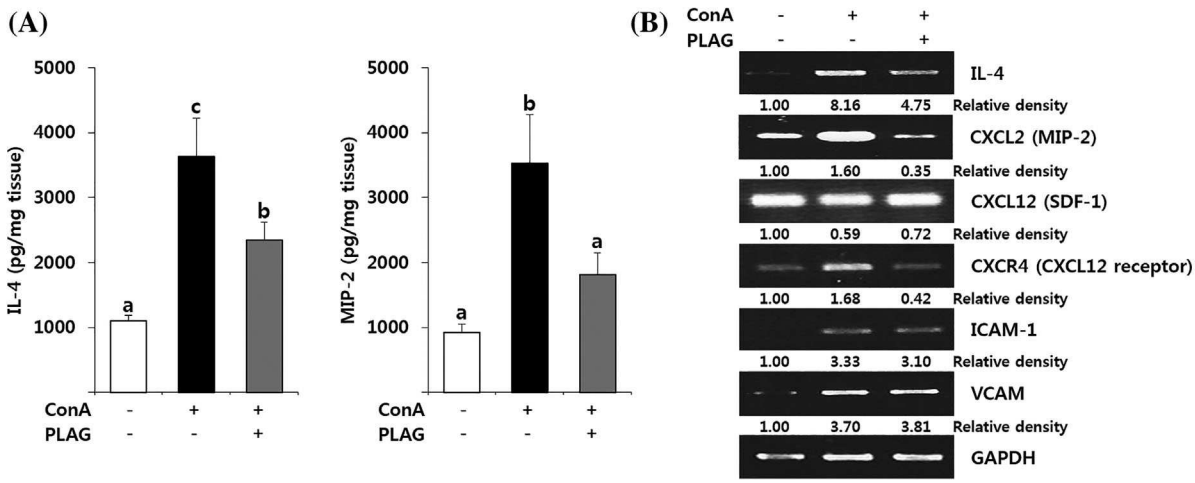

FIGURE 4 IL-4 and CXCL2 in liver tissue were decreased in PLAG-treated mice. The protein levels of IL-4 and CXCL2 (A), and mRNA expression of IL-4, CXCL2, CXCL12, and its receptor, ICAM-1, and VCAM (B) were evaluated in Con A-induced hepatitis by ELISA and RT-PCR, respectively. The mice were treated with or without PLAG for 2 h before Con A injection. After 18 h, liver tissue was collected and cytokine levels and mRNA expression were determined. Data are expressed as the mean ± SEM. Mice were randomly divided into three groups: control ($n = 8$), Con A ($n = 10$), and PLAG pretreatment group ($n = 10$). In the figure above, significant differences among groups at $P < 0.05$ were marked with different letters (a, b, c) and groups that share the same letter are not significantly different

($P < 0.05$). Moreover, at the same condition, IL-4 also lead to cell adhesion as well as Con A. The results of the assay indicated that 10 ng/mL of IL-4 was enough to stimulate HL-60 cell activity for adhesion to coated fibronectin, and that 100, 10, and 1 μg/mL of PLAG significantly reduced the attachment of HL-60 cells in a dose- dependent manner as shown in Figures 7C and 7D ($P < 0.05$). The RT-PCR results showed that HL-60 cells stimulated with IL-4 secreted IL-8 and VCAM-1, which might stimulate the cells to adhere to fibronectin, and that the addition of PLAG effectively reduced the expression of IL-8 and VCAM-1 in a dose-dependent manner (Figure 7E).

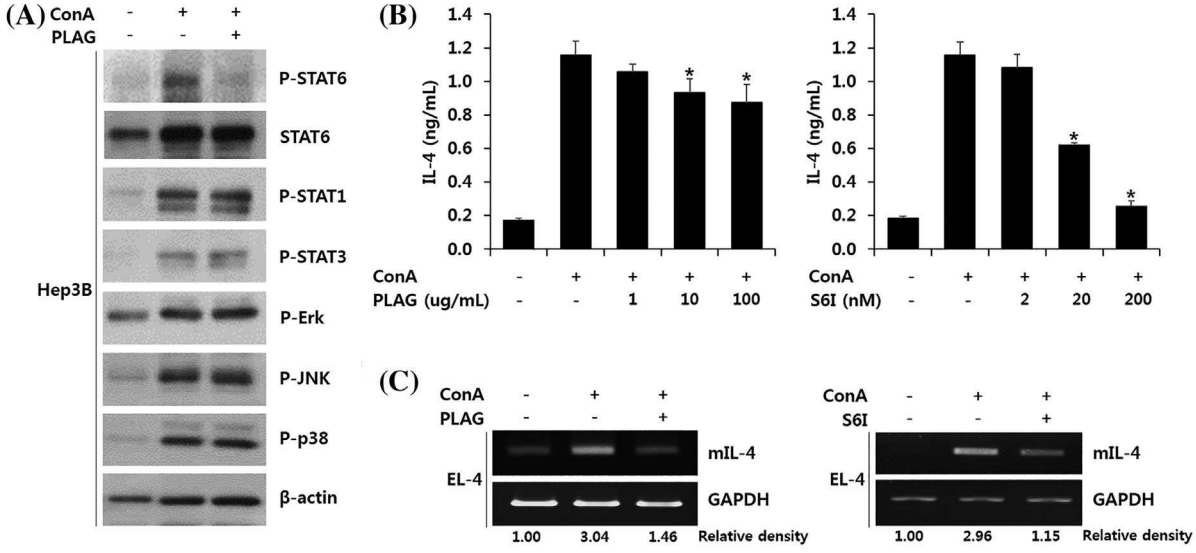

FIGURE 5 PLAG inhibited STAT6 phosphorylation in Con A induced hepatocyte. (A) Hep3B cells were pretreated with PLAG for 2 h and then stimulated with Con A for 3 h. The phosphorylation of STATs (STAT1, 3, 6) and MAPKs (ERK 1/2, JNK, p38) were determined using western blotting with GAPDH as the internal control. EL-4 cells were pretreated with PLAG and STAT6 inhibitor (AS1517499) for 2 h and then stimulated with Con A for 6 (C) or 24 h (B). Cell supernatant and pellet were collected and cytokine levels and mRNA expression were determined by ELISA and RT-PCR, respectively

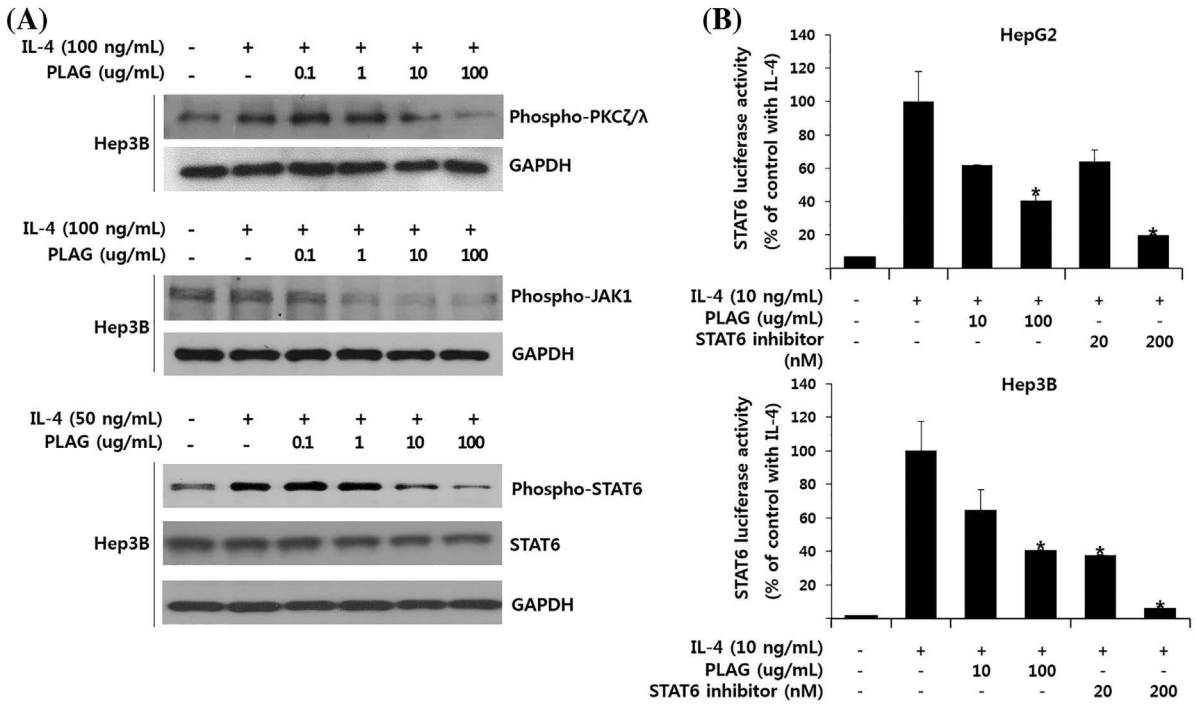

FIGURE 6 PLAG attenuated PKC-ζ/λ and STAT6 phosphorylation in activated hepatocytes. (A) Hep3B cells were pretreated with PLAG for 2 h and then stimulated with IL-4 for 2 h. The phosphorylation of PKC-ζ/λ, JAK1, and STAT6 were determined using western blotting with GAPDH as the internal control. (B) The pGL4-STAT6-Luc-Hygro vector was transfected into Hep3B and HepG2 cells for 48 h. The cells were pretreated with PLAG for 2 h and then stimulated with IL-4 for an additional incubation period of 6 h. Luciferase activity was assessed using the Dual-Glo™ Luciferase assay system. Data are expressed as the mean ± SEM. *Represents $P < 0.05$ relative to the IL-4 treated group

3.8 | PLAG inhibit novel and atypical PKC in neutrophil like HL-60 cells

As shown in Figure 8, HL-60 cells express PKCs (conventional, atypical and novel PKC) and PLAG markedly inhibited PKC-δ in a dose dependent manner. Moreover, PKC-ζ/λ also slightly blocked by PLAG. These results indicate that PLAG may block the neutrophil adhesion or activation via regulating PKC phosphorylation.

3.9 | The specificity of PLAG

The selectivity of PLAG was confirmed by a comparison of PLAG and PLG in STAT6 phosphorylation and transcriptional activity, respectively. As shown in Figure 9A, PLG is a prototype of diacylglycerol (DAG) and PLAG is a type of acetylated DAG. The specificity of PLAG function in mitigation of liver injury was indirectly examined by analysis of STAT6 activity. Western blot analysis using anti-phospho-STAT6 showed that the phosphorylation of STAT6 was significantly reduced in PLAG-treated Hep3B cells, but was not altered in the PLG-treated cells (Figure 9B). A promoter analysis also showed that STAT6 transcriptional activity induced by IL-4 was markedly reduced by half in PLAG-treated cells ($P < 0.001$), but was not altered in PLG-treated cells (Figure 9C). These results indicate that PLAG plays a unique role in the protection of the liver from injury in Con A-induced hepatitis.

4 | DISCUSSION

Hepatitis is a medical condition characterized by T cell-mediated immune response and inflammation of the liver. In hepatitis, leukocyte recruitment increases and intrahepatic localization of these cells induces pathogenesis of the disease.[31] Although much is known about the pathogenic mechanisms of hepatitis, a therapeutic target has yet to be discovered. PLAG is a synthetic monoacetyldiaglyceride that has been shown to have immunomodulatory properties.[27] However, the protective effect of PLAG on leukocyte migration and cytokine secretion in liver diseases has not yet been elucidated. Therefore, we focused on the infiltration of neutrophils to the liver following administration of Con A, which is known to induce IL-4 and IFN-γ-mediated liver injury. In the current study, we found that Con A-induced leukocyte migration and related cytokine secretion were improved in PLAG-treated mice, and we further confirmed this amelioration by analysis of the histological findings. PLAG also exerted a STAT6 pathway inhibitory effect by

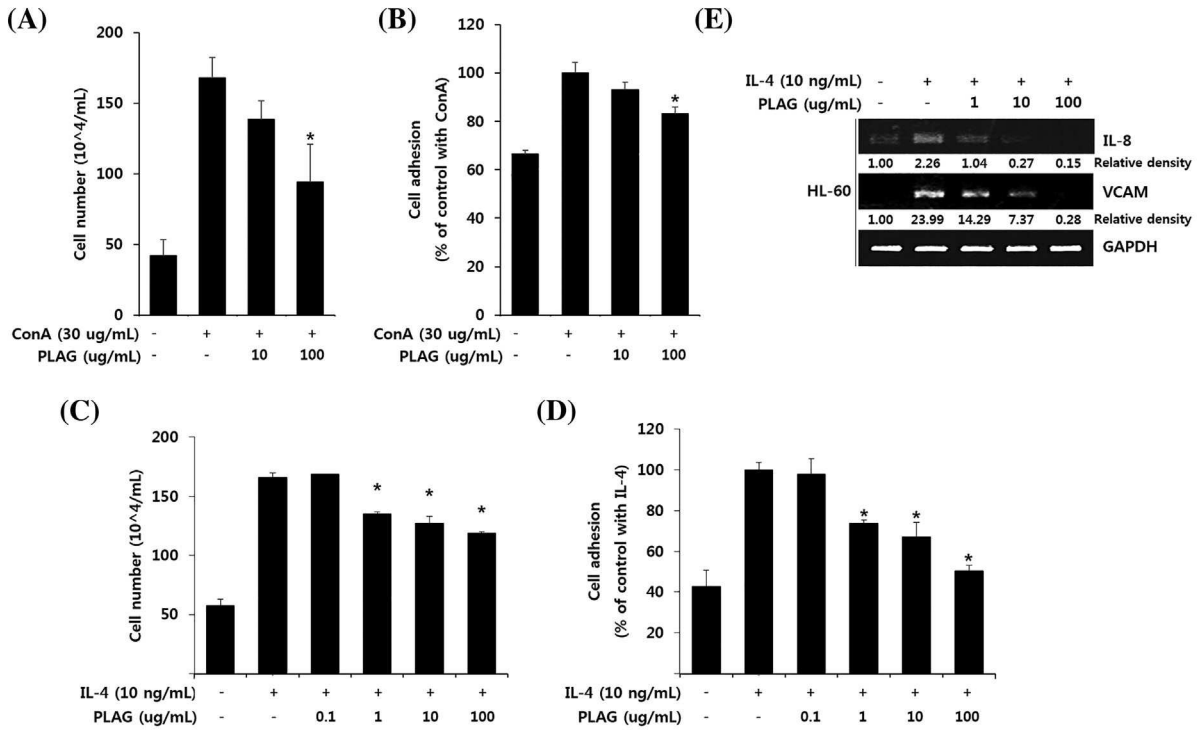

FIGURE 7 In vitro assay of the function of PLAG in neutrophil activation and adhesion. DMSO-treated HL-60 cells were treated with PLAG for 2 h and then stimulated with Con A or IL-4 for 4 h. The cell adhesion assay was performed using a hemocytometer (A and C) and a WST-1 assay (B and D). (E) HL-60 cells were pretreated with PLAG for 2 h and then stimulated with IL-4 for 2 h. The mRNA levels of IL-8 and VCAM were determined using RT-PCR. Data are expressed as the mean ± SEM. *Represents $P < 0.05$ relative to the IL-4 treated group

blocking atypical PKC phosphorylation. These results indicate that PLAG attenuates cytokine secretion and the migration of neutrophils to the liver in Con A-induced liver injury, thereby inhibiting the activation of the atypical PKC signal pathway in hepatocytes and novel PKC in neutrophil-like HL-60 cells.

Con A induces T cell activation and causes T cell-mediated hepatic injury.[32] T cells induce cytokines,

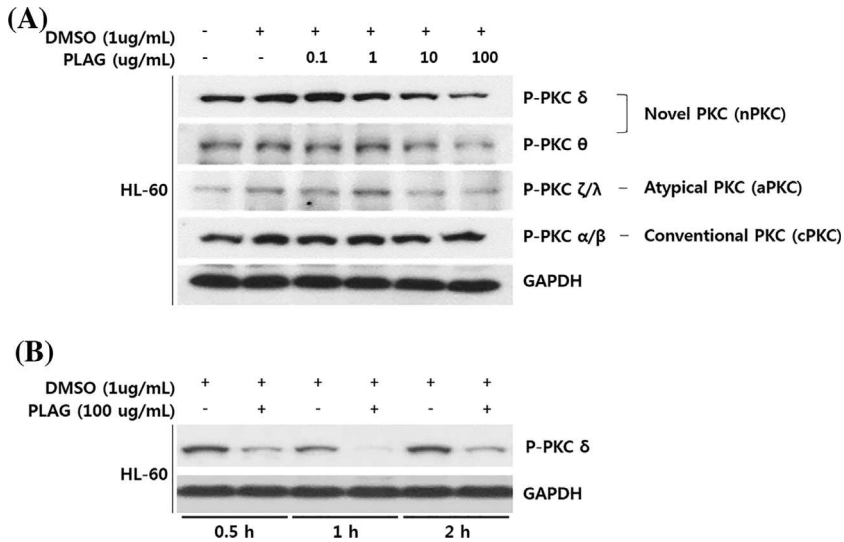

FIGURE 8 PLAG inhibited PKC δ phosphorylation in neutrophil. (A) HL-60 cells were treated with the indicated concentrations of PLAG for 2 h, and then phosphorylation of PKC δ, θ, ζ/λ, α/β, and GAPDH levels were determined using western blotting. (B) HL-60 cells were treated with 100 μg/ml PLAG at various times (0.5, 1, 2 h)

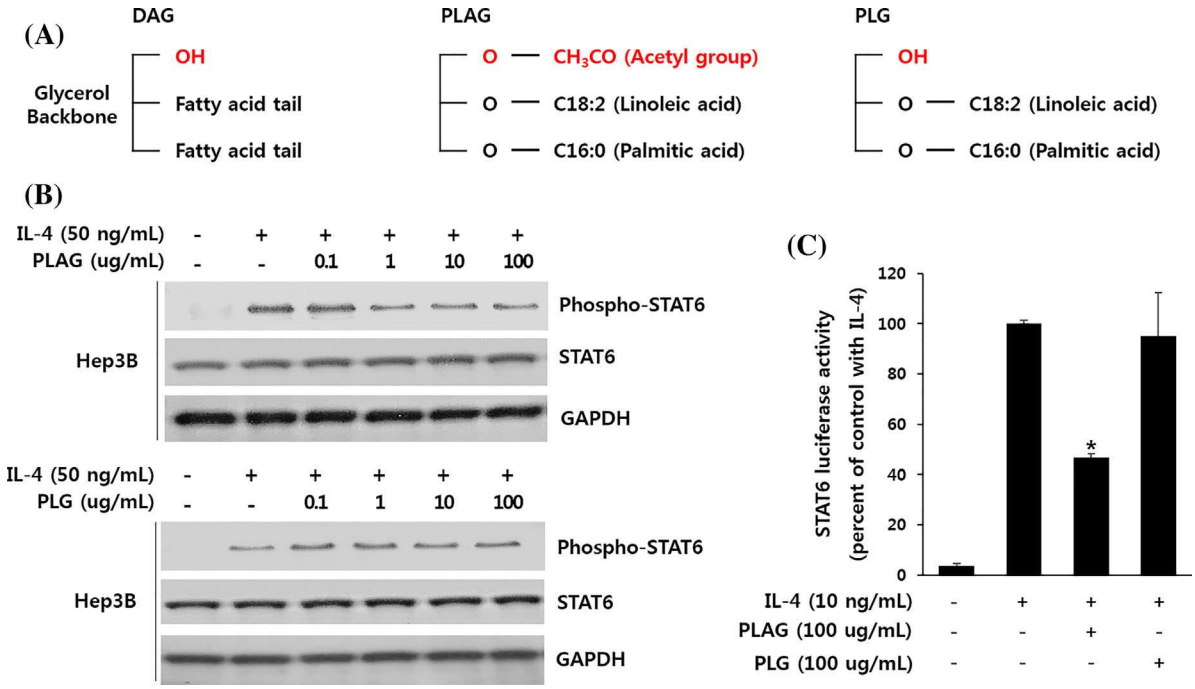

FIGURE 9 The specificity of PLAG in the activation and transcriptional activity of STAT6. (A) The simple structure of PLAG and PLG. PLAG is a type of acetylated DAG and PLG is a prototype of DAG. (B) Hep3B cells were pretreated with PLAG and PLG for 2 h and then stimulated with IL-4 for 2 h. The phosphorylation of STAT6 was determined by western blotting. (C) The pGL4-STAT6-Luc-Hygro vector was transfected into Hep3B cells for 48 h. The cells were pretreated with PLAG and PLG for 2 h and then stimulated with IL-4 for an additional incubation period of 6 h. Luciferase activity was assessed using the Dual-Glo™ Luciferase assay system. Data are expressed as the mean ± SEM. *Represents $P < 0.05$ relative to the IL-4 treated group

such as TNF-α, IFN-γ, and IL-4. In turn, IL-4 and IL-13 specifically activate STAT6, which plays a crucial role in Th2 differentiation, tissue adhesion, and inflammation, and exerts a variety of effects on hemopoietic tissues. Therefore, modulation of IL-4 mediated STAT6 also be an effective therapeutic strategy for hepatitis. The current study revealed that IL-4, IL-6, IL-10, IFN-γ, and CXCL2 were secreted into the plasma of Con A-induced mice. However, PLAG treatment decreased the secretion of these cytokines, except for IFN-γ (Figure 3). Considering the association of IFN-γ and IL-4, as well as Con A-induced hepatitis, we speculated that PLAG reduced neutrophil migration and adhesion by inhibiting IL-4/STAT6 signaling. In addition, protein levels and mRNA expression of IL-4 and CXCL2 in the liver were also inhibited by PLAG, which was similar to the plasma levels (Figures 3 and 4). This observation indicated that PLAG could potentially inhibit inflammatory responses in Con A-induced hepatitis.

IL-4 induces neutrophil maturation in HL-60.[33] and promotes the recruitment of neutrophils and monocytic cells in murine air pouch models.[7] Additionally, IL-4 directly augments the release of IL-8 from human bronchial epithelial cells[8] and human neutrophils,[9] as well as the expression of chemokines in Con A-induced hepatitis via a STAT6-dependent mechanism.[17] As well, neutrophils are known to express the type 1 IL-4 receptor, and IL-4 can play both pro- and anti-inflammatory roles in neutrophils.[7] Neutrophils are strongly activated by a variety of pathogenic stimuli, including phorbol esters,[34] lipopolysaccharide,[35] proinflammatory cytokines,[36] CXC-chemokines,[37] or IL-4.[33] Neutrophils migrate from the bloodstream through the vascular endothelium to their target[38] and subsequently induce necrotic tissue destruction.[39] Neutrophil depletion reduces leukocyte infiltration to the liver in Con A-injected mice.[1] In our study, the numbers of infiltrating neutrophils in the livers of Con A-injected mice significantly increased, but were reduced in the liver and maintained in the blood by PLAG treatment (Figuer 2). This finding indicates that PLAG blocks neutrophil infiltration to the liver via the inhibition of cytokine secretion, which could contribute to the improvement of hepatitis.

Transmigration (extravasation) of neutrophils can be mediated by chemotactic signals such as IL-8, CXCL2, or keratinocyte-derived chemokines (KC).[37] IL-8 plays a key role in the recruitment and activation of neutrophils during inflammation and serves as a potent chemoattractant. These mediators upregulate the expression of macrophage-1 antigen (MAC-1, CD11b/CD18), a member of the β2 integrin family of adhesion molecules,[40] as well as the expression of endothelial cell intracellular adhesion molecules (ICAM-1).

Previous studies have shown that ICAM-1 (CD54) and its integrin ligands contribute cooperatively to Con A-induced hepatitis by regulating leukocyte infiltration and subsequent cytokine production.[41] Moreover, IL-4 markedly induced VCAM-1 in endothelial cells via the STAT6 binding transcription factor, but did not affect ICAM-1 expression[42] and the adherence of neutrophils to hepatocytes through their β2 integrins and ICAM-1 expression on hepatocytes. In our study, Con A injection induced ICAM-1 and VCAM mRNA expression in the liver, but these adhesion molecules were not affected by PLAG (Figure 4). However, PLAG attenuated VCAM and IL-8 expression in IL-4 treated human promyelocytic HL-60 cells (Figure 7E). Moreover, PLAG suppressed the adhesion of differentiated HL-60 cells to the culture plate in a dose-dependent manner (Figures 7A-D). These findings suggest, at least in part, that PLAG protects against neutrophil adhesion.

STAT proteins are critical mediators of cytokine-inducible genes. Among the STAT proteins, STAT6, which is a transcription factor that is activated during Th2 differentiation, has been associated with immunoregulatory cytokines including IL-4 and IL-13.[43] STAT6 exists in an unphosphorylated, monomeric form in the cytoplasm. Upon the binding of IL-4 or IL-13 to their cognate receptors, STAT6 becomes activated through the phosphorylation of members of the JAK family.[14] Phosphorylated STAT6 proteins dimerize and are then translocated to the nucleus, where they bind DNA and activate transcription. STAT6 plays a critical role in T cell-mediated hepatitis and leukocyte recruitment.[17] This signal upregulates eotaxin expression in hepatocytes and induces IL-5 expression, resulting in eosinophil and neutrophil recruitment to the liver, and leading to hepatitis.[17] In contrast to the potential for STAT6 to induce T cell-mediated hepatitis, other studies have demonstrated hepatoprotective effect of STAT6 against hepatic ischemia/reperfusion injury.[44] These results imply that STAT6 activation might play either a helpful or harmful role in liver injury depending on the disease model in mice.[45] As described in our previous study,[46] PLAG regulated the transcription of IL-4 via dephosphorylation of STAT6 in human lung carcinoma epithelial cells but did not affect STAT1 in IFN-γ-treated cells. In our study, we observed that PLAG mainly down regulated the phosphorylation of STAT6 and also resulted in IL-4 reduction against Con A stimulation. From our data, we thought PLAG might specifically inhibit STAT6, because other signals does not changed by PLAG at the same condition. To verified intracellular mechanism underlying modulation of STAT6 by PLAG, we focused on IL-4 mediated STAT6 signals even though IL-4 expression is not fully dependent on STAT6. Consistent with the above findings, PLAG also blocked IL-4-mediated phosphorylation of STAT6 and transcriptional activity of STAT6 in human liver-derived Hep3B cells. In regards to the atypical PKCs in STAT6 signaling, PKC-ζ, and PKC-ι/λ downregulate two isoforms led to a decrease in STAT6 phosphorylation. Further mechanistic studies revealed that atypical PKCs, rather than the classic PKC or another signaling pathway, was pivotal in the IL-13-stimulated STAT6 activation.[47] Because PKC-ζ is reportedly associated with the phosphorylation of JAK and STAT6, PKC-ζ could be considered a potential upstream kinase. Our data provide evidence for the inhibitory effects of PLAG on PKC-ζ/ λ in the kinase cascades at the hepatocyte level (Figure 6).

PKC is a family of protein kinase enzymes that are critical to intracellular signaling in the differentiation, migration, proliferation, and activation of various cell types.[48,49] The PKCs are divided into three groups including classical (alpha, beta), novel (delta), and atypical (zeta). Neutrophils express PKC-α, PKC-β, PKC-δ, PKC-ζ, PKC-ι/λ, and PKC-θ.[49] Of those PKCs, PKC-δ is an important regulator of neutrophil adhesion and the influx of neutrophils to the lungs.[50] The inhibition or deficiency of PKC-δ causes markedly reduced adhesion in neutrophils stimulated by chemotactic factors[51] or by cecal ligation and puncture.[50] In our study, PKC-δ was strongly inhibited by PLAG in human promyelocytic HL-60 cells (Figure 8), which may block the neutrophil adhesion that accompanies liver injury. However, our study suffered from limitations. One limitation was that IL-4 was used as a stimulator of neutrophil adhesion, which might not explain the mechanism of PKC-δ adhesion clearly because the phosphorylation of PKC-δ was only confirmed in non-stimulated cells. As well, PKC-ζ has an effect on neutrophil adhesion that is similar to PKC-δ. It is implicated in the adhesion of human neutrophils to fibrinogen, and the PKC-ζ inhibitory peptide led to a marked reduction in N-formyl-methionyl-leucyl-phenylalanine (fMLP) or IL-8 induced adhesion.[52]

The 1-palmitoyl-2-linoleoyl-3-acetyl-glycerol structure of PLAG enables the regulation of cytokines via atypical PKC/STAT6 modulation. This compound is structurally similar to PLG (1-palmitoyl-2-linoleoyl-glycerol), in which an acetyl group is substituted by a hydroxyl group. We aimed to determine whether the structural difference might differentially affect the working mechanism. Our findings show that PLAG treatment results in the inhibition of STAT6 activation in Hep3B cells. On the contrary, PLG lacks any inhibitory effect on STAT6 phosphorylation (Figure 9). Thus, this significant effect on STAT6 activation might be related to differences in the chemical composition of the different glycerols.

In conclusion, our findings demonstrate the targeting of PLAG in the hepatic infiltration of neutrophils and the activation of an atypical and novel PKC pathway in hepatocytes and human promyelocytic HL-60 cells. The precise mechanism by which PKC-δ confers these effects requires further investigation, but the present findings suggest

that the ability of PLAG to suppress the phosphorylation of the PKC-ζ/STAT6 pathway resulted in inhibition of hepatic cytokine expression in the plasma, the expression of adhesion molecules, and neutrophil migration. Thus, our results suggest that PLAG has therapeutic potential for the treatment of Th2 cell-mediated liver diseases such as autoimmune hepatitis.

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CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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