European Journal of Pharmacology 682 (2012) 161–170

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Atorvastatin synergizes with IFN-γ in treating human non-small cell lung carcinomas *via* potent inhibition of RhoA activity

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ARTICLE INFO

Article history:
Received 21 September 2011
Received in revised form 30 January 2012
Accepted 9 February 2012
Available online 21 February 2012

Keywords:
IFN-γ
Non-small-cell-lung carcinomas
NSCLCs
RhoA
Atorvastatin

ABSTRACT

Interferon-γ (IFN-γ) has been widely used to treat various malignant tumors including human non-small-cell-lung carcinomas (NSCLCs). However, the tumor-inhibitory effect of IFN-γ displays not satisfactory in NSCLC treatment due to the lack of immunogenicity of NSCLCs. This study demonstrated that inhibition of RhoA activity led to significant inhibition of NSCLC cell growth accompanied by decreased expression of c-myc and cyclin D1 and increased levels of major histocompatibility complex (MHC) class I and peptide transporter protein 1 (TAP1) which are involved in tumor immunity. Combination treatment of atorvastatin and IFN-γ resulted in a synergistic inhibition of NSCLC cell growth both *in vitro* and *in vivo*. Though IFN-γ alone exerted minimal inhibitory effect on RhoA activity, additional administration of atorvastatin could result in a significant inhibition of RhoA activity, thus substantially suppressing NSCLC cell growth. Specifically, atorvastatin could induce specific deposition of endogenous IFN-γ in tumors while not in other normal tissues in LLC-harbored mice. In conclusion, atorvastatin can enhance IFN-γ sensitivity in NSCLCs both *in vitro* and *in vivo*, probably through induction of a synergistic inhibitory effect on RhoA activity. This study also suggests a potential alternative of combination of atorvastatin and IFN-γ in clinical therapy against NSCLCs.

1. Introduction

Non-small-cell-lung carcinomas (NSCLCs) are the leading cause of cancer deaths throughout the world. However, there are limited strategies to treat NSCLCs due to the high frequency of recurrence and the insensitivity of NSCLCs to most chemotherapeutics (Greco and Hainsworth, 1992).

NSCLCs are nonimmunogenic tumors that suppress immune priming. Due to the absence of priming, NSCLC cells are not subjected to immune attack (Ferrone and Marincola, 1995). The most widely accepted tumor escape mechanism is the loss or the down-regulation of the expression of tumor-derived major histocompatibility complex (MHC) class I (Ferrone and Marincola, 1995; Garcia-Lora et al., 2003). Such deficiency in the expression of MHC class I (HLA-A) has been confirmed in various murine and human tumors including human NSCLCs and is intimately correlated with tumor progression (Seliger et al., 1996). Besides, deficiency in the expression of peptide transporter protein 1 (TAP 1) by tumors has also been demonstrated to result in evasion of immune surveillance and increased tumorigenicity (Johnsen et al., 1999; Seliger et al., 1997). Thus, enhancement of the expression of MHC class I and TAP 1 would be of great interest for immunotherapeutic strategies. Interferon-γ (IFN-γ), the critical target of immunologic therapy, has been shown to increase the expression of MHC class I (Wroblewski et al., 2001) and TAP 1 (Tilkin-Mariame et al., 2005) in tumor cells, and effectively treat ovarian cancer, melanoma, renal cell carcinoma and prostate cancer (Gleave et al., 1998; Hildenbrand et al., 2007; Kirkwood et al., 1990; Pujade-Lauraine et al., 1996). In addition to its immunologic function, IFN-γ has also been shown to have anti-proliferative effects against cancer cells (Chin et al., 1996; Hobeika et al., 1999). However, the IFN-γ-mediated monotherapy is inactive in clinical practices against NSCLCs (Mattson et al., 1991).

Statins, previously known to effectively treat high cholesterol (Baigent et al., 2005), can also inhibit proliferation and induce apoptosis in various lines of tumor cells (Hoque et al., 2008; Kotamraju et al., 2007) as well as non transformed cells in lung tissue (Ghavami et al., 2011). Specifically, many studies have confirmed that statins can stimulate the IFN-γ-secreting function of CD8+ T cell and NK cell, which suggests that statins may be used as an immuno-regulatory agent to treat malignant tumors. Moreover, statins were also shown to potentiate the anti-tumor

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doi:10.1016/j.ejphar.2012.02.015

effects of some cytokines (Jakobisiak and Golab, 2003) and especially, atorvastatin was demonstrated to enhance IFN-γ-induced MHC class I expression in melanoma cells (Tilkin-Mariame et al., 2005). Nevertheless, whether atorvastatin could synergize with IFN-γ in treating NSCLCs remains unexplored.

In this study, we explored the influence of atorvastatin on the IFN-γ-induced inhibitory effect on the growth of NSCLC cells *in vitro* and *in vivo*. Our results demonstrated that atorvastatin exerted the synergistic effect on IFN-γ-induced inhibition of NSCLCs probably through inhibition of RhoA activity. Furthermore, atorvastatin could induce specific deposition of endogenous IFN-γ in tumors, which may partially contribute to its synergistic effect on IFN-γ-mediated inhibition of LCC tumors *in vivo*.

2. Materials and methods

2.1. Cell lines and cell culture

Lewis lung carcinoma (LLC)-mouse lung cancer cell line and H23, H358 and H596 NSCLC cell lines were obtained from American Tissue Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin 100 (U/ml)/streptomycin (100 μg/ml). All cell culture reagents were obtained from Invitrogen. Cell number was determined using hemocytometry.

2.2. Cell proliferation/viability

Proliferation/viability of cells was determined using MTT method as previously reported with a few modifications (Kim et al., 1993). 5×10^4 cells (H23, H358 or H596) in 100 μl of serum-free DMEM were seeded in 96-well plates and incubated overnight for cell attachment. Cells were treated with different doses of atorvastatin or/and recombinant human IFN-γ (Peprotech, Cat#AF-300-02) for 3 days before analysis. Then MTT was added to each well (with a final concentration of 0.5 mg/ml), and the plates were incubated for 4 h at 37 °C. The plates were centrifuged at 450×g for 5 min. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in dimethyl sulfoxide (150 μl/well) quantified spectroscopically at 570 nm.

2.3. Hoechst 33258 staining

Hoechst 33258 staining was performed as described previously with a few modifications (Hambrock et al., 2006). Briefly, cells were treated with different doses of atorvastatin and/or recombinant human IFN-γ for 3 days before analysis. Then confluent cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed once with PBS. Fixed cells were stained with Hoechst 33258 of 50 ng/ml and incubated for 30 min at room temperature and washed with PBS. Apoptotic cells were identified by condensation and fragmentation of nuclei examined by an Olympus IX71 fluorescence microscope. The apoptotic rate of cell population was calculated as the ratio of apoptotic cells to total cells counted ×100. A minimum of 500 cells were counted for each treatment.

2.4. SiRNA transfection

Gene silencing by RNA interference (siRNA) was used to down-regulate RhoA expression in H23, H358 and H596 cells. The siRNA (Cat#sc-29471) specific for RhoA and nonsilencing siRNA (Cat#sc-37007) were obtained from Santa Cruz. Transfection of 7×10^5 of above cells with 0.1 μM of siRNA was done in a 66-mm Petri dish (Corning, USA) using 15 μl Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After overnight incubation, cells were cultured for an additional 48 h. Expression of RhoA was determined by Western blotting.

2.5. Western blotting

Western blotting protocols were as in a previous report (Liu et al.). Briefly, cell lysates were separated by SDS/PAGE in 10% Tris-glycine gels (Invitrogen) and transferred to a nitrocellulose membrane. To determine expression levels of RhoA, the monoclonal anti-RhoA antibody (diluted with 5% BSA to 1:2000, Santa Cruz Biotechnology, Cat #sc-418) was used. For Western blot analysis of c-myc and cyclin D1, blots were probed with specific antibodies to c-myc (clone 9E11, Upstate Biotechnology) and cyclin D1 (BD Pharmingen), respectively, which was diluted with 5% BSA to 1:1000. Membranes were probed with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody (Cell Signaling, diluted with 5% BSA to 1:1000). Antibody binding was detected by enhanced chemiluminescence detection kit (ECL) (UK Amersham International plc). All Western blot exposures were in the linear range of detection.

2.6. RhoA activity assay

Cellular membrane extracts were obtained according to a previous study (Sterpetti et al., 1999). Briefly, lysates (25 μg) were incubated in Rho-GTP affinity plate (Cytoskeleton, Cat#BK121) for 45 min, and then were incubated with antibody detection reagent for 2 h. Then the change of RhoA activity was observed as luminescence signal was measured by a microplate reader.

2.7. Real-time RT PCR

For RNA isolation and real-time RT PCR analysis of MHC I related and TAP 1, RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen). cDNA was synthesized using iScript reverse transcriptase reagent from 2 μg of total RNA. RT PCR analysis was performed on an Mx3000P QPCR system (stratagene) using Sybr-green and Δσ Ct method was used to normalize transcripts to that of GAPDH and to calculate fold induction relative to control group. Primer sequences for real-time RT PCR analysis of TAP 1, MHC class I were: TAP 1, 5′-ACTGCTACTTCTCGCCGACT-3′ and 5′-CTGCGTTTTCGCTCTTGGAG-3′; MHC class I, 5′-ACCCTCGTC-CTGCTACTCTC-3′ and 5′-CTGTCTCCTCGTCCCAATACT-3′.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Lysates from LLC tumor and non-tumor specimen were obtained by mechanical homogenization in cell lysis buffer (CST, Cat#9803). Concentration of mouse IFN-γ was quantified using Quantikine mouse IFN-γ immunoassays (R&D Systems). Concentrations were expressed as ng/ml.

2.9. Xenograft studies

H358 or H596 cells (approximately 1×10^7, respectively) were subcutaneously inoculated into the right flank of 6-week-old female NU/NU Nude mice. Treatments were initiated when tumors reached 80–100 mm^3. The drug efficacy study was performed with two categories: low dose combination, including atorvastatin (1 mg/kg per day, by gavage) and/or IFN-γ (5×10^3 IU/per mouse, by i.p.), and high dose combination, including atorvastatin (10 mg/kg per day, by gavage) or/and IFN-γ (10^4 IU/per mouse, by i.p.) (n = 10 per group). Animals were randomized to receive above treatments. Tumor sizes were calculated with the formula: $(mm^3)=(L\times W^2)\times0.5$. The tumor growth-inhibitory effects of each agent alone or their combination were examined for about 3 weeks. Six-week-old female wild-type C57BL/6J mice were inoculated s.c. in the right flank with approximately 1×10^7 Lewis lung cancer (LLC) cells. For analysis of the effect of atorvastatin

on endogenous IFN-γ deposition, from the day of LLC cells implantation, animals were randomized for treatment with either saline or atorvastatin (Liptor, Pfizer) every day by gavage (n = 10 per group). Animal handling and procedures were approved by the Peking University Health Science Center Institutional Animal Care and Use Committee.

2.10. Statistical analysis

All statistical analysis was evaluated by Student's test for simple comparisons between two groups and one-way ANOVA for comparisons among multiple groups using JMP7.0 software (SAS Institute Inc, Cary, US). All data are expressed as mean ± S.D. (Junttila et al., 2009).

3. Results

3.1. The synergistic effect of atorvastatin on IFN-γ-induced inhibition of NSCLC cell growth in H358 cells

Before demonstrating the feasibility of our combination, we firstly evaluated the tumor-inhibitory effect of IFN-γ or atorvastatin treatment alone in NSCLC cells. Fig. 1A and B showed that after 3-day treatment, IFN-γ or atorvastatin dose-dependently inhibited the growth of H358 cells (an adenocarcinoma cell line), yet the inhibitory effect was not significant at the low doses of IFN-γ (10 ng/ml) or atorvastatin (0.1 μM), respectively. Additional administration of atorvastatin to IFN-γ-treated NSCLC cells led to a significant reduction in cell growth as compared with atorvastatin or IFN-γ treatment alone (Fig. 1C). Next, we determined the effect of atorvastatin and/or IFN-γ alone or their combination on the apoptosis of H358 cells by Hoechst staining. As shown in Fig. 1D, the enhancement effect on cell apoptosis was not significant at the low doses of IFN-γ (10 ng/ml) or atorvastatin (0.1 μM), respectively. Combination treatment of atorvastatin and IFN-γ in NSCLC cells led to a significant increase in cell apoptosis as compared with atorvastatin or IFN-γ treatment alone. Thus, these results suggest that atorvastatin can synergize with IFN-γ to inhibit NSCLC cell growth.

3.2. Involvement of inhibition of Rho protein activity in synergistic inhibitory effect of atorvastatin and IFN-γ on NSCLC cell growth

To explore the mechanism for the synergistic effect of atorvastatin and IFN-γ on inhibition of NSCLC cell growth, we determined whether

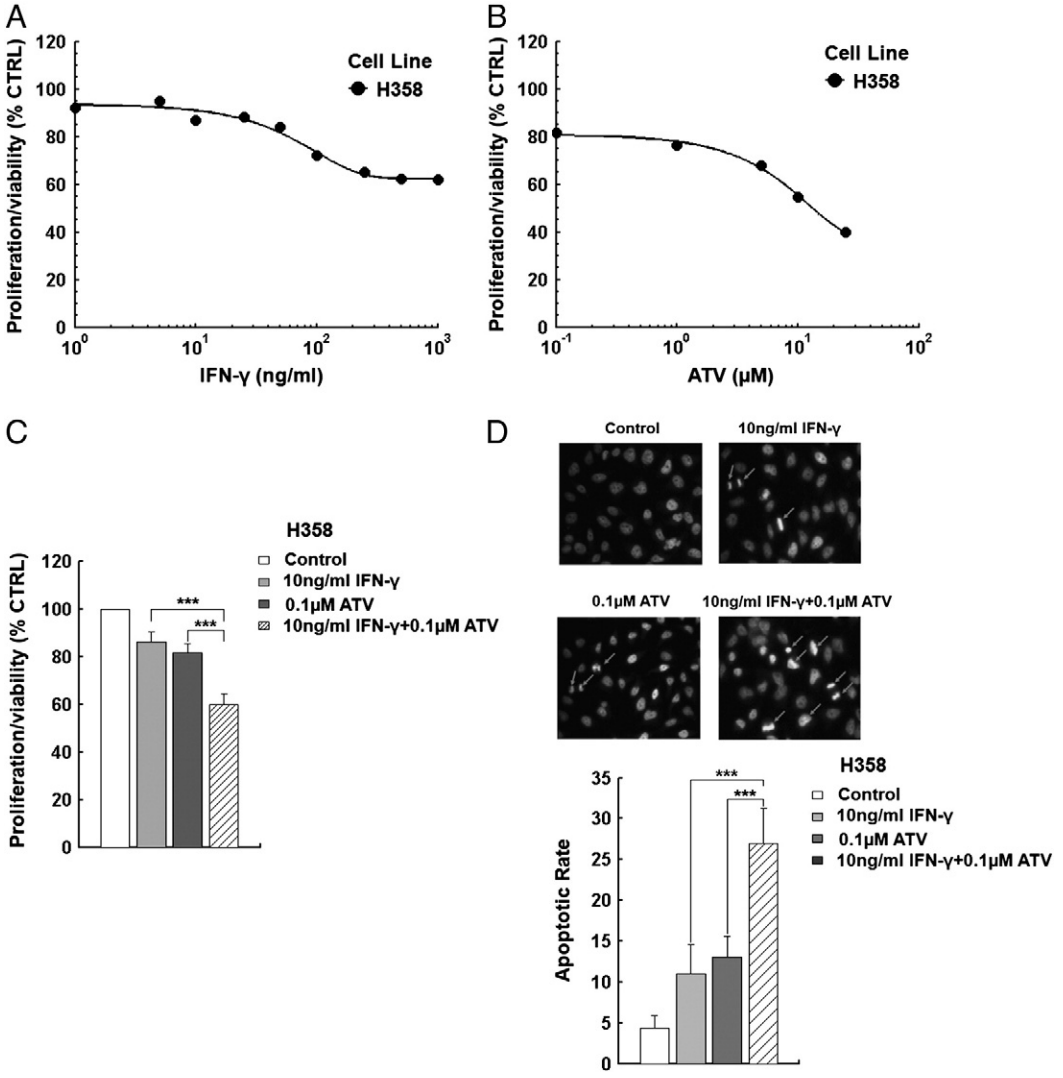

Fig. 1. Synergistic effect of atorvastatin (ATV) and IFN-γ on inhibition of NSCLC cell growth. The effect of different treatments on cell growth was determined by MTT after 72-h incubation and the data of treatment group was expressed by % control. (A) The dose-dependent inhibitory effect of IFN-γ on H358 cell growth. (B) The effect of atorvastatin on H358 cell growth at various concentrations. (C) The effect of atorvastatin at relative low doses (0.1 μM) or low dose of IFN-γ (10 ng/ml), or their combination on H358 cell growth. Bars are mean ± S.D. from six-seven independent experiments. ***P<0.001. (D) The effect of atorvastatin at relative low doses (0.1 μM) or low dose of IFN-γ (10 ng/ml), or their combination on H358 cell apoptosis. Bars are mean ± S.D. from five independent experiments. ***P<0.001.

RhoA proteins are involved in this synergistic effect, as RhoA proteins were recently shown to be one of the targets for IFN-γ-mediated anti-cancer therapy against melanoma (Tilkin-Mariame et al., 2005).

RhoA is over-expressed and activated during tumorigenesis in multiple human tumors (Fritz et al., 1999). We firstly evaluated the role of RhoA in regulation of NSCLC cell growth. Fig. 2A showed that siRNA to RhoA at the concentrations of 0.1 μM decreased the expression of RhoA to the level hardly detected. The nonsilencing siRNA had no impact on RhoA expression. Fig. 2B indicated that knockdown of RhoA expression with RhoA siRNA resulted in a significant inhibition of NSCLC cell growth and the nonsilencing siRNA had no impact on cell growth after 3-day incubation. This result demonstrates the RhoA protein-dependent NSCLC cell growth. As shown in Fig. 2C, one-hour pretreatment of NSCLC cells with C3 transferase (1 g/ml), a specific inhibitor of RhoA activity (Whitehead et al., 2009), significantly reduced the RhoA activity by approximately 80% in NSCLC cells as compared with control and inhibited NSCLC cell growth, which was similar to the effect of specific siRNA to RhoA (Fig. 2D). As c-myc and cyclin D1 were most recently confirmed to be intimately related to tumor progression (Zhou et al.), we next determined the effect of inhibition of RhoA activity on the expression of these pro-tumor proteins. As Fig. 2E showed, inhibition of RhoA activity led to a substantial decrease in the expression of all these pro-tumor proteins in NSCLC cells. These results suggest that inhibition of RhoA activity may contribute to suppression of the progression of NSCLC cells, and RhoA-dependent NSCLC cell growth inhibition is probably related

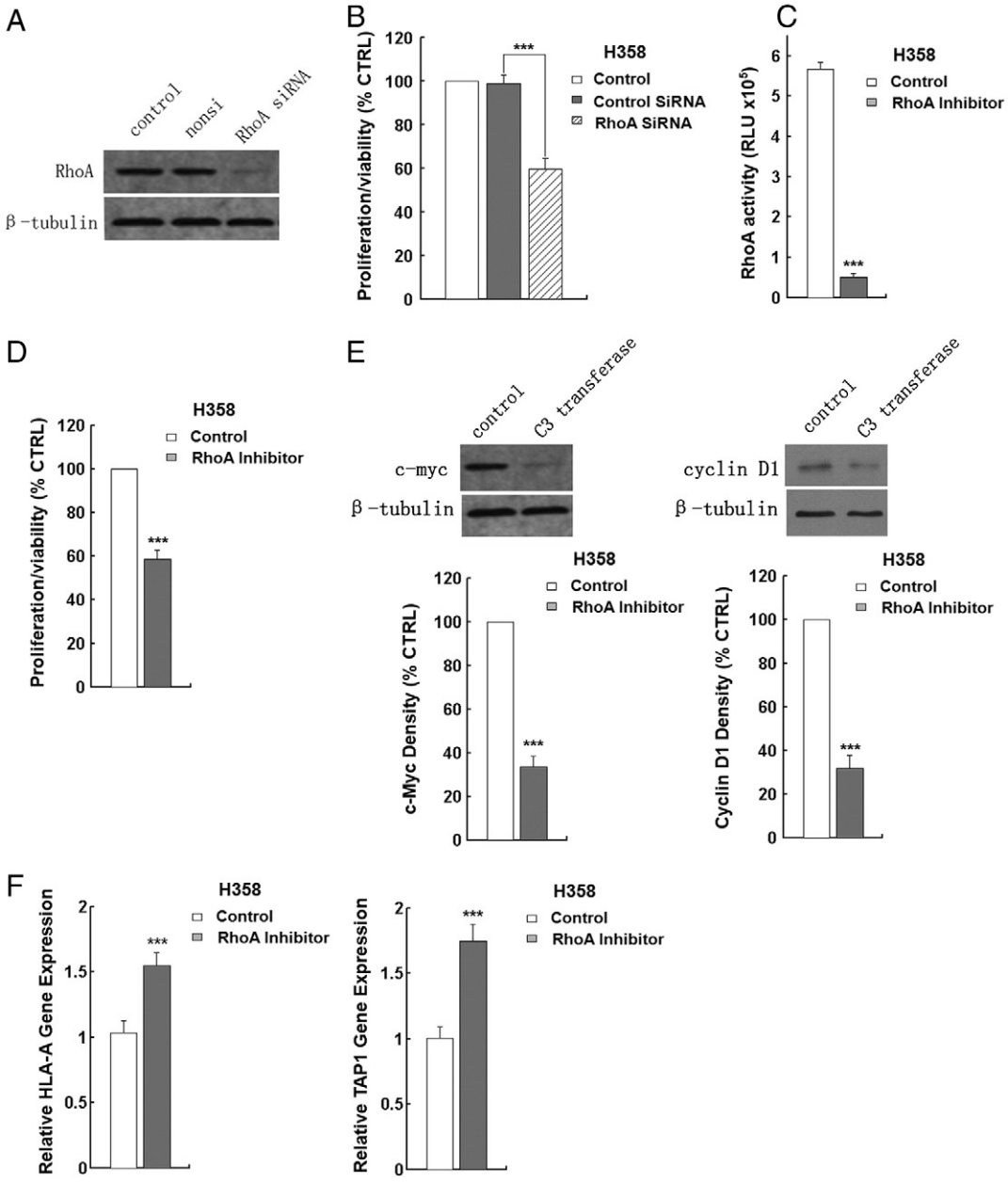

Fig. 2. The RhoA-dependent NSCLC cell growth inhibition. The effect of different treatments on cell growth was determined by MTT after 72-h incubation and the data of treatment group was expressed by % control. (A) The effect of specific siRNA to RhoA on the expression of RhoA in H358 cells determined by Western blotting. (B) The effect of specific siRNA to RhoA and Nonsilencing siRNA (control siRNA) on H358 cell growth. Bars are mean ± S.D. from six independent experiments. ***P<0.001. (C) The specific inhibitory effect of the RhoA activity inhibitior, C3 transferase (1 g/ml), on the RhoA activity determined by Rho-GTP affinity assay. Bars are mean ± S.D. from six independent experiments. ***P<0.001. (D) The effect of RhoA activity inhibitior, C3 transferase (1 g/ml), on NSCLC cell growth assayed by MTT. Bars are mean ± S.D. from six independent experiments. ***P<0.001. (E–F) The effect of inhibition of RhoA activity by C3 transferase on the expression of pro-tumor proteins c-myc and cyclin D1, as well as the tumor immunity-related MHC class I and TAP 1, determined by Western blotting and real time PCR after 48-h incubation, respectively. Bars are mean ± S.D. from six independent experiments. ***P<0.001.

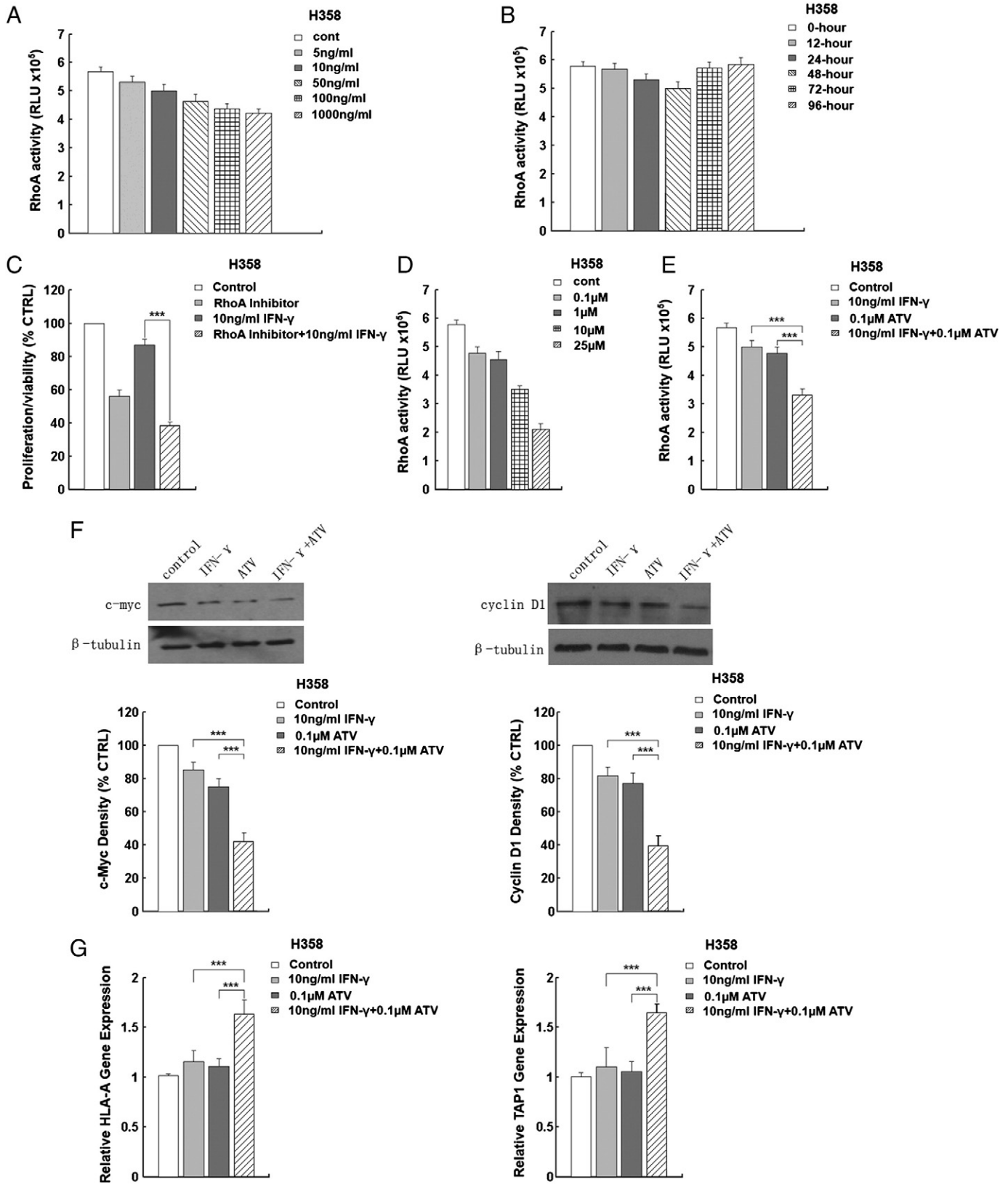

Fig. 3. Involvement of RhoA in the synergistic effect of atorvastatin (ATV) and IFN-γ on inhibition of NSCLC cell growth. The effect of different treatments on cell growth was determined by MTT after 72-h incubation and the data of treatment group was expressed by % control. (A) The effect of IFN-γ on the RhoA activity in H358 cells at the indicated concentrations. (B) The effect of IFN-γ on the RhoA activity in H358 cells at the indicated times. (C) The effect of RhoA inhibitor or low dose of IFN-γ (10 ng/ml), or their combination on H358 cell growth. Bars are mean ± S.D. from six-eight independent experiments. ***P<0.001. (D) The effect of atorvastatin on the RhoA activity in the H358 cells at the indicated concentrations. (E) The effect of low dose of atorvastatin (0.1 μM) on the IFN-γ-induced inhibition of H358 cell growth. Bars are mean ± S.D. from six independent experiments. ***P<0.001. (F–G) The effect of low doses of atorvastatin (0.1 μM) or low dose of IFN-γ (10 ng/ml), or their combination on the expression of pro-tumor proteins c-myc and cyclin D1, as well as the tumor immunity-related MHC class I and TAP 1, determined by Western blotting and real time PCR after 48-h incubation, respectively. Bars are mean ± S.D. from seven to ten independent experiments. ***P<0.001.

to its effect on the expression of c-myc and cyclin D1. Meanwhile, inhibition of RhoA activity also resulted in increased levels of MHC class I and TAP 1 in NSCLC cells (Fig. 2F), suggesting that regulation of tumor immunity may be another critical component of RhoA-dependent tumor cell growth inhibition *in vivo*.

Next we determined the effect of IFN-γ on RhoA activity in NSCLC cells. Fig. 3A showed that IFN-γ administration could dose-dependently inhibit RhoA activity in H358 cells after 48-hour incubation, though low dose of IFN-γ (10 ng/ml) treatment exerted no significant inhibitory effect on RhoA activity in NSCLC cells, as the inhibitory rate was approximately 16% in H358 cells. Fig. 3B showed that low dose of IFN-γ (10 ng/ml) administration led to suppression of RhoA activity in NSCLC cells maximally at about 48 h. However, after 72-hour treatment, the suppressive effect disappeared. Furthermore, the data from MTT assay showed that combination of C3 transferase (1 g/ml) and IFN-γ (10 ng/ml) resulted in a substantial inhibition of cell growth, and the inhibitory rate was significantly higher than that of IFN-γ or C3 transferase treatment alone (Fig. 3C). Thus, RhoA may be the critical target of IFN-γ to exert its suppressive effect on NSCLC cell growth.

Fig. 3D indicated that atorvastatin could exert an inhibitory effect on RhoA activity in a dose-dependent manner. However, the inhibitory effect of low doses of atorvastatin (0.1 μM and 1 μM) appeared minimal. Additional administration of atorvastatin (0.1 μM) to the IFN-γ (10 ng/ml)-treated H358 cells resulted in a synergistic effect with IFN-γ on inhibition of RhoA activity (Fig. 3E). Meanwhile, additional atorvastatin could also lead to a substantial inhibition of the expression of c-myc and cyclin D1 in NSCLC cells, as compared with atorvastatin or IFN-γ treatment alone (Fig. 3F). Furthermore, we also evaluated the combinatorial effect of atorvastatin and IFN-γ on the expression of the MHC class I and TAP 1 in H358 cells. Though atorvastatin or IFN-γ treatment alone produced minimal effect on the expression of the MHC class I and TAP 1, combination treatment of atorvastatin and IFN-γ could significantly upregulate the expression of the MHC-related genes (Fig. 3G).

Taken together, the above results suggest that atorvastatin exerts the synergistic effect on IFN-γ-induced inhibition of NSCLC cell growth probably through inhibition of RhoA activity and resultant downregulation of the levels of some pro-tumor proteins *in vitro*. Besides, the combinatorial effect of atorvastatin and IFN-γ on enhancement of MHC class

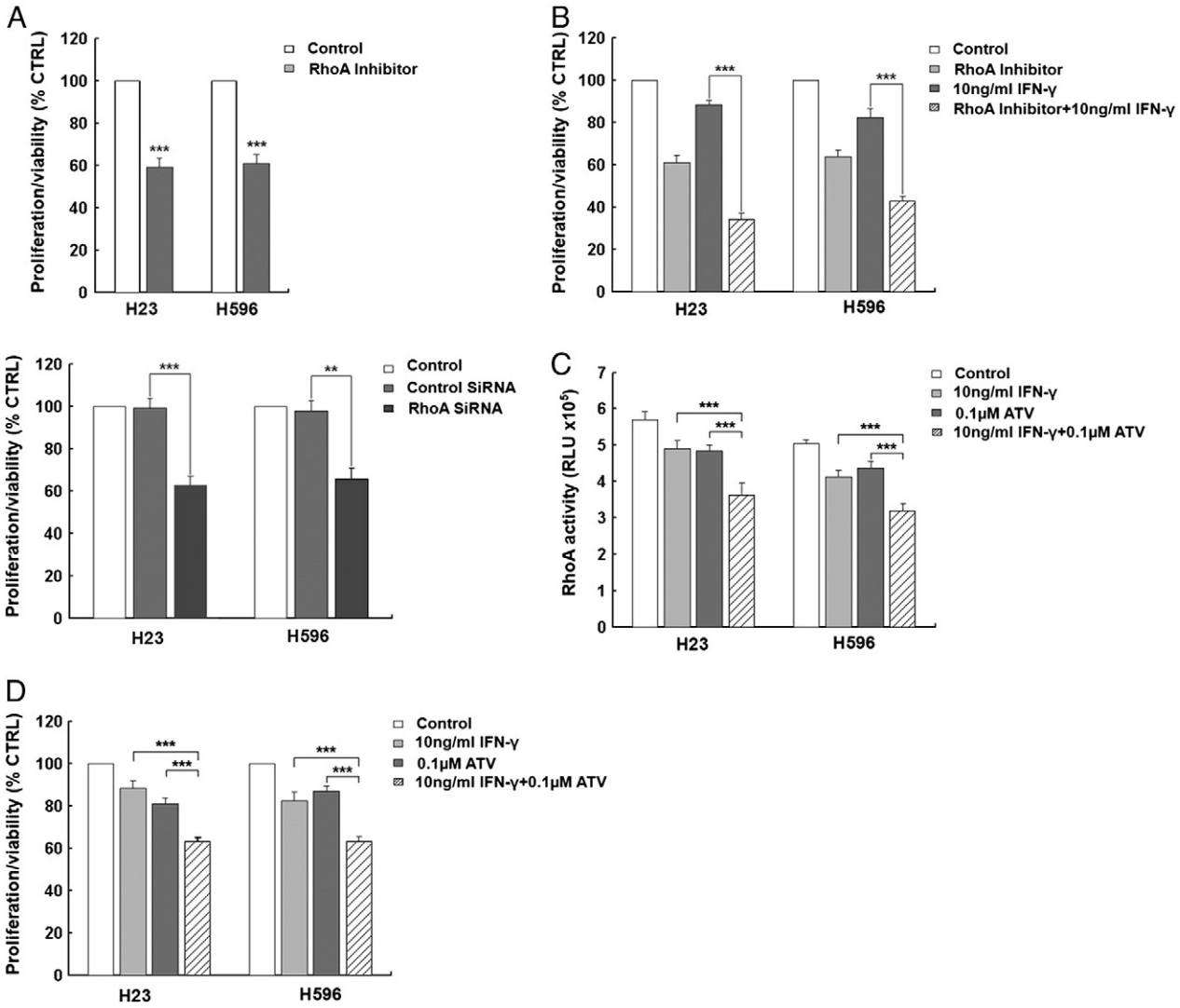

Fig. 4. Involvement of RhoA activity in atorvastatin (ATV)-mediated enhancement of cell growth inhibition by IFN-γ in other NSCLC cell lines. (A) The effect of the RhoA inhibitor and RhoA knockdown on cell growth in H23 and H596 cells. Bars are mean ± S.D. from seven independent experiments. **P<0.01, ***P<0.001. (B) The effect of inhibition of RhoA activity by the RhoA inhibitor on IFN-γ cytotoxicity in H23 and H596 cells. Bars are mean ± S.D. from six to seven independent experiments. ***P<0.001. (C) The effect of atorvastatin (0.1 μM) on IFN-γ-induced reduction in RhoA activity in H23 and H596 cells. Bars are mean ± S.D. from seven independent experiments. ***P<0.001. Bars are mean ± S.D. from five to eight independent experiments. ***P<0.001. (D) The effect of atorvastatin (0.1 μM) on IFN-γ-induced cytotoxicity in H23 and H596 cells. Bars are mean ± S.D. from five to seven independent experiments. ***P<0.001.

I and TAP 1 expression may be another contribution to atorvastatin-enhanced IFN-γ cytotoxicity in NSCLC cells *in vivo*.

3.3. Role of RhoA activity in atorvastatin plus IFN-γ-mediated enhancement of cell growth inhibition in other NSCLC cell lines

To exclude the possibility that the observed effects are restricted to H358 cells, we further examined the relationship between RhoA activity and NSCLC cell growth and the synergistic effect of atorvastatin plus IFN-γ on inhibition of RhoA activity and resultant suppression of cell growth in H23 and H596 cells (two other NSCLC cell lines). Like in H358 cells, the RhoA inhibitor and RhoA knockdown could effectively inhibit cell growth (Fig. 4A) and inhibition of RhoA activity significantly enhanced the tumor-inhibitory effect of IFN-γ (Fig. 4B), suggesting that RhoA protein is closely related to tumor cell growth and IFN-γ cytotoxicity in H23 and H596 cells. Furthermore, atorvastatin could also effectively synergize with IFN-γ to block the RhoA activity (Fig. 4C) and resultantly enhanced the IFN-γ cytotoxicity substantially in H23 and H596 cells (Fig. 4D).

3.4. Atorvastatin and IFN-γ synergize to suppress the growth of H358 and H596 tumors in vivo

The above works indicated that atorvastatin could effectively enhance IFN-γ-induced inhibition of NSCLC cell growth *in vitro*. However, it was critical to determine whether this combination could produce synergistic effect on inhibition of tumor growth *in vivo*. The use of immunodeficient animal model provides the opportunity for observing the direct inhibitory effect of IFN-γ alone, or combined with atorvastatin on the tumor growth. Firstly, H358 or H596 cells were injected subcutaneously into nude mice to develop tumor-harbored mice. When tumors grew to 80–100 mm^3, the mice were differently treated with 1) high-dose combination of atorvastatin (10 mg/kg/day, by gavage) and IFN-γ (10^4 IU/mouse, three times/week, by IP), and each agent alone as well as 2) low-dose combination of atorvastatin (1 mg/kg/day, by gavage) and IFN-γ (5×10^3 IU/mouse, three times/week, by IP), and each agent alone. The treatment period was 3 weeks.

As shown in Fig. 5A and B, after the 3-week period, the H358 tumors displayed insensitivity to IFN-γ treatment either at high dose or at low dose. High dose or low dose of IFN-γ could inhibit the growth of H596 tumors up to about 27% and 17% compared to vehicle group (Fig. 5C and D), respectively. High dose of atorvastatin could inhibit the growth of H358 (Fig. 5A) and H596 tumors (Fig. 5C) to 32% and 24.5% at 21st day, respectively. Furthermore, atorvastatin could result in an only partial inhibition of these two tumor growth at its low dose (1 mg/kg), 13% in H358 tumor (Fig. 5B) and 10% in H596 tumor (Fig. 5D), respectively. However, the high-dose combination treatment could decrease the tumor size of H358 (Fig. 5A) and H596 tumors (Fig. 5C) up to 64% and 53%, respectively. Interestingly,

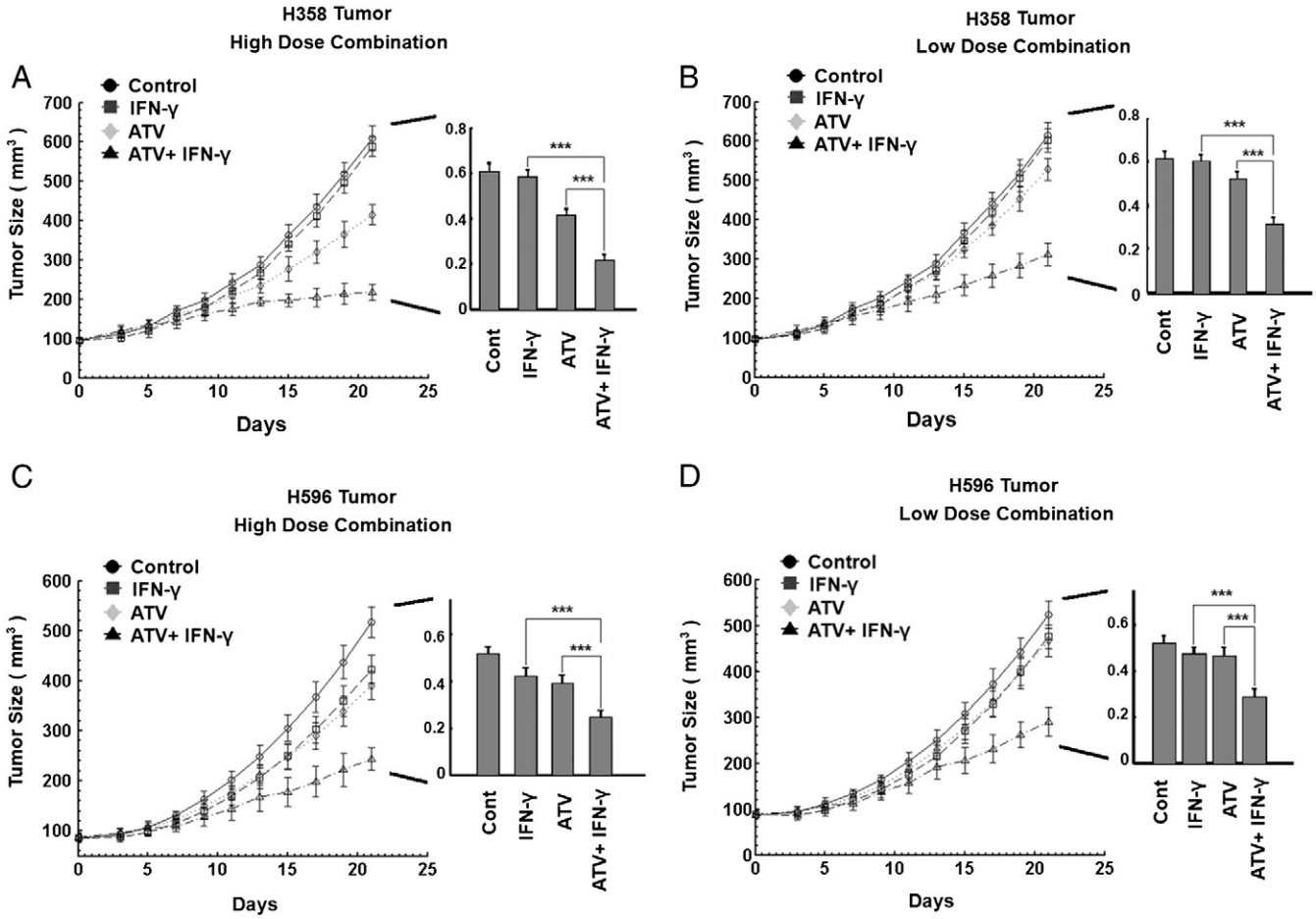

Fig. 5. The synergistic effect of atorvastatin (ATV) and IFN-γ on suppression of the growth of H358 and H596 tumors after 3-week treatment *in vivo*. H358 or H596 cells were injected subcutaneously into nude mice to develop tumor-harbored mice. When tumors grew to 80–100 mm^3, the mice were differently treated. (A) The effect of high-dose combination of atorvastatin (10 mg/kg/day, by gavage) and IFN-γ (10^4 IU/mouse, three times/week, by IP), and each agent alone on H358 tumor size. Columns, mean (n = 10 tumors in each group); bars, S.D., ***P<0.001. (B) The effect of low-dose combination of atorvastatin (1 mg/kg/day, by gavage) and IFN-γ (5×10^3 IU/mouse, three times/week, by IP), and each agent on H358 tumor size. Columns, mean (n = 10 tumors in each group); bars, S.D., ***P<0.001. (C–D) The effect of high-dose combination atorvastatin and IFN-γ or each agent alone, as well as low-dose combination atorvastatin and IFN-γ or each agent alone, on H596 tumor size. Columns, mean (n = 10 tumors in each group); bars, S.D., ***P<0.001.

the low-dose combination could also produce significant tumor-inhibitory effect in these two tumors (48.7% in H358 tumor and 43.8% in H596 tumor, respectively). These results suggest that the combination treatment of atorvastatin and IFN-γ with clinical doses can produce an effective synergization in inhibition of NSCLCs growth *in vivo*. Furthermore, it was also important to note that the combination did not show an overall increased toxic effect, since a difference in weights of the mice was not observed (data not shown). Thus, the above results demonstrate the feasibility of the combination treatment of atorvastatin and IFN-γ in treating NSCLCs *in vivo*.

3.5. Atorvastatin induced specific deposition of endogenous IFN-γ in tumors but not in other normal organs

Atorvastatin has been shown to induce secretion of IFN-γ in NK cells and $CD8^+$ T lymphocytes (Coward et al., 2006; Gruenbacher et al., 2010). However, whether atorvastatin has some influence on endogenous IFN-γ deposition *in vivo* remains unknown. Our NSCLC cells-harbored animal models could not provide the platform for our further observation of the endogenous IFN-γ deposition induced by atorvastatin, due to the loss of immune function in nude mice. As a result, immune competent Lewis lung carcinoma (LLC)-beared wild-type C57BL/6 mice established by inoculation of LLC cells into these mice with the weight of approximately 20 g were used. When LLC cells were injected, atorvastatin was orally administrated to mice simultaneously, and the effect of atorvastatin administration on endogenous IFN-γ deposition *in vivo* was determined after 21 days. Fig. 6A showed that atorvastatin of the lower clinical dose (1 mg/kg/day) could not induce the up-regulation of endogenous IFN-γ level in LLC tumors compared to saline group. However, from the higher clinical dose (10 mg/kg/day) to the 10-fold higher than this dose (100 mg/kg/day), atorvastatin could significantly up-regulate endogenous IFN-γ level in LLC tumors.

To evaluate the possibility that atorvastatin induces specific deposition of endogenous IFN-γ in tumors, the distribution of endogenous IFN-γ in LLC tumor-harbored mice was examined. Fig. 6B showed that atorvastatin administration could only up-regulated accumulation of IFN-γ in tumors. However, the levels of IFN-γ in other normal tissues, such as lung, liver, kidney, bone marrow, skin, peritoneum and muscle, were not enhanced. These results suggest that atorvastatin can induce specific deposition of IFN-γ in LLC tumors *in vivo*, which may partially contribute to its synergistic effect on IFN-γ-mediated inhibition of tumor growth *in vivo*. Nevertheless, our results do not necessarily indicate that atorvastatin can induce upregulation of endogenous IFN-γ secretion, as the levels of IFN-γ in other normal tissues after atorvastatin administration appears to be reduced.

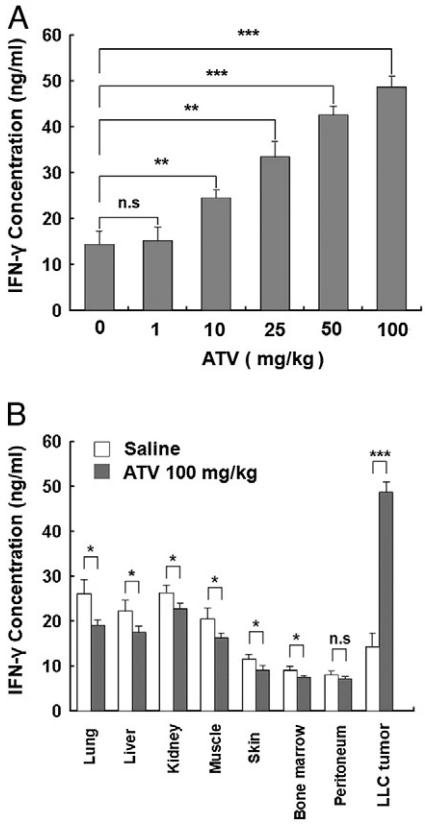

Fig. 6. The specific deposition of endogenous IFN-γ in tumors but not in other normal organs induced by atorvastatin. Immune competent Lewis lung carcinoma (LLC)-beared wild-type C57BL/6 mice established by inoculation of LLC cells into these mice with the weight of approximately 20 g was used. The effect of atorvastatin administration on endogenous IFN-γ deposition *in vivo* was determined after 21 day-treatment. (A) The effect of atorvastatin at the indicated doses on the level of IFN-γ in tumors assayed by ELISA after 21-day treatment. Columns, mean (n = 10 tumors in each group); bars, S.D., $^{**}P<0.01$, $^{***}P<0.001$. (B) The effect of atorvastatin (100 mg/kg) on deposition of endogenous IFN-γ in different tissues after 21-day treatment, as compared with saline treatment. Columns, mean (n = 10 tumors in each group); bars, S.D., $^{*}P<0.05$, $^{***}P<0.001$.

4. Discussion

This study demonstrates that atorvastatin enhances the growth inhibitory effect of IFN-γ treatment against NSCLCs *via* synergistic inhibition of RhoA activity, thus upregulating the expression of MHC class I and TAP 1, as well as suppressing the expression of some pro-tumor proteins. Specifically, atorvastatin can also induce specific deposition of endogenous IFN-γ in LLC tumor in tumor-harbored mice.

RhoA, which belongs to GTP/GDP-binding GTPases of the Ras superfamily, has been shown to promote both cell proliferation and cell apoptosis (Aznar and Lacal, 2001). RhoA is overexpressed during tumorigenesis in various human tumors. Activated RhoA triggers subsequent activation of the PI3K/AKT pathway, which is thought to be essential for cell survival and expression of genes involved in cell proliferation (Denoyelle et al., 2003). Besides, inhibition of Rho protein function was confirmed to stimulate the immune response against melanoma associated with the expression of MHC class I (Tilkin-Mariame et al., 2005). In the present study, inhibition of RhoA activity leads to both decreased levels of some pro-tumor proteins and a significant increase in the expression of MHC class I and TAP 1. This result suggests that activation of RhoA may be closely correlated with tumorigenesis as it cannot only promote the expression of pro-tumor proteins but suppress the immune surveillance *via* inhibition of MHC class I and TAP 1 expression. Furthermore, as activation of the PI3K/AKT pathway in tumor cells can induce the expression of c-myc and cyclin D1 (Zhou et al., 2010) which are related to promotion of tumor cell growth, combined with other reports, our data also suggest that the involvement of activation of PI3K/AKT pathway in RhoA-dependent NSCLC cell growth may derive from induction of subsequent expression of c-myc and cyclin D1 that are directly responsible for overgrowth of NSCLC cells.

IFN-γ has long been associated with cytotoxic and anti-tumor functions. It was suggested that IFN-γ is vital to tumor surveillance by the immune system, which involves the upregulation of MHC class I and TAP 1 (Tilkin-Mariame et al., 2005; Weber and Rosenberg, 1988). Besides, recombinant IFN-γ was also shown to be involved in direct cytotoxic and anti-proliferative effects against cancer cells through inhibition of p21 (WAF1) (Chin et al., 1996; Hobeika et al., 1999). However, our results show that IFN-γ administration can result in inhibition of RhoA activity and subsequent changes of the levels of both some pro-tumor proteins and MHC class I-related molecules. These findings suggest that the effect of immune surveillance and the direct anti-proliferative function of IFN-γ may not be independently exerted while possessing some interaction with each other.

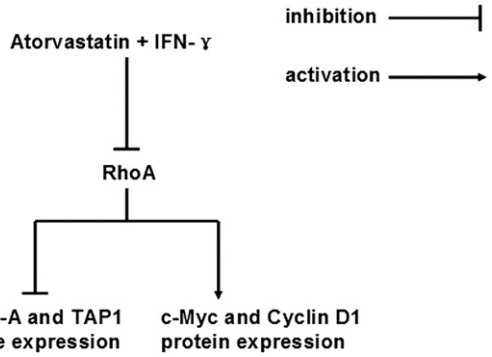

Fig. 7. Schematic diagram of the mechanism underlying the effective combined therapy of atorvastatin and IFN-γ against NSCLC cell growth.

The tumor-inhibitory effect of direct injection of IFN-γ displays not very satisfactory in NSCLC treatment. Mutation of some genes, such as *KRAS*, or *BRAF*, as well as hyperactivation of oncogenic proteins including Raf-1 or COX-2 was shown to induce activation of downstream signaling, such as PI3K and MEK, thus blunting the tumor-inhibitory or immunoactivatory effect of IFN-γ and leading to IFN-γ resistance in various tumor cell lines (Boni et al., 2010; Gollob et al., 2005; Klampfer et al., 2003; Ramana et al., 2000; Sharma et al., 2005). This study finds that IFN-γ administration leads to inhibition of RhoA activity and resultant suppression of some pro-tumor protein expression and upregulation of the levels of MHC class I and TAP 1, accounting for its cytotoxic effects on NSCLCs. However, after 72-hour incubation, the suppressive effect of IFN-γ on RhoA activity disappears *in vitro*. These results may suggest a novel mechanism underlying IFN-γ resistance in NSCLC therapy, which involves the non-persistent inhibitory effect on RhoA activity.

Previous studies have shown that high dose of atorvastatin (10 μM) alone can effectively block the RhoA-mediated MMPs activation and resultantly inhibit the tumor growth and cell invasion of osteosarcoma (Fromigue et al., 2008). However, whether clinically relevant doses of atorvastatin alone or combined with IFN-γ can regulate the activity of RhoA and subsequently inhibit the growth of NSCLCs remains still unexplored. This study indicates that atorvastatin (0.1 μM) can synergize with IFN-γ in treating NSCLCs both *in vitro* and *in vivo*. Moreover, additional atorvastatin administration can significantly reduce the RhoA activity in IFN-γ-treated NSCLC cells. These results suggest the critical component of synergistic inhibition of RhoA activity in atorvastatin-mediated enhancement in IFN-γ sensitivity in NSCLCs (Fig. 7).

Various studies have shown that atorvastatin may induce proinflammatory responses in $CD8^+$ and NK cells and resultantly enhance the secretion of endogenous IFN-γ (Coward et al., 2006). Besides, Georg Gruenbacher et al. demonstrated that simvastatin can synergize with IL-2 to induce the IFN-γ secretion from NK cells and subsequently induce the apoptosis of many cancer cell lines, such as kidney cancer, breast cancer and prostate cancer (Gruenbacher et al., 2010). This study finds that after atorvastatin treatment, the levels of IFN-γ deposited in tumor tissues are increased significantly in LLC tumor-beared mice, which may account for another novel mechanism for the synergistic inhibitory effect of atorvastatin plus IFN-γ on tumor growth that involves the induction of specific IFN-γ deposition in tumor tissues by atorvastatin. However, our study does not necessarily demonstrate that atorvastatin can induce endogenous IFN-γ secretion, as the levels of IFN-γ in other normal tissues appear to be reduced. Furthermore, previous studies have showed that IFN-γ can induce chronic lower fever in the clinical tumor therapy, due to the pro-inflammatory characteristics of IFN-γ (Schiller et al., 1987). Therefore, when IFN-γ is administrated to NSCLC patients, additional atorvastatin treatment may be used to reduce the clinical dosage of IFN-γ to diminish its side effects. More importantly, the atorvastatin-induced specific deposition of endogenous IFN-γ in tumor tissues while not in other tissues may also contribute to avoidance of damage to normal tissues.

5. Conclusions

It remains to be investigated whether RhoA is the primary target of atorvastatin plus IFN-γ to exert their synergistic effect on inhibition of NSCLC progression. Also relevant to our observations, it will be important to determine the molecular basis underlying the RhoA protein-mediated IFN-γ insensitivity in NSCLCs. Notwithstanding these drawbacks, our study does indicate that atorvastatin can enhance IFN-γ sensitivity in NSCLCs both *in vitro* and *in vivo*, probably through induction of a synergistic inhibitory effect on RhoA activity.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81020108031, 30572202, 30973558, 30772571, 30901815, 30901803, 30973902), the Major Specialized Research Fund from the Ministry of Science and Technology in China (No. 2009ZX09103-144), Research Fund from Ministry of Education of China (111 Projects No. B07001), and Province Natural Science Foundation of Hei Long Jiang (No. D200929).

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