

# 1-Methyl-L-tryptophan promotes the apoptosis of hepatic stellate cells arrested by interferon- $\gamma$ by increasing the expression of IFN- $\gamma$ R $\beta$ , IRF-1 and FAS

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**Abstract.** Liver fibrosis, a precursor to cirrhosis, is the result of the deposition of extracellular matrix (ECM) proteins and is mediated primarily by activated hepatic stellate cells (HSCs). In this study, we investigated the anti-fibrotic effects of interferon (IFN)- $\gamma$  in activated HSCs *in vitro* and whether cell viability would be decreased by the inhibition of indoleamine 2,3-dioxygenase (IDO), which is responsible for cell cycle arrest. Following treatment with IFN- $\gamma$ , cell signaling pathways and DNA content were analyzed to assess the inactivation of HSCs or the decrease in HSC proliferation. The IDO inhibitor, 1-methyl-L-tryptophan (1-MT), was used to determine whether IDO plays a key role in the regulation of activated HSCs, as IFN- $\gamma$  increases the expression of IDO. IFN- $\gamma$  significantly inhibited the growth of HSCs and down-regulated the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the HSCs. IDO expression was markedly increased by IFN- $\gamma$  through signal transducer and activator of transcription 1 (STAT1) activation and resulted in the depletion of tryptophan. This depletion induced G1 cell cycle arrest. When the cells were released from IFN- $\gamma$ -mediated G1 cell cycle arrest by treatment with 1-MT, the apoptosis of the HSCs was markedly increased through the induction of IFN- $\gamma$ R $\beta$ , interferon regulatory factor (IRF-1) and FAS. Our results thus suggest that the inhibition of IDO enhances the suppression of activated HSCs, and therefore co-treatment with IFN- $\gamma$  and 1-MT may be applied to ameliorate liver fibrosis.

## Introduction

Hepatic fibrosis is caused by an extensive accumulation of extracellular matrix (ECM) proteins during chronic hepatic injury, which ultimately results in liver cirrhosis. It is a common disease causing high morbidity and mortality worldwide (1-6). Hepatic stellate cells (HSCs), which are important cells for the production of ECM in the liver, are activated and differentiated into proliferative, contractile and ECM-producing myofibroblasts (MFs) by stimulation with platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (7,8). These profibrogenic cytokines are released by injured hepatocytes, inflammatory cells and non-parenchymal cells during liver damage. Therefore, activated HSCs or MFs have been considered as an important target for the inhibition or attenuation of hepatic fibrosis (9).

Interferons (IFNs) are a family of multi-functional cytokines that play important roles in innate and acquired immunity, host defense against viral and bacterial infections, immunomodulation and hematopoietic development, as well as tumor surveillance. Three different types of IFNs have been identified and categorized into 3 families: type I IFNs (IFN- $\alpha$ s, - $\beta$ , - $\epsilon$ , - $\kappa$  and - $\omega$ ), type II IFN (IFN- $\gamma$ ) and type III IFNs (IFN- $\lambda$ s). Of these, IFN- $\gamma$  is a very potent immunomodulatory and anti-fibrotic cytokine and has been used in the treatment of immunodeficiency disorders, chronic inflammatory diseases and tumors in preclinical trials (10,11), and hepatic and pulmonary fibrosis in clinical studies (12,13). As regards its anti-fibrotic effects, IFN- $\gamma$  inhibits the TGF- $\beta$ -mediated production of ECM through the regulation of Smad3, Smad7, YB-1 or p300/CBP (14-16), the inhibition of fibroblast-myofibroblast differentiation (17) and the growth retardation of myofibroblasts (18). IFN- $\gamma$  can increase the expression of the natural killer (NK) cell stimulatory receptor, NKG2D, and the cell death inducing ligand, TRAIL on NK cells (19) and these NK cells can induce the death of activated HSCs, but not quiescent HSCs (19,20). As another mechanism with which inhibit fibrosis, it is important to inhibit the proliferation of activated HSCs and MFs. Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme that catabolizes tryptophan to kynurenine, leading to tryptophan depletion (21) and suppresses the growth of pathogens

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or tumor cells (22), but also restricts T cell proliferation (23). Ubiquitously, IDO expression can be induced by inflammatory mediators, including interleukin (IL)-12, IL-18 (24), bacterial lipopolysaccharide (LPS) (25) and IFNs (26). The induction of IDO-1 is a potent mechanism through which IFN- $\gamma$  inhibits cell proliferation, when the microenvironment is deprived of the essential amino acid, tryptophan.

In this study, we demonstrate that IFN- $\gamma$  induces IDO expression and leads to G1 cell cycle arrest in activated HSCs, immortalized with human telomerase reverse transcriptase (hTERT). Moreover, when the HSCs were released from G1 cell cycle arrest by treatment with the IDO inhibitor, 1-methyl-L-tryptophan (1-MT), cell death was increased through the induction of IFN- $\gamma$ R $\beta$ , interferon regulatory factor (IRF-1) and FAS, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression was markedly decreased in the activated HSCs.

## Materials and methods

**Cell culture.** hTERT-immortalized HSCs were kindly provided by Dr K.S. Lee (Yonsei University, Seoul, Korea). HSCs were maintained in SteCM medium with 2% fetal bovine serum, 1% penicillin-streptomycin and 1% stellate cell growth supplement (SteCGS; ScienCell Research Laboratories, Carlsbad, CA, USA). IFN- $\gamma$  and 1-MT were purchased from R&D Systems (Minneapolis, MN, USA) and Sigma (San Diego, CA, USA), respectively. To examine cell viability, the HSCs were treated with IFN- $\gamma$  (0, 50, 100 or 200 ng/ml) for 48 h, while IFN- $\gamma$  at 100 ng/ml (R&D Systems) or 0.5 mM of 1-MT (Sigma) were used to treat the cells in the other experiments. To measure kynurenine production, the culture supernatant was collected, filtered (0.45  $\mu$ m), and stored at -80°C until use.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** The HSCs were plated at  $1 \times 10^4$  cells/cm<sup>2</sup> in 96-well plates. After 24 h, the HSCs were treated with IFN- $\gamma$  or 1-MT for an additional 48 h and then MTT (Sigma) dissolved in phosphate-buffered saline (PBS) was added to each well (final concentration, 5 mg/ml) and incubated at 37°C for 2 h. MTT formazan was dissolved in 100  $\mu$ l DMSO and incubated for a further 15 min with shaking before the optical density of each well was read at 570 nm using a microplate reader (239241; BioTek Instruments, Winooski, VT, USA).

**Cell cycle analysis.** The cellular DNA content was analyzed using the Cycle Test Plus DNA reagent kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, the HSCs were trypsinized, neutralized with SteCM medium, and centrifuged at 1,800 rpm for 5 min. The cells were washed twice with buffer solution provided with the kit and then solution A, B and C (components of the Cycle Test Plus DNA reagent kit) were sequentially added to the cells in a dark room according to the manufacturer's instructions. The DNA content was analyzed on a flow cytometer (P28200117; BD FACSAria III; BD Biosciences).

**Immunoblotting.** The HSCs were treated with IFN- $\gamma$  or IFN- $\gamma$  and 1-MT in a time-dependent manner. The cells were lysed in 1X Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol and 5%  $\beta$ -mercaptoethanol) and boiled

for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-HCl-buffered saline containing 0.1% Tween-20 (TBST) for 30 min and then incubated with primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-25778), IDO-1/2 (sc-87164/sc-25809) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA),  $\alpha$ -SMA (ab7817; Abcam, Cambridge, MA, USA), signal transducer and activator of transcription 1 (STAT1; 9176), p-STAT1 (T701/S727; 9167/9177) (both from Cell Signaling Technology, Danvers, MA, USA), IFN- $\gamma$ R $\beta$ 1 (sc-970), IRF-1 (sc-497) and FAS (sc-8009) (all from Santa Cruz Biotechnology, Inc.). Bound primary antibodies were detected with appropriate HRP-conjugated secondary antibodies (sc-2030 and sc-2031; Santa Cruz Biotechnology, Inc.), treated with west Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA), and visualized using a BioSpectrum imaging system (UVP, Upland, CA, USA).

**Measurement of kynurenine production.** Kynurenine production in the culture supernatant was analyzed following treatment with IFN- $\gamma$  with or without 1-MT for 48 h. A total of 100  $\mu$ l of culture supernatant was mixed with an equal volume of 30% trichloroacetic acid (TCA; Sigma) and incubated for 30 min at 50°C oven. After the proteins were precipitated at 3,000  $\times$  g for 10 min, 100  $\mu$ l of supernatants were recovered to a new tube. An equal volume of Ehrlich's reagent (2% p-dimethylaminobenzaldehyde in glacial acetic acid) was added and followed by incubation 10 min at room temperature. Kynurenine products were determined at 490 nm on a microplate reader (239241; BioTek Instruments).

**Annexin V/7-AAD staining.** The PE-Annexin V apoptosis detection kit I (BD Biosciences) was used according to the manufacturer's instructions. The cells were harvested and washed twice with cold PBS and then re-suspended in 1X binding buffer. The cells were stained with PE-Annexin V and 7-AAD for 15 min at room temperature in the dark. The cells were analyzed without washing on a flow cytometer (P28200117; BD FACSAria III; BD Biosciences) within 1 h.

**Statistics.** Data were analyzed by using PASW 18.0 software (SPSS Inc., Chicago, IL, USA) and expressed as the means  $\pm$  standard deviation. Differences between groups were assessed by one-way analysis of variance (one-way ANOVA) with Tukey's test against the control sample. Significance was defined as a P-value of <0.05.

## Results

**Viability of activated HSCs treated with IFN- $\gamma$ .** Type I and II IFNs appear to have anti-fibrogenic properties resulting from inhibiting the activation and proliferation of HSCs (27-29). However, little is known about the mechanisms of IFN- $\gamma$ -induced cell cycle arrest, which can mediate the anti-fibrogenic effects of IFN- $\gamma$  in HSCs. In this study, to understand these mechanisms, first of all, we examined whether IFN- $\gamma$  can regulate the proliferation of activated HSCs through cell cycle

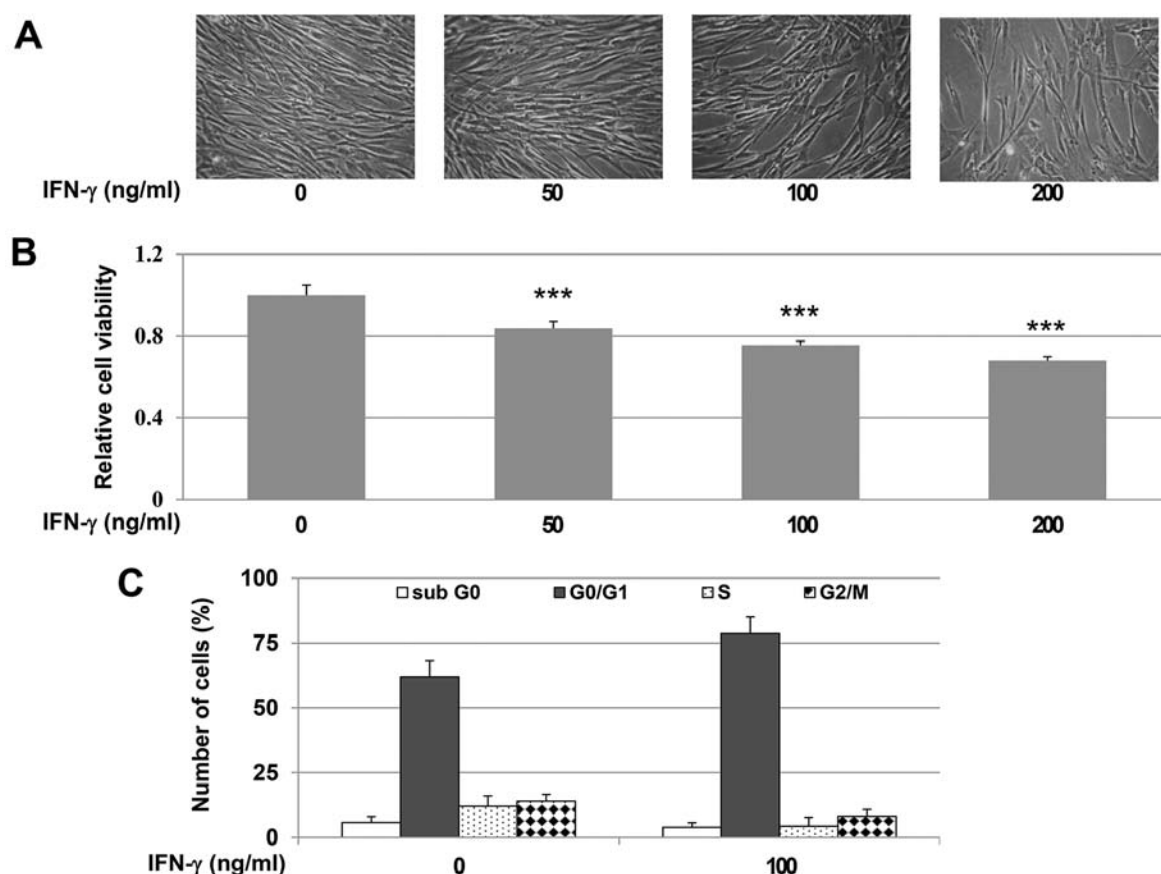


Figure 1. Suppression of viability of hepatic stellate cells (HSCs) by interferon- $\gamma$  (IFN- $\gamma$ ). (A) Growth inhibitory effects of IFN- $\gamma$  on HSCs. HSCs were treated with IFN- $\gamma$  (0, 50, 100 or 200 ng/ml) for 48 h and cell viability was then examined by light microscopy. (B) MTT assay of HSCs treated with IFN- $\gamma$  for 48 h. Error bars represent the means  $\pm$  SD of triplicate wells, Data are from one of 3 independent experiments. \*\*\* $P$ <0.001. (C) Cell cycle analysis of HSCs treated with IFN- $\gamma$  for 24 h. Cellular DNA content was analyzed by flow cytometry.

arrest. Following treatment with IFN- $\gamma$  for 2 days, the numbers of HSCs decreased in a dose-dependent manner, while dead cells were not observed under the microscope (Fig. 1A). When cell viability was assessed by MTT assay, 200 ng/ml of IFN- $\gamma$  was found to decrease the viability of the HSCs by approximately 30% after 2 days (Fig. 1B). To determine whether the decrease in cell viability following treatment with IFN- $\gamma$  is a result of cell cycle arrest, the cellular DNA content was analyzed using a flow cytometer. After 24 h, it was found that IFN- $\gamma$  decreased the S and G2/M cell population, but increased the G0/G1 cell population from 62.0 to 78.6%. However, the Sub-G0 cell population was not increased by IFN- $\gamma$  (Fig. 1C). These data suggest that IFN- $\gamma$  decreases the viability of HSCs by inducing cell cycle arrest at the G0/G1 phase, but not by inducing apoptosis.

*IDO expression is increased by IFN- $\gamma$  in activated HSCs.* It is well known that IFN- $\gamma$  is a potent inducer of IDO and inhibits the growth of pathogens, immune cells and mesenchymal stem cells through the induction of essential amino acid tryptophan depletion under the microenvironment (30,31). In this study, we investigated whether the decrease in cell growth induced by IFN- $\gamma$  results from an increase in IDO expression in activated HSCs. As shown in Fig. 2, the protein expression of IDO-1 and IDO-2 was markedly increased following treatment with IFN- $\gamma$  for over 24 h. Moreover, STAT1 expression was also increased

from 12 h and STAT1 phosphorylation (T701/S727) was also increased by IFN- $\gamma$ . However, the levels of apoptosis-related genes, such as IFN- $\gamma$ R $\beta$ 1 and IRF-1 were not altered, although FAS expression increased slightly. The expression of  $\alpha$ -SMA gradually decreased in a time-dependent manner by IFN- $\gamma$  (Fig. 2A). When the band intensity was analyzed by densitometry at 48 h,  $\alpha$ -SMA expression decreased by approximately 42%, whereas the expression of STAT1, IDO-1 and IDO-2 increased by approximately 25-, 105- and 133-fold, respectively (Fig. 2B). These data suggest that IFN- $\gamma$  induces G0/G1 cell cycle arrest by inducing IDO-1 and IDO-2 expression in activated HSCs.

*Cell cycle arrest is induced by IFN- $\gamma$  in activated HSCs through tryptophan depletion.* Subsequently, in order to determine whether the increase in the expression of IDO induced by IFN- $\gamma$  is responsible for the cell cycle arrest of HSCs, we examined the cellular DNA content following treatment with the IDO inhibitor, 1-MT in the IFN- $\gamma$ -treated HSCs. Following co-treatment with IFN- $\gamma$  and 1-MT for 24 h, the number of cells in the S and G2/M phase increased; however, the G0/G1 cell population decreased from 78.6 to 64.2% compared to the IFN- $\gamma$ -treated groups. Moreover, the cell cycle profile was changed similarly to that of the control group (Fig. 3A). In addition, we found that IFN- $\gamma$  led to the production of approximately 10 nmoles of kynurenine, whereas co-treatment with 1-MT suppressed the amount of

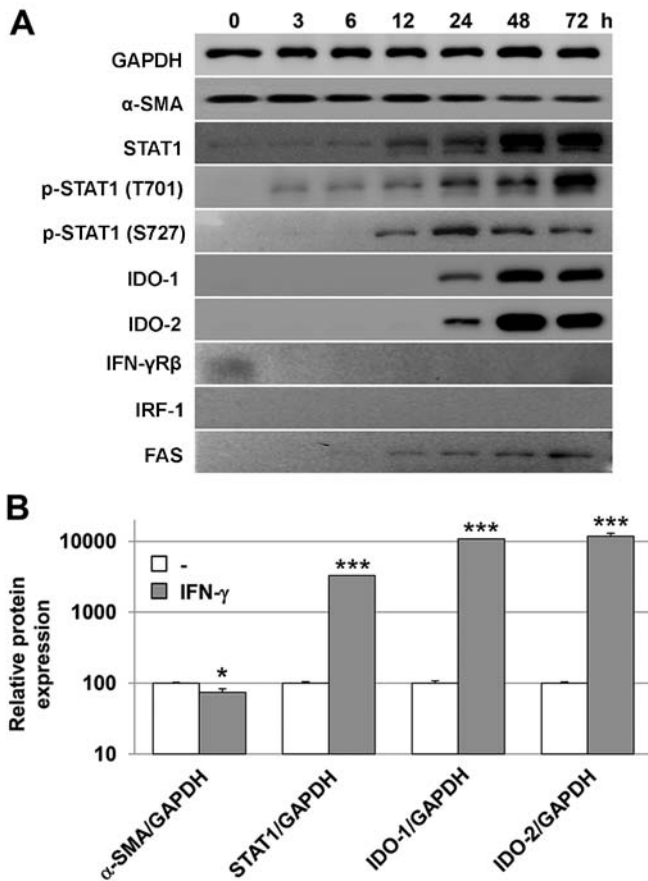


Figure 2. Expression of STAT1, indoleamine 2,3-dioxygenase (IDO)-1 and IDO-2 by interferon- $\gamma$  (IFN- $\gamma$ ) in activated hepatic stellate cells (HSCs). (A) HSCs were treated with IFN- $\gamma$  (100 ng/ml) for the indicated periods of time and the expression of proteins related to apoptosis (i.e., IFN- $\gamma$ R $\beta$ 1, IRF-1 and FAS) or cell cycle arrest (i.e., IDO-1 and IDO-2) was then evaluated by immunoblotting. Additionally, (B) STAT1 expression and phosphorylation and  $\alpha$ -SMA expression was also examined. Relative protein expression following treatment for 48 h was analyzed by densitometry to compare the expression levels of STAT1, IDO-1 and IDO-2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Data are expressed as the means  $\pm$  SDs of triplicate experiments. \* $P$ <0.05 and \*\*\* $P$ <0.001. The y axis is the logarithmic scale.

kynurenine produced by IFN- $\gamma$  by half (Fig. 3B). These data suggested that IFN- $\gamma$  induced the production of kynurenine and then induced tryptophan depletion. This depletion induced G0/G1 cell cycle arrest of the HSCs. However, treatment with 1-MT led to the release of HSCs from the G0/G1 cell cycle arrest through the attenuation of tryptophan depletion.

*The death of activated HSCs is induced by co-treatment with IFN- $\gamma$  and 1-MT in through the increase in the expression of IFN- $\gamma$ R $\beta$ 1, IRF-1 and FAS.* It should be noted that when the HSCs were treated with both IFN- $\gamma$  and 1-MT for 48 h, cell viability was markedly decreased (Fig. 4A and B). The number of viable HSCs treated with IFN- $\gamma$  and 1-MT for 48 h decreased by about half that of the HSCs treated with only IFN- $\gamma$  (Fig. 4B). We also examined the apoptosis of the HSCs. As shown by PE-Annexin V and 7-AAD staining and flow cytometry, treatment with both IFN- $\gamma$  and 1-MT for 48 h increased the apoptosis of the HSCs (Fig. 4C). Thus, we also examined the expression of apoptosis-related genes, such as

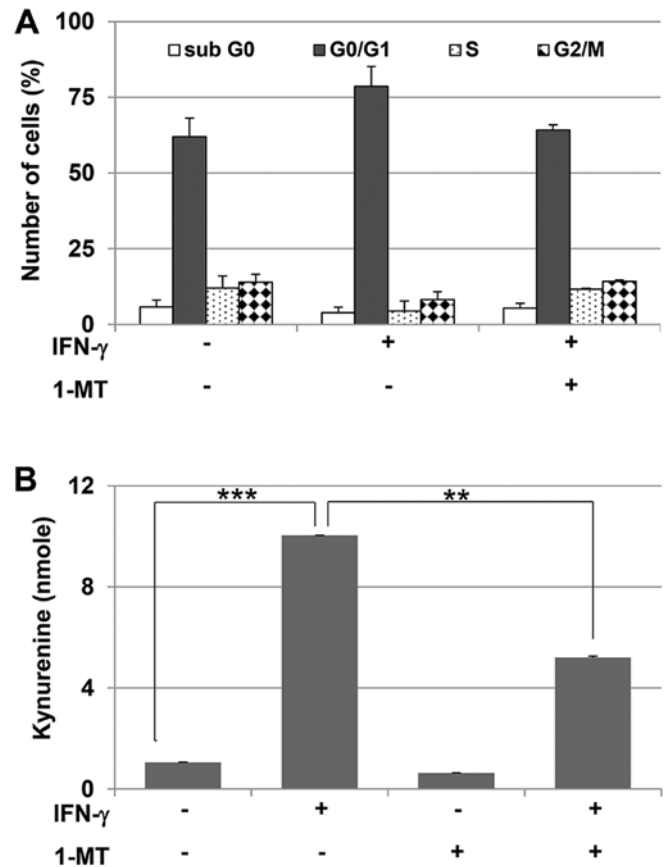


Figure 3. Hepatic stellate cells (HSCs) are released from G0/G1 cell cycle arrest induced by interferon- $\gamma$  (IFN- $\gamma$ ) by treatment with the IDO inhibitor, 1-methyl-L-tryptophan (1-MT). (A) Cell cycle analysis of HSCs treated with IFN- $\gamma$  or IFN- $\gamma$  + 1-MT for 24 h. Cellular DNA content was analyzed by flow cytometry. (B) Kynurenine production in HSCs treated with IFN- $\gamma$  or IFN- $\gamma$  + 1-MT for 48 h. Data are expressed as the means  $\pm$  SD of triplicate experiments. \*\* $P$ <0.01 and \*\*\* $P$ <0.001.

IFN- $\gamma$ R $\beta$ 1, IRF-1 and FAS in the HSCs treated with IFN- $\gamma$  and 1-MT. We found that the expression of IFN- $\gamma$ R $\beta$ 1, IRF-1 and FAS was markedly increased in a time-dependent manner by IFN- $\gamma$  and 1-MT. By contrast,  $\alpha$ -SMA expression was decreased more efficiently by IFN- $\gamma$  and 1-MT compared to the HSCs treated with IFN- $\gamma$  only (Fig. 5). These data suggested that when the HSCs were released from G0/G1 cell cycle arrest, the apoptosis-inducing ability of IFN- $\gamma$  was enhanced in the activated HSCs. Furthermore, this apoptosis may augment the anti-fibrotic effects in HSCs.

## Discussion

The present study evaluated the growth inhibitory and anti-fibrogenic effects of IFN- $\gamma$  in activated HSCs. We found that IFN- $\gamma$  significantly inhibited the growth of HSCs through the expression of IDO, which induced tryptophan depletion. These growth inhibitory effects were mediated by G0/G1 cell cycle arrest, and subsequently  $\alpha$ -SMA expression was down-regulated in the HSCs. Moreover, the cells were released from IFN- $\gamma$ -induced G0/G1 cell cycle arrest following co-treatment with the IDO inhibitor, 1-MT; apoptosis was then induced in these cells which were released from G0/G1 phase arrest by 1-MT, which led to the upregulation of IFN- $\gamma$ R $\beta$ 1, IRF-1 and

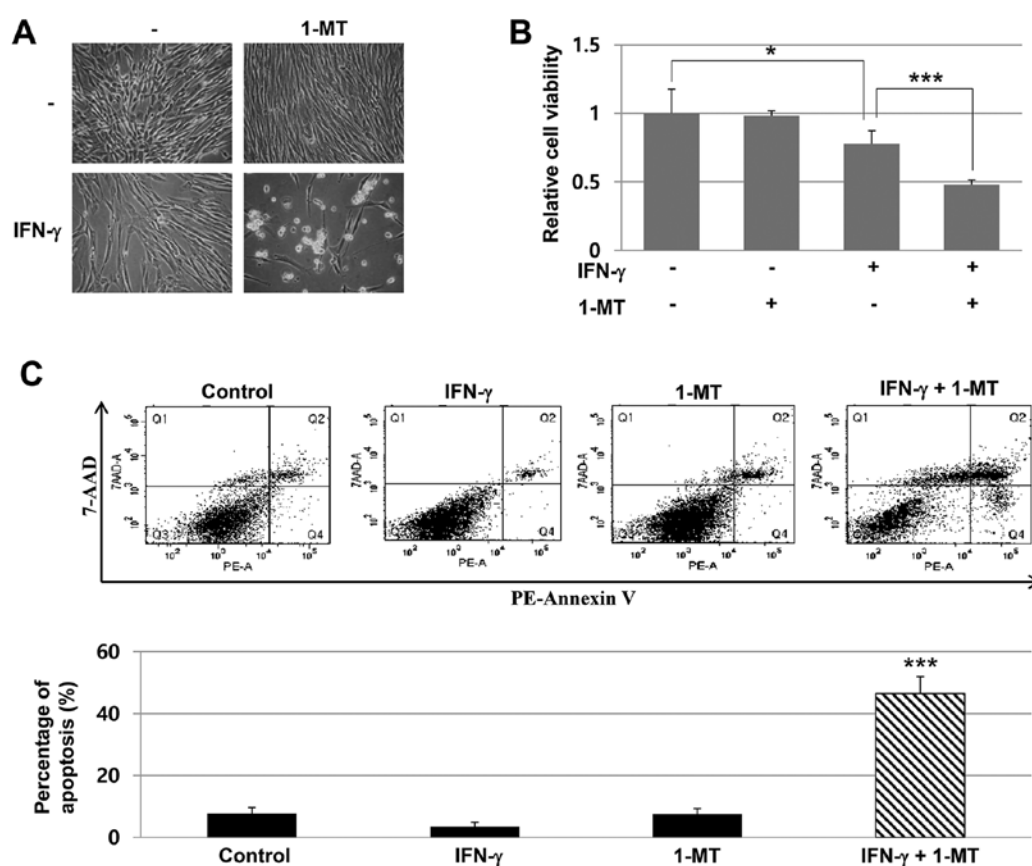


Figure 4. Induction of cell death by interferon- $\gamma$  (IFN- $\gamma$ ) and 1-methyl-L-tryptophan (1-MT) in hepatic stellate cells (HSCs). (A and B) Viability of HSCs following treatment with IFN- $\gamma$  and 1-MT for 48 h. Cell viability was observed by (A) light microscopy and (B) quantified by MTT assay. Error bars represent the means  $\pm$  SD of triplicate wells; data are from 1 of 3 independent experiments. \* $P$ <0.05 and \*\*\* $P$ <0.001. (C) Cell death of HSCs induced by IFN- $\gamma$  and 1-MT. Cell death mode was analyzed by PE-Annexin V and 7-AAD staining and then cell death population was quantified by flow cytometry. Quadrants (Q)1, Q2, Q3 and Q4 represent necrotic (PE-Annexin V/7-AAD, -/+), late apoptotic (PE-Annexin V/7-AAD, +/+), live (PE-Annexin V/7-AAD, -/-), and early apoptotic cells (Annexin V-PE/7-AAD, +/-), respectively.

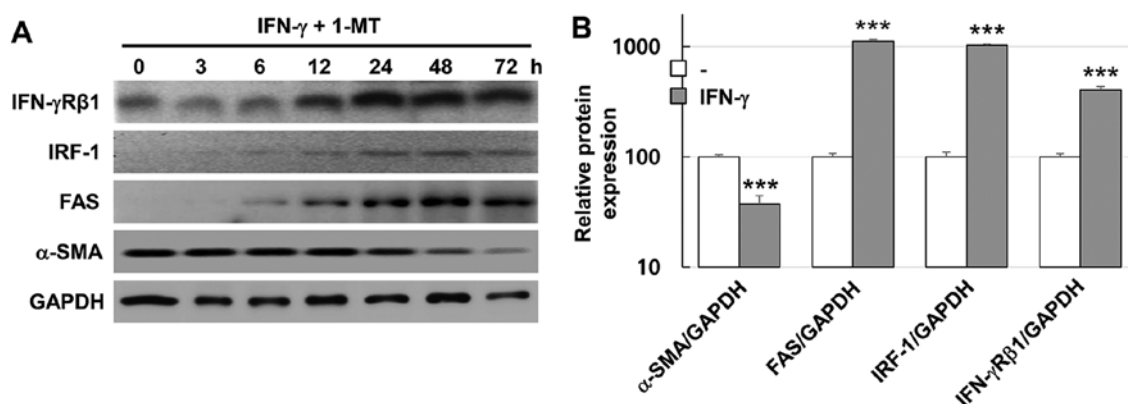


Figure 5. Expression of FAS, interferon regulatory factor (IRF-1) and interferon- $\gamma$  (IFN- $\gamma$ )R $\beta$ 1 by IFN- $\gamma$  and 1-methyl-L-tryptophan (1-MT) in hepatic stellate cells (HSCs). (A) HSCs were treated with IFN- $\gamma$  (100 ng/ml) and 1-MT (0.5 mM) for the indicated periods of time and the expression of proteins related to apoptosis (i.e., FAS, IRF-1 and IFN- $\gamma$ R $\beta$ 1) was then evaluated by immunoblotting. Additionally,  $\alpha$ -SMA was also examined. (B) Relative protein expression following treatment for 48 h was analyzed by densitometry to compare the expression levels of FAS, IRF-1 and IFN- $\gamma$ R $\beta$ 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are expressed as the means  $\pm$  SD of triplicate experiments. \*\*\* $P$ <0.001.

FAS, which induce cell death. Furthermore, co-treatment with IFN- $\gamma$  and 1-MT decreased  $\alpha$ -SMA expression more efficiently compared to treatment with IFN- $\gamma$  alone.

The anti-fibrotic effects of IFN- $\gamma$  are well-established as mentioned above. Therefore, IFN- $\gamma$  has been used to attenuate

hepatic fibrosis (13,32,33). In a randomized controlled study on hepatic fibrosis, IFN- $\gamma$  treatment for 9 months improved the fibrosis scores in patients with chronic hepatic fibrosis. However, side-effects, such as fever, headache, and muscular, skeletal and limb pain, as well as nausea and decreased white

blood cell and platelet counts were observed during IFN- $\gamma$  treatment intramuscularly (13). Another limitation of IFN- $\gamma$  is the short half-life period due to rapid renal clearance, which reduces its biological function (34). Furthermore, IFN- $\gamma$  can affect hepatocytes through the induction of apoptosis or cell cycle arrest (35). Therefore, in order to overcome these detrimental effects, it is required that IFN- $\gamma$  has to act on HSCs specifically, and the half-life of IFN- $\gamma$  must be increased, and the anti-fibrotic functions must be increased to reduce the treatment period and costs. To increase the specificity to HSCs and the half-life of IFN- $\gamma$ , therapies based on nanotechnologies for the effective and targeted delivery of IFN- $\gamma$  to HSCs have been conducted as an innovative and promising alternative to conventional therapy (36,37). Liposomes containing IFN- $\gamma$  and cyclic peptides (pPB) with a specific affinity for platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ) which is expressed in HSCs can be used to increase the specificity to HSCs efficiently (36,37). These nanotechnologies can improve the half-life and selective delivery of IFN- $\gamma$  to activated HSCs and can enhance the anti-fibrotic effects of IFN- $\gamma$  and reduce its side-effects.

In this study, we demonstrated that the anti-fibrotic effects of IFN- $\gamma$  were enhanced through the induction of cell death in activated HSCs by treatment with 1-MT. We consider that the induction of the apoptosis of HSCs by IFN- $\gamma$  and 1-MT can also reduce the treatment period and costs dramatically during clinical trials. Taken together, our data suggest that combination treatment with nanotechnologies using IFN- $\gamma$  and 1-MT may be used to increase the HSC-targeted anti-fibrotic effects and reduce the detrimental effects, including side-effects, the long treatment period and expensive treatment costs. However, more detailed studies using the combination of nanotechnologies and strategies inducing the death of HSCs are warranted in order to determine the optimal anti-fibrotic effects of IFN- $\gamma$ .

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