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Enhancing Regulatory T cell function by mevalonate pathway inhibition prevents liver fibrosis

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ABSTRACT

Liver fibrosis is a well-established risk factor for liver cancer development. Despite extensive mechanistic studies on liver fibrosis, the role of the immune cell network in fibrotic disease remains poorly understood. In this study, we demonstrate that regulatory T cells (Tregs) are involved in preventing liver fibrosis by regulating the mevalonate pathway. Blocking the mevalonate pathway increased the granzyme B secretion from Tregs, while restoring the pathway reduced it. Statin treatment, which inhibits the mevalonate pathway, alleviated liver fibrosis progression and enhanced the immunosuppressive function of Tregs *in vivo*. Mechanistically, mevalonate products, including geranylgeranyl pyrophosphate, inhibited the phosphorylation and activation of LKB1, that is a key regulator of Treg homeostasis. Furthermore, these products disrupted the interaction between LKB1 and cAMP-dependent protein kinase (PKA), leading to further reduction of LKB1 phosphorylation. These findings suggest that targeting LKB1 in Tregs through statin treatment prevents the progression of liver fibrosis, offering a promising and safe therapeutic strategy for liver disease and liver cancer.

1. Introduction

Tissue fibrosis is a reversible process associated with cancer development [1,2]. Liver fibrosis is a common outcome of liver diseases, including metabolic dysfunction-associated steatotic liver disease (MASLD or MASH) [3]. Tissue fibrosis also acts as a physical barrier, hindering drug penetration and immune cell infiltration, thereby contributing to drug resistance [4]. Immune cells, such as group 2 innate lymphoid cells (ILC2), T helper 2 (Th2) cells and Kupffer cells, play pivotal roles in liver fibrosis progression, highlighting the importance of precise immune regulation to prevent liver fibrosis [5,6]. Understanding the immune mechanisms driving liver fibrosis is essential for developing effective therapeutic interventions.

Tregs, a subpopulation of $CD4^+$ T lymphocytes, are crucial for maintaining peripheral tolerance and preventing autoimmune diseases [7,8]. Tregs secrete immunosuppressive cytokines, such as interleukin (IL)-10 and IL-35, and exert cytolytic effects through the release of granzyme B [9,10]. However, their role in tissue fibrosis remains controversial due to their cytokine profiles [11,12]. Several studies have shown that damaged hepatocytes promote Treg infiltration in the liver, suggesting that modulating Treg function or recruitment could serve as a potential therapeutic strategy to mitigate the severity of liver fibrosis [13].

The mevalonate pathway is a key metabolic pathway involved in the production of sterols and lipids essential for cell growth [14]. Several studies have demonstrated that targeting the mevalonate pathway can improve tissue fibrosis in various organs, including the heart, lungs and liver [15,16]. Statins, inhibitors of HMG-CoA reductase, are known to block the mevalonate pathway and reduce tissue fibrosis in the liver and lungs [17,18]. However, the specific immune mechanisms underlying the anti-fibrotic effects of statins remain poorly understood.

In this study, we investigated the statin-induced modulation of Tregs in alleviating liver fibrosis, focusing on enhancing their cytolysis function. Statin treatment promoted granzyme B secretion from Tregs by inhibiting the mevalonate pathway. This treatment significantly reduced the severity of liver fibrosis compared to untreated mice in the carbon tetrachloride (CCl_4)-induced fibrosis model, primarily by activating granzyme B-secreting Tregs. Furthermore, we demonstrated that this regulation depends on the phosphorylation of LKB1, a key regulator of Treg homeostasis. Inhibition of the mevalonate pathway enhanced

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LKB1 phosphorylation, while the addition of geranylgeranyl pyrophosphate (GGPP) inhibited it. The phosphorylation site was located near the GGPP-dependent prenylation site in LKB1, suggesting that these two different post-translational modifications interfere with each other for regulation of the cytolytic function of Tregs. Our findings suggest that Tregs modulation through statin treatment represents a promising therapeutic strategy and a valuable approach for managing liver fibrosis, including MASH.

2. Materials and methods

2.1. Animal

All mice were housed in a specific pathogen-free (SPF) facility and provided standard feed, following established animal care regulations. C57BL/6 wild-type (WT) mice were obtained from Hyochang Science (Daegu, Republic of Korea) and sterol regulatory element-binding protein 1c (SREBP1c) knockout (1cKO) mice were generously provided by Prof. SS Lim. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IRB No. KM-2023-18) of Keimyung University in Daegu, Republic of Korea.

2.2. CCl_4-induced liver fibrosis

Six-week-old WT female mice received intraperitoneal (IP) injections of CCl_4 (Sigma-Aldrich, St. Louis, MO, Catalog no. 289116) at a dose of 1 mL/kg, dissolved in olive oil, twice weekly for four weeks. Mice were sacrificed the day following the final injection. Pitavastatin (from Selleck Chemicals) was administered twice weekly via IP injection, on alternate days, until harvest.

2.3. Cell culture and treatment

Jurkat cells were cultured at 37 °C in a 5 % CO_2 atmosphere in RPMI-1640 medium (Welgene, Gyeongsan, Republic of Korea, Catalog no. LM01103) supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, Catalog no. 16000044) and 1X penicillin–streptomycin (antibiotics, Welgene, Catalog no. LS20202) (referred to as 'R10'). Cells were activated using a cell activation cocktail (BioLegend, Catalog no. 423301) according to the manufacturer's instructions and treated with pitavastatin (5 mg/mL), GGPP (1 mM) (Echelon Bioscience, Salt Lake City, UT, Catalog no. I-0200) or GGTI-2147 (5 mM) (Sigma-aldrich, Catalog no. 345885). AML12 cells were cultured in a complete medium (Elabscience, Houston, Texas, Catalog no. CM-0602).

2.4. In vitro T cell and Treg activation

Spleens were harvested from WT mice, and single-cell suspensions were prepared using a 70 μM cell strainer followed by centrifugation at 1500 rpm for 5 min. $CD4^+CD25^+$ regulatory T cells were isolated using a MojoSort™ mouse $CD4^+CD25^+$ regulated T cell isolation kit (BioLegend, San Diego, CA, Catalog no. 480137) according to the manufacturer's instructions. Isolated cells were plated in a 6-well plate precoated with anti α-CD3 (Bio X cell, Lebanon, NH, Catalog no. BE0001-1) and cultured in R10 medium supplemented α-CD28 (Bio X cell, Catalog no. BE0015-1). The next day, pitavastatin was added to the Treg cultures and the control group was treated with DMSO. The supernatant from the Treg cultures was collected and applied to AML12 cells treated with doxorubicin (Dox, Sigma-aldrich, Catalog no. D1515). $CD4^+$ T cell were isolated using the MojoSort™ mouse $CD4^+$ T cell isolation kit (BioLegend, San Diego, CA, Catalog no. 480006).

2.5. Western blotting

Equal amounts of total protein from cell lysates were loaded onto gels prepared with 1X Tris/glycine/SDS buffer (Bio-rad, Hercules, CA, USA, Catalog no. 1610772). Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland, Catalog no. 03010040001) using Tris-glycine transfer buffer (Bio-rad, Catalog no. 1610771). Membranes were incubated overnight at 4 °C with appropriate primary antibodies, followed by incubation with secondary antibodies the next day. Details of all primary and secondary antibodies are provided in the Supplementary Table 1.

2.6. Hepatic lymphocytes isolation

Hepatocytes were discarded, and cell suspensions were resuspended in 40 % Percoll (Cytiva, Uppsala, Sweden, Catalog no. 17089101) and layered over 70 % Percoll. Density gradient centrifugation was performed at 2400 rpm with low deceleration. Lymphocytes from the middle layer were carefully collected using a transfer pipette and resuspended in a pre-warmed R10 medium. Brefeldin A (BioLegend, Catalog no. 420601) and monensin (BioLegend, Catalog no. 420701) were added for further analysis of liver immune cells.

2.7. Flow cytometry

Cells were washed with PBS containing FBS and 0.5 mM EDTA (Bio Basic, Toronto, CA, Catalog no. SD 8135) and stained on ice for 30 min with antibodies to label surface markers (Supplementary Table 1). After surface marker staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Catalog number 00-5523-00). Intracellular antibodies (Supplementary Table 1) were then used for staining. For intracellular cytokine expression analysis, cells were pre-treated with brefeldin A and monensin for 4 h prior to granzyme B staining.

2.8. Protein modeling

The protein structure of LKB1 was retrieved from the AlphaFold Protein Structure Database (ID: AF-Q9WRK7-F1-v4) in PDB format with 3D coordinates. The structure was analyzed using UCSF CHIMERA (Version 1.17.13) and the distances between internal residues were measured with its structure analysis tools.

2.9. Statistical analysis

Data were analyzed using GraphPad Prism version 10 (GraphPad, San Diego, CA). Unpaired t-tests were performed to compare the two groups, and one-way ANOVA followed by Tukey's multiple comparisons test was used for grouped variables. p-values <0.05 were considered statistically significant. Bar graphs represent the mean +SD.

3. Results

3.1. The mevalonate pathway modulates granzyme B secretion from Tregs

To investigate the role of the mevalonate pathway in cytolytic function of immune cells, particularly Tregs, we first analyzed granzyme B secretion *in vitro*. Statin treatment significantly increased granzyme B secretion from Tregs compared to carrier-treated controls (Fig. 1A). Notably, the addition of GGPP inhibited statin-mediated increase in granzyme B secretion from Tregs, demonstrating that the granzyme B secretion is modulated through the mevalonate pathway (Fig. 1A). In contrast, granzyme B secretion from $CD8^+$ T cells was not dependent on the mevalonate pathway (Fig. 1B), highlighting a unique regulatory mechanism in Tregs. To further confirm the involvement of the mevalonate pathway in the cytolytic function of Tregs, we assessed the effect of SREBP1c, a known inducer of the mevalonate pathway [19]. Tregs lacking SREBP1c exhibited enhanced granzyme B secretion compared to WT Tregs (Fig. 1C). Collectively, these findings suggest that the cytolytic function of Tregs, including granzyme B secretion, is modulated by the

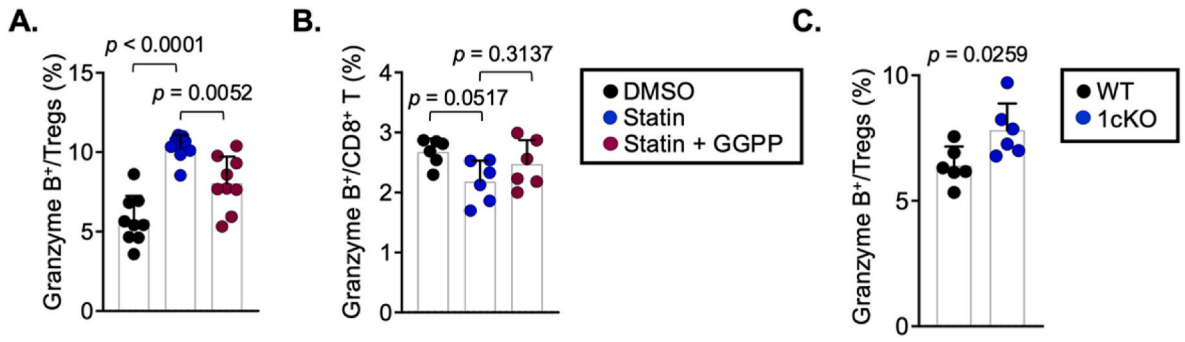

Fig. 1. The mevalonate pathway regulates granzyme B secretion from Tregs.
(A) Granzyme B^+ Tregs frequency as % total WT male splenic $CD4^+$ Tregs after incubation with statin plus GGPP, statin versus no treatment control (n = 9 culture wells per condition). (B) Granzyme B^+ $CD8^+$ T cells frequency as % total WT male splenic $CD8^+$ T cells after incubation with statin plus GGPP, statin versus no treatment control (n = 6 culture wells per condition). (C) Granzyme B^+ Tregs frequency as % total WT and 1cKO male splenic $CD4^+$ Tregs (n = 6 culture wells per condition). Graphs show mean +SD, One-way ANOVA, Unpaired *t*-test.

mevalonate pathway.

3.2. Statins alleviate CCl_4-induced liver fibrosis

To evaluate whether statin treatment induces granzyme B secretion from Tregs *in vivo*, we utilized a CCl_4-induced liver fibrosis model [20]. Four weeks after CCl_4 injections, collagen deposition was evident in the livers of treated mice (Fig. 2A). However, statin treatment significantly reduced collagen deposition compared to PBS-treated controls (Fig. 2A). Additionally, the levels of fibrotic markers, such as *α-smooth muscle actin (Sma)* and *Il13* were significantly decreased in statin-treated mice compared to controls (Fig. 2B) [21,22], indicating that statins mitigate the severity of CCl_4-induced liver fibrosis. Statin treatment also enhanced granzyme B secretion and promoted Treg proliferation compared to the PBS-treated group (Fig. 2C). Furthermore, statins markedly reduced $CD3^+$ and $CD8^+$ T cell infiltration in the liver, likely due to the enhanced cytolytic function of Tregs. These findings suggest that statins enhance the cytolytic activity of Tregs, contributing to the alleviation of liver fibrosis.

3.3. LKB1 is regulated by phosphorylation and prenylation

Previous studies have identified serine/threonine kinase LKB1 as a key regulator of Treg homeostasis [23]. LKB1 is tightly controlled by phosphorylation and is essential for maintaining Treg function via the mevalonate pathway [24]. We explored whether the mevalonate pathway regulates LKB1 through a feedback loop to maintain Treg homeostasis. GGPP, a final product of the mevalonate pathway, is essential for protein prenylation, a lipid post-translational modification that regulates cellular processes, such as receptor trafficking and cell growth [25]. Our findings reveal that LKB1 is a target for prenylation, as indicated by the presence of a conserved CAAX motif in its sequence across multiple species (Fig. 3A) [26]. Activation of Jurkat cells by treatment with "activation cocktail" consisting of PMA and ionomycin led to an induction of LKB1 phosphorylation (Fig. 3B). Statin treatment to the activated Jurkat cells further increased LKB1 phosphorylation compared

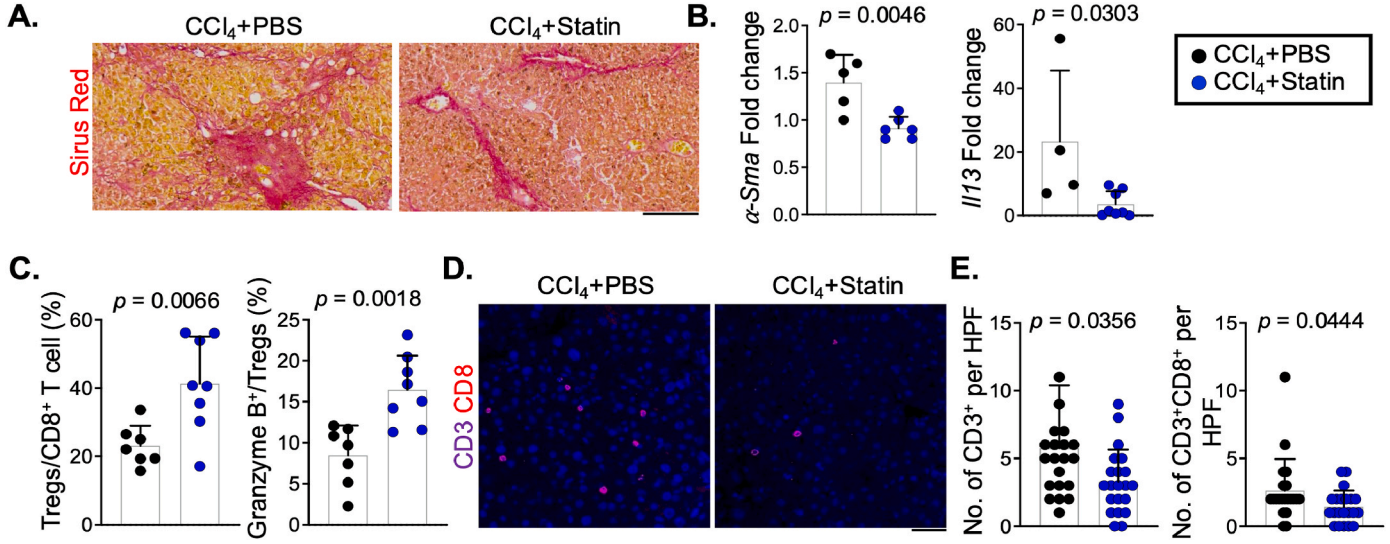

Fig. 2. Statin treatment reduces the severity of liver fibrosis.
(A) Representative images of hematoxylin and eosin (H&E)-stained liver sections from mice treated with CCl_4 combined with PBS or CCl_4 combined with statin, taken 30 days after the CCl_4 first injection. (B) (Left) *α-Sma* and (Right) *Il13* expression in CCl_4 and CCl_4 with statin-treatment mice (n = 5 for CCl_4-treated mice and n = 6 for CCl_4 with statin-treated mice in *α-Sma*, n = 4 for CCl_4-treated mice and n = 8 for CCl_4 with statin-treated mice in *Il13*). (C) (Left) Tregs frequency as % $CD8^+$ T cells and (Right) granzyme B^+ Tregs frequency as % total $CD4^+$ Tregs cells in the same groups as in Figure (A) (n = 7 for CCl_4-treated mice and n = 8 for CCl_4 with statin-treated mice). (D) Representative images of $CD3^+$ and $CD8^+$ T cells staining from liver tissues of the same groups as in Figure (A). (E) Quantification of (Left) $CD3^+$ and (Right) $CD3^+CD8^+$ T cells from Figure (D). $CD3^+$ and $CD3^+CD8^+$ cells were counted in three randomly selected high power field (HPF) images per liver sample. Each dot represents an HPF image. Graphs show mean + SD, Unpaired *t*-test, scale bars: 100um.

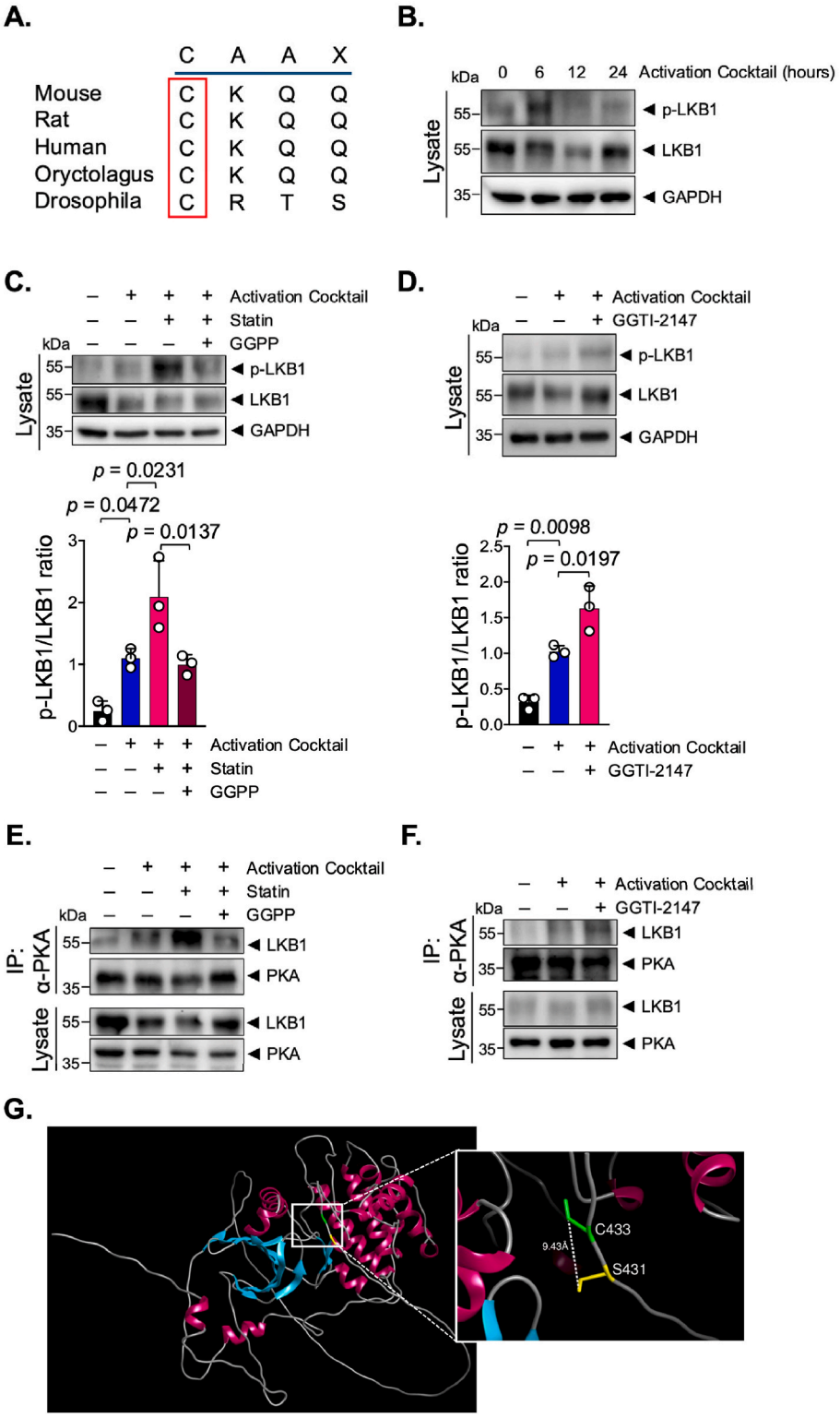

Fig. 3. LKB1 is antagonistically regulated by phosphorylation and prenylation in T cell lines.
(A) Prenylation motif (CAAX, C is a cysteine residue, AA are two aliphatic residues, and X represents any C-terminal amino acid) in LKB1 sequence across various species. (B) Time course of *p*-LKB1, LKB1, GAPDH protein expression in Jurkat cells following activation cocktail treatment. (C) (Top) Immunoblot of *p*-LKB1, LKB1 and GAPDH proteins in whole cell lysate from activation cocktail-treated Jurkat cells that received pitavastatin alone or in combination with GGPP. (Bottom) The ratio of *p*-LKB1/LKB1 protein band intensity from immunoblots (n = 3 in each group). (D) (Top) Immunoblot of *p*-LKB1, LKB1 and GAPDH proteins in whole cell lysate of activation cocktail-treated Jurkat cells that received with or without GGTI-2147. (Bottom) The ratio of *p*-LKB1/LKB1 protein band intensity from immunoblots (n = 3 in each group). (E) Immunoblot of LKB1 and PKA proteins in whole cell lysate and immunoprecipitated samples from activation cocktail-treated Jurkat cells that received pitavastatin alone or in combination with GGPP (n = 2). (F) Immunoblot of LKB1 and PKA proteins in whole cell lysate and immunoprecipitated samples from activated Jurkat cells treated with or without GGTI-2147. (G) 3D structural representation of LKB1, highlighting the spatial distance between Cys433 and Ser431. Graphs show mean + SD, one-way ANOVA.

to the cells incubated with activation cocktail alone, while GGPP treatment inhibited LKB1 phosphorylation even in the presence of statins (Fig. 3C). Interestingly, the prenylation site (Cys433) and LKB1 phosphorylation site (Ser431) of LKB1 are closely located, suggesting that prenylation and phosphorylation may interfere with each other. To test this possibility, we used GGTI-2147, a prenylation inhibitor. This inhibitor treatment led to a significant increase in LKB1 phosphorylation (Fig. 3D). PKA is responsible for LKB1 phosphorylation at Ser431 [27,28]. Significantly, statin treatment enhanced the interaction between LKB1 and PKA, whereas GGPP completely blocked their interaction (Fig. 3E). GGTI-2147 treatment further increased the LKB1-PKA interaction compared to activation cocktail treatment alone (Fig. 3F). These findings suggest that prenylation may sterically hinder the access of PKA due to close proximity between the prenylation and phosphorylation sites. To verify this possibility, the LKB1 protein model was visualized using molecular graphics, depicting its secondary structure in ribbon form (Fig. 3G). In this model, pink represents α-helices, cyan represents β-strands and gray indicates coil regions. Cys433 and Ser431 were separated the coil region of LKB1 with a distance of 9.43 Å. This close proximity of two distinct modification sites highlights the mechanism by which LKB1 can be antagonistically regulated by a process depending on the mevalonate pathway.

3.4. Statin-treated Tregs induce apoptosis in damaged hepatocytes

Previous studies have shown that damaged hepatocytes in the liver recruit Tregs [29]. To assess whether Tregs modulate damaged cells via granzyme B secretion, we used Dox to induce cellular damage. Dox treatment increased apoptosis markers, including p53 and cleaved PARP1 in a dose-dependent manner (Fig. 4A). Next, we compared the effects of conditioned medium from statin- and DMSO-treated Tregs on damaged cells. The medium from statin-treated Tregs significantly enhanced apoptosis, as indicated by elevated p53 and cleaved PARP1 levels, compared to the controls (Fig. 4B). These findings demonstrate that statins enhance the cytolytic function of Tregs by promoting apoptosis in damaged cells, such as hepatocytes, thereby contributing to the resolution of liver fibrosis.

4. Discussion

Our study demonstrates that regulating the cytolytic function of Tregs through statin treatment represents a promising therapeutic strategy for liver fibrosis. Statins enhanced granzyme B secretion from Tregs by modulating the mevalonate pathway, which alleviates the severity of liver fibrosis. This process was closely associated with the phosphorylation of LKB1, a critical regulator of Tregs function. Specifically, statins promoted LKB1 phosphorylation, whereas GGPP inhibited

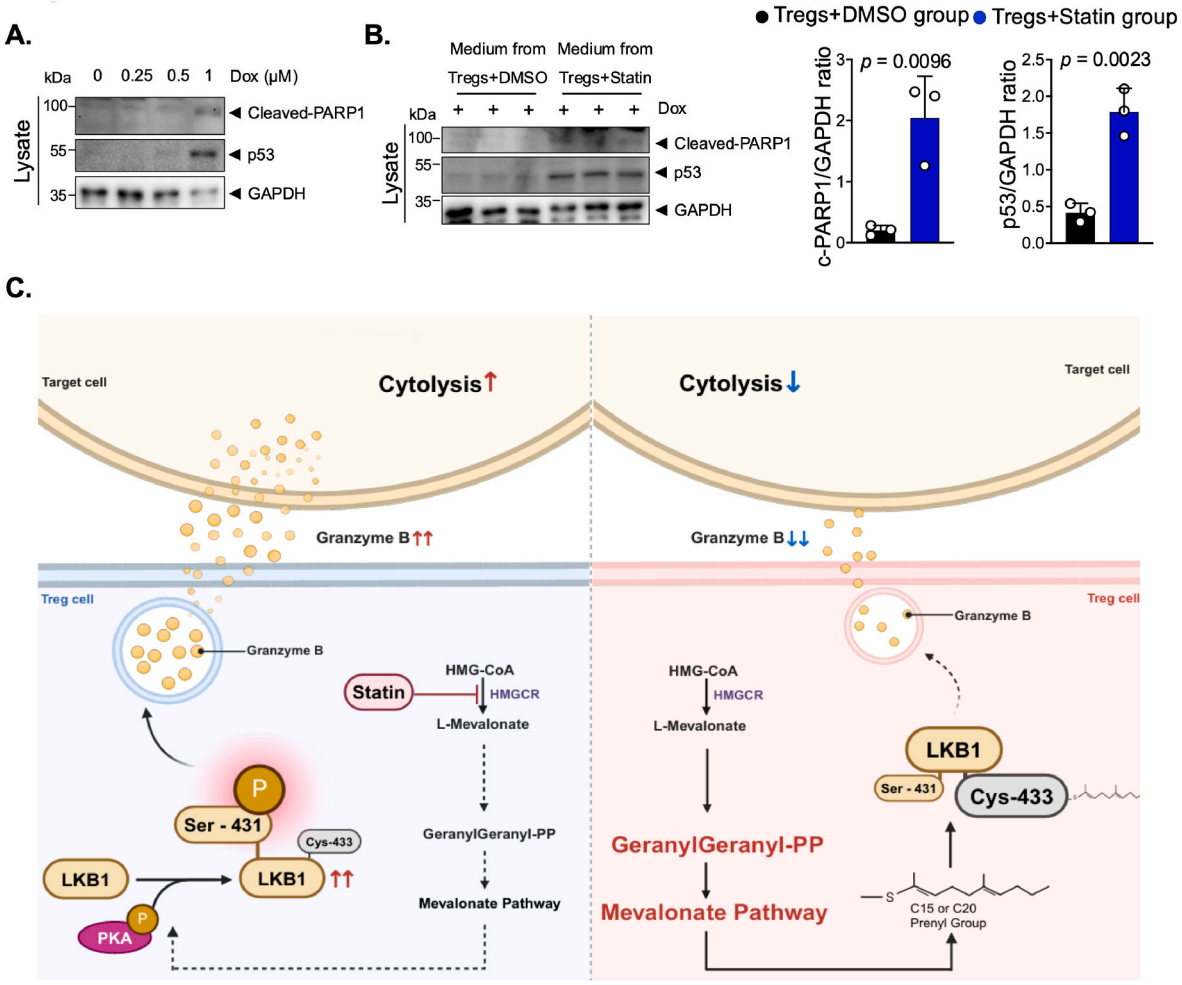

Fig. 4. Statin-treated Tregs induce apoptosis of damaged hepatocytes.
(A) Immunoblot of cleaved-PARP1, p53 and GAPDH protein expression in AML12 cells following treatment with increasing dose of Dox. (B) (Left) Immunoblot of cleaved-PARP1, p53 and GAPDH protein expression in AML12 cells after treated Dox in the presence of culture medium from DMSO-treated activated Tregs or statin-treated activated Tregs. (Right) The ratio of cleaved-PARP1/GAPDH protein and p53/GAPDH band intensity from Fig. 4B immunoblots (n = 3 in each group). (C) Modulation of Tregs cytolytic function by statin in a liver fibrosis model (created with BioRender.com). Graphs show mean + SD, Unpaired *t*-test.

its phosphorylation and altered the pattern of granzyme B secretion from Tregs. Furthermore, inhibition of protein prenylation with GGTI-2147 enhanced LKB1 phosphorylation, revealing a novel mechanism by which the mevalonate pathway regulates the cytolytic function of Tregs (Fig. 4C).

Statins are widely recognized as primary preventive agents for cardiovascular disease due to their cholesterol-lowering effects [30]. However, the role of statins in fibrosis remains controversial [31], primarily because statins influence on a wide spectrum of cells, including immune cells, fibroblasts and endothelial cells. In this study, we specifically focused on the Treg-statin axis in a liver disease model. Nevertheless, further research is necessary to explore the effects of statins on other cell populations in the context of fibrosis. For example, targeting fibroblasts or endothelial cells with statin-loaded nanoparticles could provide a more precise therapeutic approach to fibrosis and other diseases [32]. Such strategies could mitigate off-target effects and optimize the therapeutic efficacy of statins. Integrating these approaches into fibrosis research could help resolving the ongoing debates about the utility of statins in fibrotic conditions.

Previous studies have shown that Rap1B phosphorylation by PKA suppresses its prenylation [33], emphasizing the importance of tight regulation of post-translational modifications, such as phosphorylation and prenylation. Our findings suggest that similar mechanisms govern LBK1 regulation in maintaining Treg homeostasis. Future studies should investigate how phosphorylation and prenylation affect LKB1's role in Treg stability and other Treg functions. Targeting these specific modifications could present novel avenues for modulating Treg activity in liver fibrosis and other immune-related diseases.

Rezdiffra (Resmetirom), a THR-β agonist for non-alcoholic steatohepatitis, is currently the only US Food and Drug administration (FDA)-approved therapy for liver fibrosis [34,35]. Despite extensive research, many investigational drugs for liver disease have yet to gain approval, indicating the challenges in understanding the underlying mechanisms of liver disease development [36]. The limited research on Rezdiffra's interaction with the immune system highlights a potential gap in its therapeutic approach. Combining Rezdiffra with statin treatment could help addressing this limitation, as statins have immunomodulatory effects that may enhance the efficacy of liver fibrosis treatment.

CRediT authorship contribution statement

An-Na Bae: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **Hajin Lee:** Formal analysis, Investigation. **Huiseong Yang:** Data curation, Methodology. **Sulagna Mukherjee:** Methodology, Software. **Seung-Soon Im:** Methodology. **Jae-Ho Lee:** Methodology, Resources. **Jong Ho Park:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

All of authors in this study declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.151094.

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