



## Rebuilding the balance of STAT1 and STAT3 signalings by fusaruside, a cerebroside compound, for the treatment of T-cell-mediated fulminant hepatitis in mice

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### ABSTRACT

Dysregulation of signal transducer and activator of transcription (STAT) signaling is usually associated with intricate immune diseases and rebuilding the balance of STAT1 and STAT3 signaling is being explored as a useful approach for the treatment of these diseases. However, few chemicals have been reported to rebuild the balance of these two signalings for immune hepatitis therapy. In the present study, we found that fusaruside, a new kind of cerebroside isolated from an endophytic fungus *Fusarium* sp. IFB-121 in *Quercus variabilis*, significantly ameliorated concanavalin A (Con A)-induced T-cell-mediated fulminant hepatitis in mice, which was closely associated with the improvement of histopathological parameters, inhibition of activation of liver CD4<sup>+</sup> T cells and NKT cells, regulation of balance of Th1/Th2/Th17/Treg cytokines and protection of hepatocyte from apoptosis. Moreover, T-cell proliferation and activation was also notably inhibited by fusaruside *in vitro*. Furthermore, the protective effect of fusaruside was attributable to a novel regulatory mechanism through down-regulating STAT1 activation and T-bet expression in liver CD4<sup>+</sup> T cells and up-regulating STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes. In conclusion, fusaruside exhibited its capability against T-cell-mediated liver injury *in vivo*, through rebuilding the balance of STAT1 and STAT3 signalings. These results suggest that fusaruside is potentially useful for the treatment of T-cell-mediated human liver disorders.

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### 1. Introduction

The Janus kinase-signal transducer and activator of transcription factor (JAK-STAT) signaling pathway, activated by more than 40 cytokines and growth factors, plays an important role in the control of a wide variety of biological processes. Dysregulation of JAK/STAT signaling was usually associated with various human diseases, such as immune disorders and tumorigenesis [1,2]. A body of reports has demonstrated that many inflammatory diseases involve the nonrestrictive activation of the cytokine-JAK-STAT pathway [3–10]. The inhibition and/or enhancement of activation of various STAT family members could ameliorate the severeness of the diseases. For example, IFN- $\gamma$ /STAT1 had been proved essential for CD4<sup>+</sup> T-cell activation in Con A-induced liver injury model, because such activation was markedly attenuated in IFN- $\gamma$ <sup>-/-</sup> or STAT1<sup>-/-</sup> mice [11–13]. In contrast to STAT1, IL-6/STAT3 signaling played a protective role by inducing the

anti-apoptotic protein expression in the progress of many diseases [12,14,15]. Different STAT members are activated by distinct group of cytokines and a wide variety of STAT signaling involve in the pathogenesis of the same disease, which made it difficult to regulate specifically and properly in the progress of diseases. There is much evidence for the pathogenesis of various inflammatory diseases due to the imbalance of STAT1 and STAT3 signalings [3–9]. Therefore, rebuilding the balanced STAT1 and STAT3 signalings might be a useful approach for the treatment of these intricate immune diseases.

Cerebrosides are a group of glycosphingolipids and made up of essential components of various tissues and organs in biological systems. Chemically, they are composed of a hexose and a ceramide moiety, which usually consists of a long-chain aminoalcohol trivially called 'sphingoid base' (=sphingosine or sphingol) and an amide-linked long-chain fatty acid. Biologically, they are structural support and texture determinants of cell membranes, and act most likely through protein binding as mediators of biological events, such as activation, cell agglutination, intracellular communication and cell development. Moreover, they play essential biological roles in cellular membranes as cell surface antigens or receptors. A growing pile of evidence has indicated that cerebrosides possess a broad range of biological

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functions, all being potentially related to the common amphipathic and/or ionophoretic nature of this type of molecules [16]. Besides those chemically new and/or bioactive constituents from various endophytic fungi [17–19], we have extended our attention to the cerebroside constituents abundant in fungal cells. Co-bioassays with immunosuppressive tests showed that the chloroform-methanol (1:1) extract from the culture of *Fusarium* sp., an endophytic fungus in *Quercus variabilis* Bl., was significantly bioactive in the mixed lymphocyte reaction. Subsequently, a novel cerebroside compound fusaruside from the above chloroform-methanol (1:1) extract was obtained and its antibacterial and xanthine oxidase inhibitory activities were demonstrated [20]. Furthermore, our preliminary study found that fusaruside possessed an immunosuppressive activity *in vitro*. The purpose of this study, thus, was to report a novel strategy for immune hepatitis therapy involving rebuilding the balance of STAT1 and STAT3 signalings by means of this small molecule.

## 2. Materials and methods

### 2.1. Mice

Specific pathogen-free, 8- to 10-week-old female C57BL/6 mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

### 2.2. Drugs, reagents and antibodies

Fusaruside (the chemical structure is shown in Supplementary Fig. S1), with 99.2% of purity, was isolated from an endophytic fungus *Fusarium* sp. IFB-121 in *Q. variabilis* [20], without bacterial contaminants. Cyclosporin A (CsA) (Sandoz Ltd., Basel, Switzerland), Concanavalin A (Con A), carboxyfluorescein diacetate succinimidyl ester (CFSE), and mitomycin C were purchased from Sigma-Aldrich (St. Louis, MO). Kits for determining serum alanine transaminase (ALT), aspartate transaminase (AST) and lactic dehydrogenase (LDH) were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Acrylamide and bis-acrylamide were purchased from Sangon Co. Ltd. (Shanghai, China). Ac-DEVD-pNA was purchased from Alexis (San Diego, CA). RPMI 1640 and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT), Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). ELISA kits for IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-6, IL-17A and IL-10 were purchased from R&D systems (Minneapolis, MN). Murine cytokines IFN- $\gamma$  and IL-6 were purchased from Peprotech (Rocky Hill, NJ). PE-anti-mouse CD4 mAb (GK1.5) was purchased from eBioscience (San Diego, CA). FITC-anti-mouse CD69 mAb (H1.2F3) and FITC-anti-mouse CD3 mAb (17A2) were purchased from Biolegend (San Diego, CA). Anti-Bcl-X<sub>L</sub>, purified anti-mouse CD3 (145-2C11), and purified anti-mouse CD28 (37.51) were purchased from BD Pharmingen (San Diego, CA). Anti-STAT1 (9H2) and phospho-STAT antibody sampler kit were purchased from Cell Signaling (Beverly, Massachusetts). Anti-T-bet (H-210) and anti- $\alpha$  tubulin (TU-02) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse T-cell enrichment column was purchased from R&D system (Minneapolis, MN). Mouse CD4 (L3T4) MicroBeads were purchased from Miltenyi (Auburn, CA). *In situ* TUNEL Cell Death detection kit was purchased from Roche Applied Science (Mannheim, Germany). Light Shift Chemiluminescent

EMSA kit was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

### 2.3. Con A-induced hepatitis in mice: drug treatment schedules, transaminase assay and histological analysis

Acute liver injury was induced by injecting C57BL/6 mice with Con A in pyrogen-free phosphate buffered saline (PBS) at 15 mg/kg via the tail vein [21]. C57BL/6 mice were administered intraperitoneally for 3 times at an interval of 8 h with fusaruside or CsA suspended in PBS at different doses. One hour after the final administration, 15 mg/kg of Con A was injected intravenously to induce the liver damage. In control animals, the same dose of PBS was given before Con A injection. Mice were killed at the indicated time points, and blood samples, livers and spleens were collected. Serum alanine transaminase (ALT), aspartate transaminase (AST) and lactic dehydrogenase (LDH) activities were measured by commercial kits as the protocols indicated. Portion of livers from individual mice were fixed in 10% formaldehyde and embedded in paraffin. The tissue sections were stained with hematoxylin-eosin for morphological evaluation and read on a 0–3 scale (0, no change; 1, mild; 2, moderate; and 3, severe) by a pathologist who had no prior knowledge of the induction of liver injury or other experimental data.

### 2.4. Isolation of liver mononuclear cells and hepatocytes

Mouse liver mononuclear cells and hepatocytes were isolated as described previously [22]. In brief, the livers of the mice were first perfused *in situ* via the portal vein with Ca<sup>2+</sup> and Mg<sup>2+</sup> free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA and 25 mM HEPES at 37 °C. Then, the buffer was replaced with 0.1% collagenase solution in HBSS (containing 4 mM CaCl<sub>2</sub> and 0.8 mM MgSO<sub>4</sub>). After a few minutes of perfusion, the liver was excised rapidly from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered through a 100 gauze mesh. After washing twice, the pellets were resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). The supernatant obtained after isolating the above parenchymal cells was used for preparation of nonparenchymal cells by centrifugation at 300 × g for 10 min. In some cases, the nonparenchymal cell suspensions were overlaid on Percoll gradient solutions consisting of 25%, 50% and 80% Percoll and then centrifuged at 600 × g for 20 min. The resulting layers which appeared between 25% and 50% or between 50% and 80% of Percoll were named as Fraction A and Fraction B, respectively. Then, Fraction A was confirmed to be about 88–94% of Kupffer cells and Fraction B was the lymphocyte-enriched populations. Cells in the different layers were collected and washed three times with RPMI 1640 medium.

### 2.5. Preparation of spleen cells and purification of T cells

The spleen cell preparation and T-cell purification were performed as previously described [23]. Mouse CD4<sup>+</sup> T cells from splenocytes were purified using magnetic beads (Miltenyi) with more than 95% purity as assessed by flow cytometric analysis. The cells were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO<sub>2</sub> atmosphere at 37 °C.

### 2.6. Flow cytometric analysis of T-cell activation in the liver after administration of Con A

Mice were killed 0, 2, 5 and 8 h after Con A administration and liver mononuclear cells were isolated. Activation of CD4<sup>+</sup> T cells

was determined by anti-CD4 plus anti-CD69 (early activation marker) or anti-CD3 plus anti-FasL using a FACScalibur (Becton-Dickinson, San Jose, CA). The data were analyzed using CellQuest software (BD Immunocytometry Systems).

### 2.7. Cytokine assay

The cytokines in the cell supernatant or in serum were determined using ELISA kits for IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-6, IL-17A and IL-10 from R&D systems (Minneapolis, MN).

### 2.8. Western blot analysis

Western blot was done as described before [24]. Briefly, the cells were collected and lysed in lysis buffer containing protease inhibitor (protease inhibitor cocktail, Pierce). The proteins were fractionated by SDS-PAGE and electrotransferred to PVDF membranes. Different antibodies were used for blotting, and detection was done by LumiGLO chemiluminescent system (KPL, Guildford, UK).

### 2.9. Determination of STAT1 DNA binding by electrophoretic mobility shift assay (EMSA)

Nuclear extracts of the cells were subjected to EMSA assay as described before [25]. In brief, the cells ( $1 \times 10^7$ ) were collected and lysed in 500  $\mu$ l of lysis buffer A (HEPES 10 mM (pH 7.9), MgCl<sub>2</sub> 1.5 mM, KCl 10 mM, DTT 0.5 mM and PMSF 1 mM) for 10 min at 4 °C. Then 100  $\mu$ l of 10% NP40 were added. After 20 min incubation at 4 °C, the pellets were obtained by centrifugation at  $280 \times g$  for 5 min and washed by buffer A twice. Then, the pellets were lysed in 50  $\mu$ l of lysis buffer B (HEPES 20 mM (pH 7.9), MgCl<sub>2</sub> 1.5 mM, NaCl 420 mM, Glycerol 25%, EDTA 0.2 mM, DTT 0.5 mM and PMSF 1 mM) for 30 min at 4 °C. The supernatants obtained by centrifugation at  $1200 \times g$  for 5 min were nuclear extracts. The double-stranded oligonucleotide probe containing a gamma-activated sequence (GAS) (5' biotin-AGCCTGATTTCCCCGAAATGACGGC-3' and 5' biotin-GCCGTCATTTCCGGGAAATCAGGCT-3') corresponding to the STAT1 consensus DNA-binding site, was obtained from Invitrogen (Carlsbad, CA). Reactions for nuclear protein-DNA binding were performed using the Light Shift Chemiluminescent EMSA kit. Specificity of DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled GAS binding sequences (Invitrogen, Carlsbad, CA).

### 2.10. Single mixed lymphocyte reaction

Single mixed lymphocyte reaction was performed as described before [26]. The lymphocytes ( $5 \times 10^5$ ) from C57BL/6 mice, which had been treated with mitomycin C (500  $\mu$ g/ml) for 1 h, were co-cultured with the lymphocytes ( $5 \times 10^5$ ) from BALB/c mice (Model Animal Genetics Research Center of Nanjing University, Nanjing, China) in the presence or absence of the various concentrations of the drug at 37 °C in 5% CO<sub>2</sub> for 72 h. The proliferation of the lymphocytes from BALB/c mice was measured by MTT method. The value of OD<sub>570</sub> was determined by an ELISA reader and the stimulation index was counted as following formula: stimulation index = (OD<sub>sample</sub> - OD<sub>C57BL/6 alone</sub>)/OD<sub>BALB/c alone</sub>.

### 2.11. Caspase-3 activity assay

Mice were killed at 2, 5, and 8 h after Con A administration, and caspase 3-like activity was measured in liver homogenates using Ac-DEVD-pNA as a substrate (Alexis biochemicals) [27].

### 2.12. Statistical analysis

Data are presented as mean  $\pm$  SEM. The data were analyzed by one-way ANOVA with Student–Newman–Keuls (SNK) post hoc analysis. A value of  $P < 0.05$  was considered to be significant.

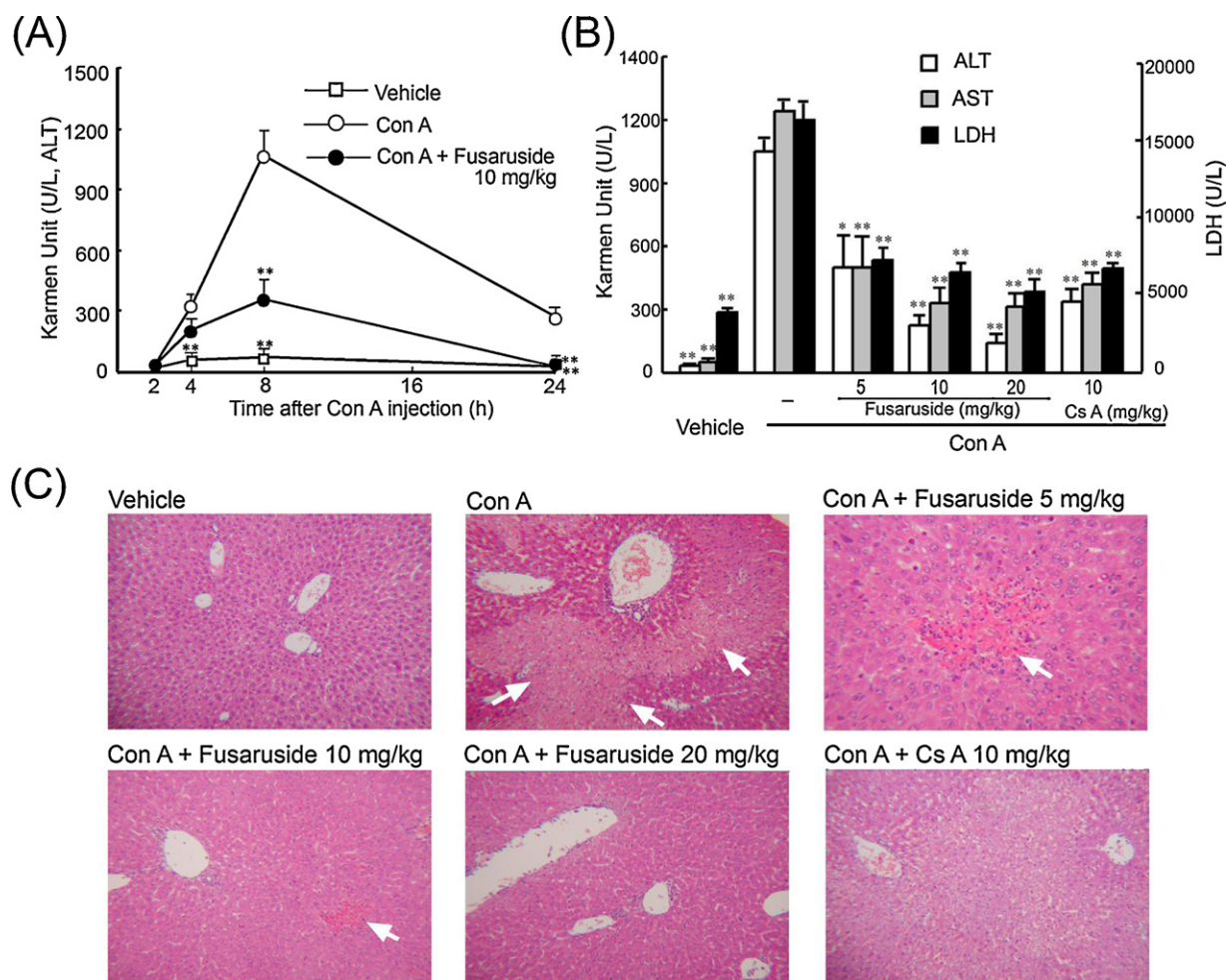
## 3. Results

### 3.1. Fusaruside ameliorated Con A-induced T-cell-mediated liver injury in mice

The chemical structure of fusaruside is cerebroside-like (molecular weight = 751; Supplementary Fig. S1). The purity of fusaruside was confirmed to be 99.2% by a high performance liquid chromatography assay. Con A-induced T-cell-mediated hepatitis in mice was used to evaluate the immunosuppressive effect of fusaruside *in vivo*. Intravenous administration of Con A significantly resulted in a time-dependent increase in serum ALT level. The ALT value began to increase at 4 h after Con A administration compared with naive group, reached a peak at 8 h, and declined thereafter. Compared with the vehicle control, fusaruside pretreatment significantly decreased the ALT level at 8 h and 24 h (Fig. 1A). Furthermore, at 8 h after Con A injection, fusaruside pretreatment dose-dependently reduced ALT, AST and LDH serum levels, among which 5 mg/kg of fusaruside showed the same intensity of inhibition with 10 and 20 mg/kg (Fig. 1B). In this case, 10 mg/kg of fusaruside showed a stronger inhibition than the same concentration of cyclosporine A, especially in ALT level. The histological examination of liver sections showed a massive necrosis with cytoplasmic swelling, inflammatory infiltration and severe hepatocyte degeneration in the Con A-treated mice without medication (Fig. 1C). Against these changes, pretreatment with fusaruside markedly reduced the extent of liver damage, and in the group treated with 20 mg/kg of fusaruside, only minor degenerative changes were observed. The detailed histological changes were shown in Supplementary Table 1. Notably, there was no significant difference in the weight and cell numbers of lymphoid tissues (thymus, spleen and lymph nodes) between the mice intraperitoneally injected with fusaruside (20 mg/kg) daily for 7 days and the mice treated with normal saline (data not shown), suggesting the safety of fusaruside to some degree.

### 3.2. Fusaruside inhibited the activation of liver CD4<sup>+</sup> T cells and NKT cells in Con A-injected mice

CD69 expression was determined by flow cytometric analysis as the early activation marker of liver lymphocyte activation. As shown in Fig. 2A and B, Con A caused a significant increase in CD4<sup>+</sup>CD69<sup>+</sup> double-positive cells (upper-right quadrant) with a peak almost at 5 h after Con A administration. Compared with the vehicle control, fusaruside completely abolished the increase in the numbers of liver T cells expressing CD69 (Fig. 2A and B). It has been shown that NKT cells are rapidly activated and consequently depleted after administration of Con A [28]. As shown in Fig. 2C and D, injection of Con A led to significant depletion of NKT cells (NK1.1<sup>+</sup>CD3<sup>+</sup> double-positive cells in upper-right quadrant) 2 h after Con A treatment in vehicle-treated mice, but not in fusaruside-treated mice. In the pre-experiment, we found that the drug concentration in the serum of mice with intraperitoneally administered 10 mg/kg of fusaruside thrice a day reached a peak at about 4.5  $\mu$ M by a high performance liquid chromatography assay. It was important to note that fusaruside at the concentrations (1–50  $\mu$ M) *in vitro* did not affect T lymphocyte's viability by MTT uptake assay (Fig. 5F) and annexin V-propidium iodide assay (Fig. 5G). These results suggest that the inhibition of fusaruside on



**Fig. 1.** Fusaride protected mice from Con A-induced liver injury. C57BL/6 mice were administrated intraperitoneally for 3 times at an interval of 8 h with fusaride or CsA suspended in PBS at different doses. One h after the final administration, 15 mg/kg of Con A was injected intravenously to induce the liver damage. (A) Time-dependent inhibition on alanine transaminase (ALT) activity by fusaride. Values are shown as mean  $\pm$  SEM from four mice at each time point.  $**P < 0.01$  vs. corresponding Con A-treated groups at the same time points. (B) Dose-dependent inhibition on ALT, aspartate transaminase (AST) and lactic dehydrogenase (LDH) activities by fusaride. Each column indicates the mean  $\pm$  SEM of eight animals.  $*P < 0.05$ ,  $**P < 0.01$  vs. Con A group. (C) Photomicrographs of representative livers with H&E staining are shown (original magnification 100 $\times$ ). White arrows indicate massive necrosis observed in the liver.

the activation of liver CD4<sup>+</sup> T cells and NKT cells is not due to a cytotoxic activity.

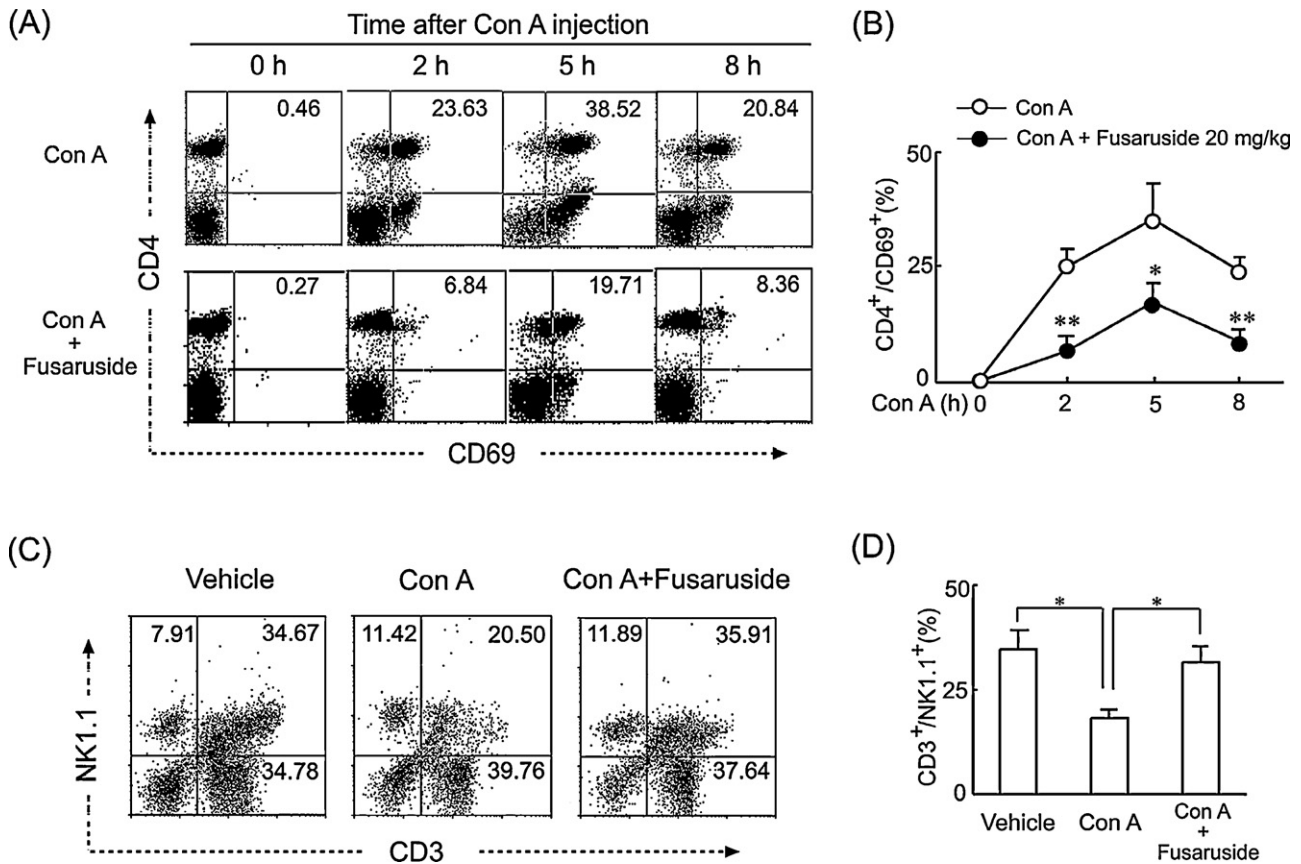
### 3.3. Fusaride modulated the cytokine profiles in Con A-induced hepatitis in mice

Th1/Th2 and Th17/Treg balance are critical for the maintenance of immune homeostasis. To determine whether the regulation of pro- and anti-inflammatory cytokines by fusaride contributed to the amelioration of Con A-induced liver injury, the effects of fusaride on the serum levels of Th1, Th2, Th17 and regulatory cytokines were examined. The productions of Th1 cytokine IFN- $\gamma$ , TNF- $\alpha$  and IL-2 as well as Th17 cytokine IL-17A were significantly inhibited by fusaride, while the productions of Th2 cytokine IL-4 and IL-6 were not markedly altered (Fig. 3). In addition, regulatory cytokine IL-10 was remarkably increased by fusaride *in vivo* (Fig. 3). It should be noted that the percentage of Treg cell population in liver lymphocytes was increased with the treatment of fusaride in the Con A-induced liver injury (Supplementary Fig. S2). These findings suggest the imbalance of Th1/Th2 and Th17/Treg is recovered by fusaride in Con A-induced T-cell-mediated fulminant hepatitis. Moreover, the mRNA expressions of IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and T-bet in the injury liver tissue were also reduced by

fusaride in a dose-dependent manner *in vivo* (Supplementary Fig. S3). Furthermore, the mRNA expressions of IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and T-bet (Supplementary Fig. S4) as well as IFN- $\gamma$  production (Supplementary Fig. S5) of splenic CD4<sup>+</sup> T cells induced by Con A were also inhibited by fusaride in a concentration-dependent manner *in vitro*.

### 3.4. Fusaride inhibited STAT1/T-bet signaling in CD4<sup>+</sup> T cells *in vivo* and *in vitro*

To further analyze the mechanism by which fusaride inhibits the production of IFN- $\gamma$ , we examined the STAT1/T-bet signaling in CD4<sup>+</sup> T cells *in vivo* and *in vitro*. Con A injection rapidly induced the expression of T-bet as well as the phosphorylation of STAT1 in liver CD4<sup>+</sup> T cells with the peak at 1–3 h and the elevations were gradually reduced thereafter. Against the elevations, fusaride administration showed a significant inhibition on the phosphorylated STAT1 in liver CD4<sup>+</sup> T cells from mice injected with Con A in a time-dependent manner (Fig. 4A). In consistent with the inhibition of STAT1 activation, fusaride also inhibited the DNA binding activity of STAT1 (Fig. 4B). As a downstream molecule of STAT1 signaling, Th1-specific transcription factor T-bet was also suppressed by fusaride *in vivo* (Fig. 4A) and *in vitro* (Fig. 4C).

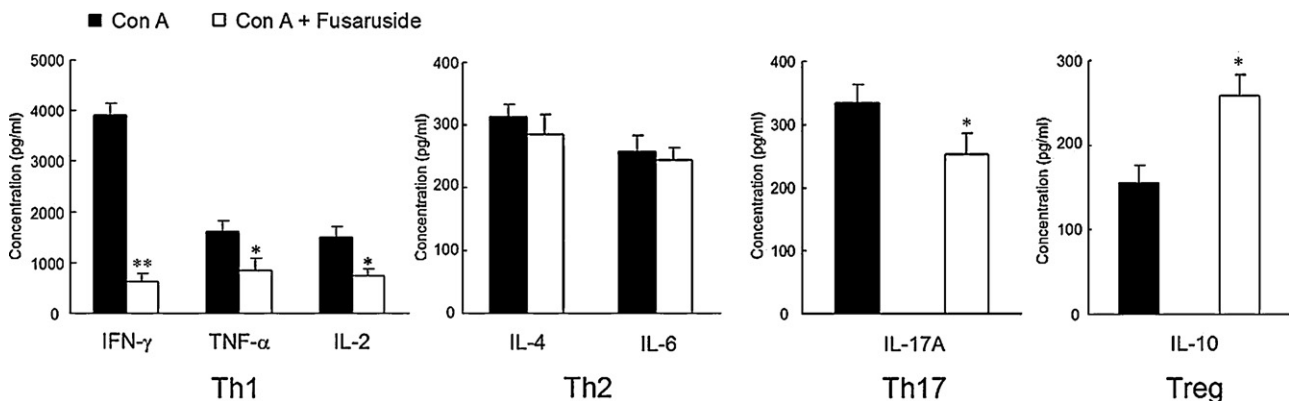


**Fig. 2.** Fusaricide suppressed the activation of liver CD4<sup>+</sup> T cells and NKT cells in Con A-injected mice. C57BL/6 mice were injected with Con A in the presence or absence of 20 mg/kg fusaricide pretreatment. (A) Hepatic lymphocytes were isolated at 2, 5 and 8 h later, respectively. The surface expression of CD4<sup>+</sup>CD69<sup>+</sup> was analyzed by flow cytometry. The panels shown here are representatives of three independent experiments. (B) Values are shown as mean ± SEM from three mice at each time point. \**P* < 0.05 and \*\**P* < 0.01 vs. corresponding Con A-treated vehicle control groups at the same time points. (C) Hepatic lymphocytes were isolated at 2 h after Con A treatment. The surface expression of CD3<sup>+</sup>NK1.1<sup>+</sup> was analyzed by flow cytometry. The panels shown here are representatives of three independent experiments. (D) Values are shown as mean ± SEM from three mice. \**P* < 0.05 vs. corresponding Con A-treated vehicle control group.

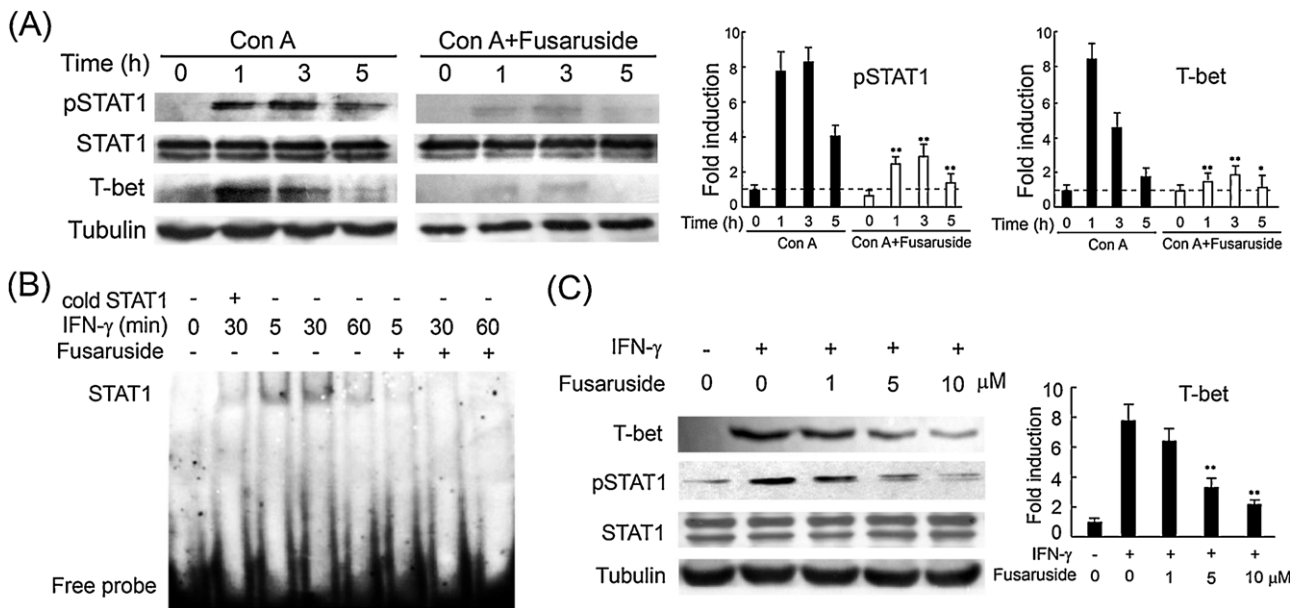
**3.5. Fusaricide inhibited proliferation and activation of murine splenic T cells in vitro**

Since STAT1/T-bet signaling was reduced by fusaricide, we wonder whether fusaricide inhibited T-cell proliferation and activation *in vitro*. As a result, fusaricide significantly inhibited splenic T-cell proliferation induced by both Con A (Fig. 5A) and

anti-CD3 plus anti-CD28 (Fig. 5B and D) in a dose-dependent manner. A similar inhibitory effect of fusaricide was also seen in the mixed lymphocyte reaction (Fig. 5C). Moreover, fusaricide dose-dependently reduced CD69 expression (Fig. 5E) of T cells activated by Con A. It was important to note that fusaricide at the concentrations mentioned above did not affect T lymphocyte's viability by MTT uptake assay (Fig. 5F) and annexin V-propidium



**Fig. 3.** Fusaricide modulated the cytokine profiles in Con A-induced hepatitis in mice. C57BL/6 mice were injected with Con A in the presence or absence of 20 mg/kg fusaricide pretreatment. The sera were collected at 3 h after Con A injection and determined by ELISA. Data are presented as mean concentration (pg/ml ± SEM) of 5 mice. \**P* < 0.05, \*\**P* < 0.01 vs. Con A group.



**Fig. 4.** Fusaruside inhibited STAT1/T-bet signaling in CD4<sup>+</sup> T cells *in vivo* and *in vitro*. (A) The liver CD4<sup>+</sup> T cells from Con A-treated vehicle control and 20 mg/kg fusaruside-pretreated mice were isolated at the indicated time and analyzed by Western blotting. The bands were measured and quantified by ImageQuant analysis (Amersham Biosciences). The values are shown as mean ± SEM from three independent experiments at each time point. \*P < 0.05, \*\*P < 0.01 vs. corresponding Con A-treated vehicle control groups at the same time points. (B) Splenic CD4<sup>+</sup> T cells isolated from C57BL/6 mice were cultured with 10 μM fusaruside for 3 h, then treated with murine IFN-γ for indicated time. After the incubation, nuclear proteins were extracted and assessed by EMSA analysis. The data shown here are representatives of three independent experiments. (C) Splenic CD4<sup>+</sup> T cells isolated from C57BL/6 mice were cultured with 1–10 μM fusaruside for 3 h, then treated with murine IFN-γ for 6 h (detection of T-bet) or 30 min (detection of pSTAT1). After the incubation, proteins were extracted and assessed by Western blotting. The bands were measured and quantified by ImageQuant analysis and the values are shown as mean ± SEM from three independent experiments. \*\*P < 0.01 vs. IFN-γ-treated group.

iodide assay (Fig. 5G). These results also indicate that the inhibition of fusaruside on T-cell proliferation and activation is not due to a cytotoxic activity.

### 3.6. Fusaruside enhanced and prolonged STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes and protected hepatocyte from apoptosis

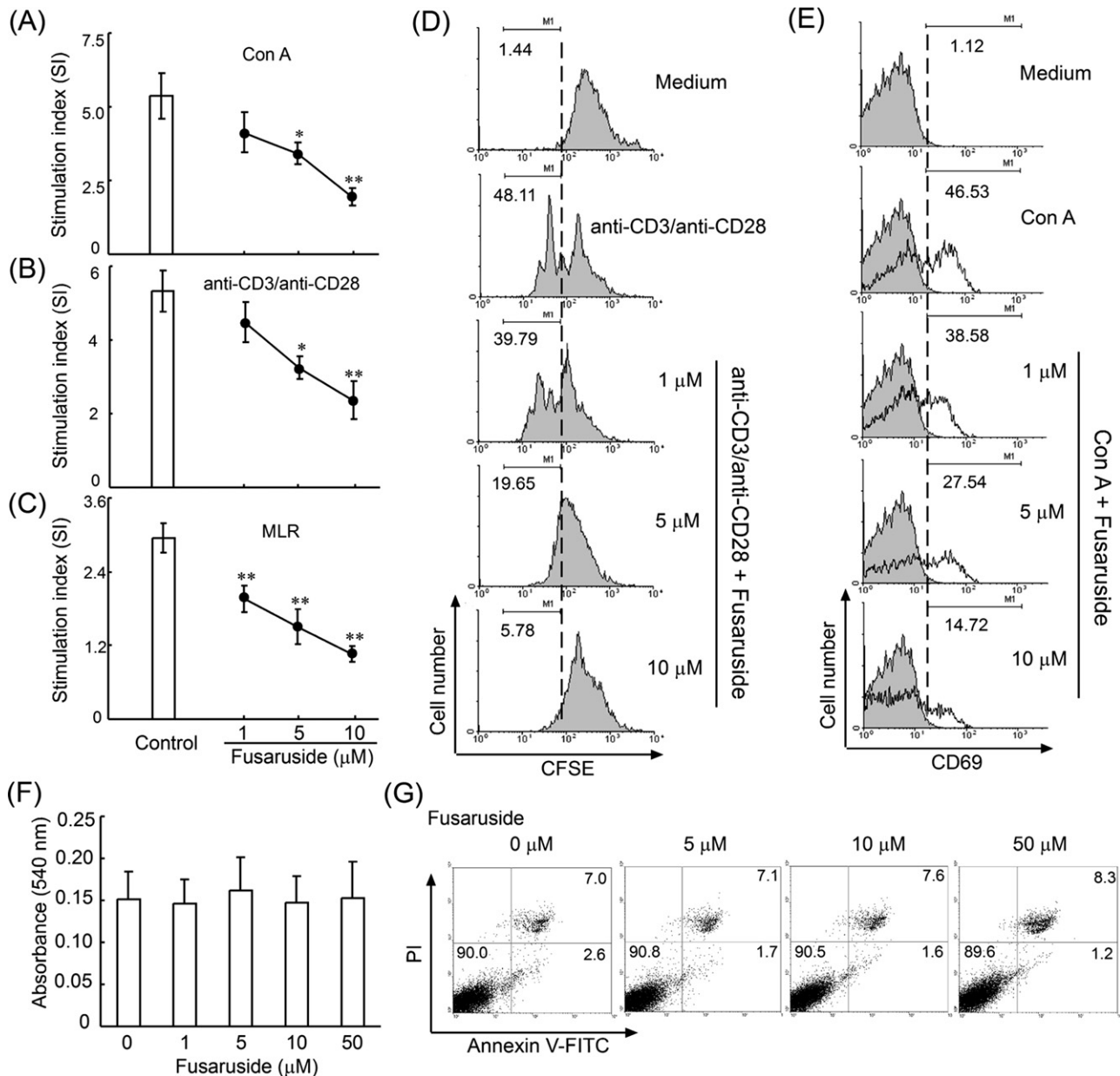
As shown in Fig. 6A and B, Con A administration caused a time-dependent increase in caspase-3 activation and activity. Pretreatment with 10 mg/kg of fusaruside significantly reduced caspase-3 activation and activity in hepatocytes in a time-dependent manner. Consistent with the data in caspase-3, the TUNEL staining assay also showed the reduction of apoptosis in hepatocytes from the fusaruside-treated group against those from vehicle-treated group (Fig. 6C). Interestingly, this compound significantly enhanced and prolonged the expressions of phosphorylated STAT3 and Bcl-X<sub>L</sub> in hepatocytes (Fig. 6D). Moreover, the enhancement in phosphorylated STAT3 and Bcl-X<sub>L</sub> was also seen in IL-6-stimulated hepatocytes treated with fusaruside *in vitro* (Fig. 6E). These results suggest that enhancing and prolonging STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes by fusaruside accounts for the amelioration of liver injury induced by Con A.

## 4. Discussion

Many studies have documented that STATs are important and essential to modulate various inflammatory diseases [3–10]. The STAT1 and STAT3 signaling pathways play a key pathogenic role in T-cell-mediated hepatitis and the down-regulation of STAT1 signaling has been shown to prevent or terminate the development of the inflammation, which suggests that the regulation of STATs activation may be a useful approach for the treatment of T-cell-mediated immune diseases [11,12]. However, only a few drug candidates have been reported working through such regulations of STATs so far. In this study we provided experimental evidence of an *in vivo* therapeutic effect for fusaruside in a murine model of

T-cell-mediated liver injury. As a result, we found the unique characteristics of fusaruside on the modulation of STAT1, STAT3 and their downstream signalings, which were distinct from the present immunosuppressants, such as antimetabolites and glucocorticoids.

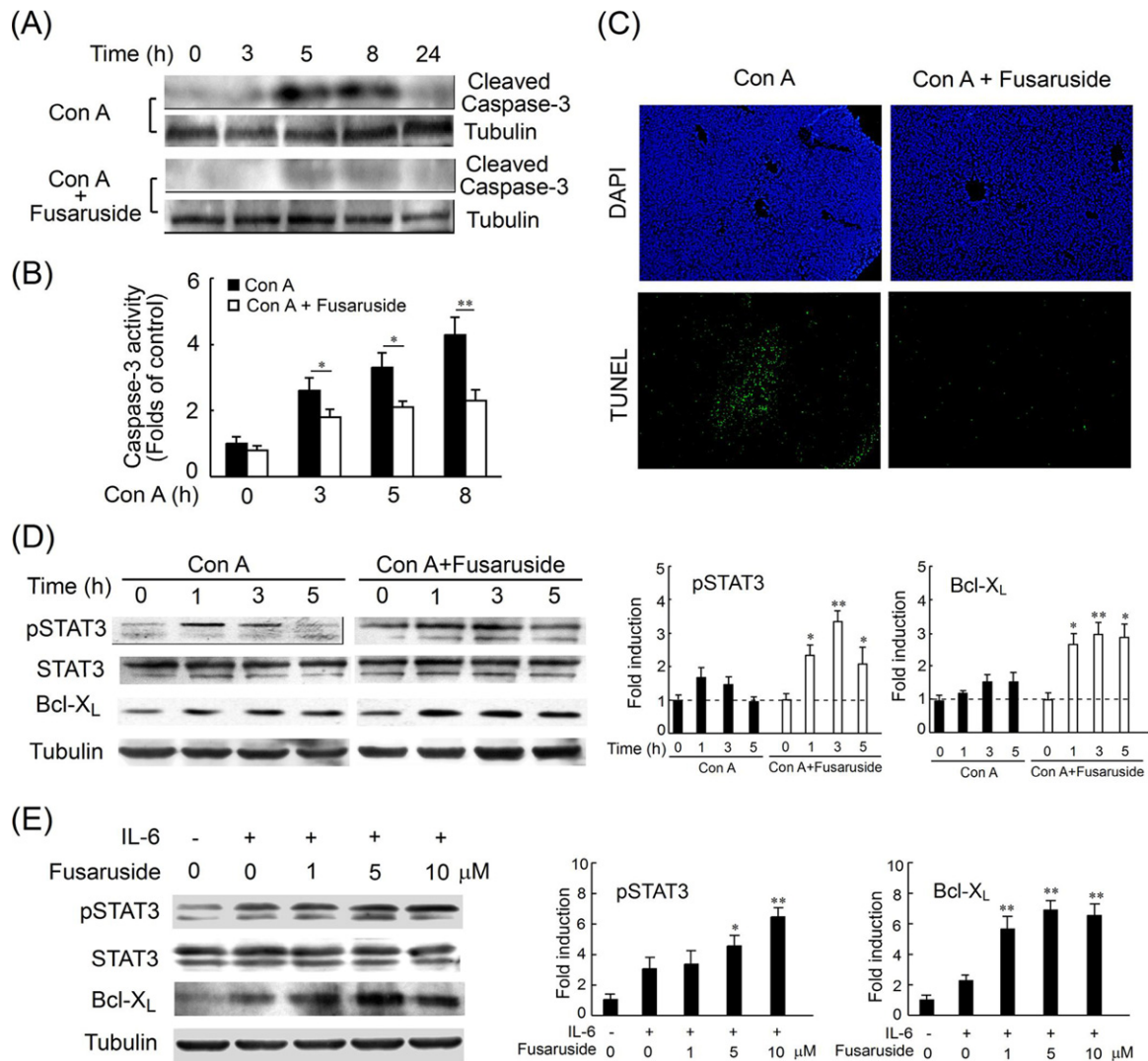
In the present study, we first investigated the *in vivo* effect of fusaruside in a Con A-induced T-cell-dependent murine model of hepatitis, which is considered to be an appropriate model for mimicking human immunomediated liver diseases [21,29]. The biochemical and histopathological data indicated that pretreatment with fusaruside was sufficient to protect mice from Con A-induced liver damage (Fig. 1). The activation of CD4<sup>+</sup> T cells also plays a critical role in Con A-induced hepatitis [12,21]. In this study, CD4<sup>+</sup> T-cell activation as well as the serum levels of Th1 cytokines, such as IFN-γ, TNF-α, IL-2 and Th17 cytokine IL-17A, were markedly attenuated by fusaruside (Figs. 2 and 3). This effect was of relevance, because IFN-γ and TNF-α might alone or synergistically cause a direct cytotoxic effect on hepatocytes *in vitro* [30,31]. In order to elucidate how fusaruside exerted its protective effect, we next examined the effect of fusaruside on STAT1, STAT3 and their regulating proapoptotic and antiapoptotic proteins in the Con A-induced hepatitis in mice. It has been well known that STAT1 and STAT3 play a critical role in regulating the progress of the inflammation including hepatitis [12] and ischaemia/reperfusion injury [32]. In Con A-induced liver failure, IFN-γ-induced activation of STAT1 has been demonstrated to play a harmful role by activating CD4<sup>+</sup> T cells and NKT cells, along with directly inducing the hepatocyte death. Additionally, T-bet, a Th1 specific transcription factor, is regulated by STAT1 [33,34] and plays a harmful role in Con A-induced hepatitis through directly inducing hepatocyte death by the activation of CD4<sup>+</sup> T cell, because the disruption of T-bet gene abrogated the elevated ALT activity and necrosis [11]. In contrast, IL-6-induced activation of STAT3 protected mice against Con A-induced liver injury by the suppression of IFN-γ signaling and induction of Bcl-X<sub>L</sub>, which could protect hepatocytes against cell death induced by many



**Fig. 5.** Fusaruside inhibited proliferation and activation of murine splenic T cells. Spleen cells were prepared from naïve C57BL/6 mice and T cells were purified using commercial enrichment columns. Mouse CD3<sup>+</sup> T cells were stimulated with 5  $\mu\text{g}/\text{ml}$  of Con A (A) or plate-bound anti-CD3 (10  $\mu\text{g}/\text{ml}$ )/soluble anti-CD28 (1  $\mu\text{g}/\text{ml}$ ) (B) or mixed lymphocyte reaction (C) in the presence of 1–10  $\mu\text{M}$  fusaruside for 72 h and cell proliferation was measured by MTT uptake. (D) Spleen cells were prepared from naïve mice and CD4<sup>+</sup> T cells were purified using commercial MACS columns. Mouse CD4<sup>+</sup> T cells ( $2 \times 10^5/\text{well}$ ) from C57BL/6 mice were labeled with 2.5 mM CFSE for 10 min, then they were stimulated with anti-CD3 (10  $\mu\text{g}/\text{ml}$ ) plus anti-CD28 (1  $\mu\text{g}/\text{ml}$ ) in the presence of fusaruside for 96 h. The proliferation of CFSE-labeled CD4<sup>+</sup> T cells was evaluated using flow cytometry. Data are one of three independent experiments with similar results. (E) Mouse CD3<sup>+</sup> T cells were stimulated with 5  $\mu\text{g}/\text{ml}$  of Con A in the presence of fusaruside for 24 h and CD69 expression was measured by flow cytometric analysis. Data are one of three independent experiments with similar results. (F) Mouse CD3<sup>+</sup> T cells were treated with various concentrations of fusaruside for 24 h and cell viability was measured by MTT assay. Data are shown as mean  $\pm$  SEM from three independent experiments. (G) Mouse CD3<sup>+</sup> T cells were treated with various concentrations of fusaruside for 24 h and apoptosis was measured by annexin V-propidium iodide assay. Data are one of three independent experiments with similar results.

hepatotoxins, including CCL<sub>4</sub>, TNF- $\alpha$ , alcohol and Fas ligand [35,36]. In the present study, Con A administration rapidly triggered the activations of STAT1 and STAT3 with the peak effect occurring at 1–3 h. Selectively, fusaruside pretreatment time-dependently attenuated the STAT1 activation and T-bet expressions in liver CD4<sup>+</sup> T cells (Fig. 4A), but significantly enhanced and prolonged STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes from Con A-treated mice (Fig. 6D). To further confirm the regulation on STAT1/T-bet and STAT3/Bcl-X<sub>L</sub> signaling, we isolated primary CD4<sup>+</sup> T cells and hepatocytes and examined the effect of fusaruside *in vitro*, respectively. As the result, fusaruside notably repressed T-bet

expression in IFN- $\gamma$ -activated CD4<sup>+</sup> T cells (Fig. 4C) whereas significantly upregulated Bcl-X<sub>L</sub> expression in IL-6-activated hepatocytes (Fig. 6E) in a dose-dependent manner. Consequently, resembling its *in vivo* effect, fusaruside also induced the specific loss of STAT1 activation and enhancement of STAT3 activation *in vitro*. It has been proved that intravenous application of the lectin Con A in mice induced T-cell-mediated liver damage with necrosis and hepatocyte apoptosis [21,29]. As a result of protective effect of fusaruside, hepatocyte apoptosis and caspase-3 activity in the injured liver were also inhibited in fusaruside-pretreated mice (Fig. 6A–C). All the findings suggest that fusaruside protects mice



**Fig. 6.** Fusaraside protected hepatocyte from apoptosis through enhancing STAT3 activation and Bcl-X<sub>L</sub> expression. (A) Fusaraside time-dependently reduced caspase-3 activation in hepatocytes from Con A-induced liver injury mice. The hepatocytes from Con A-treated vehicle control and fusaraside (20 mg/kg)-pretreatment mice were analyzed by Western blotting. The data shown here are representatives of three independent experiments. (B) Fusaraside down-regulated caspase-3 activity of hepatocytes. The hepatocytes from Con A-treated vehicle control and fusaraside (20 mg/kg)-pretreatment mice were analyzed by caspase-3 activity analysis. \*P < 0.05, \*\*P < 0.01 vs. corresponding Con A-treated groups at the same time point. (C) Fusaraside protected hepatocyte from apoptosis *in vivo*. TUNEL assay was done according to the manufacturer's instructions. The nuclei of hepatocytes in each group were stained with DAPI. Original magnification, 100×. (D) Fusaraside enhanced STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes *in vivo*. The hepatocytes from Con A-treated vehicle control and fusaraside (20 mg/kg)-pretreatment mice were analyzed by Western blotting. The bands were measured and quantified by ImageQuant analysis and the values are shown as mean ± SEM from three independent experiments at each time point. \*P < 0.05, \*\*P < 0.01 vs. corresponding Con A-treated vehicle control groups at the same time points. (E) Fusaraside enhanced STAT3 activation and Bcl-X<sub>L</sub> expression in hepatocytes *in vitro*. The primary hepatocytes isolated from naive C57BL/6 mice were cultured with fusaraside for 3 h, then treated with murine IL-6 for 30 min (detection of pSTAT3) or 6 h (detection of Bcl-X<sub>L</sub>). After the incubation, proteins were extracted and assessed by Western blotting. Values are shown as mean ± SEM from three independent experiments. \*P < 0.05, \*\*P < 0.01 vs. IL-6-treated group.

from liver injury by recovering the balance between IFN- $\gamma$ /STAT1 and IL-6/STAT3 signalings, which exerts a mutual functional antagonism role in T-cell-induced hepatitis.

This is the first time, to our knowledge, that evidence has been presented for the bidirectional regulation of STAT1 and STAT3 by a small molecule compound, which revealed a potential strategy for the therapy of T-cell-related diseases by regulating the balance of STAT signalings. STATs are a family of nuclear proteins mediating the actions of numerous cytokines. Among them, STAT1 plays a critical role in the signal transduction pathway of IFN- $\gamma$ . STAT1 cascade is one major signaling pathway converting the IFN- $\gamma$  signal into gene expressions, such as T-bet, Fas receptor, caspases, inducible nitric oxide synthase, cyclooxygenase, and intercellular cell

adhesion molecules, all of which are critically involved in different pathologies correlated to the inflammatory process. Thus, STAT1 may potentially be claimed as a new molecular target of an anti-inflammatory treatment [37]. As far as it concerns, few natural compounds have been reported targeting the inhibition of STAT1. For example, green tea polyphenol epigallocatechin-3-gallate inhibited IFN- $\gamma$ -elicited STAT1 activation but had no effect on IL-6-elicited STAT3 activation [38,39]. The peroxisome proliferator-activated receptor  $\gamma$  agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15dPGJ<sub>2</sub>) exhibited a strong anti-STAT1 as well as anti-STAT3 activities [40]. On the other hand, STAT3 has been demonstrated to be an anti-apoptotic signaling factor in T-cell-mediated hepatitis [12] and ischemia/reperfusion injury [32]. One feasible way to ameliorate

the inflammation is to recover the balanced STAT signaling by enhancing STAT3 activation. However, there is no corresponding drug candidate except cytokine cardiotropin-1 [41]. Up to now, to our knowledge, there is no report on a small compound that bidirectionally regulating STAT1 and STAT3. Therefore, we demonstrated that fusaruside fulfilled its immunosuppressive effect on T-cell-mediated hepatitis by downregulating STAT1 whilst upregulating STAT3 and their respective downstream signaling. Previously, the immunosuppressant fludarabine and the glucocorticoid dexamethasone also inhibited STAT1 activation via suppressing STAT1 expression at the mRNA level [42,43]. Nevertheless, fusaruside worked through inhibiting the phosphorylation of STAT1, without influencing the total STAT1 expression, which is distinct from the above immunosuppressants in down-regulating STAT1 activity. As a result, by displaying a usual character, fusaruside might have significant advantages for the treatment of immune diseases, including hepatitis.

In conclusion, fusaruside can effectively protect mice from Con A-induced liver injury through inhibiting proinflammatory immune response in T cells and preventing hepatocyte from apoptosis, contributing to rebuilding the balance between STAT1 (in inflammatory T cells) and STAT3 (in hepatocytes) signalings. The protective effect of fusaruside on T-cell-mediated immune reactions through the cytokine and cytokine-mediated STAT signaling modulation also suggests that the study of this compound might provide a new and specific approach for developing novel immunomodulatory drugs distinct from the present immunosuppressants, such as antimetabolites and glucocorticoids.

### Conflict of interest

The authors have no financial conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bcp.2012.08.006>.

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