Astragaloside IV augments anti-PD-1 therapy to suppress tumor growth in lung cancer by remodeling the tumor microenvironment

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ABSTRACT

Programmed cell death protein-1 (PD-1) inhibitors are increasingly utilized in the treatment of lung cancer (LC). Combination therapy has recently gained popularity in treating LC. This study aimed to assess the efficacy of combining Astragaloside IV (AS-IV) and anti-PD-1 in LC. C57BL/6J mice were subcutaneously injected with Lewis lung carcinoma (LLC) cells. After 3 weeks, the animals were sacrificed, and the tumors were harvested for analysis. Ki-67 immunolabeling and TUNEL assay were used for evaluating cell proliferation and apoptosis in tumor tissues. In addition, anti-cleaved caspase 3 was used for immunolabelling of apoptotic cells. Immune cell infiltration (macrophages and T cells) and gene expression in tumor tissues were also investigated by using immunofluorescence staining. Compared to treatment with anti-PD-1 or AS-IV, the combination of AS-IV and anti-PD-1 notably reduced tumor volume and weight of LLC-bearing mice. Additionally, the combination treatment strongly induced the apoptosis and suppressed the proliferation in tumor tissues through inactivating PI3K/Akt and ERK signaling pathways, compared to single treatment group. Moreover, the combination treatment elevated levels of the M1 macrophage marker mCD86, reduced levels of the M2 macrophage marker mCD206, as well as upregulated levels of the T cell activation marker mCD69 in tumor tissues. Collectively, the combination treatment effectively inhibited tumor growth in LLC mice through promoting M1 macrophage polarization and T cell activation. These findings showed that combining AS-IV with anti-PD-1 therapy could be a promising therapeutic approach for LC.

Key words: lung cancer; PD-1; immunotherapy; Astragaloside IV; T cell activation; macrophage.

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Introduction

Lung cancer (LC) is a malignant cancer that originates from the bronchial mucosa or lung glands.[1] In 2022, LC was the most commonly diagnosed cancer accounting for nearly 2.5 million new cases. It is also the primary cause of cancer-related death worldwide.[2] LC is classified into small-cell LC (SCLC) accounting for 15% of LC cases and non-small-cell LC (NSCLC) making up the remaining 85%.[3] Current treatment options for LC encompass surgery, radiotherapy, chemotherapy, immunotherapy, targeted therapy, *etc.*[4] Immunotherapy has shown significant advancements in the treatment of LC, offering new hope for individuals with advanced stages of the disease and emerging as a crucial therapeutic approach for advanced LC.[5]

Programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) are key targets in cancer immunotherapy.[6] PD-1, an immunosuppressive molecule, is widely expressed on immune cells,[7] while PD-L1, a ligand of PD-1, is predominantly found on the surface of tumor cells.[8-10] The interaction between PD-L1 and PD-1 inhibits T-cell receptor signaling and suppresses antitumor immune response.[11,12] Anti-PD1 therapy, which blocks the interaction between PD-L1 and the PD1, reactivates anti-tumor immune responses and significantly enhances the survival of patients with cancer.[13,14]

The efficacy of the combination therapy in treating tumors has been demonstrated to be superior to single treatments.[15] Recent advancements in the field have shown that immunotherapy in conjunction with radiotherapy or chemotherapy has significantly improved patient outcomes.[15,16] Clinical research has highlighted the notable benefits of traditional Chinese medicine (TCM) as a complementary anti-tumor treatment.[17] *Astragalus membranaceus,* a kind of TCM, has been previously studied for its anti-tumor properties, metabolic regulation, and immune enhancement.[18] Astragaloside IV (AS-IV), a key active compound derived from Astragali,[19] has been identified for its ability to inhibit LC invasion and metastasis.[20].However, the potential of AS-IV to enhance the anti-tumor effects of anti-PD1 therapy remains largely unexplored. Our study reveals that the combination of anti-mPD-1 with AS-IV demonstrates anti-tumor effects in Lewis lung carcinoma (LLC) mice by influencing macrophage polarization and T cell activation. These findings suggest a promising new approach for the treatment of LC.

Materials and Methods

Cell culture

The mouse lung cancer LLC cell line was obtained from Procell (Wuhan, China) and STR profiling was used to validate the authentication of cell line. Cell lines were maintained in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% FBS and 1% Penicillin-Streptomycin and cultured at 37°C in a humidified 5% CO_2 atmosphere.

Animal study

Female, 6–8-week-old C57BL/6J mice were obtained from SiPeiFu (Beijing, China), and kept in specific pathogen-free (SPF) conditions. Male C57BL/6J mice are prone to fight comparing with female one, thus female mice were used in this study. All experimental procedures were conducted in compliance with the guidelines of NIH and the Ethics Committee of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine Hospital approved this research. Animals were randomly assigned to four groups: control; anti-mPD1 10 mg/kg intraperitoneally (i.p.), twice weekly (BIW); AS-IV 40 mg/kg, every three days (Q3D) and anti-mPD1 + AS-IV groups. LLC cells (5×10^6 cells per mouse) were subcutaneously injected into the left flank of each mouse. Anti-mPD-1 antibody was administered intraperitoneally at a dose of 10 mg/kg twice a week. AS-IV (40 mg/kg) was injected into the mice Q3D. Tumor size was measured every 3 days using Vernier calipers and tumor volume was calculated (length × width × width × 0.5). After 3 weeks, tumors and spleen were collected for analysis. AS-IV was purchased from MCE (Shanghai, China).

Immunofluorescence staining assay

Tumor and spleen tissues were fixed in 4% paraformaldehyde at 4°C for 24 h, embedded in paraffin and cut into 4-µm sections. Paraffin-embedded tumor or spleen tissue sections were dewaxed in xylene and dehydrated in ethanol. Following this, sections were blocked with 5% BSA for 1 h and were incubated overnight at 4°C with primary antibodies including anti-Ki-67 (1:500, No. ab15580; Abcam, Waltham, MA, USA), anti-cleaved caspase 3 (1:500, No. 25128-1-AP; Proteintech, Wuhan, China), anti-caspase 3 (1:500, No. ab32351; Abcam), anti-mCD86 (1:400, No. 13395-1-AP; Proteintech), anti-mCD206 (1:400, No. 18704-1-AP; Proteintech), anti-mCD3 (1:400, No. 17617-1-AP; Proteintech) and anti-mCD69 (1:400, No. 10803-1-AP; Proteintech) antibodies. Nuclei were counterstained with DAPI (10 µg/mL). Signal visualization was achieved using fluorescently labeled secondary antibodies (1:1000, No. ab150077, ab150079; Abcam) and images were captured using a fluorescence microscope (Nikon Eclipse Ci-L, Monato, Tokyo, Japan) and the fluorescence intensity was quantified using the Image-Pro Plus software. Stained sections incubated without primary antibodies were used as negative controls.

TUNEL staining assay

Sections were stained with the TUNEL mixtures (Boster Biological Technology, Wuhan, China) at 37°C with no light for 1.5 h. Subsequently, cell nuclei were stained with DAPI, and the result was examined using a fluorescence microscope (Nikon Eclipse Ci-L). There fields of TUNEL staining were randomly selected, and the positive rate of TUNEL-positive cells in each group were counted. Then, a mean of positive rate in each group was counted.

Western blot assay

Proteins (30 µg/lane) were separated *via* 10% SDS-PAGE and then transferred onto a PVDF membrane (Millipore). Later on, the membrane was probed with primary antibodies against p-PI3K (1:1000, No. #AF3241, Affinity Biotech, Houston, TX, USA), PI3K (1:1000, No. #AF6241; Affinity), p-Akt (1:1000, No. #AF0016; Affinity), Akt (1:5000, No. 60203-2-Ig, Proteintech;), p-ERK1/2 (1:1000, No. ab201015; Abcam), ERK1/2 (1:1000, No. ab184699; Abcam), and GAPDH (1:50000, No. 60004-1-Ig; Proteintech) separately at 4°C overnight. Subsequently, the membrane was probed with the corresponding secondary antibody for 1 h. Subsequently protein bands were visualized using ECL reagent.

Statistical analysis

Data are expressed as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's tests were used to determine the differences between three or more groups. All data were independently repeated at least three times; p values <0.05 were considered as statistically significant.

Results

AS-IV enhances the anti-tumor effect of anti-mPD1 in LLC mice

The anti-tumor effects of AS-IV in combination with anti-mPD1 treatment were evaluated in subcutaneously transplanted mice with LLC cells. As shown in Figure 1 A,B, compared to control group, anti-mPD1 or AS-IV alone treatment showed a partial reduction in tumor volume of LLC mice. However, the combination treatment demonstrated significantly greater inhibition of tumor growth and weight compared with the single treatment group (Figure 1 A,B). Additionally, neither anti-mPD1 or AS-IV treatment led to a decrease in Ki-67 positive cells in tumor tissues of LLC mice (Figure 1C). As expected, the combination treatment further reduced the percentage of Ki-67-positive cells in tumor tissues of LLC mice compared with the single treatment group (Figure 1C). Moreover, neither anti-mPD1 or AS-IV treatment elevated TUNEL positive cell rate and upregulated cleaved caspase 3 protein expression in tumor tissues (Figure 2 A,B). As expected, the combination treatment further increased TUNEL positive cell rate and cleaved caspase 3 expressions in tumor tissues of LLC mice compared with the single treatment group (Figure 2 A,B). Collectively, AS-IV could enhance the anti-tumor effect of anti-mPD1 in LLC mice.

Combining anti-mPD-1 with AS-IV exhibits a significant anti-tumor effect in LLC mice through modulating macrophage polarization

It has been shown that tumor-associated macrophages (TAMs) play crucial roles in cancer progression.[21] Thus, we explored the potential of AS-IV to augment the anti-tumor efficacy of anti-mPD1 by influencing macrophage polarization. As indicated in Figure 3 A,B, anti-mPD1 treatment had little effect on the expressions of macrophage M1 marker mCD86 and macrophage M2 marker mCD206 in tumor tissues. However, AS-IV alone treatment significantly increased mCD86 protein expression and reduced mCD206 protein expression in tumor tissues of LLC mice (Figure 3 A,B). Notably, compared with the single treatment group, these effects were further potentiated by the combination treatment (Figure 3 A,B). Collectively, AS-IV could enhance the anti-tumor properties of anti-mPD1 through promoting the transition macrophages from pro-tumor M2 phenotype to anti-tumor M1 phenotype.

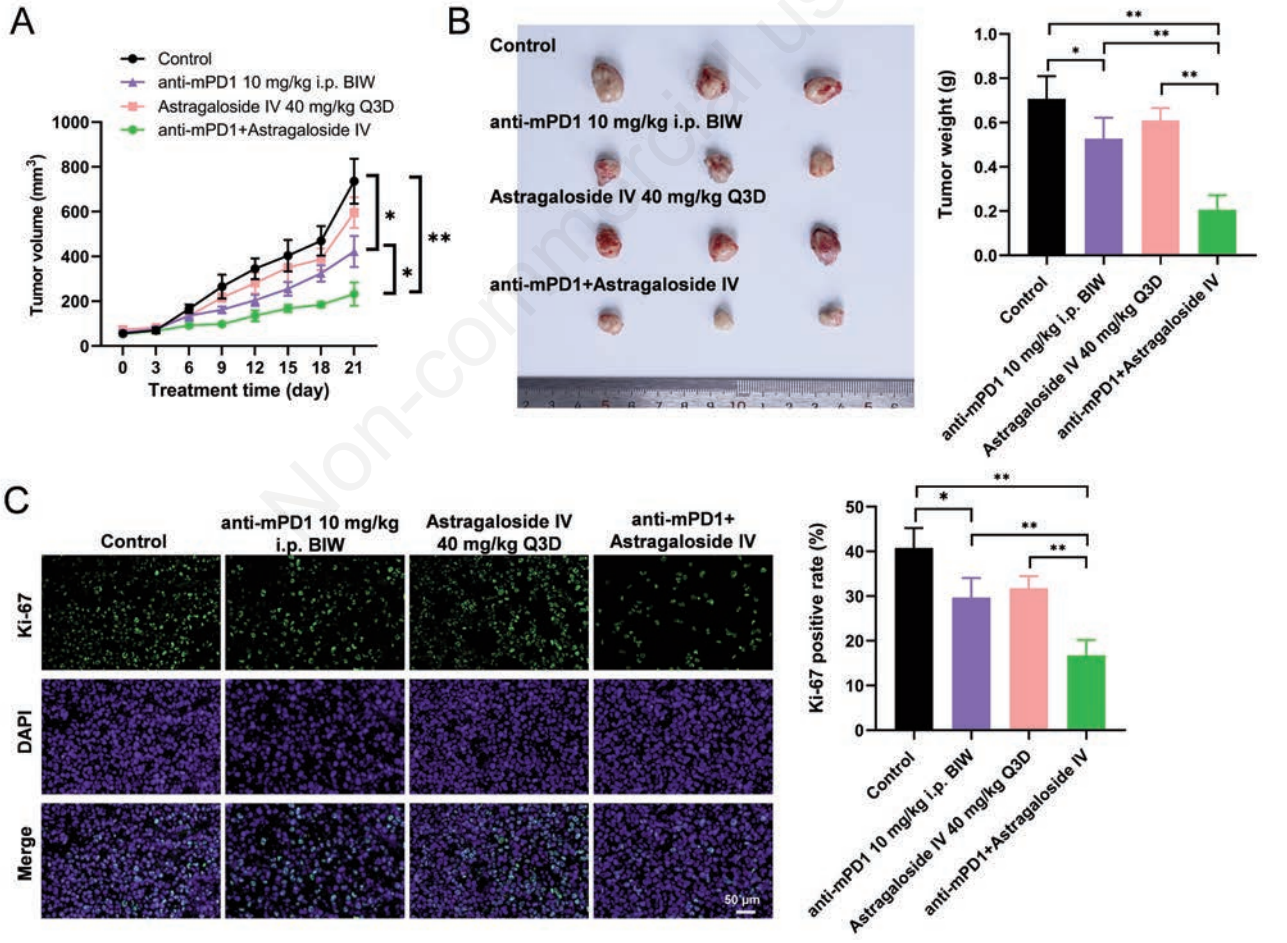

Figure 1. AS-IV enhances the anti-tumor role of anti-mPD1 in LLC mice. **A**) Volume of tumor in each group. **B**) Representative pictures of tumors; the weight of tumor in each group was monitored. **C**) Expression of Ki-67 in tumors of LLC mice was assessed by immunofluorescence analysis. *p<0.05, **p<0.01.

Combining anti-mPD-1 with AS-IV exhibits a significant anti-tumor effect in LLC mice through promoting T cell activation

T cell activation plays an important role in anti-cancer immunity and immunotherapy efficacy.[22,23] Thus, we assessed mCD3 (a T cell marker) and mCD69 (a marker of activated T cells) expression levels in tumor and spleen tissues of LLC mice. As shown in Figure 4 A-D, anti-mPD1 or AS-IV treatment led to a significant increase in mCD3 and mCD69 expressions in both tumor and spleen tissues of LLC mice. As expected, the combination treatment resulted in a further enhancement of mCD3 and mCD69 expressions in these tissues when compared to the single treatment group (Figure 4 A-D). These results suggested that combining anti-mPD-1 with AS-IV could exhibit a significant anti-tumor activity in LLC mice by promoting T cell activation.

Combining anti-mPD-1 with AS-IV exerts anti-tumor effects through inhibiting PI3K/Akt and ERK signaling pathways

To delve deeper into the detailed anti-cancer mechanism of anti-mPD-1 and AS-IV co-treatment, we evaluated the effect of the combination therapy on AKT and ERK signaling pathways. As shown in Figure 5 A-D, anti-mPD1 or AS-IV treatment led to a significant decrease in p-PI3K, p-Akt and p-ERK levels in tumor tissues of LLC mice. As expected, the combination treatment further reduced p-PI3K, p-Akt and p-ERK levels in tumor tissues of LLC mice in comparison to the single treatment group (Figure 5 A-D). These results showed that combining anti-mPD-1 with AS-IV could exert anti-tumor effects through inhibiting PI3K/Akt and ERK signaling pathways.

Discussion

AS-IV demonstrated a range of pharmacological properties including immunomodulatory, antioxidant, and anti-hypoglycemic activities.[24] Additionally, recent research has also highlighted its potential anti-tumor effects in various cancer types, such as cervical cancer, breast cancer and NSCLC.[25-2]. Furthermore, previous studies have shown that combining AS-IV with chemotherapy can enhance tumor sensitivity to chemotherapeutic drug.[25,26,28] For example, Zheng *et al.* found that AS-IV could improve taxol chemo-sensitivity in breast cancer.[25] Similarly, Liu *et al.* demonstrated that AS-IV could enhance the anti-tumor effects of propofol in NSCLC cells through affecting cell autophagy.[26] Additionally, Lai *et al.* discovered that AS-IV was capable of sensitizing NSCLC cells to cisplatin through preventing endoplasmic reticulum stress.[28] However, the potential of AS-IV to enhance the anti-tumor effects of anti-PD-1 therapy in LC remains unclear. Our study revealed that the combination of AS-IV and anti-mPD-1 exhibited significant anti-tumor effects in LLC mice when compared to the single treatment group. These results highlighted the effectiveness of the combination treatment as a strategy to increase the vulnerability of tumor cells.

TAMs are one of the most prevalent types of immune cells that infiltrate the tumor microenvironment (TME).[29,30] Macrophages play crucial roles in immunity and cancer development, and have a controversial role in pro- and anti-tumoral effects.[21] Specifically, pro-inflammatory macrophages (M1 phenotype) are capable of engulfing tumor cells, whereas anti-inflammatory macrophages (M2 phenotype), also known as TAMs, tend to support tumor growth.[30] Research has shown that TAMs significantly impact the

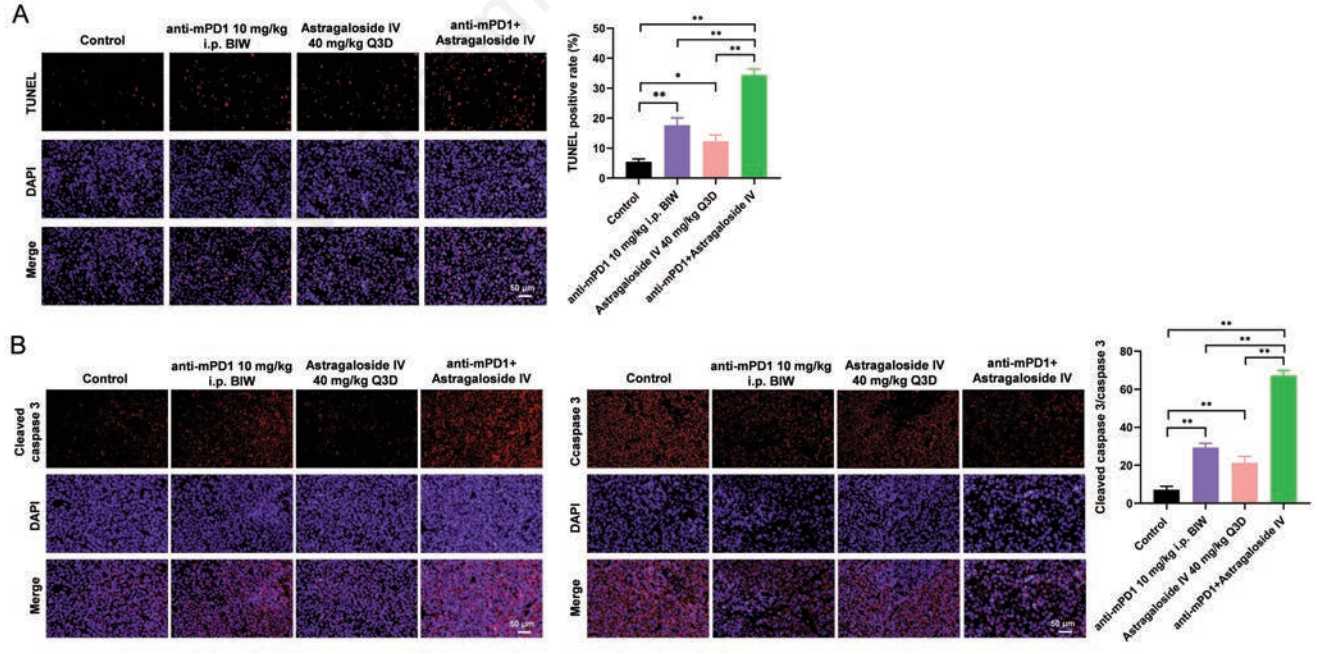

Figure 2. Combining anti-mPD-1 with AS-IV induces the apoptosis in tumor tissues of LLC mice. **A**) Cell apoptosis in tumors of LLC mice was assessed by TUNEL staining analysis. **B**) The expression of Ki-67 in tumors of LLC mice was assessed by immunofluorescence analysis. *$p<0.05$, **$p<0.01$.

effectiveness of PD-1/PD-L1 inhibitors in cancer treatment.[31] TAMs, as a type of immunosuppressive cells, impede the infiltration and activation of T cells, thereby restricting the effectiveness of immune checkpoint blockade in combating cancer.[32] Consequently, targeting TAMs represents a promising approach to bolster anti-tumor immunity.[33] Previous studies have demonstrated that AS-IV has the ability to transition from a pro-tumor macrophage phenotype to an anti-tumor macrophage phenotype in the TME in cancer.[27,34] For instance, Shen *et al.* observed that AS-IV could inhibit the migration and epithelial-mesenchymal transition in cervical cancer cells through suppressing macrophage M2 polarization.[27] Similarly, Yu *et al.* reported that AS-IV could inhibit the polarization of macrophages towards the M2 phenotype, consequently impeding breast cancer progression.[34] Additionally, Min *et al.* indicated that AS-IV could prevent the migration and invasion of liver cancer by inhibiting M2 macrophage polarization.[35] In this study, it was observed that anti-mPD1 treatment had very limit effect on the expressions of mCD86 and mCD206 in tumor tissues of LLC mice. However, AS-IV treatment led to a significant increase in CD86 protein expression and a decrease in mCD206 protein expression in tumor tissues. Interestingly, compared to anti-PD1 or AS-IV single treatment group, combination of anti-PD1 and AS-IV further increased mCD86 expression and reduced mCD206 expression in tumor tissues of LLC mice. These findings suggested that AS-IV has the potential to augment the anti-tumor effects of anti-mPD1 through promoting the transition of macrophage from a pro-tumor M2 phenotype to an anti-tumor M1 phenotype.

T lymphocytes are crucial components in adaptive immunity and serve as central players in the immune system.[36] Within tumor sites, T lymphocytes are referred to as tumor-infiltrating lymphocytes (TILs), and play a significant role in anti-tumor immune responses.[37,3]. Specifically, CD8+ TILs are essential for eliminating tumor cells.[39] Nevertheless, in the TME, TILs can become functionally impaired, leading to a state of T cell exhaustion that hinders anti-tumor immunity. Thus, enhancing T-cell activation could potentially improve immune responses. Research has demonstrated that PD-1 can negatively regulate T-cell activation.[40] Interestingly, AS-IV has been shown to enhance the activity of cytotoxic T lymphocytes.[41,42] For instance, Zhang *et al.* discovered that AS-IV was shown to promote the immune response of tumors in LC by enhancing the activity of cytotoxic T lymphocyte function.[41] Our study revealed that anti-PD1 or AS-IV treatment led to increased levels of mCD3 and mCD69 expression in tumor and

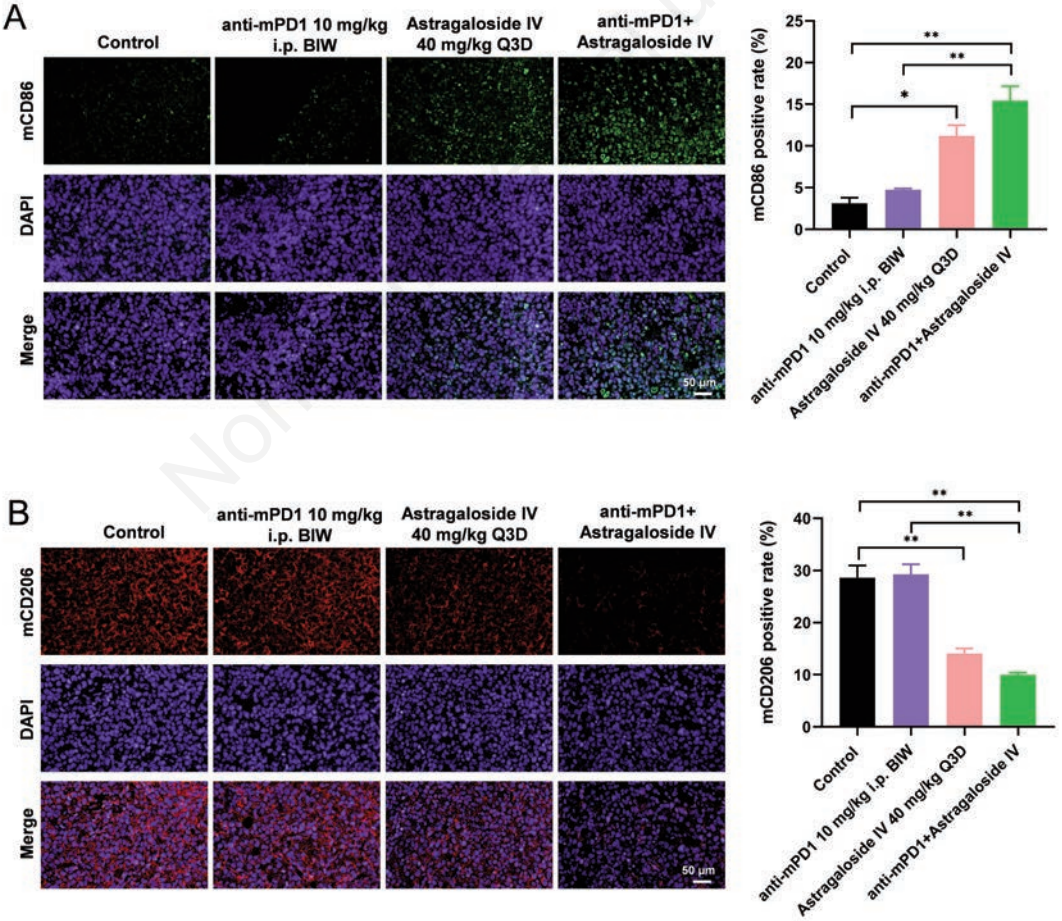

Figure 3. Combining anti-mPD-1 with AS-IV exhibits anti-tumor effects in LLC mice through shifting macrophages from pro-tumor M2 phenotype to anti-tumor M1 phenotype. **A,B**) The expressions of mCD86 and mCD206 in tumors of LLC mice were assessed by immunofluorescence analysis. *p<0.05, **p<0.01.

spleen tissues of LLC mice, suggesting that these treatments could enhance T cell activation in LC. As expected, the combination treatment further upregulated mCD3 and mCD69 levels in tumor and spleen tissues of LLC mice, suggesting that combination treatment could effectively combat tumors in LLC mice through enhancing T cell activation.

Research has demonstrated that hyperactivation of PI3K/AKT and MAPK/ERK signaling pathways were commonly detected in various cancers including LC.[43-45] AS-IV has been shown to inhibit NSCLC progression *via* inhibiting the Akt signaling.[46] Meanwhile, a study by Li *et al.* has revealed that AS-IV could impede glioma progression through suppressing MAPK/ERK signaling.[47] Our study aligns with these findings; we observed that AS-IV treatment led to decreased levels of phosphorylated PI3K, Akt and ERK in tumor tissues of LLC mice, suggesting that AS-IV could suppress tumor growth in LLC mice through inhibiting PI3K/Akt and ERK signaling pathways. Moreover, compared to anti-PD1 alone group, the combination treatment resulted in further reductions in phosphorylated PI3K, Akt and ERK levels in tumor tissues of LLC mice. These findings showed that AS-IV could enhance the anti-tumor effects of anti-PD1 in LC through inhibiting PI3K/Akt and ERK signaling pathways.

The novelty of this study is that we firstly demonstrated that the combination of anti-mPD-1 with AS-IV could exert anti-tumor effects in LLC mice by promoting M1 macrophage polarization and T cell activation. Moreover, AS-IV was able to enhance the anti-tumor effects of anti-PD1 in LLC mice by inhibiting PI3K/Akt and ERK signaling pathways. This finding may shed new light on the treatment of LC. Collectively, AS-IV could enhance the anti-tumor effects of anti-PD1 in LC through influencing immune cell

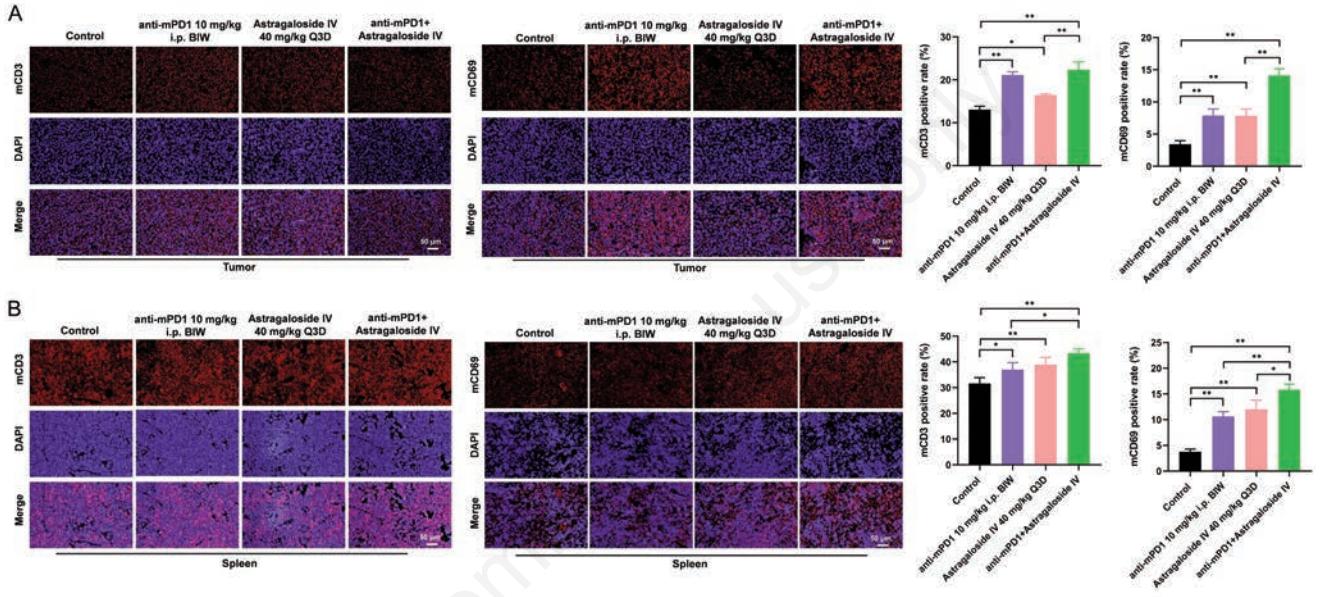

Figure 4. Combining anti-mPD-1 with AS-IV exhibits anti-tumor effects in LLC mice through promoting T cell activation. **A-D**) Expressions of mCD3 and mCD69 in tumors and spleen of LLC mice were assessed by immunofluorescence analysis. *p<0.05, **p<0.01.

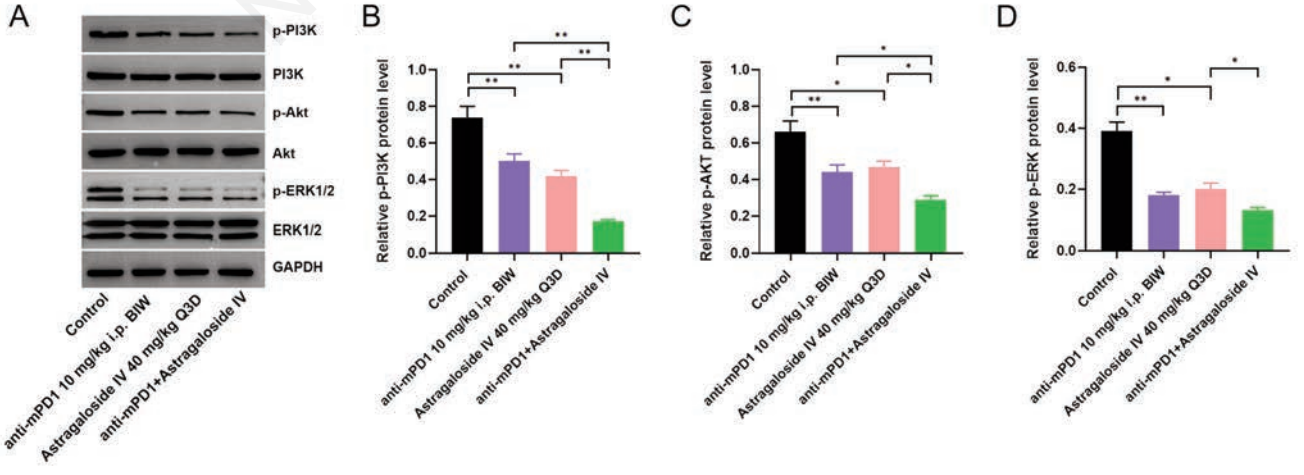

Figure 5. Combining anti-mPD-1 with AS-IV exerts anti-tumor effects through inhibiting PI3K/Akt and ERK signaling pathways. **A-D**) Western blot was used to determine p-PI3K, PI3K, p-Akt, Akt, p-ERK1/2 and ERK protein expressions in tumor tissues of LLC mice. The relative expressions of p-PI3K, p-AKT and p-ERK1/2 were normalized to PI3K, AKT and ERK1/2, respectively. *p<0.05, **p<0.01.

infiltration in the TME and pro-survival signaling pathways within the tumor. However, we only explored the effect of AS-IV on the T cell activation. It is possible that, AS-IV may affect other immune cells including T regulatory cells, dendritic cells and so on. Thereby, more investigations are needed in future.

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