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Myrothecine A modulates the proliferation of HCC cells and the maturation of dendritic cells through downregulating miR-221

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ABSTRACT

Myrothecine A, characterized from the extracts of myrothecium roridum strain IFB-E012, isolated as endophytic fungi found in the traditional Chinese medicinal plant *Artemisia annua*. Here we investigated its roles on anti-tumor and immune regulation in vitro. Dendritic cells (DCs) are the most potent antigen presenting cells in immune responses. Recent studies have indicated that miRNAs are indispensable in regulating the development, differentiation, maturation and function of DC. MiR-221, acted as an oncogene, is an important regulator in cancer development by binding to 3′ untranslated regions (3' UTR) of target mRNA. Here, we investigated whether myrothecine A could inhibit cell proliferation in hepatocellular carcinoma (HCC) cell line SMMC-7721 by regulating miR-221. The HCC cells were treated with myrothecine A at different concentration, and the cell growth ability was measured by MTT assay. Then we observed whether myrothecine A could affect the maturation of DC by regulating miR-221. The HCC cell line was co-cultured with immature DC from mice bone marrow, and the levels of CD86 and CD40 was detected by FCM. Our results showed that myrothecine A could rescue miR-221-induced cell proliferation and influence the protein level of p27 by inhibiting the expression of miR-221. In addition, myrothecine A could enhance the expression of CD86 and CD40 by reversing the function of miR-221. Therefore, myrothecine A may be acted as an anti-tumor drug to promote the maturation of DC in the microenvironment of hepatocellular carcinoma.

1. Introduction

The trichothecenes are a group of over 180 sesquiterpenoids that are produced by fusarium, stachybotrys, myrothecium, and other fungal genera. The trichothecene family is divided into four groups (A through D) based on modifications to the parent trichothecane ring system [1]. Most trichothecenes possess an epoxide ring at C12–13, which appears to be critical for bioactivity, including anti-proliferating action, ability to induce apoptosis, antimalarial activity, and antiviral effects [2]. These macrocyclic trichothecene mycotoxins are known metabolites of M. verrucaria [3]. Strains of myrothecium verrucaria and its related taxa myrothecium roridum have been intensely studied for their content of the trichothecene class of sesquiterpenes and their macrocyclic analogues [2]. Myrothecine A is a trichothecene macrolide compound isolated from the fermentation broth of myrothecium roridum IFB-E012 [4]. Although the molecular structure of myrothecine A is clear, its biological function and anti-tumor mechanism is rarely known.

MicroRNAs (miRNAs) are a family of ∼22 nt noncoding RNAs that post-transcriptionally regulate diverse biological functions by promoting degradation or inhibition of target mRNAs translation [5,6]. Since the first report in 1993 [7], it has been identified that miRNAs played critical roles in many biological processes, including apoptosis, cell cycle, proliferation, differentiation, migration and invasion [5,8]. Moreover, several studies indicated that the specific expression of miRNAs was associated with clinical features and affected the occurrence and development of tumors [9]. Previous study showed that miR-221 was differentially expressed in subtypes of breast cancer and correlated with clinical outcome [10]. Garofalo et al. demonstrated that miR-221 played oncogenic roles in lung cancer [11], human thyroid papillary carcinomas [12], and prostate carcinoma [13]. In vivo study showed that delivery of anti-miR-221 oligo nucleotides leads to a significant reduction of the number and size of tumor nodules [14], and in vitro study showed miR-221 promoted angiogenesis and metastasis of glioma cells by targeting TIMP2 [15]. The identification of target

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mRNAs is a key step for assessing the role of aberrantly expressed microRNAs in human cancer. Several important target proteins of miR-221 have been identified, including p57, Bmf, PTEN, and TIMP3 [16–18]. In addition, p27 also turned out to be regulated by miR-221 in some human malignancies. The downregulation of p27 was associated with poor survival, advanced tumor stage and recurrence of hepatocellular carcinoma (HCC) [16].

Dendritic cells (DCs) are antigen-presenting cells with the ability to induce primary immune responses. DCs are not only critical for the induction of primary immune responses, but may also be important for the induction of immunological tolerance, as well as for the regulation of the type of T cell–mediated immune responses [19]. A number of cytokines and other factors have been proposed to promote DC growth and differentiation from myeloid progenitor cells, including GM-CSF, TNF, Flt3L, IL-4, IFN-γ, TGF-β, PGE2, ionomycin and others. Of all culture conditions tested, bone marrow-derived dendritic cells (BmDC) generated in the presence of CD40L mediate the most potent immune responses in vivo, including the generation of protective and therapeutic tumor immunity. In general, the capacity to induce a tumor-specific immune response in vivo correlates to the degree of DC maturation [20]. It is identified that functional miRNA-protein networks regulate human monocytederived dendritic cell (MDDC) differentiation [21]. Inhibition of miR-511 and miR-99b resulted in reduced DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) level [22]. Silencing of c-Fos expression by microRNA-155 is critical for dendritic cell maturation and function [23]. Inhibition of microRNA let-7i depresses maturation and functional state of dendritic cells via targeting suppressor of cytokine signaling 1. Downregulation of let-7i significantly impeded DC maturation as evidenced by reduced CD80 and CD86 expression [24]. MiRNA-146a regulates the maturation process and pro-inflammatory cytokine secretion in DCs by targeting CD40L in ox-LDL-stimulated DCs [25].

Previous studies have found that macrolide compounds have potential value in anti-tumor therapeutics. Macrolides can not only inhibit the proliferation and differentiation of tumor cells, but also have important immunomodulatory effects. However, there are few studies on the anti-tumor mechanism and immunoregulation of trichothecenes macrolides in vitro, especially for studies on DC maturation. In our study, we investigated the effect of the trichothecene macrolide compound myrothecine A on HCC cell proliferation and DC maturation. We found that myrothecine A could modulate the proliferation of HCC cells and the maturation of DC in liver cancer microenvironment through regulating miR-221. Taken together, our study may provide experimental basis for myrothecine A to become a new anti-tumor drug and immunotherapeutic drug.

2. Materials and methods

2.1. Cell lines and culture

HCC cell lines SMMC-7721 and mice hepatocarcinoma cell lines Hepal-6 were obtained from the American Type Culture Collection (ATCC). SMMC-7721 and Hepal-6 cells were cultured in RPMI-1640 (GIBCO, US). The culture media were all supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin at 37 °C, in a humidified air with 5% CO2.

2.2. Tissue samples

24 patients with histologically conformed hepatocellular carcinoma tissues (HCT) were obtained from the first affiliated hospital of Yangzhou University. The cancer tissues and adjacent cancerous tissues were collected from patients. All samples were acquired at the time of surgery and were frozen in liquid nitrogen immediately. This investigation was approved by the medical ethics committee of Yangzhou University and informed consent was gained from patients before recruitment.

2.3. Extraction and isolation of Myrothecine A

Myrothecines A was characterized from the extracts of Myrothecium roridum strain IFB-E012, isolated as endophytic fungi found on the traditional Chinese medicinal plants *Artemisia annua*. The extraction and isolation method details were shown in the previous paper [34]. And the purity of this drug is > 99%.

2.4. DC isolation ex vivo

The mouse bone marrow cells were flushed from the femur and tibiae, and then treated with ACK lysis buffer. Cells (2×10^6 cells/ml) were cultured in RPMI1640 complete medium supplemented with 10% FCS, 50 μM mercaptoethanol, 10 mM HEPES, 100 U/ml streptomycin/penicillin, 1 ng/ml rmIL-4 and 10 ng/ml rmGM-CSF at 37 °C and 5% CO2. The non-adherent cells were removed by mild pipetting on alternate days, and the adherent cells were cultured in the complete medium containing rmGM-CSF and rmIL-4. On the 6th day, BMDCs were harvested for the experiments.

2.5. Cell transfection

The miR-221 overexpression SMMC-7721 cell lines, miR-221 overexpression Hepal-6 cell lines and control SMMC-7721 cell lines, control Hepal-6 cell lines were established by infecting with lentivirus packing miR-221 mimic vector and control vector respectively (GenePharma, Shanghai, China). Expression of miR-221 was driven by the promoter U6. The generation of LV-miR-221 was at the titer of 9×10^8 TU/ml and the LV-Vector was at the titer of 7×10^8 TU/ml. Target cells were infected with lentivirus at a multiplicity of infection (MOI) of 100 for 48 h and then selected with puromycin (Santa Cruz) for 3 weeks. Total RNA and protein were collected and used for western blot or qRT-PCR analysis. For the interfering assay, IP10 siRNA was obtained from GenePharma (Shanghai, China). The oligonucleotides of si-IP10 and miR-221 inhibitor were transfected into human HCC cells by siLentFect Lipid Reagent (Bio-Rad, Hercules, IN, USA) and incubated for 48 h. After 48 h of transfection, the efficiency of knocking down was assayed by western blot or qPCR.

2.6. MTT assay

Cell viability was accessed using the MTT assay. After transfected with miR-221 or treated with the varying concentrations of myrothecine A, cultivated cells were cultured with 50 μL of MTT solution (5 mg/ml) at 37 °C for 4 h and centrifuged with 400 *g* for 5 min. In each well, 150 μL DMSO were applied to melt the crystallization. The absorbance was assessed at 550 nm. All consequences were acquired from 3 self-governing experimentation that were executed in quadruplicate.

2.7. Flow cytometry

DCs were harvested, washed once with phosphate-buffered saline, and were counterstained immunophenotypically for anti-CD86 and anti-CD40. Analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using the Cell Quest Pro software.

2.8. RNA extraction and Quantitative Real-time PCR (qRT-PCR)

Cells were washed with ice-cold phosphate-buffered saline twice and lysed with TRIZOL reagent to isolate total RNA, which was followed by DNase digestion. Single-strand cDNA was synthesized using the Ncode miRNA First-Strand cDNA Synthesis kit (Invitrogen). miRNA-specific primers were designed following the kit instruction and

Table 1
Primer sequences for qRT-PCR.

Name	Primer sequences
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
miR-221	F:5'-ACACTCCAGCTGGGAGCTACATTGTCTGCT-3' R:5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTG-3'
Arg-1	F: 5'-CAGAAGAATGGAAGAGTCAG-3' R: 5'-CAGATATGCAGGGAGTCACC-3'
TGF-β	F: 5'-AAACGGAAGCGCATCGAA-3' R: 5'-GGGACTGGCGAGCCTTAGTT-3'
iNOS	F: 5'-GAGCCACAGTCCTCTTTGCTA-3' R: 5'-TGTCACCACCAGCAGTAGTTG-3'
IL-10	F: 5'-ACTCTTCACCTGCTCCACTG-3' R: 5'-GCTATGCTGCCTGCTCTTAC-3'
IP-10	F: 5'-GGACGGTCCGCTGCAACTGCATCC-3' R: 5'-GCAGCCTGGGCATGGCACATGGTG-3'

synthesized. U6 was used as endogenous controls. Primer sequences are detailed as Table 1. Comparative real-time polymerase chain reaction (PCR) using SYBR Green SuperMix (Invitrogen) was performed in a 96-well plate and run in a 7500 Real-Time PCR System (Applied Biosystems) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 57 °C for 1 min. Each sample was analyzed in triplicate. The relative expression was calculated using the relative quantification equation (RQ) = 2 − ΔΔCt.

2.9. Cell cycle analysis

Myrothecine A was dissolved in medium as a 1 μg/ml, 5 μg/ml, 10 μg/ml stock solution, then the cells were harvested, washed in PBS, and fixed with 70% cold ethanol at 4 °C overnight. Cells were incubated in RNase (0.4 mg/ml) and stained with 0.4 mg/ml propidium iodide in hypotonic fluorochrome buffer for 30 min at 37 °C. Samples were then analyzed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). To determine the cell cycle distribution, we used ModFit LT 3.0 software to analyze the data.

2.10. Immunohistochemistry

Immunohistochemistry was performed according to the avidin biotinalated–HRP complex (ABC) method using a standard ABC kit (Zhongshan biotech, Beijing, China). The slides were incubated with anti-IP-10 antibody (1:1000) (abcam) overnight at 4 °C, and diaminobenzidine (DAB; Zhongshan Biotech, Beijing, China) was used to produce a brown precipitate. The immunoreactivity was assessed blindly by two pathologists using light microscopy (Olympus BX-51 light microscope), and the image was collected by Camedia Master C-3040 digital camera. The expression of IP-10 was graded as high when ≧5% of tumor cells showed immunopositivity. Biopsies with < 5% tumor cells immunostaining were considered low.

2.11. ELISA assay

Cell culture supernatants were collected from cultures after miR-221 mimic transfection. The levels of IP-10 were determined by ELISA according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). These assays were performed in triplicate.

2.12. Western blot analysis

Protein extracts were denatured and the solubilized proteins (80 μg) were subjected to electrophoresis on 10% polyacrylamide SDS gels. These were followed by probing with antibodies (Abcam) for rabbit anti-p27 (diluted 1:1000 in TBST), or mouse anti-actin (diluted 1:2000 in TBST). Secondary antibodies (anti-mouse/rabbit IgG 1:10,000) were incubated and washed under similar conditions. Finally, the bands on the membrane were scanned with Odyssey (Li-COR Lincoln, NE).

2.13. Statistical analysis

Data is presented as mean ± S.D. of at least three independent experiments and were compared using the two-tailed paired Student's *t*-test or one-way ANOVA, a difference with a $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of miR-221 on growth in HCC cell lines or tissues

To investigate the effect of miR-221 on the proliferation of HCC cells, lentiviral plasmid with miR-221 was infected in HCC cell line SMMC-7721. MTT assay results showed that miR-221 significantly promoted cell proliferation, and the Lv-miR-221 group had a growth rate 1.5 times than that of the Lv-GFP group (Fig. 1A). Moreover, we collected 24 human HCC tissue samples and found that miR-221 mRNA levels were higher in HCC tissues (HCC) than in adjacent non-cancerous tissues (Para) (Fig. 1B). Thus, these results may demonstrate that higher miR-221 expression was associated with HCC.

3.2. miR-221 inhibits the maturation of DCs

To investigate the role of miR-221 on DCs maturation, mouse bone marrow-derived DCs were obtained and co-cultured with miR-221 overexpressing mouse hepatoma cell line Hepa1–6 or human HCC cell line SMMC-7721. After 3 days, cells were collected and flow cytometry was used to detect the expression of DC surface molecules CD86 and CD40. Then we used the marker CD11c to validate the cells. And it is identified that the cells we evaluated are DC but not monocytes. The upper Fig. 2A illustrated the gating strategy. As shown in Fig. 2A-B, the expression of CD86 and CD40 was significantly increased in the LPS (induction of DC maturation) group compared to DC-Con group. When DC group was co-cultured with stably transfected GFP (negative control of miR-221) overexpressing SMMC-7721, the expression of CD86 and

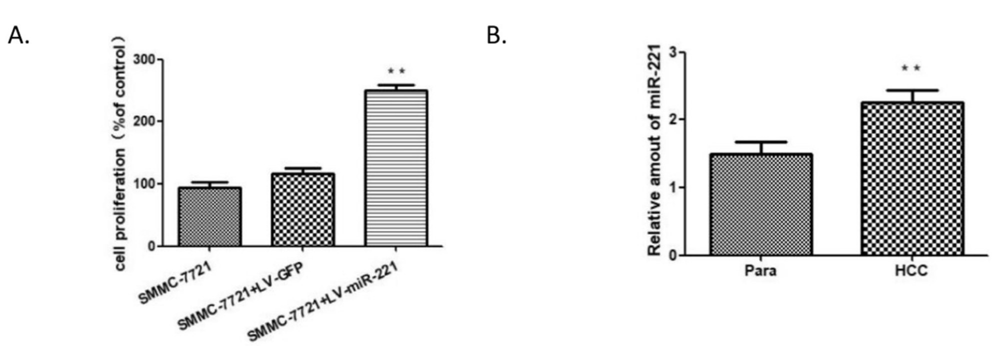


Fig. 1. MiR-221 promotes cell proliferation in SMMC-7721 cell lines and has higher mRNA level in HCC tissues. A. The MTT assays were performed after miR-221 overexpression in SMMC-7721 cells. B. RNA extraction was prepared from 12 paired tumor adjacent normal hepatocellular tissues (Para) and HCC tissues (HCC). The miRNA-221 level was determined by q-PCR. Data are shown as mean ± SD. ** $P < 0.01$.

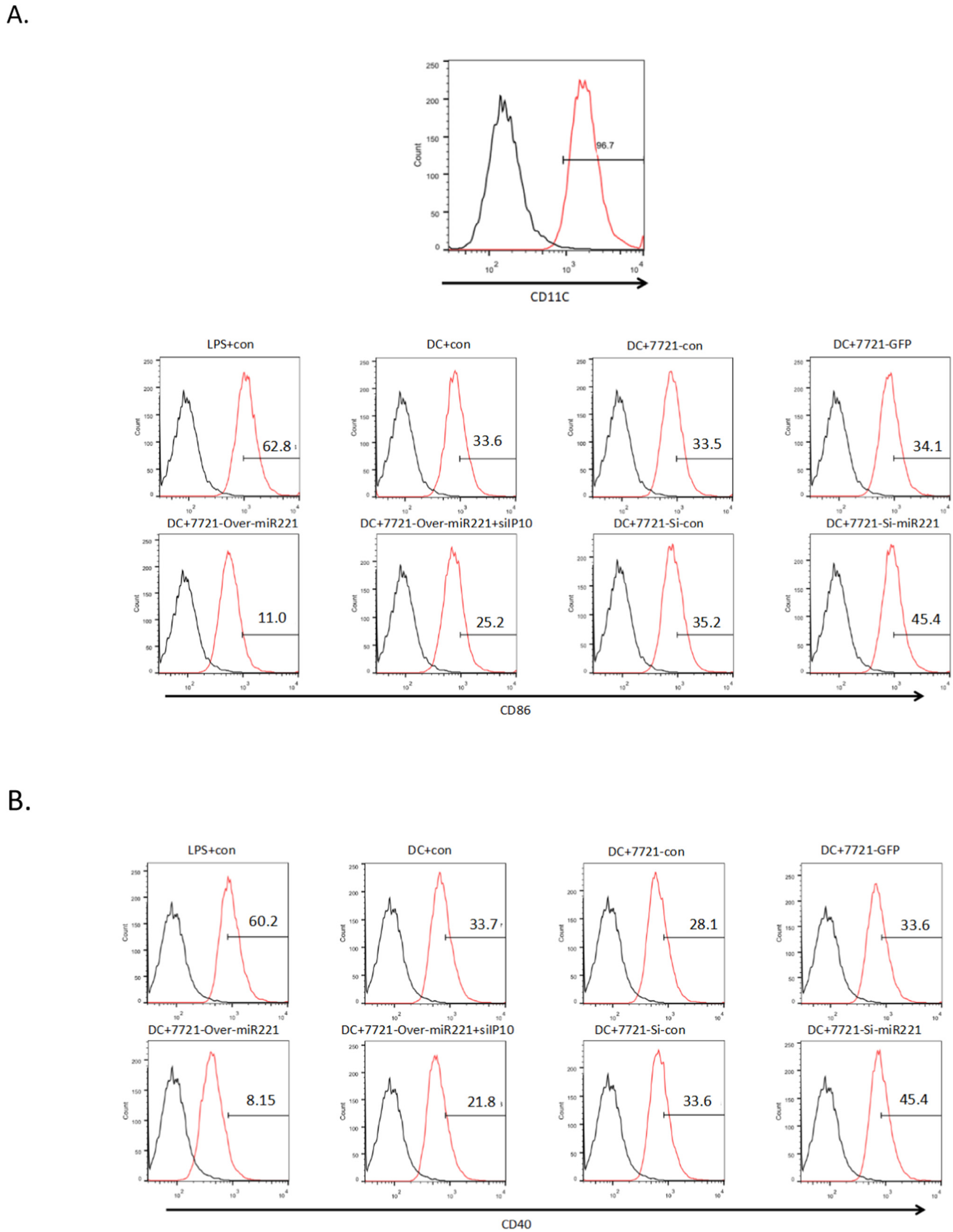
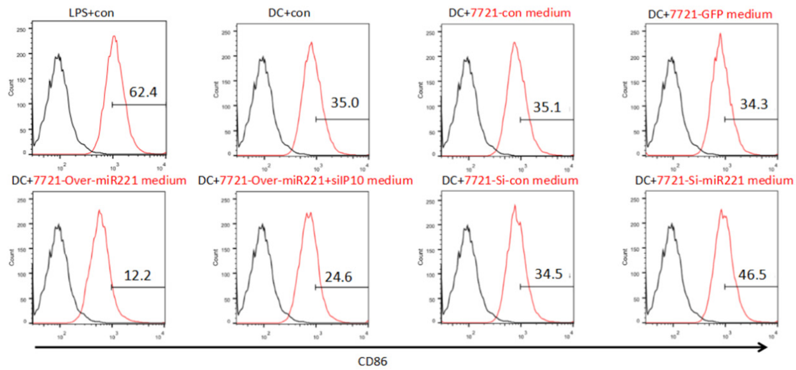


Fig. 2. Ectopic miR-221 expression or miR-221 silencing affects dendritic cells (DC) maturation. A-B. Flow cytometric analysis of DC after treated with lipopolysaccharide (LPS) or co-cultivation with si-miR-221 SMMC-7721 cells and miR-221-overexpression SMMC-7721 cells transfected with IP-10 siRNA. Cells were stained for expression of CD86 or CD40. C-D. Flow cytometry analysis of DCs cultured in the conditioned medium from the si-miR-221 SMMC-7721 cells or miR-221-overexpression SMMC-7721 cells treated with IP-10 siRNA. Cells were stained for expression of CD86 or CD40. E-F. Flow cytometric analysis of DC after administration with lipopolysaccharide (LPS) or co-cultivation with miR-221-overexpression Hepal-6 cells. Cells were stained for expression of CD86 or CD40.

C.



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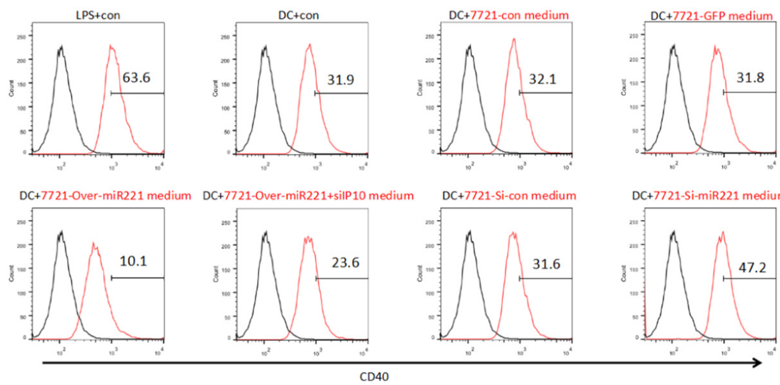

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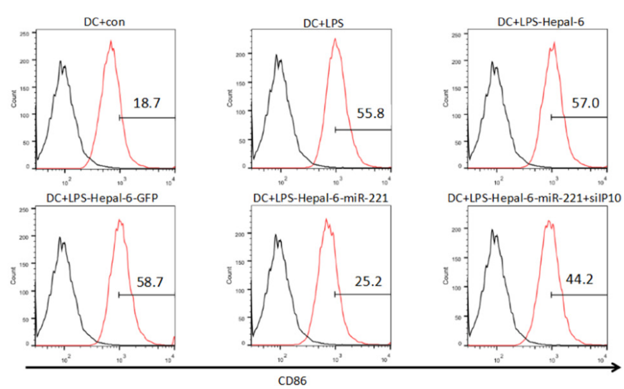

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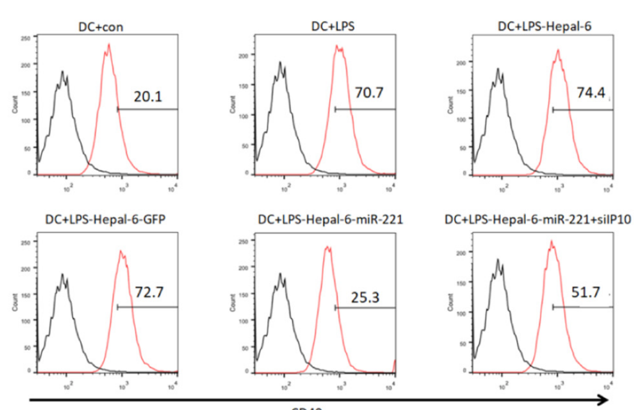

Fig. 2. (*continued*)

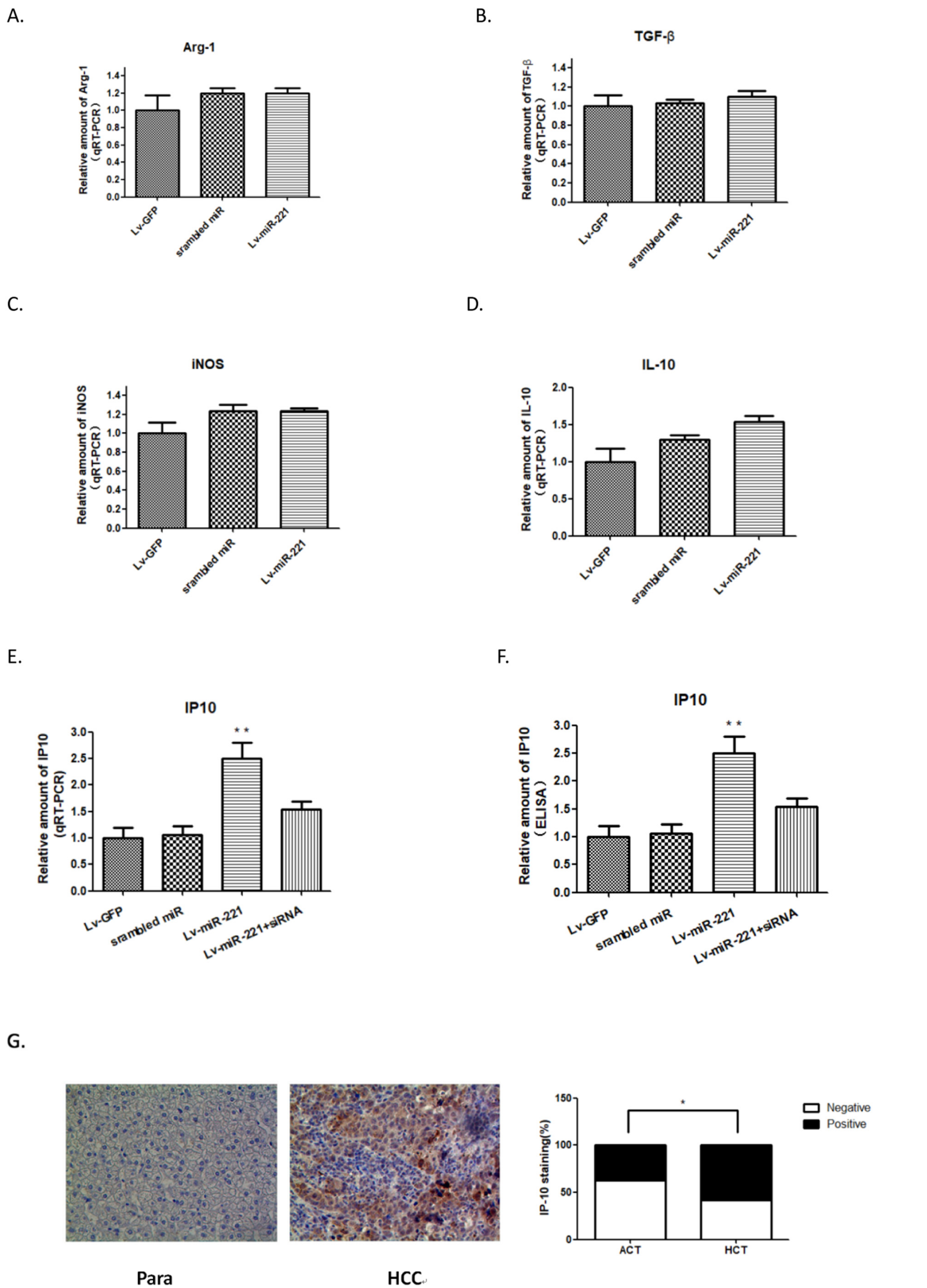

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Fig. 3. qRT-PCR analysis of the cytokine expression in Hepal-6 cell lines transfected with miR-221. A-E. mRNA levels of Arg-1, TGF-β, iNOS, IL-10 in miR-221 overexpression Hepal-6 cell lines and control cells. Data are shown as mean ± SD. **P < 0.01. F. Results of ELISA assay of IP-10 gene in miR-221 overexpression Hepal-6 cell lines and control cells. Data are shown as mean ± SD. **P < 0.01. G. Representative photograph showed IP-10 immunohistochemical staining in HCC tissues and paired non-cancerous tissues, which were taken at ×200 magnifications. Compared with that in the adjacent cancer tissue, the overall expression level of IP-10 in the hepatocellular carcinoma tissues was significantly higher ($P < 0.05$, $\chi 2$ test).

CD40 on DC surface did not change significantly. However, when DC was co-cultured with miR-221 overexpressing stable cell line SMMC-7721, the expression of CD86 and CD40 on DC surface was significantly lower than that of the control group (DC + 7721 + GFP). Meanwhile, when DC was co-cultured with si-miR-221 stable cell line SMMC-7721, the expression of CD86 and CD40 on DC surface was significantly higher than that of the control group (DC + 7721 + si-miR-221) (Fig. 2A-B). A similar expression trend of CD40 and CD80 was observed after DCs cultured in the conditioned medium from the SMMC-7721 cells with various treatments as in the Fig. 2 A and B (Fig. 2C-D). These data may demonstrate that DC maturation was affected by secretion of soluble factors after miR-221 was transfected into hepatoma cells. In addition, miR-221 inhibits the expression of CD86 and CD40 of DC cultured with Hepal-6 cell line (Fig. 2E-F). These results suggest that miR-221 inhibits the maturation of DCs in the mouse liver cell microenvironment.

3.3. Regulation of inflammatory cytokine via miR-221

Differentiation and maturation of immune cells can be regulated by cytokines such as IL-12p40, IL-6 and TGF-α [26] to promote DC maturation, while Arg-1, TGF-β, iNOS, IL-10 and IP-10 [27–31] is an inhibitory regulator of immune cells. The previous results have confirmed that miR-221 could inhibit the maturation of DC. To study the functional role of miR-221, we extracted the mRNA of miR-221 overexpressing Hepal-6 cells and examined the mRNA level of cytokine Arg-1, TGF-β, iNOS, IL-10 and IP-10. Results showed that Arg-1, TGF-β, iNOS and IL-10 was not significantly affected by modulation of miR-221 expression (Fig. 3A-D). However, overexpression of miR-221 significantly increased the expression of IP-10 (Fig. 3E-F). To validate whether miR-221 inhibits the maturation of DCs through regulating IP-10, Flow cytometry analysis was carried out following miR-221-overexpression SMMC-7721 cells treated with IP-10 siRNA. Results in Fig. 2 showed that the interference of IP-10 increased the expression of CD86 and CD40 on DC surface. In adjacent cancerous tissues, high IP10 staining was recorded in 37.5% (9 of 24 cases). In HCC tissues, high expression of IP10 was observed in 58.3% (14 of 24 cases). Higher expression of IP10 was detected in carcinoma tissues compared to the adjacent cancerous tissues ($P < 0.05$, paired $\chi 2$ test) (Fig. 3G). These results suggest that IP-10 may be involved in miR-221 induced DC mature inhibition.

3.4. Myrothecine A inhibits the proliferation of HCC cells

To investigate the effect of myrothecine A on the proliferation of HCC cells, we treated SMMC-7721 cells with different concentrations of myrothecine A (0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, 5 μg/ml, and 10 μg/ml, 20 μg/ml, 50 μg/ml) and the same concentration of 5-fluorouracil (5-FU) was used as a positive control. After 24 h, the proliferation of SMMC-7721 cells was detected by MTT assay. As shown in Fig. 4A, the growth inhibition on HCC cells was not significant when the concentration of myrothecine A was 0.01 μg/ml and 0.1 μg/ml. When the drug concentration reached 1 μg/ml, myrothecine A could significantly inhibit the cell proliferation and the inhibition rate showed a dose-dependent manner with increasing concentration (Fig. 4A). In order to minimize the toxicity of the drug to cells, myrothecine A was used at a concentration of 1 μg /ml in subsequent studies. To investigate whether myrothecine A inhibits proliferation of HCC cells concerning the cell cycle, flow cytometry was used to detect the cell cycle of SMMC-7721 cells at different concentrations of myrothecine A. In the control group, 8.38% of SMMC-7721 cells were in S phase, while cells were treated with 1 μg/ml, 5 μg/ml, and 10 μg/ml of myrothecine A, the proportion of S phase cells increased to 11.86%, 22.78%, and 39.46% respectively. At the same time, the proportion of cells in G0/G1 phase showed a significant decreasing inclination (Fig. 4B). These results suggest that myrothecine A could induce S phase arrest in SMMC-7721 cells, thereby leading to the inhibition of cell proliferation.

3.5. Myrothecine A rescues the cell proliferation induced by miR-221

As mentioned above, miR-221 could significantly promote the proliferation of SMMC-7721 cells. However, when miR-221 overexpressing cells were treated with 1 μg/ml myrothecine A for 24 h and we found that cell proliferation rate decreased by 23% (compared to the Lv-miR-221 group) (Fig. 4C). This suggests that myrothecine A could rescue the cell proliferation induced by miR-221. To further investigate the relationship of myrothecine A and miR-221, we treated miR-221 overexpressing or non-transfected HCC cells with myrothecine A at 1 μg/ml for 24 h and then detected the mRNA level of miR-221. The results showed that the expression of miR-221 was significantly reduced with the treatment of myrothecine A (Fig. 4D).

p27 is an inhibitor of cyclin-dependent kinase and a direct target molecule of miR-221 that negatively regulates cell proliferation and differentiation. Our results showed that miR-221 overexpression significantly downregulated the protein level of p27 (Fig. 4E left panel). Then, we added 1 μg/ml myrothecine A to treat the miR-221 overexpressing cells and used western blot to detect the protein level of p27. We found that myrothecine A significantly reversed the downregulation of p27 expression induced by miR-221 (Fig. 4E right panel). These results may suggest that myrothecine A could enhance the protein level of p27 through reducing the expression of miR-221, thereby inhibiting the proliferation of SMMC-7721 cells.

3.6. Myrothecine A rescues the DC maturation inhibition induced by miR-221

To investigate whether myrothecine A have any effect on miR-221-induced DC maturation inhibition, we co-cultured DC or DC + LPS with miR-221 overexpressing cell line Hepa1–6 for 3 days. Then, 1 μg/ml myrothecine A was added. After 24 h, the cells were collected and subjected to flow cytometry. Results showed (Fig. 5A-B) that the addition of myrothecine A to DC or DC-Hepa1–6 co-culture systems has little effect on the change of CD86 and CD40 molecules. When DC was co-cultured with miR-221 overexpressing stable cell lines, the expression of CD86 and CD40 on DC surface was significantly decreased, which is consistent with the previous results. On this basis, the addition of myrothecine A increased the expression of CD86 and CD40. This result indicated that myrothecine A partially reversed the inhibition of DC maturation induced by miR-221.

4. Discussion

HCC is the third leading cause of cancer-related death worldwide. Although the traditional treatment was effective by interfering with cell division such as chemotherapies, they usually cause severe side effects such as myelotoxicity [32]. Previous study has identified a multi-target anti-angiogenic agent (sorafenib) to be the first systemic therapy approved for the treatment of advanced HCC [33]. The achievement of

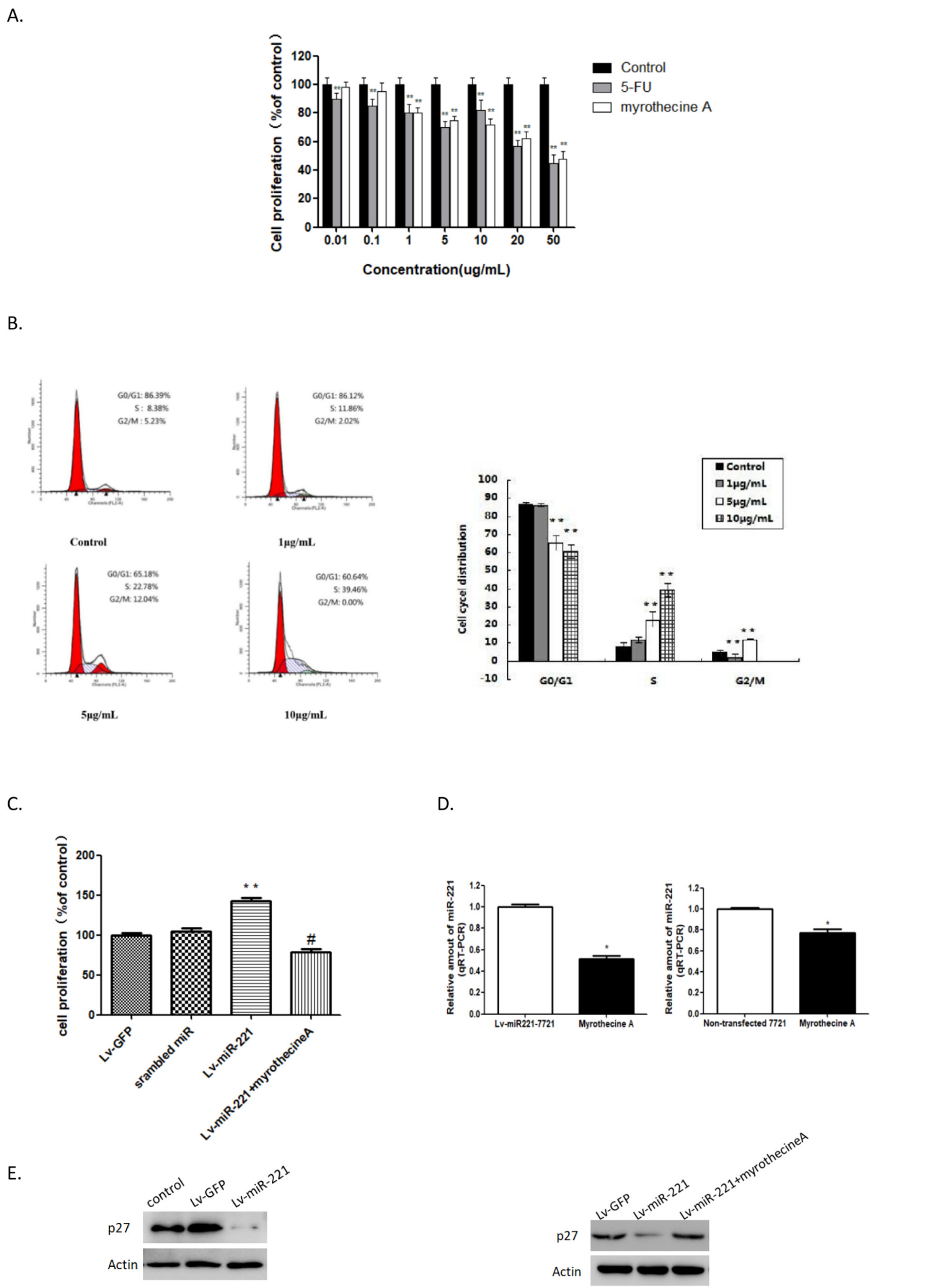

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Fig. 4. Myrothecine A inhibits the cell proliferation and rescues the cell proliferation induced by miR-221 in SMMC-7721 cell lines. A. MTT assays were performed in SMMC-7721 cell lines upon different concentrations of 5-FU and myrothecine A. B. Myrothecine A arrested cell cycle in SMMC-7721 cells. The representative pictures of cell cycle in SMMC-7721 cells with different concentrations of myrothecine A. The percentages of G1/G0, S, G2/M population cells were calculated in SMMC-7721 cells with the different myrothecine A concentrations. Data were presented as mean ± SD, $^{**}P < 0.01$. C. Myrothecine A rescues miR-221 induced cell proliferation (compared with Lv-GFP group, $^{**} p < 0.01$. compared with Lv-miR-221 group, $\# p < 0.05$). D. qRT-PCR was used to detect the endogenous and exogenous miR-221 level in SMMC-7721 cells with myrothecine A (1 μg/ml), $^{*}p < 0.05$. E. Overexpression of miR-221 decreased the expression of p27 (left panel) and myrothecine A rescued the decrease of p27 in SMMC-7721 cell lines (right panel).

sorafenib has aroused an explosive increase of studies concerning novel molecular targets and other agents in the treatment of HCC. Myrothecines A was characterized from the extracts of myrothecium roridum strains IFB-E012 and has potential implications for endophytism (or symbiosis) and biocatabolism [34]. However, its study on anti-tumor activity has rarely been reported. Therefore, this study focuses on the anti-tumor activity of myrothecine A and attempts to elucidate its mechanism associated with miR-221 in the regulation of proliferation of HCC cell line SMMC-7721.

Cancers have been one of the major life-depriving diseases in both developed and developing countries owing principally to the shortage of efficacious antitumor agents. Some microbial metabolites were proven to be very toxic to cancerous cells and tissues [35,36]. In our study, we found that low concentration of myrothecine A had little effect on the proliferation of SMMC-7721 cells. However, when the concentration reached 1 μg/ml, myrothecine A significantly inhibited the proliferation of liver cancer cells. We further investigated the effect of myrothecine A on the cell cycle of SMMC-7721. The results showed that myrothecine A blocked SMMC-7721 cells in S phase. Tumor development is a complex process that is often accompanied by aberrant expression of multiple miRNAs including miR-221. We first constructed overexpressing miR-221 HCC cell model, and found that miR-221 overexpression could significantly promote the proliferation of HCC cells. In the previous study, we found that myrothecine A inhibits the proliferation of SMMC-7721 and induces S-phase arrest in SMMC-7721 cells. Then we investigated whether the mechanism of cell proliferation inhibition was related to miR-221. Therefore, we next determined the effect of myrothecine A on miR-221 expression. The results showed that myrothecine A was able to inhibit the expression level of miR-221. In addition, we also found that myrothecine A rescued cell proliferation induced by miR-221. Previous studies showed that miR-221 could affect HCC prognosis through targeting p27 [16,37]. p27 is a cyclin dependent kinase (CDK) inhibitor, identified as a critical factor in the cell cycle regulatory cascade, and identified to be involved in the prognosis of several types of cancer [38]. Therefore, we further explored the mechanism by which myrothecine A inhibits the proliferation of SMMC-7721, and found that overexpression of miR-221 in hepatoma cells can reduce the expression level of p27. After treatment with 1 μg/ml myrothecine A, the level of p27 expression increased significantly. These results demonstrated that myrothecine A could rescue the down-regulation of p27 expression by miR-221.

Recent studies indicated that miRNAs have unique expression profiles in cells of the innate and adaptive immune systems. Emerging data have identified an important contribution of miRNAs to the function and development of DCs [39]. Moreover, the regulation of miR-22 showed significant effects on the mRNA abundance of Irf8, which plays essential roles in DC development [40]. Inhibition of microRNA let-7i depresses maturation and functional state of DCs as evidenced by reduced CD80 and CD86 expression [24]. In addition, a large number of studies have shown that miRNAs could regulate the maturation process of DCs. Chen et al. [25] found that miR-146a negatively regulates the maturation of DC and the production of pro-inflammatory factors through targeting CD40L. Ceppi et al. [41] found that the expression of miR-155 was significantly increased during LPS-induced DC maturation. Numerous studies have showed that miRNAs were involved in DC maturation, including miR-146a, miR-155, miR-142-3p [42], miR-148 family [43] and miR-221 [44]. To further study the effect of miR-221 on the DC maturation process, we co-cultured mouse hepatoma cell line transfected with miR-221 overexpression plasmid and mouse bone marrow-derived DC. And we found that overexpression of miR-221 downregulated the expression of CD86 and CD40 on DC surface, indicating that miR-221 could inhibit the maturation of DC in the microenvironment of liver cancer cells.

Macrolides have received considerable attention for their anti-inflammatory and immune modulatory actions beyond the antibacterial effect [45]. As one category of macrolides, the effect of Myrothecine A has not been explored on DC. Previous study showed some drug such as pemetrexed may target miRNAs to increase its cytotoxicity [46]. Our study showed that Myrothecine A could downregulate the expression of miR-221 in human hepatoma cells both in endogenous and exogenous. In addition, Myrothecine A could reverse cell proliferation induced by miR-221. To investigate whether myrothecine A could reverse the DC maturation inhibition caused by miR-221. We co-cultured miR-221 overexpressing mouse hepatoma cell line with mouse bone marrow-derived DC and added myrothecine A. Then CD86 and CD40 molecules on DC surface were detected by flow cytometry. The results showed that myrothecine A rescued DC maturation inhibition by miR-221.

Cytokines are a class of small molecule peptides secreted by cells that play a pivotal role in the immune response. IL-6, IL-12 and TNF-α are considered an important pro-inflammatory cytokine and promote the maturation of DCs [26]. However, some other cytokines can inhibit the differentiation, maturation and function of immune cells. Arg-1 could inhibit the antigen-specific T-cell responses via regulating NOS2 expression [27]. When activated by DCs in the presence of TGF-β, CD41 T cells exhibited a reduced capacity to proliferate [28]. IL-10 impaired the capacity of DCs to stimulate cell proliferation [30]. Injection of IP-10 specific antibody into adenovirus-infected mice inhibits the accumulation of T cells [31]. In our study, qRT-PCR was used to detect the expression of some cytokines (Arg-1, TGF-β, iNOS, IL-10 and IP-10) which inhibited immune cells after miR-221 overexpression. The results showed that there was no significant change in the expression of Arg-1, TGF-β, iNOS, IL-10 compared with the control group, and the expression of IP-10 was significantly increased. This suggests that IP-10 may be involved in the inhibition of DC maturation by miR-221.

In our study, we found that myrothecine A could increase the protein level of p27 by inhibiting the expression of miR-221, thereby inducing S phase arrest in cells and inhibiting SMMC-7721 cell proliferation. MiR-221 could regulate the maturation of DC in the microenvironment of hepatoma cells, while myrothecie A could reverse the inhibitory effect of miR-221 on DC maturation. This result provides us with further study on the immunotherapy of tumors and the anti-tumor mechanism of macrolides. Taken together, these results demonstrated a theoretical basis for myrothecine A to become a novel tumor treatment drug, and also contribute to the development of molecular targeted therapy for liver cancer.

Declaration of Competing Interest

The authors declare no conflicts of interest, financial or otherwise.

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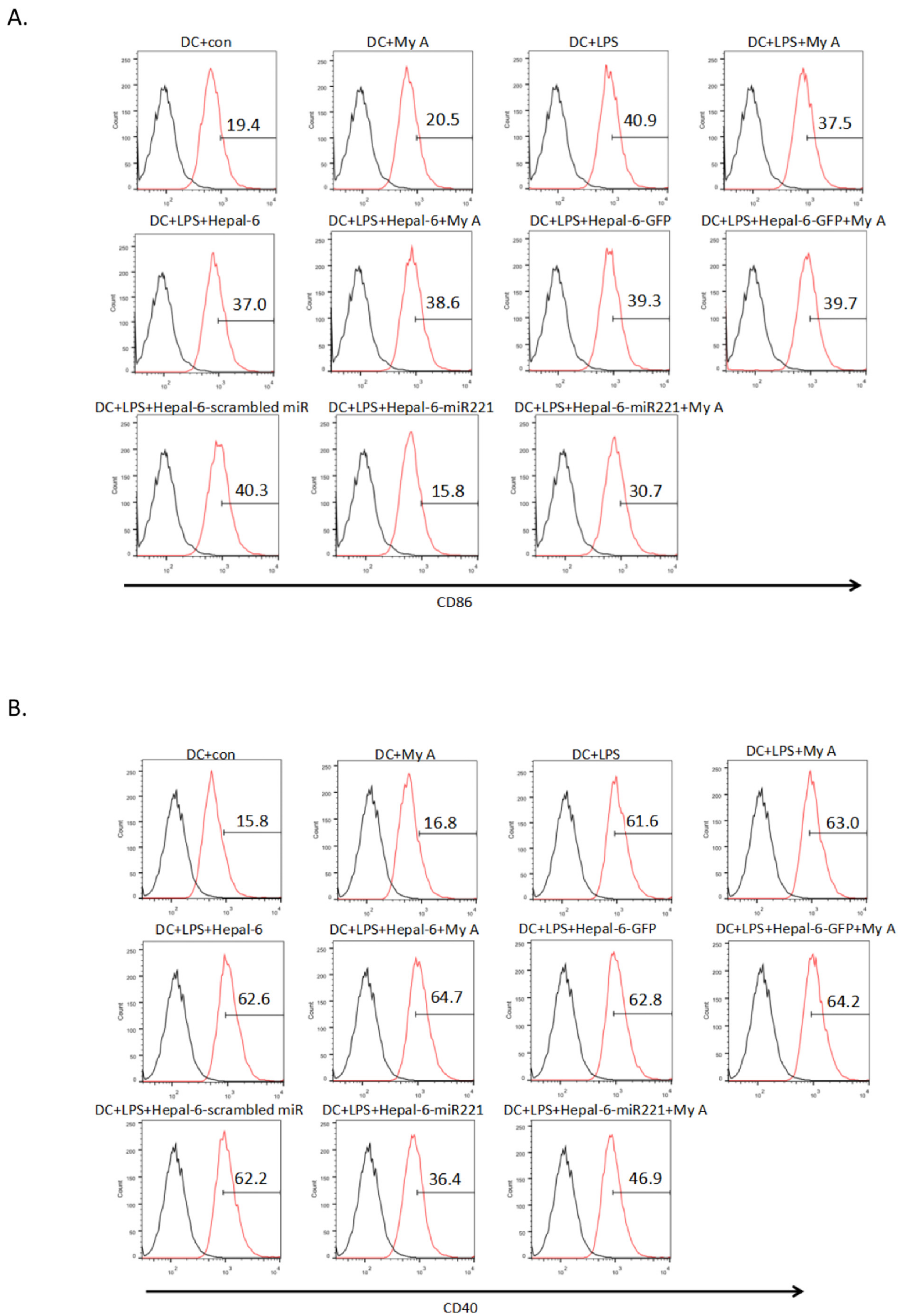

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Fig. 5. Myrothecine A rescues the inhibition of the maturation of dendritic cells in liver cancer microenvironment of mice. A-B. Flow cytometric analysis of DC after treated with lipopolysaccharide (LPS) or co-cultivation with miR-221-overexpression Hepal-6 cells and myrothecine A. Cells were stained for expression of CD86 or CD40. Myrothecine A rescues the inhibition of DC maturation induced by miR-221.

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