Solamargine inhibited the progression of non-small cell lung cancer *in vitro* by activating T cells

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ABSTRACT

Lung cancer is the leading cause of cancer-related death globally and the most common cancer type. Solamargine is an extract from the traditional Chinese medicine, Long Kui, which exhibits antitumor effects in a number of cancer types, including lung cancer. However, the possible association between solamargine and the tumor microenvironment (TME) in non-small cell lung cancer (NSCLC) remains to be elucidated. In the present study, Cell Counting Kit-8 and 5-Ethynyl-2'-deoxyuridine (EdU) assays were used to evaluate the viability and proliferation of NSCLC cells, respectively. In addition, NSCLC cells were co-cultured with peripheral blood mononuclear cells with or without prior solamargine treatment to evaluate the possible association between solamargine and the TME. The results indicated that solamargine can inhibit NSCLC cell proliferation and migration directly. In addition, it was demonstrated that solamargine can prevent the progression of NSCLC indirectly *via* activating the function of T cells. These findings may provide a novel theoretical basis in drug discovery for the treatment of NSCLC.

Key words: solamargine; non-small cell lung cancer; T cell; tumor microenvironment.

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Introduction

Lung cancer is the leading cause of cancer-related death globally, and the most common type is non-small cell lung cancer (NSCLC).[1-3] NSCLC originates from the alveolar epithelium and bronchial mucosa. The pathogenesis of NSCLC is complex and may be related to the interaction of the patients' own genetics and external factors.[3] A previous study has shown that patients with NSCLC have no typical early clinical symptoms, which may include cough, dyspnea, hemoptysis and fever, all of which lack specificity, leading to the development of middle and late stage disease before diagnosis.[4] Thus, the early diagnosis and treatment of NSCLC is essential to improve the long-term prognosis of patients. In terms of the treatments available for patients with lung cancer, surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy are the main methods for NSCLC; however, the therapeutic effect and prognosis of patients with advanced disease harboring negative driver genes remains poor, and the 5-year survival rate is low.[4]

The tumor microenvironment (TME) refers to the local biological environment of solid tumors, which includes cancerous cells,[5] nearby stromal cells (such as immune cells and fibroblasts), microvessels, cytokines and extracellular matrix.[6] The external mechanism of tumor drug resistance is closely related to the TME, which prevents the immune clearance of tumor cells, impedes drug absorption, stimulates paracrine factors and sends signals for cancer cell growth; it also promotes drug resistance without causing gene mutations and epigenetic changes.[7] Targeting the TME compared with directly targeting cancer cells has a notable therapeutic advantage as cancer cells can easily develop drug resistance due to their genomic instability, whereas non-tumor cells in the TME are genetically more stable and are thus more vulnerable.[7]

Traditional Chinese medicines can be used for tumor treatments due to their immune-regulation functions, multiple targets and fewer side effects.[8] Solamargine is an extract from the traditional Chinese medicine, Long Kui, which has been reported to exhibit antitumor effects in a number of cancer types, including cervical, renal and lung cancer.[9-11] For instance, solamargine has been shown to inhibit growth and induce cell cycle arrest in NSCLC cell lines.[12] Zhou *et al*. reported that solamargine inhibits the proliferation of lung cancer cells through the p38 MAPK-mediated pathway.[13] In addition, solamargine possess other bioactive effects including anti-inflammatory effects.[14] However, to the best of our knowledge, the possible association between solamargine and the TME in NSCLC remains to be elucidated. Therefore, the present study aimed to explore the mechanism underlying the antitumor effects of solamargine on NSCLC.

Materials and Methods

Cell culture

The NSCLC cell lines, A549 and NCI-H441, were provided by the American Type Culture Collection. The A549 and NCI-H441 cells were cultured in F-12K medium and RPMI 1640 medium (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively, in an incubator with 5% CO_2 at 37°C. All mediums were supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 µg/mL streptomycin and 100 µg/mL penicillin. To evaluate the effect of solamargine (MedChemExpress, Princeton, NJ, USA) on NSCLC cells, the A549 and NCI-H441 cells were treated with 2.5, 5 or 10 µM solamargine for 48 h at 37°C.

CCK8 assay

A549 or NCI-H441 cells were seeded into culture plates and incubated at 37°C overnight. Following treatment with 2.5, 5 or 10 µM solamargine at 37°C for 48 h, the cells were further incubated with 10 µL CCK8 reagent (Dojindo Laboratories, Inc., Rockville, MD, USA) at room temperature for 4 h. Subsequently, the absorbance of each well was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.).

Transwell assays

For cell migration analysis, A549 or NCI-H441 cells were seeded into the upper chamber (with serum-free medium) of the transwell insert (3 µm; Corning, Inc., Corning, NY, USA) without Matrigel. Regarding as cell invasion analysis, the upper chamber was pre-coated with Matrigel (1:20; Corning, Inc.) at 37°C for 30 min. After incubation for 12 h at 37°C, cells in the lower chamber (with medium containing 10% FBS) were fixed with 100% anhydrous ethanol for 30 min at room temperature. Then, the cells were stained with 1% crystal violet for 1 h at room temperature. The migrated or invasive cells were subsequently counted on 200× magnification under an inverted light microscope (IX51; Olympus Corporation, Tokyo, Japan). A total of five randomly selected fields of view were quantified.

EdU staining assay

A549 or NCI-H441 cells were incubated in a 24-well plate at 37°C for 48 h. The cells were then treated with EdU (Invitrogen, Waltham, MA, USA; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized in 1.0% Triton X-100 for 10 min and then blocked with a blocking buffer for 1 h. DAPI solution (1:1000; Thermo Fisher Scientific, Inc.) was used to stain the cell nuclei for 15 min at 37°C. The EdU-positive cells were observed and counted on 200× magnification under a fluorescence microscope (IX51; Olympus Corporation). A total of five randomly selected fields of view were quantified.

Cell apoptosis assay

Cell apoptosis was investigated using the TUNEL (TdT-mediated dUTP Nick End Labeling) staining kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, the cells were incubated with 4% paraformaldehyde for 30 min at room temperature, followed by treatment with 0.5% Triton X-100. The cells were subsequently incubated with 50 µL TUNEL reaction buffer for 1 h at 37°C, and then the nuclei were stained with DAPI (1:1000; Thermo Fisher Scientific, Inc.) for 15 min at 37°C. The cells were then blocked with anti-fade mounting medium (Beyotime Institute of Biotechnology). The images from five randomly selected fields were visualized and captured on 200× magnification using a fluorescence microscope (IX51; Olympus Corporation). TUNEL positive cell rate = TUNEL positive cell number / DAPI positive cell number × 100%.

Immunofluorescence staining

A549 or NCI-H441 cells were fixed with methanol for 30 min, permeabilized with 0.1% Triton X-100 for 1 min and then blocked with 10% goat serum (Thermo Fisher Scientific, Inc.) for 2 h at room temperature. Next, the cells were incubated with anti-cleaved caspase-3 primary antibody (1:1000; Abcam, Cambridge, UK) overnight at 4°C. After washing with PBS, the cells were incubated with goat anti-rabbit IgG (Immunoglobulin G) secondary antibody (1:2000; Abcam) for 1 h. Omission of the primary antibody was used for negative controls. After staining with DAPI for 15 min at 37°C, the cells were observed under 200× magnification a IX51

fluorescence microscope (Olympus Corporation). The images from five randomly selected fields were visualized and the immunofluorescence intensity per microscope field was quantified using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Co-culture system

The peripheral blood mononuclear cells (PBMCs) purchased from Shanghai Saili Biopharmaceutical Co., Ltd. (Shanghai, China) were stimulated with 2 µg/mL CD3/CD28 (Thermo Fisher Scientific, Inc.) or 2.5 µM solamargine. Then, A549 or NCI-H441 cells were co-cultured with the stimulated PBMCs (at a 1:10 ratio) for 6 h at 37°C. The viability of the A549 or NCI-H441 cells was subsequently detected with the CCK8 assay and the CD3 and CD69 expression levels in the PBMCs were evaluated by flow cytometry analysis (see below).

Flow cytometry analysis

A549 or NCI-H441 cells were collected and diluted to $1x10^5$ cells/100 µl. Then, the cells were incubated with anti-CD3 and anti-CD69 antibodies (both from BioLegend, Inc., San Diego, CA, USA) at 4°C for 20 min. After washing with PBS, the cells were stained with Live/Death (BioLegend, Inc.) for 15 min to exclude any dead cells from the analysis. Stained cells were analyzed using a flow cytometer (CytoFLEX; Beckman Coulter, Inc., Brea, CA, USA) and FlowJo 7.6 software (FlowJo LLC).

Statistical analysis

The quantitative data generated in the present study were analyzed using GraphPad Prism software (version 8.0; Dotmatics). All experiments were replicated three times to ensure reliability. Data are presented as the mean ± SD. For multiple group comparisons, one-way ANOVA followed by Tukey's *post-hoc* test was used. A *p*-value <0.05 was considered to indicate a statistically significant difference.

Results

Solamargine dose-dependently inhibited the proliferation of NSCLC cells

The effect of solamargine on the proliferation of NSCLC cells was evaluated using CCK8 and EdU staining assays. As indicated in Figure 1A, solamargine dose-dependently decreased the viability of A549 and NCI-H441 cells. In addition, 5 µM solamargine significantly prevented the proliferation of NSCLC cells (Figure 1 B,C). These results suggested that solamargine dose-dependently inhibited the proliferation of NSCLC cells.

Solamargine dose-dependently induced the apoptosis of NSCLC cells

Next, to explore the mechanism underlying the antitumor effects of solamargine on NSCLC cells, TUNEL and immunofluorescence staining were conducted. As shown in Figure 2 A,B, 5 µM solamargine notably induced the apoptosis of NSCLC cells. In addition, solamargine dose-dependently upregulated the expression of cleaved caspase-3 in NSCLC cells (Figure 2 C,D). Caspase-3 is an essential executor in apoptosis and its activation has been regarded as a biomarker of cell apoptosis. These results suggested that solamargine dose-dependently induced the apoptosis of NSCLC cells.

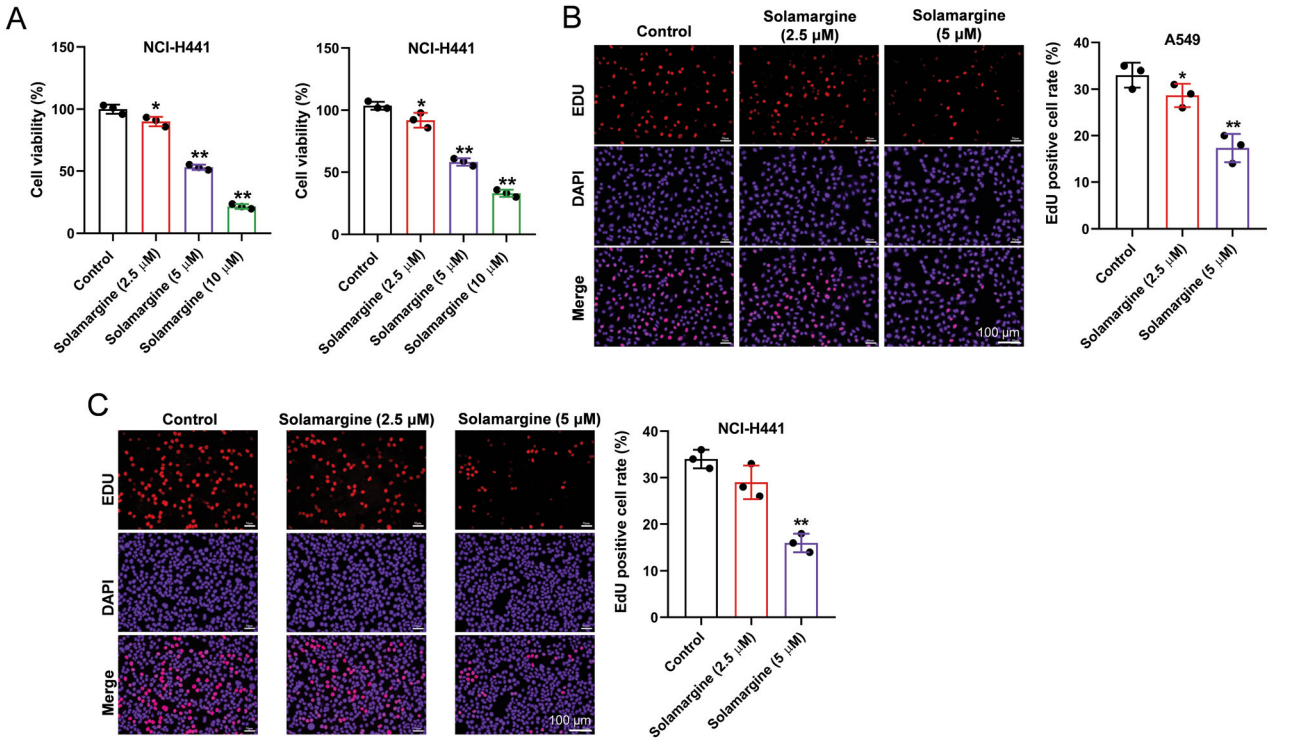

Figure 1. Solamargine dose-dependently inhibited the cell proliferation of NSCLCs. A549 or NCI-H441 cells were treated with 2.5 µM, 5 µM or 10 µM solamargine for 48 h. **A,B**) Cell viability was evaluated using with CCK8 assay. **C**) Cell proliferation was detected with EdU staining. ***p*<0.01, comparing with control group, n=3.

Solamargine inhibited the migration and invasion of NSCLC cells

The effects of solamargine on the migration and invasion of NSCLC cells were explored using the transwell assay. The results of the transwell assay suggested that 5 μM solamargine notably inhibited the migration of NSCLC cells (Figure 3 A,B). The inhibitory rate on cell migration and invasion in the 5 μM solamargine group was >50% (Figure 3 A,B).

Solamargine enhanced the T cell killing effect on NSCLC cells

To explore the effect of solamargine on the TME of NSCLC *in vitro*, A549 or NCI-H441 cells were co-cultured with stimulated PBMCs. As indicated in Figure 4A, PBMCs alone slightly inhibited the viability of A549 cells, while solamargine significantly enhanced the T cell killing effect on NSCLC cells. In addition, TUNEL staining suggested that the pro-apoptotic effect of PBMCs was notably enhanced when stimulated with CD3/CD28 or with solamargine (Figure 4B). Similar results were observed in NCI-H441 cells (Figure 4 C,D). These results suggested that solamargine enhanced the killing effect of T cells on NSCLC cells.

Solamargine promoted the proliferation and activation of T cells

Finally, to explore the mechanism underlying the effect of solamargine on the TME of NSCLC, the expression levels of CD3 and CD69 (T cell activation markers) in PBMCs were detected by flow cytometry. The results of the flow cytometry analysis indicated that solamargine promoted the expression of CD3 and CD69 in PBMCs (Figure 5 A,B), suggesting that solamargine may promote the proliferation and activation of T cells.

Discussion

Previous research has demonstrated that the incidence and motility rates of lung cancer have been continuously increasing.[15] Despite advancements in the treatments for lung cancer, less progress has been made in improving the survival of patients with advanced NSCLC.[16] Therefore, developing more effective adjunctive therapeutics with maximized efficacy and minimized adverse effects are urgently needed.

The natural compound, solamargine, an extract and glycoalkaloid of *Solanum lycocarpum*, has exhibited antitumor properties in a number of cancer types.[13,17,18] A previous study reported that solamargine inhibits the growth of lung cancer cells *via* inactivation of the PI3K/Akt pathway.[19] In addition, Tang *et al*. found that solamargine inhibits NSCLC cell growth through downregulating the HOTAIR, miR-214-3p and 3-phosphoinositide-dependent protein kinase-1 axis.[20] Fu *et al*. reported that solamargine inhibits gastric cancer progression by regulating the expression of lncNEAT1_2 *via* the MAPK signaling pathway.[21] Consistent with these findings, the present study demonstrated that solamargine directly inhibits the progression of NSCLC by inducing cell apoptosis.

As is well known, T cell immunity maintains body homeostasis by selectively clearing pathogens and abnormal cells; nevertheless, the uncontrolled hyperactivation of T cells can injure normal cells.[22] Cytotoxic T lymphocytes are effector T cells that can specifically lyse target cells *via* secreting cytokines.[23] However, upon prolonged antigenic stimulation, T cells may lose their effector functions even in the presence of the antigens that they target.[24] Programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) can regulate the activation of T cells under normal conditions, with the aim of preventing autoimmune reactions.[25] The present study explored the mechanism underlying the

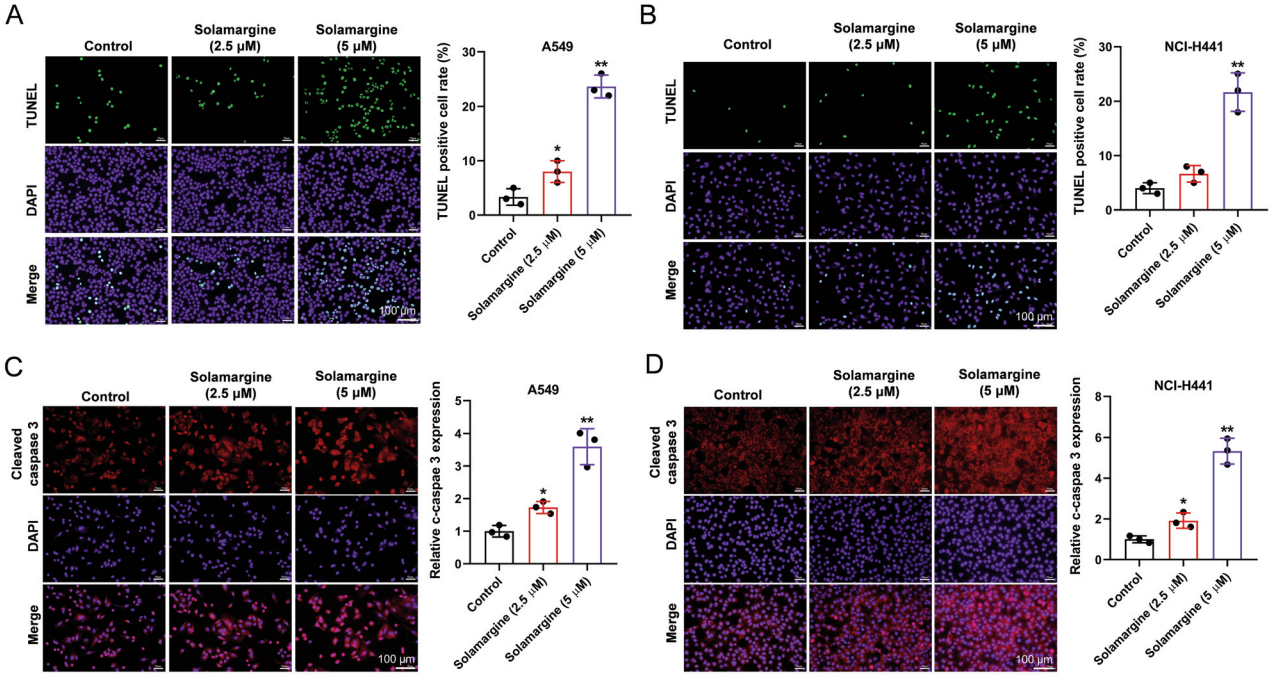

Figure 2. Solamargine dose-dependently induced the cell apoptosis of NSCLCs. A549 or NCI-H441 cells were treated with 2.5 μM or 5 μM solamargine for 48 h. **A,B**) Cell apoptosis was detected using with TUNEL assay. C,D) The expression of cleaved caspase 3 in NSCLCs was evaluated with immunofluorescence staining. *p<0.05, **p<0.01, comparing with control group, n=3.

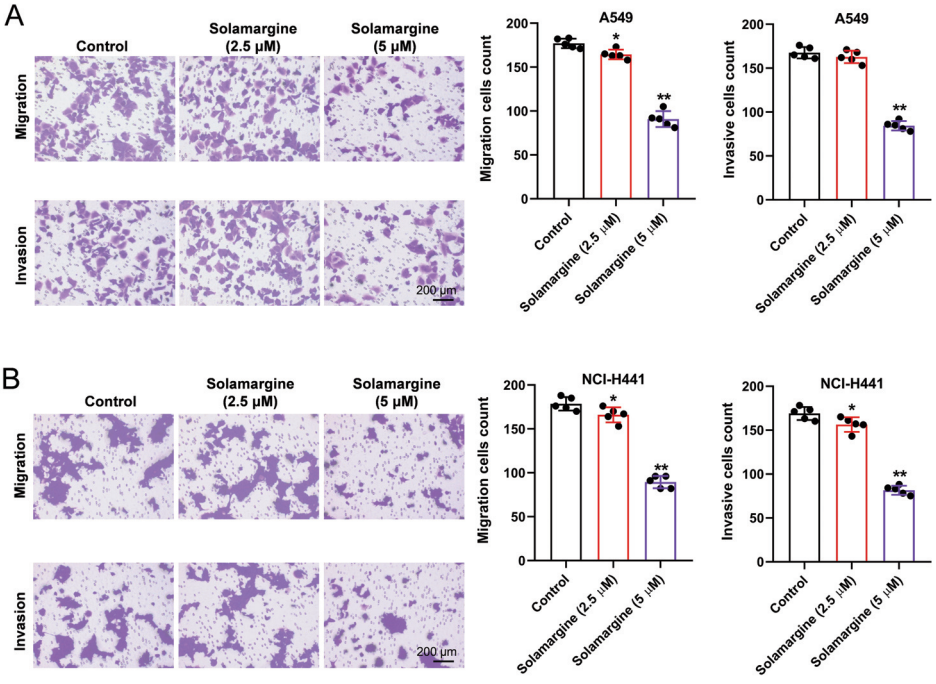

Figure 3. Solamargine inhibited the cell migration and invasion of NSCLCs. A549 or NCI-H441 cells were treated with 2.5 μM or 5 μM solamargine for 24 h. **A,B**) Cell migration and invasion were detected using with transwell assay. *p<0.05, **p<0.01, comparing with control group, n=3.

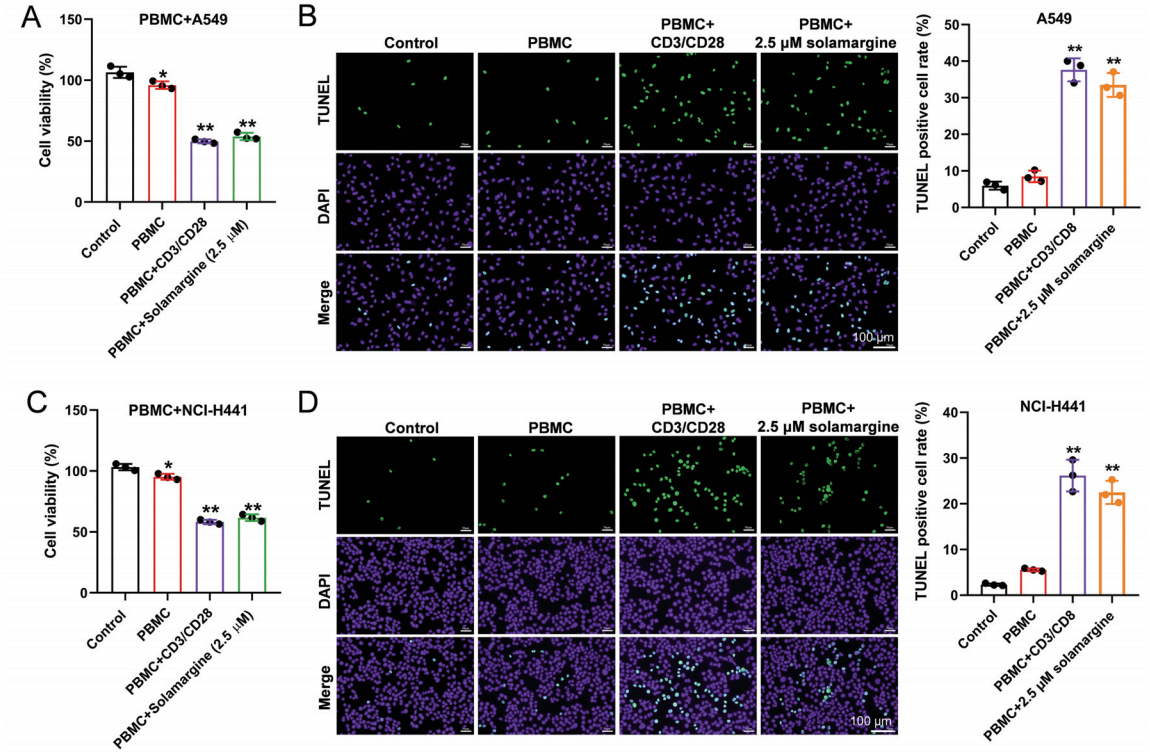

Figure 4. Solamargine enhanced T cell killing effect on NSCLCs. A549 cells were co-cultured with the stimulated PBMC (1:10 ratio) for 6 h at 37°C. **A**) Cell viability was detected with CCK8. **B**) Cell apoptosis was evaluated with TUNEL staining; NCI-H441 cells were co-cultured with the stimulated PBMC (1:10 ratio) for 6 h at 37°C. **C**) Cell viability was detected with CCK8. **D**) Cell apoptosis was evaluated with TUNEL staining. *p<0.05, **p<0.01, comparing with control group, n=3.

effect of solamargine on the TME of NSCLC. It was found that solamargine promoted the expression of CD3 and CD69 in PBMCs, suggesting that solamargine may promote the proliferation and activation of T cells. These data suggested that solamargine not only inhibited the progression of NSCLC cells directly but also activated T cell immunity. This finding was confirmed by a previous study in which it was reported that solamargine inhibits gastric cancer progression via inactivation of STAT3/PD□L1 signaling, suggesting an interaction between solamargine and the TME.[21] The novelty of the present study is that, to the best of our knowledge, it is the first to discover that solamargine can inhibit the proliferation of NSCLC cells by regulating the TME.

However, the present study has several limitations. The interaction between solamargine and PD-1 requires further investigation. In addition, the detailed mechanisms underlying the activating effect of solamargine on T cells should be investigated in the future. Moreover, the TME contains diverse immune cell populations, including T cells, macrophages and natural killer cells, and the potential effects of solamargine on other immune subsets remain unexplored.

In summary, the present study confirmed that solamargine can inhibit NSCLC cell proliferation and migration directly. In addition, it was found that solamargine could prevent the progression of NSCLC indirectly *via* activating the function of T cells. These findings may provide a novel theoretical basis in drug discovery for the treatment of NSCLC.

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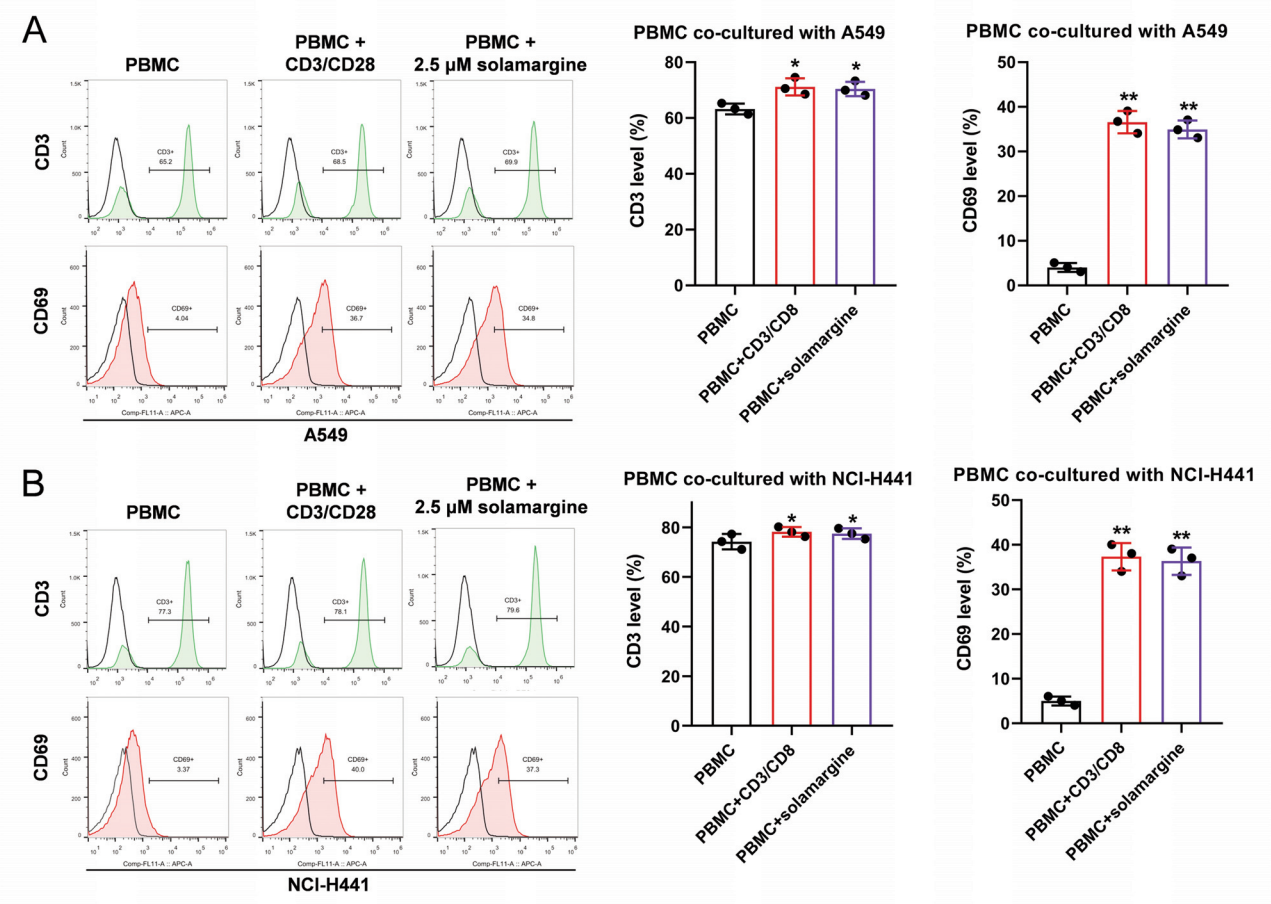

Figure 5. Solamargine promoted the proliferation and activation of T cells. **A**) A549 cells were co-cultured with the stimulated PBMC (1:10 ratio) for 6 h at 37°C; the expressions of CD3 and CD69 in PBMC were detected with flow cytometric. **B**) NCI-H441 cells were co-cultured with the stimulated PBMC (1:10 ratio) for 6 h at 37°C; the expressions of CD3 and CD69 in PBMC were detected with flow cytometric. **p*<0.05, ***p*<0.01, comparing with control group, n=3.

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