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The farnesyltransferase inhibitor tipifarnib protects against autoimmune hepatitis induced by Concanavalin A

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ABSTRACT

No effective treatment has been established for autoimmune hepatitis (AIH), except for liver transplantation in the fatal stage. Little is known about the roles and mechanisms of farnesyltransferase inhibitors (FTIs) in treating AIH. Thus, we investigated the specific role of the FTI, tipifarnib, in a Concanavalin A (Con A)-induced model of hepatitis. The effects of tipifarnib (10 mg/kg, intraperitoneal injection) were studied in Con A (20 mg/kg, intravenous injection)-challenged mice by histological, biochemical, and immunological analyses. Tipifarnib-treated mice were compared to phosphate-buffered saline (PBS)-treated mice. Con A caused liver injury characterized by increased plasma alanine aminotransferase (ALT) levels and marked histological changes. The increased serum ALT, interleukin-6, or interferon-γ (IFN-γ) levels were observed at 2 or 8 h; tumor necrosis factor-α levels at 2 h post-Con A administration decreased significantly in the tipifarnib group. Tipifarnib also suppressed Con A-induced activation of $CD4^+$ cells (but not $CD8^+$ T cells) in the liver and spleen, and also reversed the Con A-induced decrease of natural killer T (NKT) cells in the liver. Tipifarnib significantly inhibited IFN-γ production and STAT1 phosphorylation from $CD4^+$ T cells (but not $CD8^+$ T and NKT cells) in the liver at 2 h post-Con A administration. Tipifarnib significantly inhibited IFN-γ production by splenic $CD4^+$ T cells at 48 h post-Con A injection *in vitro*. Tipifarnib also inhibited the expression of farnesylated proteins induced by Con A administration. In conclusion, tipifarnib inhibited IFN-γ derived from Con A-induced $CD4^+$ T cell activation due to downregulated STAT1 phosphorylation, suggesting that Tipifarnib can protect against AIH.

1. Introduction

Autoimmune hepatitis (AIH), characterized by rapid progression, acute onset, and high mortality, is a type of chronic hepatitis that affects both children and adults of all ages [1–4]. Clinical strategies for treating AIH are limited because the mechanisms of AIH are not fully understood. The only available treatments for AIH involve immunosuppressants and liver transplantation [5–7]. However, the possibility of liver transplantation is limited by the shortage of liver donors, immunological suppression, and high costs [8]. Therefore, developing safe and effective therapies is urgently needed [9,10].

Members of the statin family (atorvastatin, simvastatin, and lovastatin) as well as 3-hydroxy-3-methylglutary-coenzyme (HMG-CoA) inhibitors can reversibly inhibit cholesterol biosynthesis, leading to a reduction in both farnesylation and geranylgeranylation [11,12]. Farnesyltransferase inhibitors (FTIs), which specifically inhibit farnesylation, have been investigated in breast cancer and leukemia in clinical trials [13,14]. Moreover, the effects of FTIs and related analogs have been studied in multiple pre-clinical animal models of autoimmune diseases. For example, FTIs can attenuate disease manifestations with experimental colitis [15], multiple sclerosis [16], systemic lupus erythematosus [17], human immunodeficiency virus protease inhibitors [18], and arthritis [19]. FTIs were also reported to down-modulate T cell responses in previous studies [19,20].

Concanavalin A (Con A) is a plant lectin purified from *Canavalia brasiliensis* [21] that serves in a well-established model for investigating T-cell dependent liver injury in mice, which closely mimics the pathogenic mechanisms and pathological changes occurring in patients with

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AIH. Con A-induced liver injury is characterized by T lymphocyte activation, resulting in the production of proinflammatory cytokines and liver necrosis [22,23]. Furthermore, interferon-γ (IFN-γ) acts as main factor in Con A-induced liver injury [24]. Thus, this model differs from other models such as the D-galactosamine (GalN)/lipopolysaccharide (LPS) [25] model, which showed enhanced liver sensitivity to tumor necrosis factor-α (TNF-α) or acetaminophen-induced liver injury model and cause damage to the hepatic parenchyma [26]. In previous reports, anti-IFN-γ [27,28], serotonin 2A receptor [29], and demethyleneberberine [30] were shown to inhibit AIH induced by Con A injection. However, although FTIs were reported to prevent GalN/LPS-induced acute liver failure [25], the role of FTIs in the AIH model was not elucidated. Therefore, we investigated the effects and therapeutic potential of the FTI tipifarnib in a mouse model of AIH.

2. Materials and methods

2.1. Animals

Male C57BL/6N mice (8–10 weeks of age and weighing 20–25 g) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were maintained in controlled chambers (22 °C ± 2 °C and 12-h light/dark cycle) and provided with water and food ad libitum. The Ethics Committee for Animal Experiments in Kyushu University approved all animal experiments (approval number A30-117-0). All methods were performed in accordance with the Guidelines for Animal Experiments of Kyushu University.

In this study, mice were randomly divided into three groups: a sham, PBS, and tipifarnib group. Mice in the sham and PBS groups were initially administrated 10 ml/kg phosphate-buffered saline (PBS) intraperitoneally. Mice in the tipifarnib group were initially subjected to intraperitoneal injection with tipifarnib (10 mg/kg, Selleckchem, Houston, TX). At 1 h after PBS or tipifarnib administration, the mice in tipifarnib or PBS groups were administered 20 mg/kg Con A (Sigma-Aldrich, USA) and 10 ml/kg normal saline; or the mice in shame group were administered 20 mg/kg PBS and 10 ml/kg normal saline. Tipifarnib was dissolved in DMSO and then mixed with PBS.

2.2. Serum liver enzymes

Before the animals were sacrificed, blood was taken from right ventricle and centrifuged at 3000 rotations per min (rpm) for 5 min twice to obtain the serum. At 2, 8, and 24 h after Con A administration, serum levels of aminotransferase (ALT) were measured using a chemical analyzer, namely the Fuji-Drychem NX500V system (Fuji Film, Tokyo, Japan). Time points were determined by a preliminary study in which ALT peaked around 8–12 h, and decreased gradually after Con A injection.

2.3. Histopathology and immunohistochemistry

Liver tissues were dissected and fixed in 10% formalin and paraffin-embedded at 2, 8, or 24 h after Con A administration. Next, 3-μm sections were stained with hematoxylin and eosin (H&E) using a standard protocol and then analyzed with a microscope. Liver sections were routinely deparaffinized and evaluated for farnesyltransferase activity by immunostaining for farnesyl. For immunostaining, we used 2 μg/ml of a primary anti-farnesyl antibody (Abnova, Taiwan) and a peroxidase–avidin complex as the secondary antibody (EnVision + Kits; Dako Japan Co., Ltd., Kyoto, Japan). The stained sections were visualized using an Axio Scan Z1 slide scanner (Carl Zeiss AG, Ltd., Thornwood, NY, USA) and Zen software (Carl Zeiss AG, Ltd.).

2.4. Cytokine secretion in the liver

Interleukin-6 (IL-6), TNF-α, and IFN-γ concentrations at 2, 8, and 24 h post-Con A administration were measured by performing cytometric bead array immunoassays (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocols.

2.5. Western blotting analysis

Liver tissue was dissected and frozen 24 h after Con A injections. The protein levels in liver homogenates were determined using standard immunoblot techniques. Primary antibodies (Cell Signaling Technology, Inc.) against cleaved-caspase 3 (1:1000) or caspase 3 (1:1000), and β-actin (1:1000; CST, MA, USA) were used. Bound antibody signal was detected with a horseradish peroxidase-linked antibody against rabbit IgG (1:10,000; VECTOR laboratories, CA, USA) and chemiluminescence was visualized using the ECL advance kit (GE Healthcare Bioscience). The images were digitized, and the intensity of each band was quantified using densitometric scanning with Image J software.

2.6. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The TUNEL assay was performed using a TACS2 TdT in situ Apoptosis Detection Kit (R&D Systems, Minneapolis, USA) to observe the apoptotic cells. According to the manufacturer's instructions, paraffin-embedded liver sections (3 μm) were dewaxed by washing with Pathoclean (Wako Chemicals, Tokyo, Japan) 5 min 2 times, and rehydrated through a graded series of ethanol (100, 95 and 70%) and double distilled. Following permeabilization and PBS wash, the sections were incubated in 50 μl TUNEL reaction mixture for 1 h at 37 °C. The slides were stained with 3,3′-diaminobenzidine following sample quality evaluation. A total of 1% Methyl Green was used as a counterstain. The cells were visualized using an Axio Scan Z1 slide scanner (Carl Zeiss AG, Ltd., Thornwood, NY, USA) and Zen software (Carl Zeiss AG, Ltd.).

2.7. Flow cytometry

Isolated mouse livers and spleens were washed with PBS and incubated in RMPI 1600 medium for 1 min, followed by pressing through a 180-gauge steel mesh. Then, the splenocytes were suspended in RBC lysis buffer for 5 mins, then the cells were washed twice with RMPI 1600 medium and centrifuged at 2000 rpm. The hepatocytes were suspended in RMPI 1600 medium, and centrifuged at 2000 rpm for 5 min. The cell pellets were collected and suspended in 40% Percoll (Sigma), overlaid gently on 70% Percoll, and then centrifuged for 20 min at 2200 rpm. Liver mononuclear cells were taken from the interphase, suspended in RMPI 1600 medium, and then centrifuged at 2000 rpm for 5 min. The cell pellets were collected, suspended in RBC lysis buffer, and then vortexed for 1 min at maximum speed. Then, the cells were washed with RMPI 1600 medium and centrifuged at 2000 rpm. The cell pellets were collected.

Cell suspensions from the liver or spleen were obtained after the mice were sacrificed. For phenotype staining, cells were washed twice with cell staining buffer (BioLegend, California). The cells were then incubated for 40 min at 4 °C with antibodies against the targets of interest. For staining, we used FITC-labeled anti-mouse CD4 (clone RM4-4) and PE-labeled hamster anti-mouse CD69 (clone H1.2F3) from BD Pharmingen; FITC-labeled anti-mouse CD8 (clone 53–5.8), APC-labeled anti-mouse CD3 (clone 17A2), and Alexa Fluor®488-labeled anti-mouse

NK 1.1 (clone PK136) from BioLegend; and Brilliant Violet 421™-labeled anti-mouse IFN-γ and PE-labeled anti-phosphoSTAT1 (Ser727) from BioLegend. To detect intracellular cytokines, cells were pretreated with Brefeldin A (BioLegend) for 3 h. The cells were stained with monoclonal antibodies for 40 min at 4 °C to evaluate the cell surface-expression levels of $CD4^+$, $CD8^+$, and $NK1.1^+$, after which they were fixed and permeabilized using the Fixation/Permeabilization Solution Kit (BD Biosciences), and stained with anti-cytokine antibodies.

2.8. $CD4^+$ T cell activation and proliferation

Flow cytometry was performed to investigate the effects of tipifarnib on the cellular immune responses to Con A. Briefly, activated $CD4^+$ T cells and $CD8^+$ T cells in the liver or spleen were determined by measuring CD69 co-expression, and activated $NK1.1^+$ T cells in the liver were determined by measuring CD3 co-expression.

2.9. IFN-γ production in vivo

The levels of IFN-γ produced by $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells in the liver of tipifarnib-treated or PBS-treated mice were analyzed by flow cytometry. Liver lymphocytes were isolated from mice treated with PBS or tipifarnib at 2 h post-Con A injection. Intracellular staining of IFN-γ was performed with the liver $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells.

2.10. STAT1 phosphorylation

The effect of tipifarnib on STAT1 activation in $CD4^+$ liver T cells was analyzed by flow cytometry. Liver lymphocytes from mice treated with PBS or tipifarnib were isolated 2 h after Con A administration. Intracellular staining of p-stat1(727) in liver $CD4^+$ T cells was performed.

2.11. IFN-γ production in vitro

To confirm the effect of tipifarnib on the functions of $CD4^+$ T cells, $CD4^+$ T cells were enriched from spleen mononuclear cells via negative-selection beads using the MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (BioLegend), according to the manufacturer's instructions, and the purity reached above 90% purity in some cases. Next, 2×10^5 mouse splenic lymphocytes per well were pretreated with tipifarnib (5 μg/ml or 10 μg/ml) or PBS for 1 h, then activated with Con A (5 μg/ml). The IFN-γ-production levels were analyzed by enzyme-linked immunosorbent assay (ELISA) 2, 24, 48 or 72 h after Con A administration.

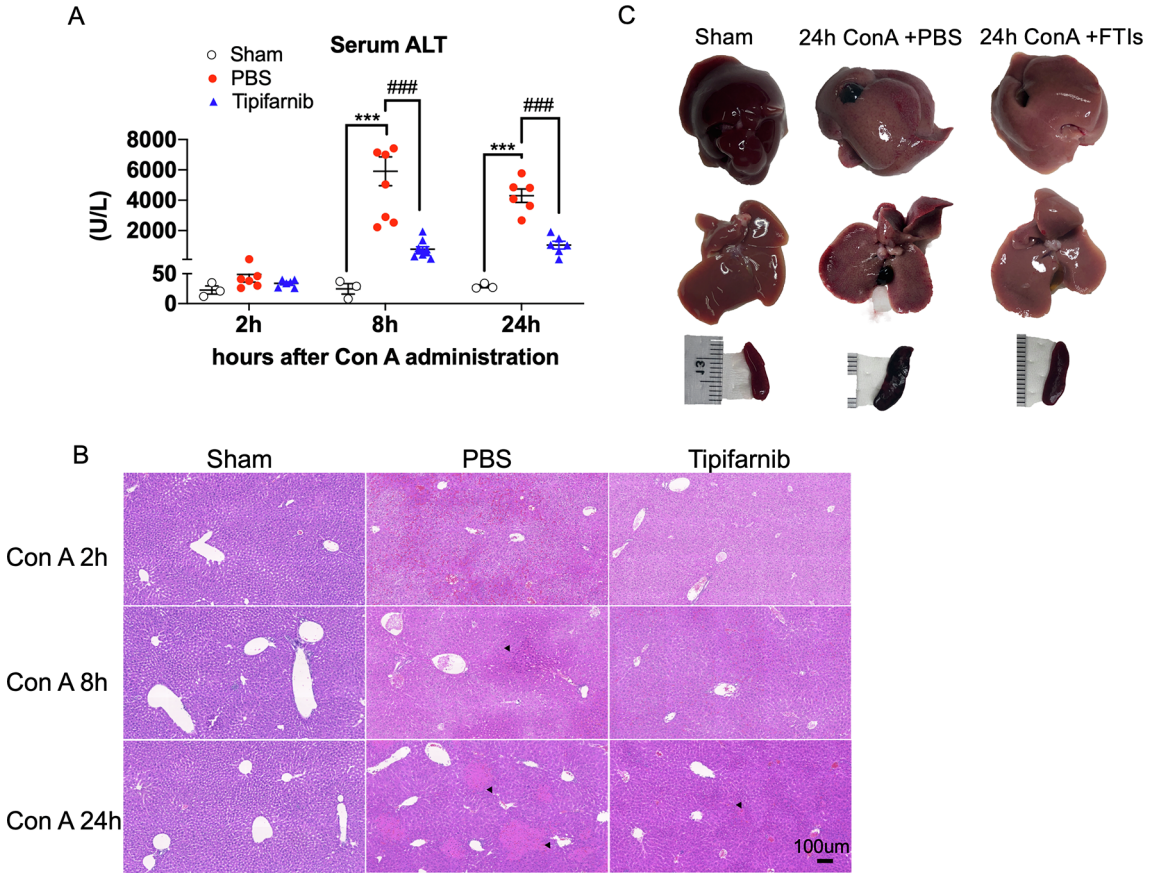

Fig. 1. The effects of tipifarnib on liver enzyme increments or histological alterations induced by Con A. (A) Serum ALT levels at 2, 8, and 24 h after Con A administration ($N = 3$ in the sham group and $N = 6$ in the PBS and tipifarnib groups). The data shown are expressed as the mean ± SEM. $^{***}p < 0.001$ between the sham and PBS groups. $^{###}p < 0.001$ between the PBS and tipifarnib groups. (B) H&E staining was performed with six animals in each group. Representative photographs are shown. (C) Representative hepatic architecture (scale bar = 100 μm). Arrows, necrotic areas. Red dots, PBS group. Blue dots, tipifarnib group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.12. Statistical analysis

All data shown are presented as the mean ± SEM. The data shown were analyzed by analysis of variance (ANOVA) using the Prism 8 software package (GraphPad Software, La Jolla, CA). Newman–Keuls multiple-comparison post-hoc test or Bonferroni's post-hoc test were performed for one-way or two-way ANOVA, respectively. Kaplan–Meier survival analysis was performed using a log-rank test. $P < 0.05$ was considered to reflect a statistically significant difference.

3. Results

3.1. Tipifarnib attenuated liver damage induced by Con A administration

Plasma ALT levels were significantly elevated at 8 h and 24 h in PBS-treated mice when compared with the sham group. Tipifarnib did not increase plasma ALT levels in the preliminary study (8 h:15.7 ± 2.9 U/L, n = 2; 24 h:17.0 ± 2.0 U/L, n = 2) (data not shown). Treatment with tipifarnib significantly suppressed Con A-induced increases in the ALT levels at 8 h (5907 ± 953.0 vs. 773.7 ± 166.8 U/L, $p < 0.0001$) and 24 h (4302.0 ± 442.6 vs. 1042 ± 256.2 U/L, $p = 0.0003$) after Con A administration (Fig. 1A). Histological analysis with H&E-stained liver sections taken after Con A administration showed the appearance of pyknotic nuclei and massive necrotic hepatocytes at 2 h and slightly increased hepatocyte necrosis at 8 h and 24 h in PBS-treated mice. Treatment with tipifarnib significantly ameliorated Con A-induced histological alterations (Fig. 1B). Con A induced erythrocyte agglutination in the liver and spleen after 24 h, but tipifarnib treatment attenuated this alteration (Fig. 1C).

3.2. Tipifarnib attenuated serum cytokine levels induced by Con A administration

Tipifarnib markedly attenuated the serum IFN-γ levels at 2 h (826.5 ± 326.2 vs. 1667.0 ± 421.1 pg/mL, $p = 0.0002$) and 8 h (927.3 ± 415.2 vs. 1987.0 ± 200.5 pg/mL, $p < 0.0001$) after Con A administration, compared with the PBS-treated group (Fig. 2A). Tipifarnib markedly attenuated the serum IL-6 levels at 2 h (282.1 ± 36.2 vs. 372.4 ± 70.5 pg/mL, $p = 0.0115$) and 8 h (220.8 ± 92.4 vs. 360.7 ± 8.359 pg/mL, $p = 0.0001$) after Con A administration,

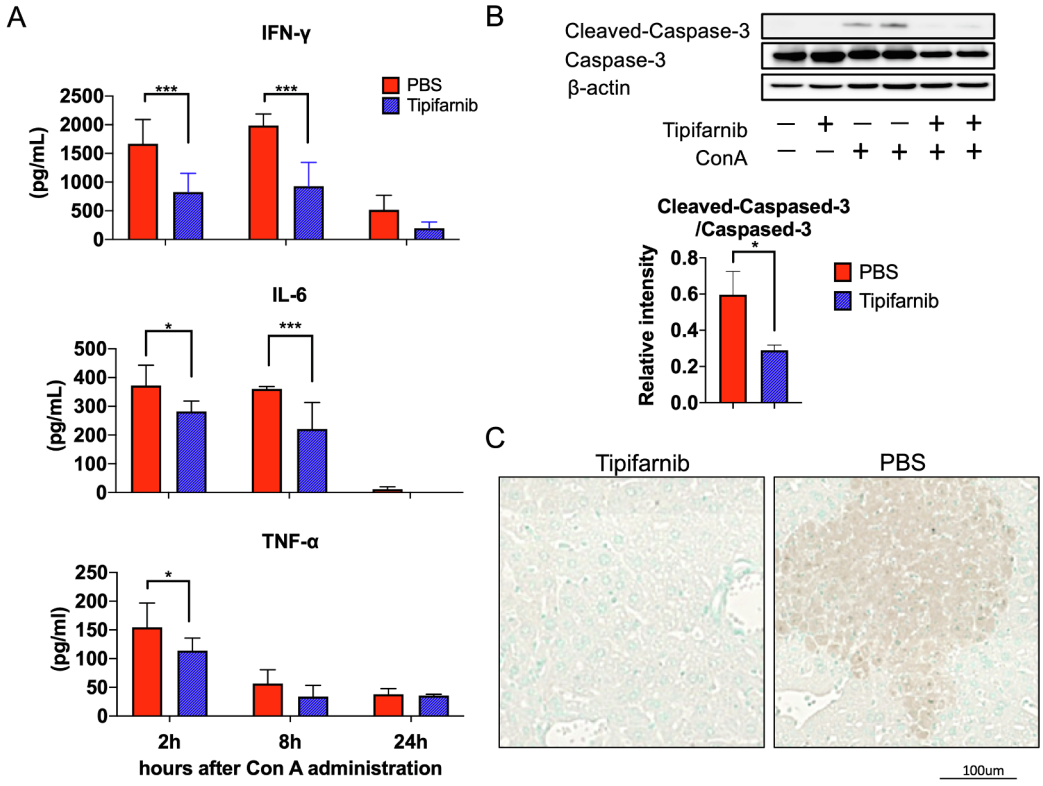

Fig. 2. The effect of tipifarnib on cytokine increments induced by Con A. (A) The serum protein-expression levels of IFN-γ, IL-6, and TNF-α were quantified by ELISA at 2, 8, and 24 h after Con A administration ($N = 6$ in each group). (B) Representative immunoblots and densitometric analysis of cleaved caspase 3. The expression levels of cleaved caspase 24 h after Con A administration. Relative intensity was normalized to uncleaved caspase 3 expression levels ($N = 6$). (C) The liver section stained by TUNEL assay. The data shown are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ between the PBS and tipifarnib groups. Red columns, PBS group. Blue columns, tipifarnib group. Scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared with the PBS-treated group (Fig. 2B). Tipifarnib markedly attenuated the serum TNF-α levels at 2 h (113.9 ± 21.9 vs. 154.6 ± 42.2 pg/mL, $p = 0.001$) after Con A administration, compared with the PBS-treated group (Fig. 2C).

3.3. Tipifarnib attenuated apoptosis induced by Con A administration in the liver

Levels of cleaved caspase 3 and the number of apoptotic cells were increased 24 h after Con A administration in the liver. Tipifarnib significantly attenuated increments of cleaved caspase 3 and the number of apoptotic cells (Fig. 2B and C).

3.4. Tipifarnib suppressed Con A-induced activation of $CD4^+$ T cells from isolated livers and spleens, as well as the consumption of NKT cells the isolated livers

Con A significantly increased the activation of $CD4^+$ or $CD8^+$ T cells in the liver (Fig. 3A) and spleen (Sup 1A), compared to baseline at 2 and 8 h post-Con A administration. At 8 h post-Con A administration, the activated $CD4^+$ T cells, but not $CD8^+$ T cells (Supplementary 1A and B), in the liver (Fig. 3A; 14.6 ± 3.3 vs. 28.0 ± 4.2, $p = 0.046$) and spleen (Fig. 3B; 14.2 ± 3.4 vs. 19.4 ± 5.1, $p = 0.03$) had decreased significantly in the tipifarnib group, compared to the PBS group. The percentage of NKT ($NK1.1^+CD3^+$) cells had significantly decreased at 2 h post-Con A administration. This depletion was

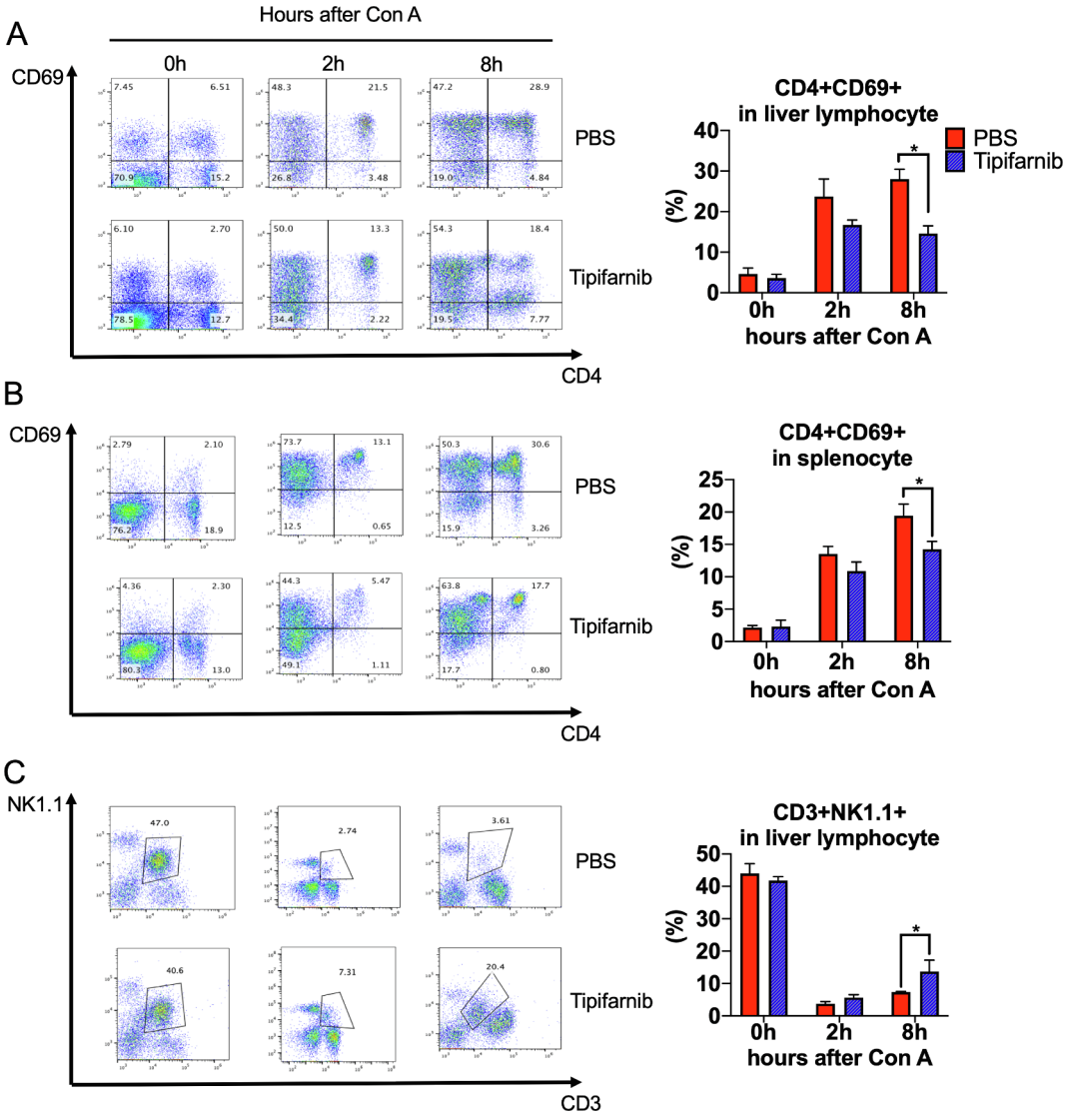

Fig. 3. The effects of tipifarnib on $CD4^+$ T and NKT cell activation induced by Con A. (A) The percentage of $CD69^+$ cells among hepatic $CD4^+$ T cells from livers isolated from mice treated with tipifarnib or PBS at 0, 2, and 8 h after Con A administration. The data shown are presented as the mean ± SEM. *$p < 0.05$ between both groups. (B) The percentage of $CD69^+$ cells among splenic $CD4^+$ T cells from isolated from mice treated with tipifarnib or PBS at 0, 2, and 8 h after Con A administration. The data shown are presented as the mean ± SEM. *$p < 0.05$ between both groups. (C) The percentage of $NK1.1^+$ cells among hepatic $CD3^+$ T cells from the isolated livers of mice treated with tipifarnib or PBS at 0, 2, and 8 after Con A administration. *$p < 0.05$ between the PBS and tipifarnib groups. Red columns, PBS group. Blue columns, tipifarnib group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

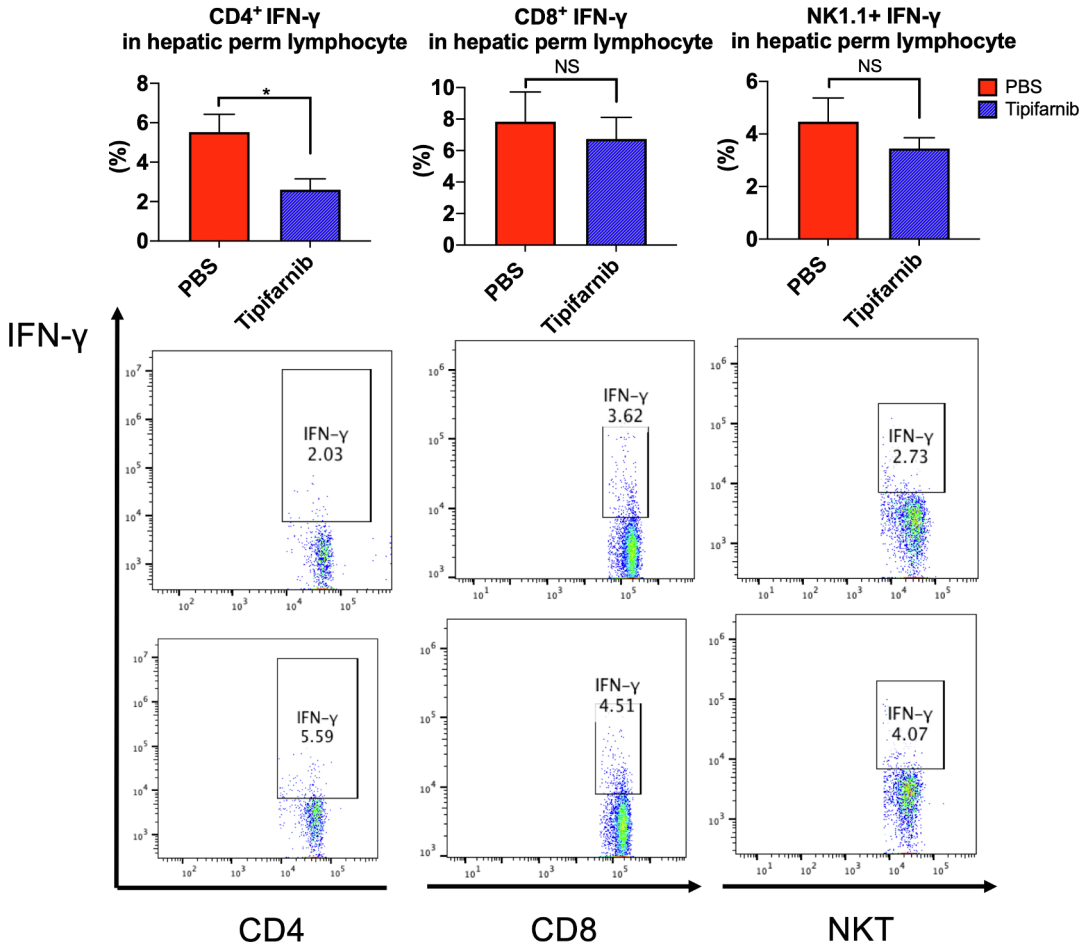

Fig. 4. The effect of tipifarnib on IFN-γ production induced by Con A in hepatic lymphocytes. IFN-γ production in (left) $CD4^+$ T cells, (center) $CD8^+$ T cells, and (right) NKT cells from the isolated livers of mice treated with tipifarnib or PBS at 2 h after Con A administration. The data shown are representative of two independent explements ($N = 6$ per group) and are presented as the mean ± SEM. *$p < 0.05$ between the PBS and tipifarnib groups. NS, not significant. Red columns, PBS group. Blue columns, tipifarnib group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly suppressed in the tipifarnib-treated group compared to the PBS group at 8 h post-Con A administration (Fig. 3C; 13.7 ± 6.1 vs. 7.4 ± 0.3, $p = 0.03$).

3.5. Tipifarnib decreased the amount of IFN-γ secreted by $CD4^+$ T cells from isolated livers

IFN-γ secretion from liver $CD4^+$ T cells isolated 2 h after Con A administration was significantly inhibited in the tipifarnib group, when compared to the PBS-treated group (Fig. 4 left; 2.6 ± 1.1 vs. 5.5 ± 1.8, $p = 0.03$). Tipifarnib did not inhibit IFN-γ secretion from liver $CD8^+$ T or NKT cells isolated 2 h after Con A administration, when compared to the PBS-treated group.

3.6. Tipifarnib inhibited STAT1 phosphorylation in $CD4^+$ T cells from isolated livers

The expression of phosphorylated STAT1 in $CD4^+$ T cells 2 h after Con A administration was significantly inhibited in the tipifarnib treated mice compared with the PBS treated mice (Fig. 5; 32.8 ± 7.1 vs. 53.6 ± 10.6, $p = 0.046$).

3.7. Tipifarnib inhibited the amount of IFN-γ secreted by splenic $CD4^+$ T cells

Tipifarnib dose-dependently and significantly inhibited the amount of IFN-γ secreted from splenic $CD4^+$ T cells at 48 h (72.0 ± 2.4 vs. 40.0 ± 3.3, $p = 0.0002$) and 72 h (81.37 ± 11.5 vs., 7.0 ± 3.3, $p < 0.0001$) after Con A injection (Fig. 6).

3.8. Tipifarnib inhibited farnesylated-protein expression induced by Con a in the liver

Comparing farnesylated-protein expression in the liver tissue of tipifarnib and PBS groups 24 h after Con A administration, tipifarnib inhibited the expression of farnesylated-protein induced by Con A administration in the liver, especially around the portal duct (Fig. 7).

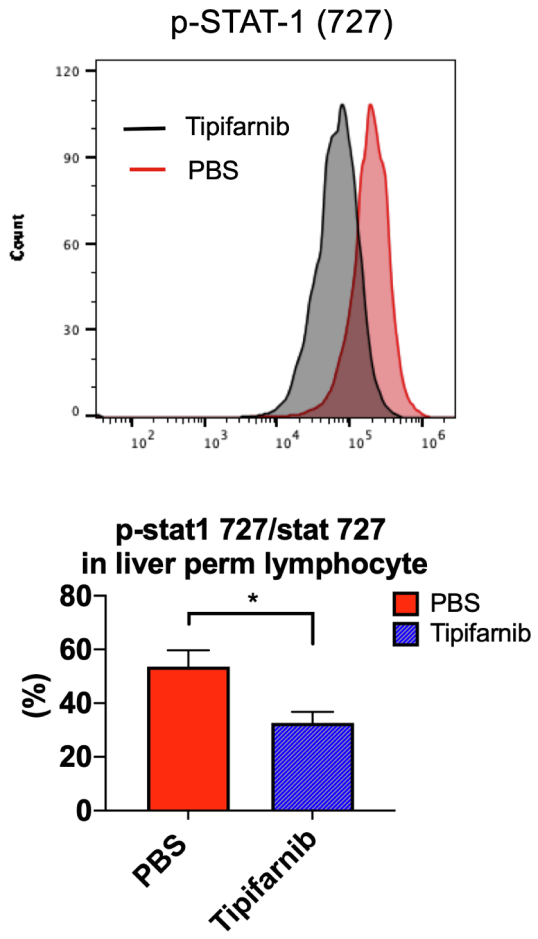

Fig. 5. The effect of tipifarnib on Con A-induced STAT1 phosphorylation in $CD4^+$ T cells. The expression of phosphorylated stat1(7 2 7) in $CD4^+$ T cells from the isolated livers of mice treated with tipifarnib or PBS at 2 h. The data shown are presented as the mean ± SEM. $^*p < 0.05$ between the PBS and tipifarnib groups. Red column, PBS group. Blue column, tipifarnib group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

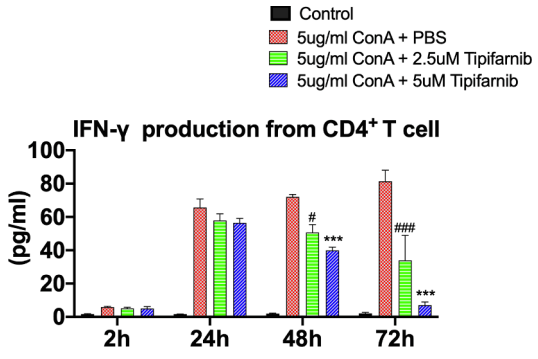

Fig. 6. The effect of tipifarnib on Con A-induced IFN-γ production in splenic $CD4^+$ T cells *in vitro*. IFN-γ secretion by splenic $CD4^+$ T cells at 2, 24, 48, and 72 h after Con A injection. The data shown are presented as the mean ± SEM. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.005$ when compared with the 5 μg/mL Con A + PBS group and the 5 μg/mL Con A + 2.5 μM tipifarnib group. $^{***}p < 0.005$ when compared with the 5 μg/mL Con A + 2.5 μM tipifarnib group and the 5 μg/mL Con A + 5 μM tipifarnib group. Black columns, control group. Red columns, 5 μg/mL Con A + PBS group. Green columns, 5 μg/mL Con A + 2.5 μM tipifarnib group. Blue columns, 5 μg/mL Con A + 5 μM tipifarnib group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

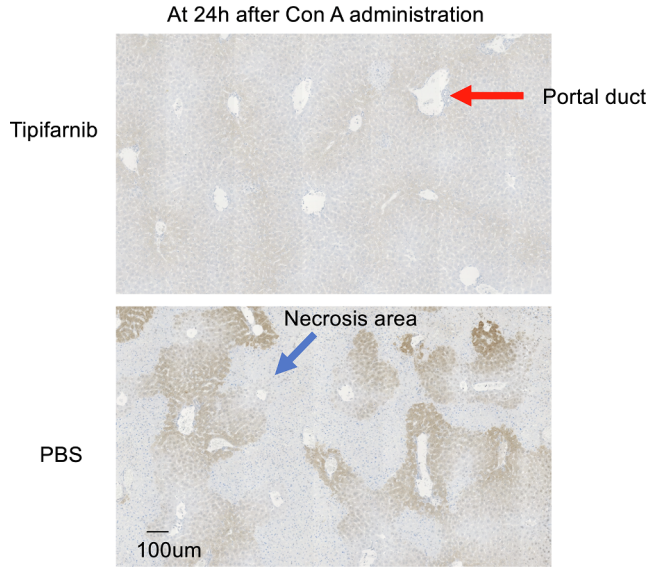

Fig. 7. The effects of tipifarnib and Con A on the expression of farnesylated proteins in the liver. Representative liver histology and the amounts of farnesylated proteins at 24 h post-Con A administration. Farnesylated liver proteins were evaluated by immunohistochemical analysis. The brown areas indicated proteins that were farnesylated. Red arrow, portal duct. Blue arrow, necrotic area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

To the best of our knowledge, this report is the first to show that an FTI can exert therapeutic effects on the AIH induced by Con A *in vivo*. Tipifarnib significantly inhibited liver enzyme increases, histological changes, the hepatic infiltration of inflammatory $CD4^+$ T cells, NKT cell consumption, and inflammatory cytokines or apoptotic cells increments induced by Con A administration. Furthermore, tipifarnib decreased IFN-γ production and STAT1 phosphorylation in hepatic $CD4^+$ T cells induced by Con A. *In vitro*, tipifarnib also inhibited the amount of IFN-γ secreted from splenic $CD4^+$ T cells.

FTIs can inhibit the function of Ras, which plays a curial role in T cell activation and function given that T cell receptor ligation led to Ras activation in T cells [31–33]. Furthermore, FTIs could augment RAF/MEK/ERK signaling, which has beneficial effects with disorders of the lymphoproliferative syndrome [34]. Our results also revealed that tipifarnib potently suppressed immune responses by inhibiting IFN-γ production via suppressed STAT1 phosphorylation in hepatic $CD4^+$ T cells. In a previous study, an anti-CD4 monoclonal antibody and tacrolimus could alleviate Con A-induced liver injury [23]. Additionally, stimulating naïve $CD4^+$ T cells with Con A resulted in IFN-γ overexpression, and development along the Th1 lineage is consequently thought to require signaling through the IFN-γ receptor, which further up-regulates T-bet expression and causes high-level production of IFN-γ by Th1 effectors [35]. Additionally, a previous report showed that apoptotic cell death induced by Con A is not observed in the livers of IFN-γ deficient mice, and IFN-γ plays a central role in Con A hepatitis by activating apoptosis of liver cells [28]. Taken together, IFN-γ seem to be important for the liver deterioration observed in Con A-induced hepatitis. Therefore, IFN-γ produced by CD4 T^+ cells could serve as a good therapeutic target in Con A-induced hepatitis.

The transcription factor STAT1 plays a vital role in the production of IFN-γ [31]. Previous reports demonstrated that IFN-γ/STAT1 contributes to Con A-induced T cell hepatitis [27,36]. Additionally, it was revealed that anti-IFN-γ treatment reduced TNF-α serum levels and liver damage, but not the infiltration of $CD4^+$ and $CD45^+$ T cells, or STAT 1 activation after Con A injection *in vivo* [27]. This previous

report indicated that the protective effect of FTIs against Con A-induced hepatitis could be due to inhibited STAT1 phosphorylation. Although it was also reported that FTIs inhibited IL-6-induced STAT3 and ERK1/2 phosphorylation in human myeloma cells [37], no previous reports have shown that FTIs inhibit STAT1 phosphorylation.

Tipifarnib dose-dependently inhibited IFN-γ production from $CD4^+$ cells, both *in vitro* and *in vivo*. This inhibitory effect significantly decreased at 48 h post-tipifarnib injection and at later time points. The difference in the onset of effects *in vivo* and *in vitro* indicated that tipifarnib was capable of systemic effects in different organs, including the liver, spleen, or blood.

It was previously reported that activated $CD8^+$ T cells and NKT cells, which produce a high amount of IFN-γ, also contribute to the development of Con A-induced hepatitis [38,39]. The data generated in this study revealed that tipifarnib had little effect on IFN-γ production in $CD8^+$ T cells and NKT cells. In a previous report, FTIs were shown to revert a sepsis-induced increase in $CD4^+Foxp3^+$ Tregs in the spleen [40], inhibit alloantigen-driven expansion of $CD4^+$ T cells but not $CD8^+$ T cells [41], or block TCR/CD28-mediated T cell activation [42]. It was also reported that FTIs significantly delayed the rejection of skin allografts in mice and that FTIs affected both $CD4^+$ and $CD8^+$ T cells [43]. The difference of these findings may be a consequence of differences in the models used or the domination of $CD4^+$ T cells response in the early stage of AIH, followed by a cytotoxic $CD8^+$ T cells response [44].

Additionally, $CD4^+$ T cells are known to be enriched in the portal tract, whereas $CD8^+$ T cells are known to be enrich in the hepatic lobe [45]. In this immunostaining study, more protein farnesylation was detected in the PBS group than in the tipifarnib group around the portal duct after Con A administration (Fig. 7). Taken together, the anti-farnesylation effects of tipifarnib in $CD4^+$ T cells might be related to the hepatoprotective effects in our AIH model. Although further research is needed in this regard, our findings and previous findings collectively demonstrate that FTIs can at least inhibit $CD4^+$ T cell function.

As a limitation, although, there is evidence that farnesyltransferase affects RAS in lymphocytes, a possible key protein for protecting AIH association with farnesylation could not be revealed in this study. However, previous reports also could not reveal the non-RAS protein, which was affected by farnesylation. Further research is required to address this concern.

In conclusion, we revealed that the FTI tipifarnib suppressed liver damage induced by Con A *in vitro* and in a mouse model of AIH model. Further research is needed to characterize the protective ability of tipifarnib against AIH and to investigate its safety for potential clinical applications.

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6. Role of the findings source

None.

CRediT authorship contribution statement

Jie Guo: Data curation, Formal analysis, Writing - original draft. **Kazuhiro Shirozu:** Conceptualization, Funding acquisition, Writing - review & editing. **Tomohiko Akahoshi:** Conceptualization. **Yukie Mizuta:** Data curation. **Masaharu Murata:** Funding acquisition. **Ken Yamaura:** Writing - review & editing.

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Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106462.

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