Secoemestrin C inhibits activation of NKT/conventional T cells and protects against concanavalin A-induced autoimmune hepatitis in mice

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Abstract

Background and Purpose: We previously found that secoemestrin C, an epitetrathiodioxopiperazine isolated from Aspergillus nidulans, has a potent immunosuppressive effect on splenocyte proliferation in drug screening. Here, we determined the immunomodulatory and hepatoprotective effects of secoemestrin C in a mouse model of acute autoimmune hepatitis. Experimental Approach: In an in vitro assay, purified hepatic mononuclear cells (MNCs) from C57BL/6J mice were stimulated with concanavalin A (Con A, 2 μg·mL-1) in the presence of secoemestrin C, and cell proliferation and cytokine production were measured. In an in vivo assay, mice with or without secoemestrin C pretreatment were injected with Con A (12 mg·kg-1) to induce acute hepatitis. Blood samples and liver tissues were harvested 8 h after Con A injection. Liver injury, serum levels of proinflammatory cytokines, hepatic lymphocyte subset ratios, and the functional status of NKT and conventional T cells were analyzed. Key Results: Secoemestrin C treatment dose-dependently suppressed cell proliferation and proinflammatory cytokine secretion in Con A-stimulated hepatic MNCs in vitro. In Con A-challenged mice, pre-injection with secoemestrin C significantly decreased the generation of proinflammatory cytokines and ameliorated liver injury. Furthermore, pretreatment with secoemestrin C significantly inhibited the Con A-induced activation of NKT and conventional T cells and decreased the production of IFN-γ by these two cell populations. Conclusion and Implications: Secoemestrin C has an immunosuppressive effect on NKT and conventional T cells and has hepatoprotective activity in mouse autoimmune hepatitis. These findings provide new insights into the use of fungus-derived natural products for the treatment of autoimmune diseases.

Introduction

Autoimmune hepatitis (AIH) is a chronic self-perpetuating liver inflammatory disease that affects all ages, both genders, and all ethnicities (Mack *et al.* , 2019). AIH may start with an episode of acute hepatitis and eventually lead to liver cirrhosis, liver cancer, the need for liver transplantation, or death (Manns *et al.* , 2015). The precise etiology of AIH is unknown, but it is been widely believed that natural killer T (NKT) cells and conventional T cells contribute to AIH-induced liver damage (Mattner, 2013; Mieli-Vergani *et al.* , 2018). The prominent features of these two cell populations during AIH include an accumulation in the liver, aberrant activation, and imbalanced Th1/Th2 responses (Senaldi *et al.* , 1992; Santodomingo-Garzon *et al.* , 2011). Once a T cell-mediated autoimmune response is initiated, hepatocytes can potentially be destroyed directly through Fas/Fas ligand (FasL) interactions and/or the action of TNF-α and perforins/granzyme B, or indirectly through the release of Th1-biased IFN-γ (Mieli-Vergani *et al.* , 2018). Therefore, T cells are expected to be the therapeutic targets in the treatment of AIH.

The mainstay of treatment for AIH is immunosuppression (Vergani *et al.* , 2012). Concanavalin A (Con A)-induced acute hepatitis in mice is considered to be a well-characterized model for studying the pathogenesis of AIH and screening immunosuppressive agents (Tiegs*et al.* , 1992). Con A primarily stimulates the recruitment and activation of NKT and conventional T cells, coinciding with the generation of large amounts of proinflammatory Th1 cytokines (Heymann *et al.* , 2015). In recent years, based on the administration of Con A to model animals, many new therapeutic drugs, antibodies, and proteins have been developed to attenuate liver injury by inhibiting NKT/T cell activation and skewing (Wang *et al.* , 2012).

Fungus-derived natural products and their derivatives, such as cyclosporine and mycophenolate mofetil, are widely used in the treatment of autoimmune diseases (Lee *et al.* , 2008; Fakih *et al.* , 2018). Preparations of fungi and their metabolites have a low synthetic cost, high practical value, and broad application in medicine, thanks to their wide availability and high biological activity (Sharma *et al.* , 2016). In a previous screening of anti-inflammatory agents, we found that secoemestrin C (Fig. 1A), isolated from *Aspergillus nidulans* , had a potent inhibitory effect on Con A-induced splenocyte proliferation, suggesting potential immunosuppressive and anti-inflammatory activity. In the present study, we sought to investigate whether secoemestrin C could protect against Con A-induced autoimmune hepatitis in mice and to explore potential mechanisms for such an effect.

Materials and Methods

Experimental animals. Specific pathogen-free C57BL/6J (6-8 weeks, 18-23g) female mice were purchased from Charles River Laboratories (Beijing, China) and maintained in controlled conditions (22, 50% humidity, 12 h light/dark cycle, with lights on at 7:00 AM). The mouse care and experimental protocols conducted in this study were approved by the Huazhong University of Science and Technology Animal Care and Use Committee.

Isolation and identification of secoemestrin C. The fungus*Aspergillus nidulans* was growing on rice culture medium (40kg) at room temperature for 21 days. The fermented medium was then extracted with 95% ethanol, and the extract was suspended in water and further treated with ethyl acetate (AcOEt) to obtain a dark brown extract (300g). The AcOEt extract was chromatographed using silica gel (100-200 mesh) and eluted with petroleum ether/AcOEt (50:1-0:100) to give six fractions (Fr1-Fr6). Secoemestrin C (5.2g) was isolated from Fraction 4, and the purity ([?] 99.0%) was determined via HPLC with a UV detector. The structure of secoemestrin C was elucidated based on NMR and HR-ESI-MS (Supplemental Table 1 and Supplemental Fig. 1) and found to be identical to that reported previously (Ooike *et al.* , 1997).

Reagents and antibodies. Carboxy-fluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen (ThermoFisher Scientific). Concanavalin A type IV from *Canavalia ensiformis*(C2010) was obtained from Sigma-Aldrich. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured with an automated biochemical analyzer BS-200 (Mindray, China). GolgiStop brefeldin A solution and Fix/Perm Buffer Set were obtained from Biolegend. Flow cytometry antibodies: antibodies against mouse TCRβ (H57-597), NK1.1 (PK136), CD4 (GK1.5), CD8 (53-6.7), CD69 (H1.2F3), and IFN-γ (XMG1.2) were purchased from BD Biosciences and Biolegend, with fluorescence conjugation. Cytokines in serum or in culture supernatants were determined by using a cytometric bead array-based mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences).

Isolation of hepatic mononuclear cells and splenocytes. Hepatic mononuclear cells (MNCs) and splenocytes were isolated as previously described (Zhou *et al.* , 2017). In brief, each mouse liver was perfused with phosphate buffer saline to eliminate blood, and the liver tissue was removed and pressed through a 70-μm cell strainer (BD Biosciences). The liver cell suspension was then collected. Hepatic MNCs were purified by density gradient centrifugation in a 38% Percoll solution (GE Healthcare). Purified splenocytes were prepared by homogenizing with a syringe, followed by passage through a 0.1-mm sterile nylon mesh and erythrocyte depletion.

Hepatitis induction and treatment. C67BL/6J mice in the Con A-challenged and secoemestrin C-treated groups were injected intravenously with a single dose of Con A (12 $mg \cdot kg^{-1}$) to simulate autoimmune he-

patitis. In the case of the secoemestrin C-treated group, secoemestrin C (1 mg·kg^{-1} or 10 mg·kg^{-1}) was intraperitoneally injected for 3 days, and then the mice were injected with Con A 1 h after the last secoemestrin C injection.

Blood samples were collected from the orbital venous plexus under mild pentobarbital anesthesia 8 h after the Con A injection, and the serum was separated by centrifugation. Later, mice were sacrificed by cervical dislocation under mild pentobarbital anesthesia, and livers were harvested.

Cytokine assay. The levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α in the serum 8 h after Con A challenge or in culture supernatants 72 h after Con A stimulation were measured with a CBA kit for mouse Th1/Th2/Th17 cytokines.

Flow cytometry. Procedures for surface staining, intracellular cytokine staining, and CBA kit use were performed according to the manufacturer's instructions. Flow cytometry data were acquired on a BD FACSCelesta and analyzed with FlowJo software (TreeStar).

Histological examination. Liver samples were fixed in 4% formaldehyde and dehydrated with xylene, absolute ethyl alcohol, and 75% alcohol, then embedded in paraffin wax. Four micron-thick sections were cut and stained with hematoxylin-eosin (H&E) and observed by three pathologists in a blinded manner. The extent of the pathology was scored from 0 (no pathology) to 3 (severe pathology) as previously described (Yan *et al.* , 2011).

Immunohistochemistry and immunofluorescent staining. Liver sections were stained with primary antibody (anti-Fas or anti-cleaved caspase-3) according to a standard protocol (Servicebio, China). Apoptotic cells were evaluated using a TUNEL assay kit (Roche Applied Science) according to the manufacturer's instructions.

Statistical analysis. Graphs were generated and analyzed by GraphPad Prism 6.0 (GraphPad Software). Data were analyzed by Student's*t* -test for comparisons of groups with normal distribution and by equal variance or Mann-Whitney U test for non-normally distributed variables among groups. Data are presented as means ± standard error of the mean (SEM). $P<0.05$ was considered significant.

Results

Secoemestrin C suppresses the proliferation of Con A-stimulated splenocytes

A total of 5.2 g of secoemestrin C was isolated from *Aspergillus nidulans,* which had been growing on rice culture medium (40 kg). The structure of the compound was elucidated by NMR and HR-ESI-MS (Fig. 1A, Supplemental Table 1 and Supplemental Fig. 1). The purity of the secoemestrin C was [?]99.0%, as determined via high performance liquid chromatography with a UV detector. To investigate whether secoemestrin C has the potential to suppress immunological reactions, a Con A-induced splenocyte proliferation assay was carried out in the presence of secoemestrin C. Mouse splenocytes strongly proliferated in response to the Con A stimulation *in vitro* (Fig. 1B, C). However, secoemestrin C treatment potently inhibited this Con A-stimulated splenocyte proliferation (Figure 1B, C).

Secoemestrin C inhibits the proliferation of liver mononuclear cells (MNCs) and the production of cytokines induced by Con A in vitro

Tissue-resident immune cells play a key role in local and systemic immune responses. To determine whether secoemestrin C can also inhibit the activation of liver-resident lymphocytes, liver MNCs were isolated from normal mice and stimulated by Con A *in vitro* . FACS analysis showed that the isolated hepatic MNCs included 30.74 +- 4.08% natural killer T (NKT) cells and 23.78 +- 1.18% conventional T cells (Fig. 2A, B). In response to Con A stimulation, hepatic MNCs strongly proliferated and released large amounts of cytokines, including IFN-γ, IL-2, IL-6, IL-17, TNF-a, and IL-4 (Fig. 2C-J). Interestingly, secoemestrin C treatment significantly attenuated Con A-stimulated liver MNC proliferation and cytokine production in a dose-dependent manner (Fig. 2C-H, J). Above all, secoemestrin C was able to restrain hepatic lymphocyte

proliferation and cytokine production *in vitro* , which may enable it to exert an *in vivo* protective effect on Con A-induced and T cell-mediated autoimmune hepatitis in mice.

Secoemestrin C ameliorates liver injury in Con A-challenged mice

Next, we evaluated the effects of secoemestrin C on Con A-induced hepatitis *in vivo* . A severe liver injury was observed in mice 8 h after Con A administration, which manifested itself as a significant elevation in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) when compared to the normal (control) mice (Fig. 3A-C). In contrast, pretreatment with secoemestrin C at a higher dose ($10mg{\cdot}kg^{-1}$) almost completely inhibited the increase in the serum levels of ALT, AST, and LDH induced by the Con A challenge (Fig. 3A-C).

To further confirm the hepatoprotective effect of secoemestrin C, hematoxylin-eosin (H&E)-stained liver tissue sections were evaluated for histopathology. In the Con A-treated group, massive bridging coagulative necrosis of hepatocytes was visualized by light microscopy (Fig. 3D). However, the hepatic pathological changes induced by Con A were dramatically reduced in the mice treated with high-dose secoemestrin C (Fig. 3D, E).

The apoptotic response plays an important role in Con A-induced liver injury. To investigate the pathway of secoemestrin C involved in the regulation of hepatocyte apoptosis, we used TUNEL staining as well as Fas and cleaved caspase-3 staining to analyze hepatocyte apoptosis in the livers of the mice. The area of apoptotic cells in the Con A group was significantly larger than that in the normal control group (Figure 3F, G). Mice pretreated with a high dose of secoemestrin C showed a much smaller apoptotic area in the liver (Fig. 3F, G). Furthermore, the expression of two apoptosis-related proteins, Fas and cleaved caspase-3, was markedly elevated in Con A-challenged mice, whereas expression of the two proteins was almost completely suppressed in the mice who received the high-dose secoemestrin C treatment (Supplemental Fig. 2). Together, these results indicate that pretreatment with secoemestrin C provides significant protection against Con A-induced hepatic damage.

Secoemestrin C reduces serum levels of proinflammatory cytokines in mice with Con A-induced hepatitis

A balance betweeen proinflammatory and anti-inflammatory factors is essential for maintaining liver homeostasis. Proinflammatory cytokines play a critical role in the initiation and propagation of the autoimmune response. To investigate the effect of secoemestrin C treatment on the generation of inflammatory cytokines in mice with Con A-induced liver injury, we examined the serum levels of IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-17 using a cytometric bead array kit. In the Con A-challenged group, we found that the levels of IFN-γ, IL-2, TNF-α, IL-17, and IL-6 were dramatically increased when compared to those in the normal control mice. However, pretreatment with secoemestrin C significantly suppressed the production of these cytokines induced by Con A (Fig. 4A-E). There were no significant differences in the serum level of IL-4 among the groups (Fig. 4F).

Secoemestrin C inhibits Con A-induced activation of NKT cells and conventional T cells in liver

The recruitment and activation of NKT cells and conventional T cells contribute remarkably to the development and progression of Con A-induced hepatic inflammation. To investigate whether secoemestrin C has any effect on lymphocyte subsets, we analyzed liver MNCs 8 h after Con A injection. As compared to the normal control group, the percentage of hepatic NKT cells in Con A-challenged mice was significantly decreased, whereas the percentages of conventional $CD4^+$ and $CD8^+$ T cells in the liver were relatively stable (Figure 5). Notably, secoemestrin C treatment largely rescued hepatic NKT cell depletion, as evidenced by a significantly increased ratio of hepatic NKT cells to liver MNCs in the treatment group when compared to that of the Con A-challenged group (Fig. 5A).

To determine whether secoemestrin C can suppress the activation of hepatic NKT cells and conventional T cells *in vivo* , we measured the expression of CD69 and IFN-γ by FACS analysis. Secoemestrin C treatment significantly inhibited the increase in CD69 expression on hepatic NKT cells and conventional $CD4^+$ and $CD8^+$ T cells that was seen in the Con A-challenged group (Fig. 6A, C). In addition, treatment with

secoemestrin C also down-regulated the elevated expression of IFN-γ in hepatic NKT and conventional $CD4^+$ T cells after Con A challenge*in vivo* (Figure 6B, D).

Discussion

Fungi are widely distributed across the globe and have been identified as a rich source of new pharmacologically active ingredients. The members of the *Aspergillus* genus are already an important source of novel pharmacological metabolites such as alkaloids, meroterpenoids, and lignans. Secoemestrin C is an epitetrathiodioxopiperazine extracted from *Aspergillus nidulans* . The biological activity of secoemestrin C and its analog has only been reported in terms of inhibiting tumor cell proliferation (Onodera *et al.* , 2004). Here, we first present evidence to show that secoemestrin C can inhibit Con A-induced mouse splenocyte proliferation as well as hepatic MNC activation and proliferation *in vitro* , and then we demonstrate that this molecule has a protective effect *in vivo* on Con A-induced autoimmune hepatitis in mice.

Accumulating evidence suggests that $CD4^+$ T cells (Mizuhara *et al.* , 1994; Wang *et al.* , 2012), $CD8^+$ T cells (Watanabe*et al.* , 1996), and NKT cells (Takeda *et al.* , 2000a; Kumar, 2013) are critical components of Con A-induced liver damage. After Con A challenge, NKT cells and conventional T cells are activated and either directly participate in perforin/granzyme- and/or Fas/Fas ligand-mediated cytotoxicity or indirectly release large amounts of proinflammatory cytokines, thus leading to hepatocyte death. Therefore, agents that can inhibit the activation of T cells and/or NKT cells are most likely capable of alleviating hepatitis induced by Con A. In our *in vitro*experiments, we found that secoemestrin C can potently suppress the proliferation of splenocytes and activation of hepatic MNCs induced by Con A, indicating that it may have a significant protective effect on Con A-induced liver damage. As predicted, pretreatment with secoemestrin C markedly protected against liver injury in Con A-challenged mice, as evidenced by decreased serum levels of liver enzymes and proinflammatory cytokines, attenuated bridging coagulative necrosis of hepatocytes, and a lessened apoptotic response in the livers. In addition, the levels of serum transaminase and hepatocyte apoptosis in secoemestrin C-treated mice were similar to those in normal control mice, indicating that secoemestrin C has limited hepatic toxicity. Thus, secoemestrin C appears to be a good prospect for clinical application in immunosuppression.

In addition to aggravating inflammation and liver injury, activation of NKT cells also leads to Fas/FasL-mediated apoptotic elimination of NKT cells during Con A challenge (Takeda*et al.* , 2000b). Our results have confirmed the rapid disappearance of activated NKT cells in Con A-challenged livers. Although we did not detect any expression of FasL on NKT cells, our observation of a lowered CD69 expression level, together with a restored NKT cell ratio, suggest that the activation-induced cell death (AICD) of NKT cells is largely abolished in the secoemestrin C-treated mice.

Given that the depletion of NKT cells begins as early as 4 h after Con A administration (Takeda *et al.* , 2000b), it appears that an NKT cell-derived proinflammatory cytokine response, rather than NKT cell-mediated cytotoxicity, plays a particularly important role in the latter phase of inflammation induced by Con A. In the treatment of autoimmune disease, targeting the production of proinflammatory cytokines has been approved as a potential therapy (Danese *et al.* , 2019; Salvi *et al.* , 2019). It has been reported that SOCS3 suppresses IFN-γ production from NKT and T cells, thereby protecting against Con A-induced liver injury (Nakaya *et al.* , 2009). Our results show that secoemestrin C can down-regulate IFN-γ production by NKT and conventional T cells after Con A challenge. In addition, we have found that secoemestrin C is able to suppress cytokine production by Con A-stimulated hepatic MNCs. The regulatory mechanism(s) by which secoemestrin C inhibits IFN-γ production remain to be further explored.

Overall, we have demonstrated for the first time that secoemestrin C can inhibit NKT- and conventional T-cell activation stimulated by Con A and thus can protect against Con A-induced autoimmune hepatitis in mice. Our results provide evidence for the immunosuppressive activity of secoemestrin C and offer novel insights regarding the development of fungi-derived metabolites for the treatment of T cell-dependent autoimmune diseases.

Author contributions: X. Tan and G. Chen conceived and designed the experiments. X. Tan, L. Sun,

C. Qi, C. Fu, X. Yang. H. Feng, and Y. Li performed the experiments. Q. Li and Y. Zhang isolated and identified secoemestrin C. X. Tan, L. Sun, Y. Zhang, and G. Chen analyzed the data. X. Tan, L. Sun, C. Qi, Y. Zhang, and G. Chen wrote the manuscript.

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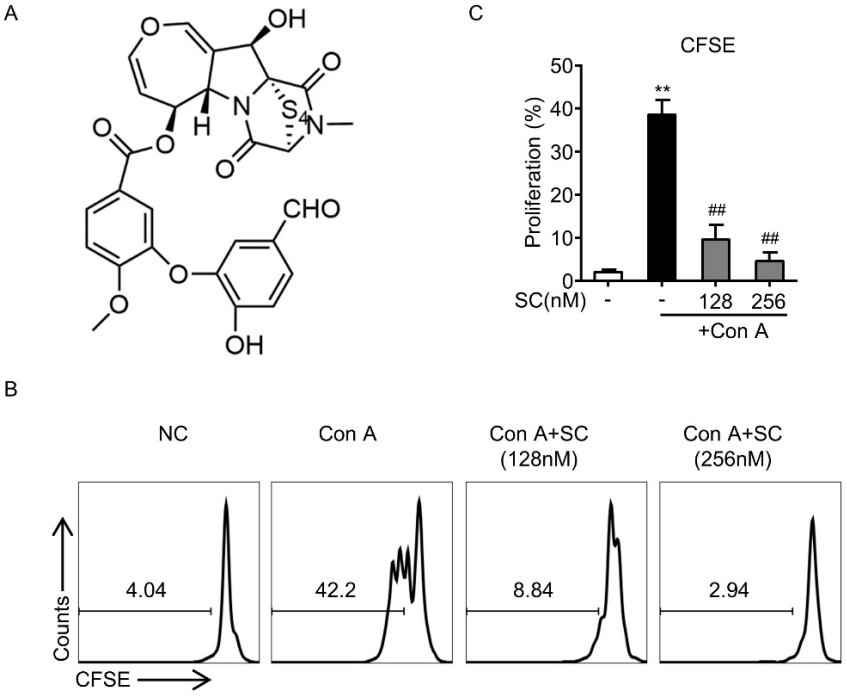

Figures and figure legends

Figure 1. Inhibitory effect of secoemestrin C in Con A-stimulated splenocyte proliferation. (A) Chemical structure of secoemestrin C (SC) isolated from *Aspergillus nidulans.* (B, C) Splenocytes from C57BL/6J mice were stained with CFSE and stimulated with Con A (2 μg·mL^{-1}) in the presence of secoemestrin C (SC) at the indicated concentration, and the cells were cultured for 72 h; splenocyte proliferation was detected by flow cytometry. A representative histogram (B) and summary bar graph (C) are shown. Data are means ± SEM of three independent experiments (n=5).$^{**}p < 0.01$ vs. the control group;$^{\#\#}p < 0.01$ vs. the Con A-treated group.

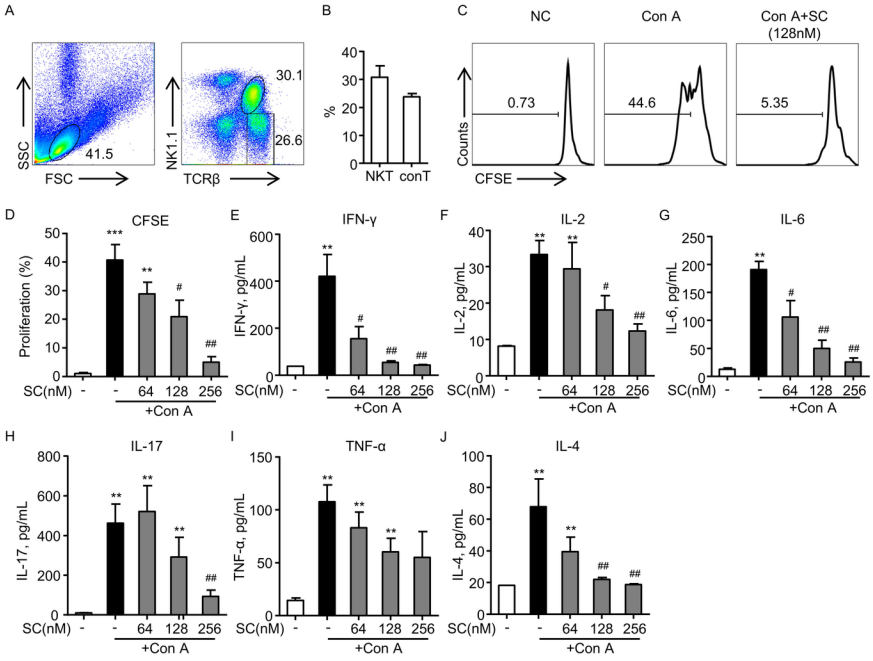

Figure 2. Inhibitory effect of secoemestrin C on the proliferation and cytokine production of Con A-stimulated liver mononuclear cells (MNCs). Liver MNCs from C57BL/6J mice were stimulated with 2 μg·mL^{-1}Con A in the presence of secoemestrin C (SC) at the indicated concentrations. After 72 h of culture, hepatic MNC proliferation was determined by CFSE-dilution assay. Cytokine concentrations in culture supernatants were quantified by cytometric bead array kits. (A) The gating strategy for hepatic NKT and conventional T cells is displayed. (B) Bar graphs depict the percentages of TCRβ^{+}NK1.1^{+} NKT cells (NKT) and TCRβ^{+}NK1.1^{-} conventional T cells (conT) in liver MNCs. (C, D) A representative histogram (C) and summary bar graph (D) depict the percentages of proliferated cells. (E-J) Levels of IFN-γ (E), IL-2 (F), IL-6 (G), IL-17 (H), TNF-α (I), and IL-4 (J) for the various groups are shown in the bar graphS. Bar graphs indicate means ± SEM. Data are from five separate experiments (five mice per group). $^{**}P<0.01$ and$^{***}P<0.001$ vs. the control (NC) group.$^{\#}P<0.05$ and$^{\#\#}P<0.01$ vs. the Con A-treated group.

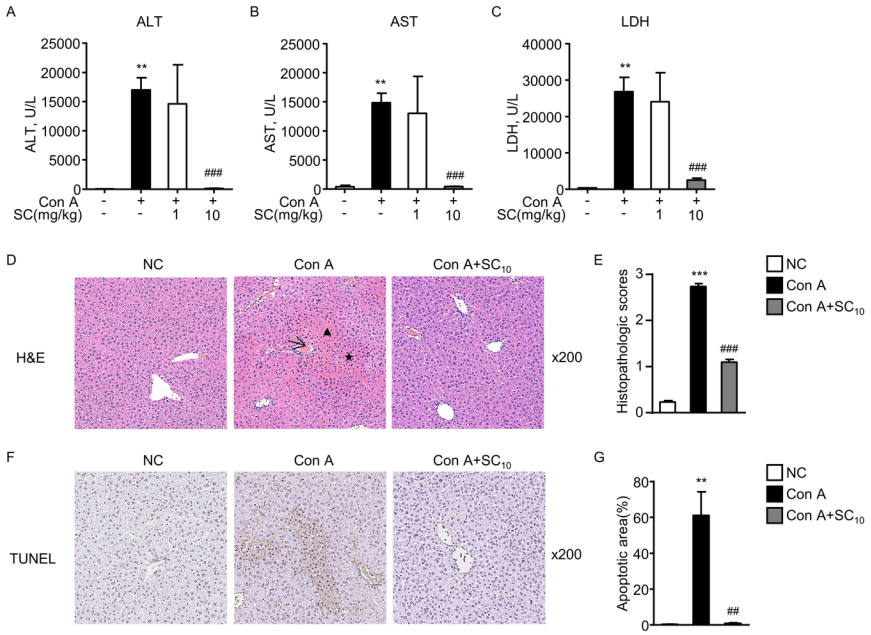

Figure 3. Protective effect of secoemestrin C in Con A-induced liver injury. Secoemestrin C (SC) was dissolved in DMSO and further diluted with normal saline. Female C57BL/6J mice were pretreated by intraperitoneal injection with SC at 10 mg·kg^{-1} (SC_{10}) or 1 mg·kg^{-1} on days -2, -1, and 1 h before i.v. injection with Con A (12 mg/kg). Mice without Con A injection served as controls (NC). (A-C) Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured 8 h after Con A injection. Data shown represent means ± SEM (six mice per group). (D) Liver sections were stained with hematoxylin and eosin (H&E). The arrow indicates hepatic ecchymosis; the star indicates infiltrated leukocytes, and the triangle shows an area of necrosis (magnification: ×200). (E) Histopathologic scores were evaluated, and the results are presented as the means ± SEM for three mice per group. (F and G) Liver sections were stained with TUNEL staining. Representative images (F) and a bar graph of apoptotic area (G) are shown (magnification: ×200). Data represent the mean ± SEM of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control (NC) group;$^{\#\#}p < 0.01$ and$^{\#\#}p < 0.001$ vs. the Con A-treated group.

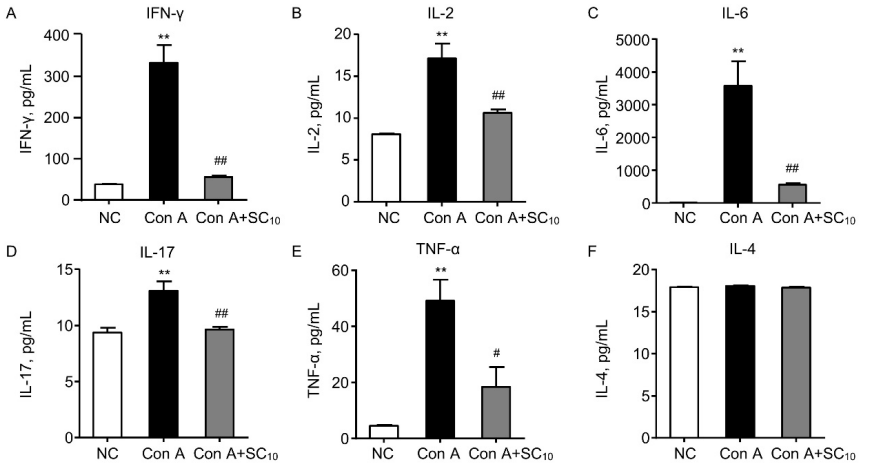

Figure 4. Effect of secoemestrin C on serum proinflammatory cytokines in Con A-challenged mice . Serum was prepared from blood collected 8 h after Con A injection. Serum levels of IFN-γ (A), IL-2 (B), IL-6 (C), IL-17 (D), TNF-α (E), and IL-4 (F) were quantified by cytometric bead array kit. Bar graphs depict the concentrations of the indicated cytokines from the various groups. Values are presented as means ± SEM. Data shown are pooled from three separate experiments (six mice per group).**P<0.01 vs. the control (NC) group.$^{\#}$P<0.05 and$^{\#\#}$p < 0.01 vs. the Con A-treated group.

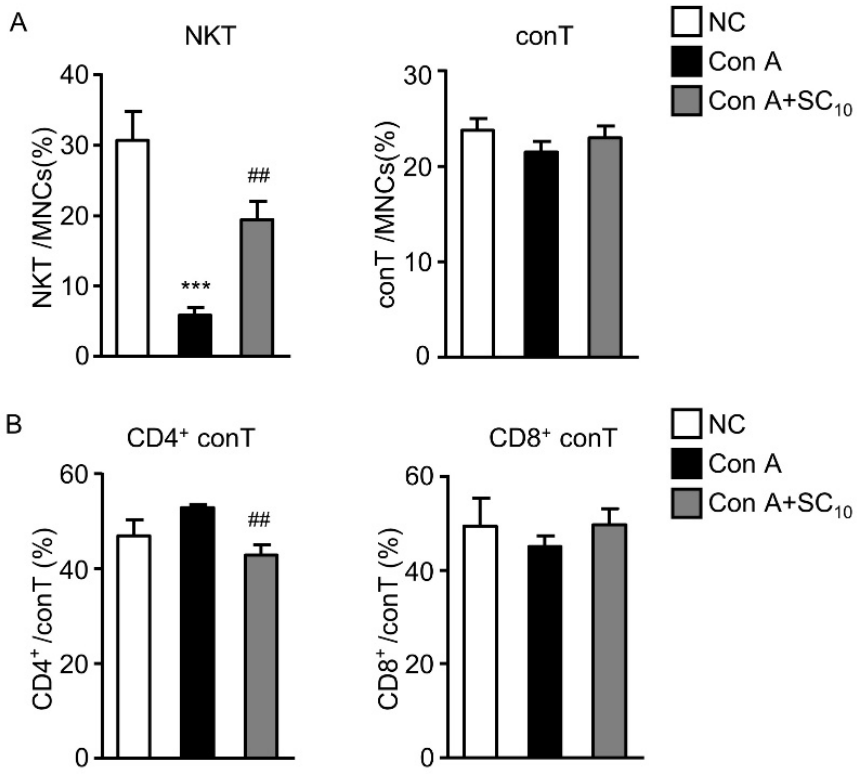

Figure 5. Effect of secoemestrin C on the percentages of hepatic NKT cells and conventional T cells in Con A-challenged mice. C57BL/6J mice were sacrificed 8 h after being injected with Con A, and liver mononuclear cells (MNCs) were collected. The cells were stained with mAbs against TCRβ, CD4, CD8, or NK1.1, then analyzed by flow cytometry. (A) Bar graphs depict the percentages of TCRβ^{+}NK1.1^{+} NKT cells (NKT) vs. MNCs and of TCRβ^{+}NK1.1^{-} conventional T cells (conT) vs. MNCs. (B) Summary bar graphs display conventional CD4^{+} and CD8^{+} T cell (CD4^{+} conT and CD8^{+} conT) proportions in conventional T cells. Values shown are means ± SEM and are pooled from three independent experiments (five mice per group).***P<0.001 vs normal the control (NC) group.$^{\#\#}$P<0.01 vs. the Con A-treated group.

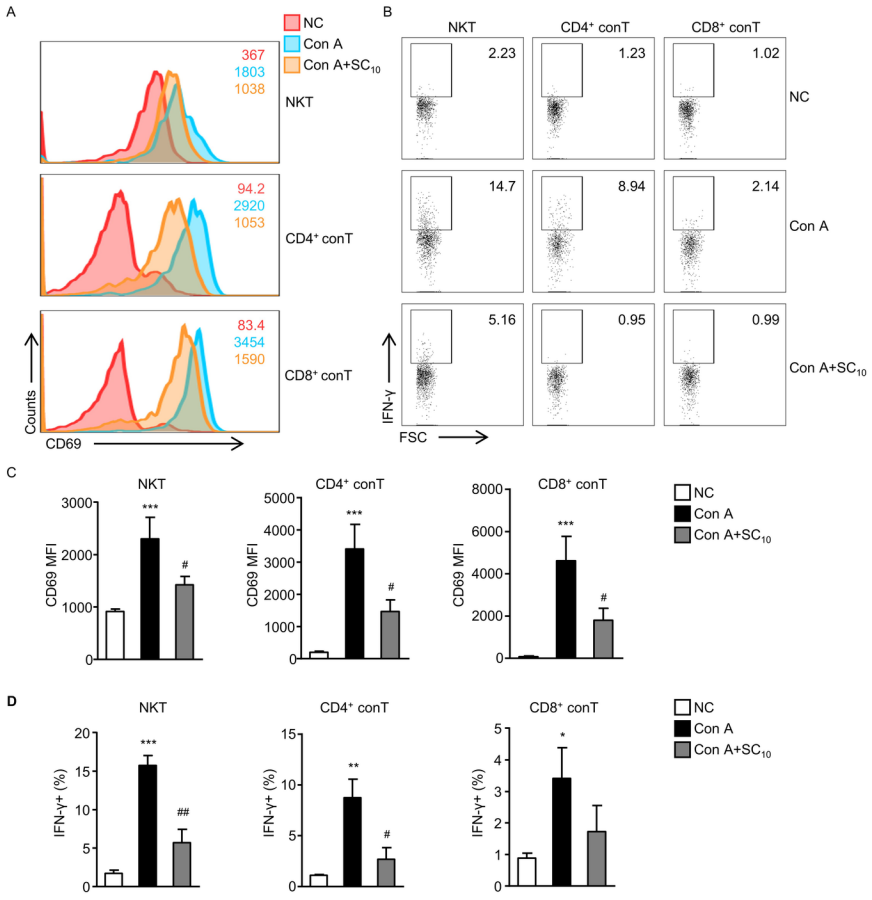

Figure 6. Effect of secoemestrin C on hepatic NKT cell and conventional T cell activation and proinflammatory cytokine secretion in Con A-challenged mice. Mice were sacrificed 8 h after Con A injection, and hepatic MNCs were prepared. (A, B) NKT cells as well as $CD4^+$ and $CD8^+$ conventional T cells were analyzed by flow cytometry for CD69 expression. Histogram with numbers (A) shows the mean fluorescence intensity (MFI) of CD69 on every subset of the normal control (NC), Con A-treated, and Con $A+SC_{10}$-treated groups. A summary bar graph (B) displays the CD69 levels on NKT cells as well as $CD4^+$ and $CD8^+$ conventional T cells. (C, D) Liver MNCs were cultured in medium with GolgiStop for a further 4 h, and intracellular staining for IFN-γ was performed. Representative dot plots (C) show IFN-γ expression by NKT cells (left panel), $CD4^+$ conventional T cells ($CD4^+$ conT, middle panel), and $CD8^+$conventional T cells ($CD8^+$ conT, right panel). Bar graphs (D) depict the percentages of IFN-γ-producing cells in the corresponding liver MNC subsets. Data are shown as means ± SEM and pooled from three independent experiments (five mice per group).$^*P<0.05$,$^{**}P<0.01$, and$^{***}P<0.001$ vs. the normal control (NC) group. $^{\#}P<0.05$ and$^{\#\#}P<0.01$ vs, the Con A-treated group.