

The Mechanism of Alisol B23 Acetate Inhibiting Lung Cancer: Targeted Regulation of CD11b/CD18 to Influence Macrophage Polarization

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Background: Tumor microenvironment has attracted more and more attention in oncology. Alisol B23 acetate (AB23A) inhibits the proliferation of tumor cells including non-small cell lung cancer (NSCLC) cells. However, whether AB23A plays a role in the tumor microenvironment of NSCLC still remains obscure.

Methods: After THP-1 cells were polarized to M0 type by PMA, M0 macrophages were differentiated into M1 by LPS and IFN γ , and were differentiated into M2 by IL-4 and IL-13. The differentiation of THP-1 cells was detected by flow cytometry. After AB23A was given to macrophage RT-qPCR and ELISA detected the expressions of IL-6, IL-1 β , IL-10 and TGF- β . Western blot and RT-qPCR detected the expressions of CD11b and CD18 at both mRNA and protein levels. Lung cancer cell A549 cells were induced by above related macrophage culture medium. Cell proliferation was detected by CCK-8. TUNEL, wound healing and Transwell detected the apoptotic, migration and invasion capabilities. Next, M0 and M1-type macrophages were cultured in the cell culture medium of conventional A549 cells, to which AB23A was added. Subsequently, cell differentiation and inflammatory response were measured. Finally, the expression of CD18 in A549 cells was knocked down to construct NSCLC tumor-bearing mice and AB23A was applied for intragastric administration. Immunohistochemistry detected the polarization of macrophages in tumor tissues. Western blot detected the expressions of CD11b, CD18, invasion-, migration- and apoptosis-related proteins.

Results: AB23A promoted the polarization of macrophages towards M1, thus promoting the apoptosis and inhibiting the invasion and migration of A549 cells. The tumor cell culture medium induced M0 macrophages to M2, while AB23A reversed this effect. AB23A targeted CD11b/CD18 and improved the polarization of macrophages, thereby affecting tumor invasion, migration and apoptosis.

Conclusion: AB23A affected the polarization of tumor-associated macrophages through the targeted regulation of CD11b/CD18, thus inhibiting the development of lung cancer.

Keywords: Alisol B23 acetate, lung cancer, CD11b/CD18, tumor microenvironment

Introduction

Lung cancer is one of the most common tumors in the world, among which non-small-cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases. According to the 2018 Global Cancer Statistics Report, the incidence of lung cancer accounted for 11.6% of all tumors, ranking the first, and it also has the highest mortality rate, accounting for about 18.4% of all tumor mortality.¹

In the past, tumor research tended to focus on tumor cells themselves; however, tumor microenvironment has attracted extensive attention in tumor field in recent years.² Tumor cells do not exist in isolation, but constantly interact with various components in the microenvironment surrounding the tumor tissue.³ It was acknowledged that microenvironment plays a key role in tumor evolution, including tissue hypoxia, acidosis, formation of interstitial hypertension, production of numerous growth factors and proteolytic enzymes, along with immune inflammatory response.⁴ Moreover, such property of tumor

microenvironment plays an important role in tumor cell proliferation, migration, adhesion and tumor neovascularization and acts as a key factor in the formation of immunosuppression, immune escape as well as drug resistance.⁵

Tumor-associated macrophages (TAMs) are one of the most important immunosuppressive cells. TAMs in tumor immune microenvironment often exhibit phenotypic characteristics of immunosuppressive M2-type macrophages, which can promote tumor development and lead to poor prognosis of patients by secreting tumor growth factors and participating in extracellular matrix remodeling, angiogenesis, cell invasion and metastasis.⁶ In addition, a case of previous study has shown that induction of M2-TAMS to M1-TAMS can inhibit the proliferation of NSCLC.⁷ Therefore, the transformation of immunosuppressive TAMs into M1 anti-tumor cells is an important direction for tumor research.

Alisol B23 acetate (AB23A) is a triterpenoid compound extracted from the root of Alisol B. Current studies have found that it has many pharmacological activities, such as inducing tumor cell apoptosis, enhancing cell sensitivity to anti-tumor drugs, regulating immune stress and inhibiting allergic reaction.^{8,9} An in vitro study has shown that AB23A could inhibit the proliferation and promote the apoptosis in non-small cell lung cancer.¹⁰ However, the effect of AB23A on the tumor microenvironment of NSCLC has not been reported so far.

As a heterodimer glycoprotein, complement receptor 3 (CD11b/CD18) is a member of $\beta 2$ integrin family, which is composed of α and β peptide chains bonded by non-covalent bonds.¹¹ A case of study has shown that the abnormal expression of CD11b/CD18 is correlated with the diagnosis, clinical stage, lymph node metastasis, prognosis and immune status of malignant tumors.¹² Moreover, according to BATMAN prediction, AB23A may have the potential to affect CD18 (ITGB2). Therefore, it is reasonable to speculate that the anticancer effect of AB23A may be realized by targeting the expressions of CD11b/CD18 and affecting the tumor microenvironment.

In this study, we investigated the effect of AB23A on CD11b/CD18 expressions and polarization phenotype of macrophages from in vitro and in vivo.

Materials and Methods

Cell Culture

Human THP-1 cells were obtained from American Type Culture Collection and cultured in RPMI1640 containing 10% FBS (with 1% penicillin plus streptomycin, all from Gibco) at 37°C with 5% CO₂.

To induce the polarization of THP-1 cells, cells were initially treated with Phorbol-12-myristate-13-acetate (PMA, 10ng/mL, Sigma-Aldrich) for 24h. The THP-1 cells were treated with IFN- γ (20 ng/mL, Sigma-Aldrich) and lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich) for M1 polarization for 18h. The THP-1 cells were treated with IL-4 (20 ng/mL, Sigma-Aldrich) and IL-13 (20 ng/mL, Sigma-Aldrich) for M2 polarization for 72h. While inducing M2 polarization, AB23A with final concentration of 9mM was added for culture for 24h.¹⁰ In other words, THP-1 cells were divided into 5 groups: Control, M0, LPS+IFN γ (M1), IL-4+IL-13 (M2) and IL-4+IL-13 +AB23A. All polarized cells were cultured in RPMI1640 medium containing 10% FBS for 48h, which culture medium was used for subsequent experiments.

Human lung cancer cell line A549 cells that obtained from American Type Culture Collection were cultured in RPMI 1640 (HyClone) supplemented with 10% FBS (with 1% penicillin plus streptomycin, all from Gibco) at 37°C with 5% CO₂.

The polarized THP-1 cell culture medium was used to cultivate A549 cells and the cells were divided into Control, M0 medium, M1 medium, M2 medium and IL-4+IL-13+AB23A medium groups. Accordingly, the polarized cell culture medium was used to cultivate A549 cells, and cells that induced by M2 medium were treated with AB23A. The Control group does no special processing.

A549 cells were conventionally cultured and the culture medium was collected to culture the induced M0 and M1 THP-1 cells. In other words, M0 and M1 cells were grouped into M0, M0+A549 medium, M1, M1+ A549 medium and M1+A549 medium +AB23A groups. AB23A was added to the M1 cells with A549 culture medium.

Flow Cytometry

After polarization induction of THP-1 cells, the cells were prepared into a single cell suspension and subsequently stained with antibodies targeting CD86, CD206, CD11b and CD14 (1:500, Abcam) at 4 °C for 30 min. After the rinse with PBS, cells were collected by BD Accuri C6 and data were analyzed with FlowJo software.

RT-qPCR

Total RNA that extracted from cells with Trizol method (Invitrogen) was reversely transcribed into cDNA using a PrimeScript™ RT Reagent Kit (TAKARA Bio Inc. Shiga, Japan). After reverse transcription, expression of the target gene was quantified by real-time PCR with a SYBR™ Green PCR Master Mix (Invitrogen) in light of recommended instructions. RT-qPCR was performed with an Applied Biosystems™ real time PCR system (Thermo Fisher Scientific). The data were quantified using the $2^{-\Delta\Delta C_t}$ method.¹³ The sequences were as follows: IL-1 β sense: 5'- CCAAACCTCTTCGAGGCACA -3', antisense: 5'- AGCCA TCATTTCACTGGCGA -3'; IL-6 sense: 5'-GTCCAGTTGCCTTCTCCCTGG-3', antisense: 5'- CCCATGCTACAT TTGCCGAAG-3'; IL-10 sense: 5'- TGCTCTTGCAAACCAAACCA-3', antisense: 5'- GGGAGGTCAGGGAAAACAGC-3'; TGF- β sense: 5'-ACCTGCCACAGATCCCCTAT-3', antisense: 5'- GAGCAACACGGGTTTCAGGTA-3'; CD11b sense: 5'- GCTTTGGTGGCTTCCTTGTG-3', antisense: 5'-TAGTCGCACTGGTAGAGGCT-3'; CD18 sense: 5'- CAGGGCAGACT GGTAGCAAA-3', antisense: 5'- GCGTCACTTTTTGTGGGGAC-3'; GAPDH sense: 5'- AATGGGCAGCCGTTAGGAAA-3', antisense: 5'- GCGCCAATACGACCAAATC-3'.

Western Blot

Cells were lysed in protein extraction solution (Beyotime, China). The BCA assay kit (SinoBio Biotech, China) was used to calculate protein concentration. Cell lysates were collected and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp). The membranes that blocked with 5% skim milk powder were incubated with primary antibodies (1:1000, Abcam) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Abcam) at 37°C for 1 h. Then, the proteins were visualized with ECL chemiluminescent kit (ECL-plus, Thermo Scientific, USA) and analyzed using ImageJ software (version 1.8.0; National Institutes of Health). The following were the antibodies used in this study: CD11b (1:1000, ab133357, Abcam); CD18 (1:1000, ab13219, Abcam); Bax (1:1000, ab32503, Abcam); Bcl-2 (1:1000, ab182858, Abcam); C-Casp3 (1:1000, ab214430, Abcam); Casp3 (1:1000, ab184787, Abcam); MMP2 (1:1000, ab92536, Abcam); MMP9 (1:1000, ab76003, Abcam); ICAM-1 (1:1000, ab171123, Abcam); CXCL12 (1:1000, ab157772, Abcam); GAPDH (1:1000, ab8245, Abcam).

ELISA

ELISA was applied for the estimation of TNF- β , IL-10, IL-6 and IL-1 β according to the protocol. Absorbance was read at 450 nm with a reference wavelength of 570 nm through a microplate reader (Tecan US, Inc., Morrisville, NC).

CCK8

Cell Counting Kit-8 (CCK-8, Nanjing Jiancheng) assay was used to detect cell viability. After indicated treatment, the cells that plated into a 96-well plate (1×10^4 cells/well) were exposed to 10 μ L of CCK-8 solution (KeyGen, Nanjing, China) for 4 h. The absorbance at 450 nm was measured using a microplate spectrophotometer reader (Bio-Tek Instruments, Winooski, VT, USA).

TUNEL

The apoptosis of A549 cells was evaluated by terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay in accordance with the manufacturer's instructions. Cells were seeded in 24-well plates. After indicated treatment, cells were fixed in 4% paraformaldehyde for 1 h and permeabilized with 0.1% Triton X-100 for 5 min. Then, the cells were incubated with TUNEL reaction mixture for 1 h at 37°C in a humidified atmosphere in the dark. After the seal with anti-fluorescence quenching solution, the fluorescence was observed under fluorescence microscope.

Lung tumor tissue was fixed in paraformaldehyde and prepared in paraffin sections. 3% H₂O₂-methanol solution was used for 10 min of rinse. The slices were subsequently incubated with 0.2% Triton for 5 min. After that, 50 μ L TUNEL reaction mixture was added into each tissue slice, followed by the incubation at 37°C in the dark according to the manual instructions. Images were taken under a fluorescent microscope.

Wound Healing

Cells were seeded into a 6-well plate (1×10^5 cells/well). When cells grew to 75% confluence in a complete medium, a wound was made by a sterile pipette tip. Then, cells were washed with PBS for several times to remove cell debris, after which was 48 h of incubation with serum-free medium. The wound healing in vitro was photographed by an inverted fluorescence microscope and the rate of closure was assessed.

Transwell

Transwell chambers (Corning Incorporated) that coated with matrigel were used to evaluate the invasive ability of cells. The transfected cells in serum-free medium were added to the top of transwell chamber. Then, 500 μ L of complete medium was added to the lower chamber as chemoattractant. After 24 h, cells on the upper surface were removed. The cells that invaded to the bottom were fixed and stained with 0.1% crystal violet. The number of apoptotic cells in five randomly selected fields was counted using light microscope (Olympus).

Cell Transfection

The plasmids (short hairpin RNA (shRNA)-ITGB2#1, shRNA-ITGB2#2, and their blank control shRNA-NC) featured in the article were designed by Ribo Bio. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The shRNA sequences were as follows: sh-ITGB2#1: TGAGTCAGGGAAGCAAGAATG; sh-ITGB2#2: GGGCTGTGAAATCTTCCTTTG; sh-NC: TTCTCCGAACGTGTACGT.

Mice Xenograft Models

A549 cells or A549 cells with ITGB2 knockout were subcutaneously injected into nude mice at the density of 2×10^6 per mL and AB23A (0.4g/kg) was administered orally.^{14,15} The mice were divided into 6 groups: A549, A549+AB23A, A549+ sh-NC, A549+ sh-NC +AB23A, A549+ShRNA-ITGB2 and A549+ShRNA-ITGB2 +AB23A groups. All mice were examined every 3 days and sacrificed 21 days after tumor inoculation.

Immunohistochemistry (IHC)

Tissue sections were dewaxed in xylene, followed by the treatment with 0.3% H_2O_2 to block endogenous peroxidase activity. Slides were placed in an autoclave cooker which was filled with 10 mM citrate buffer (pH 6.0) and antigen retrieval was heated for 10 min at 95–100°C. To block non-specific protein binding, 10% normal goat serum was used. The corresponding primary antibodies iNOS and Arg-1 (1:100 diluted, Abcam) were incubated at room temperature overnight. On the next day, the slides were incubated with biotinylated secondary antibody (1:250; Abcam) and streptavidin-peroxidase conjugate (1:1000; Abcam). Antigen-antibody reaction was visualized using streptavidin-horseradish peroxidase conjugated with diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin.

Statistical Analysis

Each experiment in this study was conducted in triplicate. All data were shown as mean \pm SD and analyzed with SPSS 18.0. Comparisons in two groups were analyzed via Student's *t*-test while comparisons in multiple-groups were analyzed via one-way ANOVA. $p < 0.05$ was considered as statistically significant.

Results

AB23A Inhibits the Polarization of Macrophage to M2 and Promotes Partial Polarization of Macrophages to M1, Thus Presenting a Certain Form of Pro-Inflammatory Response

CD11b and CD14 are monocyte or macrophage surface markers. F4/80+ antibody marks macrophages. CD86 is a surface marker of M1 macrophages and CD206 is a surface marker of M2 type macrophages. After THP-1 cells were polarized, AB23A was added to detect the phenotypic changes of macrophages by flow cytometry. The results showed that PMA

successfully induced THP-1 cells to form M0 polarization state (Figure 1A). LPS and IFN γ successfully induced the differentiation of M1-type macrophages. At the same time, IL-4 and IL-13 successfully induced the differentiation of M2-type macrophages. Compared with IL-4+IL-13 (M2) group, macrophages in IL-4+IL-13 +AB23A group changed from M2 polarization state to M1 polarization state (Figure 1B). Subsequently, RT-qPCR was used to detect the mRNA expression levels of M1-type macrophage-related markers IL-6 and IL-1 β , as well as M2-type macrophage-related markers IL-10 and TGF- β . The results showed that the expressions of IL-6 and IL-1 β in M0 macrophages were significantly increased compared with the Control group. Compared with M0, the expressions of IL-6 and IL-1 β were markedly increased in M1 group while the expressions of IL-10 and TGF- β were greatly decreased. Compared with M0

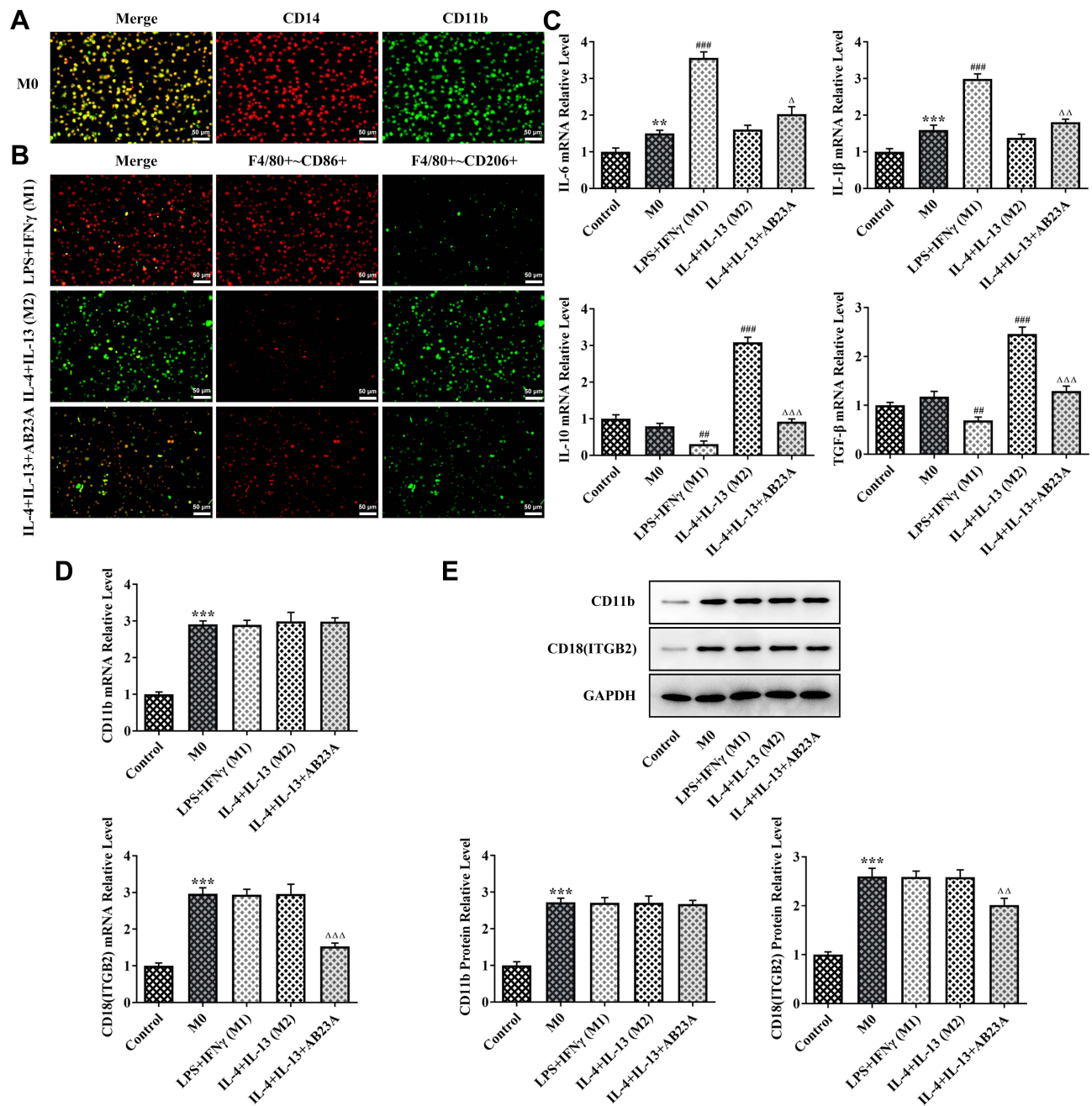


Figure 1 AB23A inhibits the polarization of macrophage to M2, but promotes partial polarization of macrophages to M1, presenting a certain form of pro-inflammatory response. (A and B) Cell differentiation was detected by flow cytometry. (C) RT-qPCR detected the mRNA expressions of IL-6, IL-1 β , IL-10 and TGF- β . (D) RT-qPCR detected the mRNA expressions of CD11b and CD18. (E) Western blot detected the protein expressions of CD11b and CD18. ** $p < 0.05$, *** $p < 0.001$ vs control; ## $p < 0.05$, ### $p < 0.001$ vs M0; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ vs IL-4+IL-13 (M2).

group, the expressions of IL-6 and IL-1 β stayed unchanged in M2 group while the expressions of IL-10 and TGF- β were significantly increased. Compared with the IL-4+IL-13 (M2) group, the expressions of IL-6 and IL-1 β were increased in IL-4+IL-13 +AB23A group but the expressions of IL-10 and TGF- β were remarkably reduced (Figure 1C). The expression levels of CD11b and CD18 (ITGB2) in THP-1 cells were detected by RT-qPCR and Western blot. We found that the expressions of CD11b and CD18 were significantly increased in the M0 group compared with the Control group. Compared with M0, the expressions of CD11b and CD18 in M1 and M2 polarization states did not change. Compared with the IL-4+IL-13 (M2) group, the expression of CD18 was significantly decreased after AB23A administration (Figure 1D and E). AB23A could inhibit the induction of macrophage polarization to M2 by IL-4+IL-13, and promoted partial polarization of macrophages to M1, presenting a certain form of pro-inflammatory response.

AB23A Promotes the Polarization of Macrophages Towards M1, Thus Promoting Apoptosis and Inhibiting the Invasion and Migration of A549 Cells

ELISA was used to detect the secretion levels of IL-6, IL-1 β , IL-10 and TGF- β in cell supernatant, and the changing trend of related factors in cell supernatant was consistent with that in cells (Figure 2A). Next, we used above differentiated macrophage culture medium to culture normal A549 cells. CCK8 kit was applied to detect the cell proliferation and the results showed that compared with the Control group, the cell proliferation in the M0 medium group did not change significantly. Compared with M0 medium, cell proliferative ability of M1 medium group was significantly decreased, while that of M2 medium group was significantly increased. Compared with M2 medium group, cell proliferative ability of IL-4+IL-13+AB23A medium group was significantly decreased (Figure 2B). TUNEL assay was employed to detect cell apoptosis and the results showed that compared with the Control group, the apoptosis of M0 medium group had no significant change. Compared with M0 medium group, cell apoptosis in M1 medium group was greatly elevated, while that in M2 medium group was hugely declined. Compared with M2 medium group, apoptosis was further increased in IL-4+IL-13+AB23A medium group (Figure 2C and D). Results obtained from wound healing and Transwell assays showed that the cell migration and invasion had no obvious change in M0 medium group when compared to the Control group. Compared with M0 medium group, the invasion and migration of M1 medium group were rapidly diminished, while those of M2 medium group were not significantly changed. Compared with M2 medium group, the invasion and migration in IL-4+IL-13+medium group were significantly decreased (Figure 2E–H). M1-type macrophages promoted the apoptosis of A549 cells and inhibited capabilities of cells to invade and migrate. M2 macrophages inhibited the apoptosis of A549 cells but promoted cell invasion and migration. In addition, AB23A could promote the polarization of macrophages towards M1, thus promoting the apoptosis of A549 cells and inhibiting the capabilities of cells to invade and migrate.

The Tumor Cell Culture Medium Induces M0 Macrophages to M2 and Inhibits M0 Macrophages to M1, While AB23A Reverses This Effect

A549 cells were conventionally cultured and the culture medium was collected for the culture of M0 and M1 THP-1 cells. The differentiation phenotype was detected by flow cytometry. The results showed that compared with M0, the expression of CD206 in M0+A549 medium group was significantly increased. Compared with M1, CD206 was greatly increased but CD86 was markedly decreased in M1+A549 medium group. Compared with M1+A549 medium group, CD206 in M1+A549 medium+AB23A group was significantly decreased (Figure 3A). The expression of cytokines in cell supernatant was detected by ELISA, and the results showed that compared with M0, the expressions of IL-6 and IL-1 β in M0+A549 medium group were not significantly changed, but the expressions of IL-10 and TGF- β were significantly increased. Compared with M1 group, the expressions of IL-6 and IL-1 β in M1+A549 medium group were significantly cut down, while the expressions of IL-10 and TGF- β significantly ascended. Compared with M1+A549 medium group, the expressions of IL-6 and IL-1 β in M1+A549 medium +AB23A group were hugely elevated, while the expressions of IL-10 and TGF- β were dramatically reduced (Figure 3B). The above studies indicated that tumor cell culture medium induced M0 macrophages to M2 and inhibited M0 macrophages to M1, while AB23A reversed this effect.

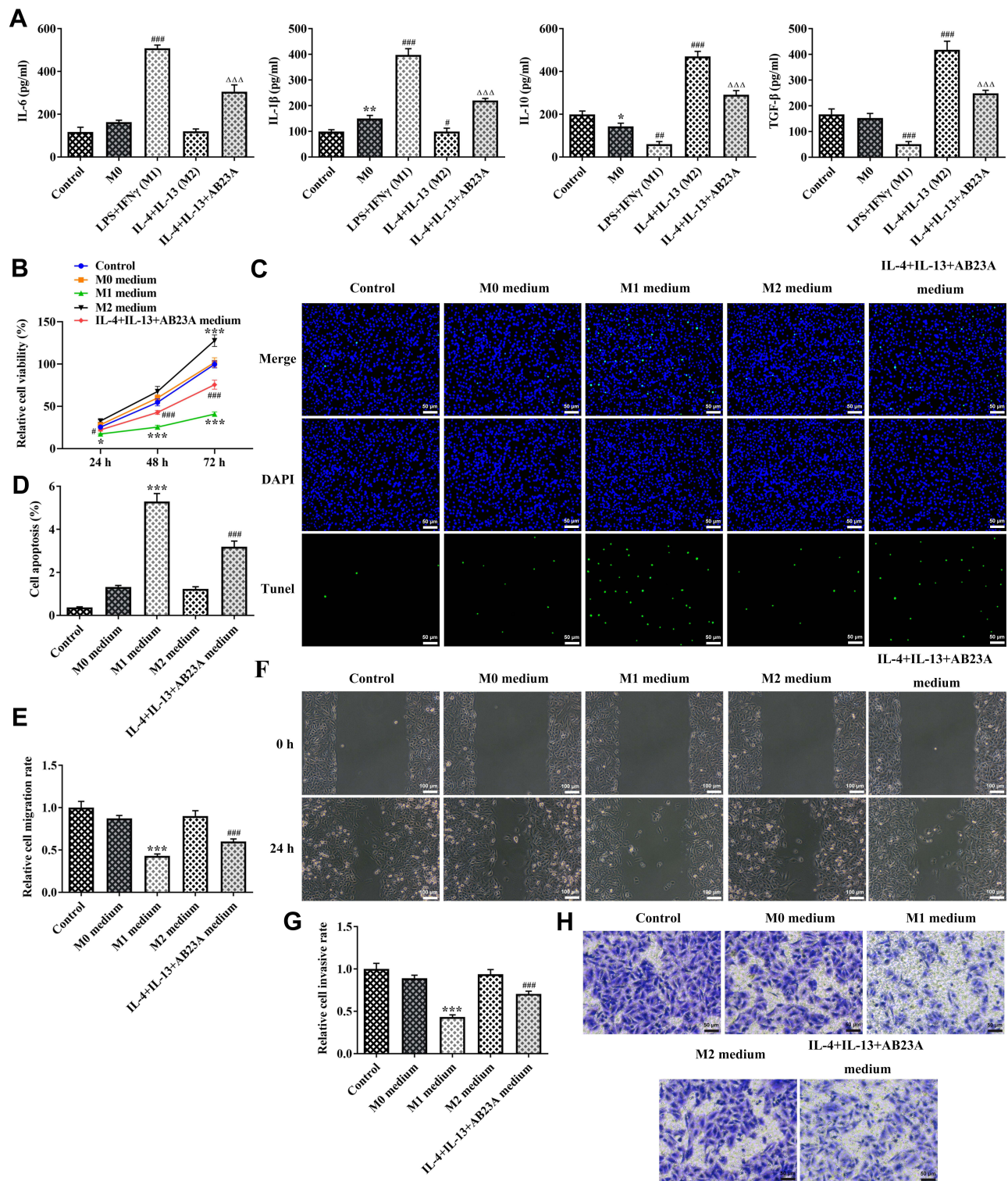


Figure 2 AB23A promotes the polarization of macrophages towards M1, thus promoting apoptosis and inhibiting cell invasion and migration of A549 cells. **(A)** ELISA was used to detect the secretion levels of related markers in cell supernatant. * $p < 0.05$, *** $p < 0.001$ vs control; ### $p < 0.01$, #### $p < 0.001$ vs M0; $\Delta\Delta\Delta p < 0.001$ vs IL-4+IL-13 (M2). **(B)** CCK-8 detected the cell viability. **(C)** TUNEL assay detected the cell apoptosis. **(D)** Statistical analysis diagram of apoptosis rate. **(E and F)** Wound healing detected the cell migration. **(G and H)** Transwell detected the cell invasion. * $p < 0.05$, *** $p < 0.001$ vs M0 medium; # $p < 0.05$, #### $p < 0.001$ vs M2 medium.

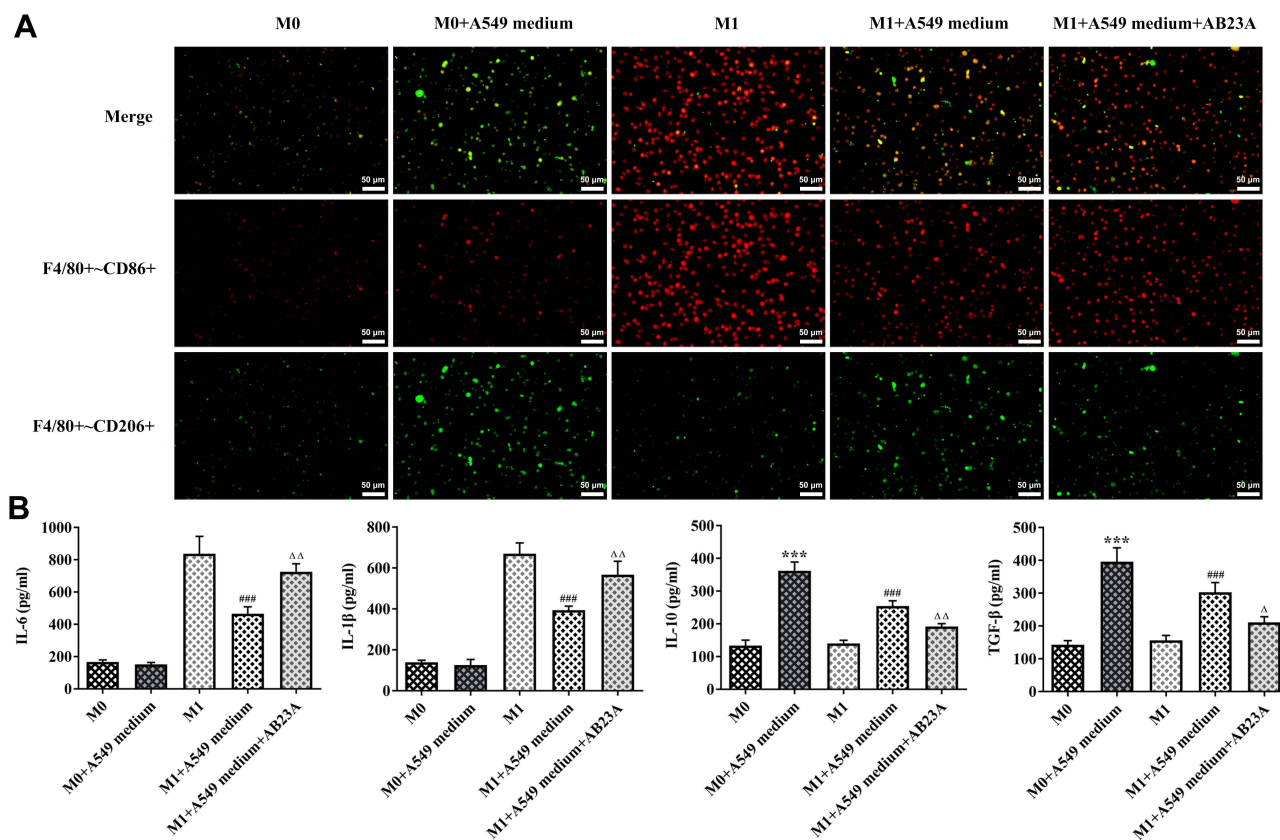


Figure 3 The tumor cell culture medium induces M0 macrophages to M2 and inhibits M0 macrophages to M1, while AB23A reverses this effect. **(A)** Cell differentiation was detected by flow cytometry. **(B)** ELISA was used to detect the secretion levels of related markers in cell supernatant. *** $p < 0.001$ vs M0; #### $p < 0.001$ vs M1; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ vs M1+A549 medium.

AB23A Targets CD11b/CD18 and Improves Polarization of TAMs, Thereby Affecting Tumor Invasion, Migration and Apoptosis

The expression of CD18 (ITGB2) in A549 cells was knocked down and the transfection efficiency was measured by RT-qPCR (Figure 4A). ShRNA-ITGB2#1 was selected for follow-up experiments. During modeling, the differences in tumor size were routinely recorded. We found that AB23A administration significantly inhibited tumor volume and mass in mice. Compared with A549+sh-NC group, tumor volume and mass of mice in A549+ shRNA-ITGB2 group were significantly increased, which were subsequently reduced by AB23A administration (Figure 4B–F). Subsequently, IHC was employed to detect the expressions of M1 marker iNOS and M2 marker Arg-1 in tumor tissues. The results showed that oral administration of AB23A significantly increased the expression of iNOS in tumor tissues and inhibited the expression of Arg-1. Compared with the A549+sh-NC group, the expression of iNOS in the A549+ shRNA-ITGB2 group was decreased and the expression of Arg-1 was increased, which were then counteracted after AB23A administration (Figure 5A and B). ELISA results showed that AB23A administration could significantly increase the expressions of IL-6 and IL-1 β , and inhibit the expressions of IL-10 and TGF- β in tumor tissues. Compared with the A549+ shRNA-ITGB2 group, the expressions of IL-6 and IL-1 β were increased and the expressions of IL-10 and TGF- β were inhibited in the A549+ shRNA-ITGB2 +AB23A group (Figure 5C). Results obtained from TUNEL showed that oral administration of AB23A significantly increased the cell apoptosis, accompanied with elevated contents of Bax and cleaved caspase3 as well as reduced content of Bcl-2. Compared with A549+ sh-NC, apoptosis was significantly decreased, the expressions of Bax and cleaved caspase3 were significantly decreased, but Bcl-2 expression was significantly increased in A549+ shRNA-ITGB2 group, which were reversed by AB23A administration (Figures 5D, E and 6A). Western blot was applied for the estimation of invasion- and migration-related proteins and the results demonstrated that the expressions of MMP2, MMP9, ICAM-1 and CXCL12 in tumor tissues of mice were

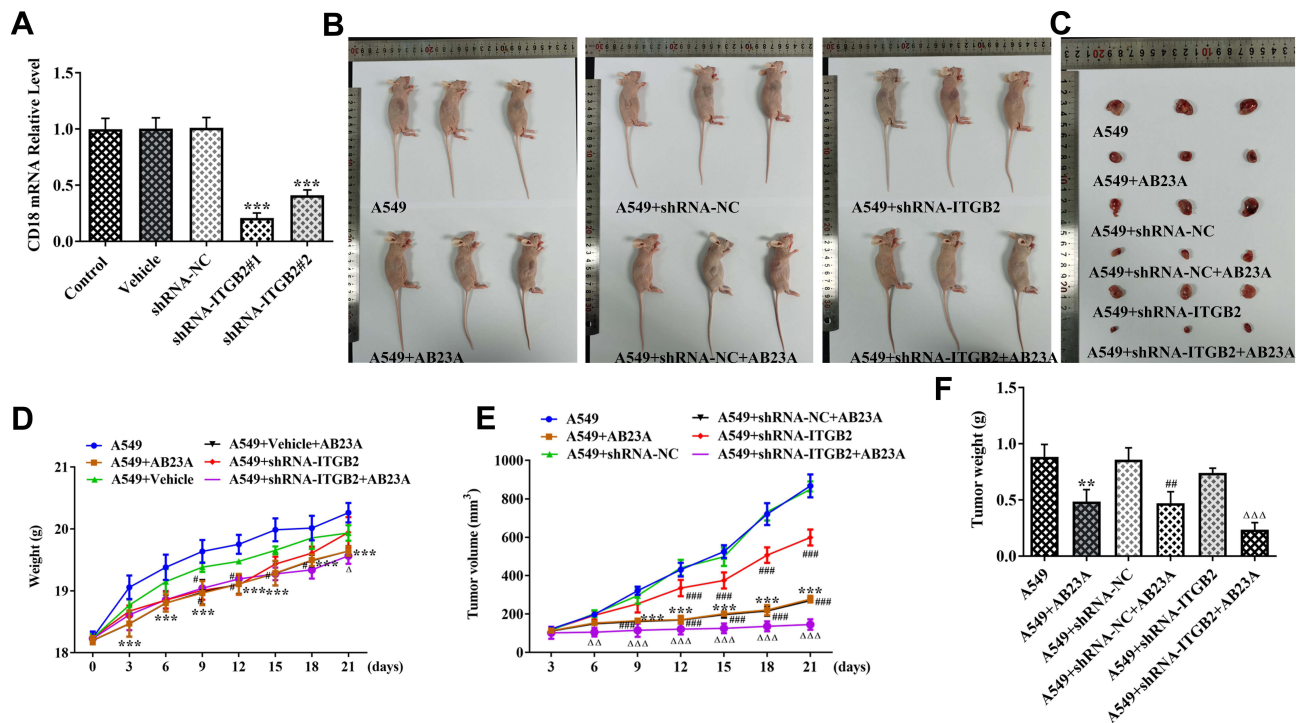


Figure 4 AB23A targets CD11b/CD18 and improves polarization of TAMs, thereby affecting tumor size. **(A)** RT-qPCR detected the mRNA expression of CD18. *** $p < 0.001$ vs shRNA-NC. **(B)** Pictures of mice. **(C)** Pictures of tumor tissue in mice. **(D)** Body weight of mice. **(E)** Tumor volume in mice. **(F)** Tumor weight of mice. ** $p < 0.01$, *** $p < 0.001$ vs A549; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs A549+shRNA-NC; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ vs A549+shRNA-ITGB2.

significantly decreased after oral administration of AB23A. Compared with the A549+sh-NC group, the expressions of invasion- and migration-related proteins were increased in the A549+ shRNA-ITGB2 group, which were reversed after AB23A administration (Fig6). At the same time, the expressions of CD11b and CD18 (ITGB2) were detected and the results showed that the expressions of CD11b and CD18 (ITGB2) were decreased significantly after oral administration of AB23A. CD18 (ITGB2) expression was significantly decreased in A549+shRNA-ITGB2 group when compared to the A549+ sh-NC group. CD18 (ITGB2) expression was inhibited in the A549+ shRNA-ITGB2 + AB23A group compared with the A549+ shRNA-ITGB2 group (Figure 6). These results showed that the changes in apoptosis, invasion and migration caused by ITGB2 inhibition alone were not as significant as those caused by AB23A addition. Combining the results of iNOS and Arg-1, we speculated that AB23A-induced changes in TAMs might play a key role.

Discussion

The role of tumor microenvironment in tumor is mainly composed of the interaction of adhesion molecules, tumor cells and stromal cells, the release of growth factors and angiogenesis.¹⁶ Therefore, exploring the molecular mechanism of adhesion molecules, tumor microenvironment and interaction between tumor cells can further clarify the mechanism of tumorigenesis and provide new strategies for tumor treatment.

In most tumor microenvironment, macrophages are the cell component with the highest proportion of immune cells, and studies have shown that in lung cancer, macrophages even account for 50% of the entire microenvironment.^{17,18} Therefore, the exploration of the mechanism of macrophages promoting tumor growth has attracted more and more attention. Also, TAMs has been considered as hypothesized target for therapeutic intervention.¹⁹ Therefore, the targeted regulation of the polarization types of TAMs provides a new idea for the treatment with drug in tumors.

Macrophages are activated by environmental factors and different extracellular signals can lead to the transformation of macrophages into different types.²⁰ Macrophages activated by LPS and IFN- γ are classically activated macrophages which are also known as M1-type macrophages. M1 macrophages mainly secreted pro-inflammatory factors, such as IL-6

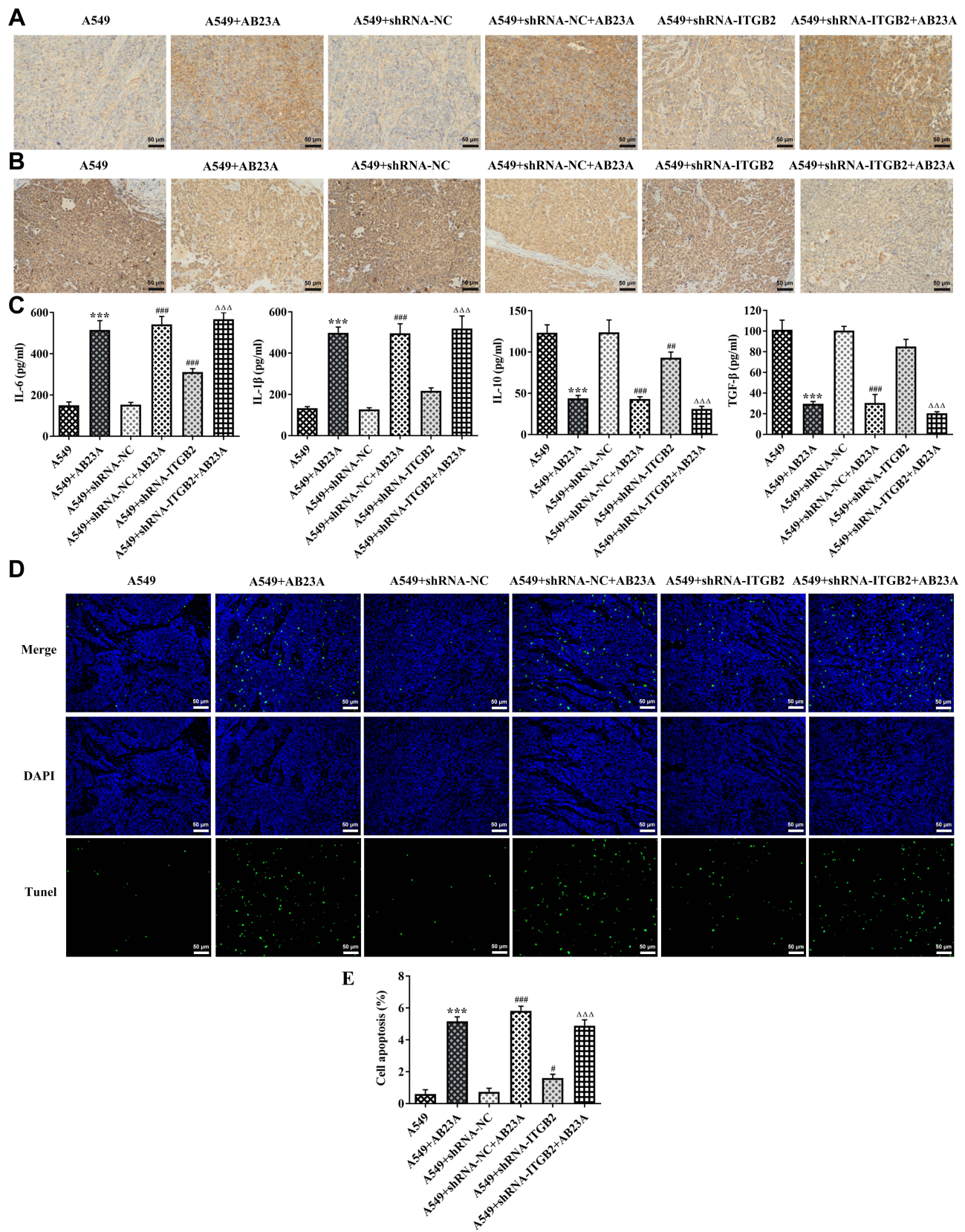


Figure 5 AB23A targets CD11b/CD18 and improves polarization of TAMs, thereby affecting tumor apoptosis. **(A and B)** IHC detected the expressions of iNOS and Arg-1. **(C)** ELISA was used to detect the levels of related markers in tumor tissue. **(D)** TUNEL assay detected apoptosis. **(E)** Statistical analysis diagram of apoptosis rate. *** $p < 0.001$ vs A549; # $p < 0.05$, ### $p < 0.001$ vs A549+shRNA-NC; $\Delta\Delta\Delta p < 0.001$ vs A549+shRNA-ITGB2.

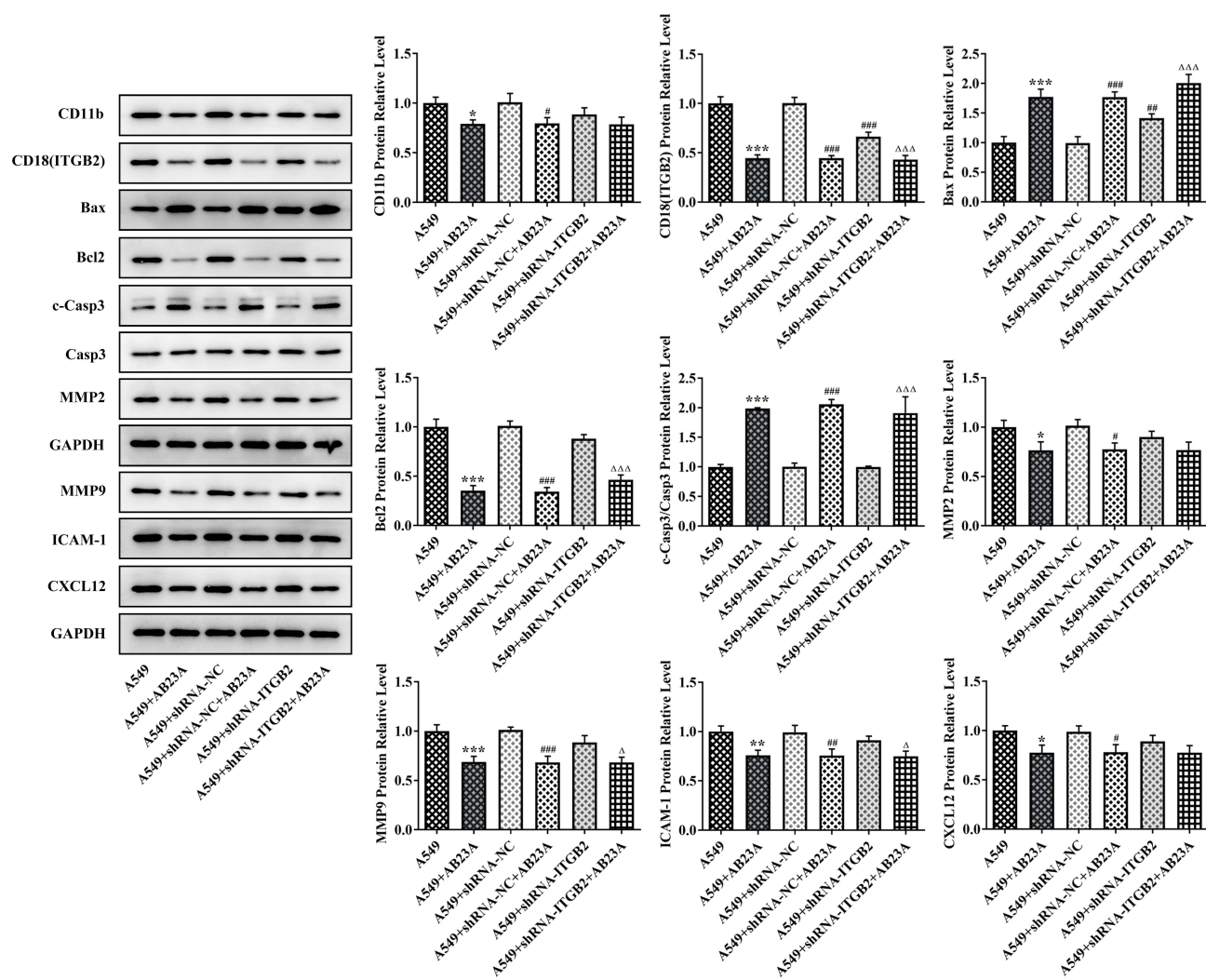


Figure 6 AB23A targets CD11b/CD18 and improves polarization of TAMs, thereby affecting tumor invasion, migration and apoptosis related proteins. Western blot detected the protein expressions of tumor invasion-, migration- and apoptosis-related proteins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs A549; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs A549+shRNA-NC; Δ $p < 0.05$, ΔΔ $p < 0.001$ vs A549+shRNA-ITGB2.

and IL-1 β , and promoted the expression of inducible nitric oxide synthase (iNOS).²¹ Macrophages that activated by Th2 cytokines (eg, IL-4 and IL-13) are called surrogate activated macrophages which are also known as M2-type macrophages. M2-type macrophages secrete anti-inflammatory cytokines, such as IL-10 and TGF- β as well as highly expressed Arg1 (Arginase-1), which play an important role in tissue damage repair and remodeling caused by pathogenic microorganisms in the late stage of inflammation.²² Therefore, in our experiment, THP-1 cells were induced by PMA. M0 cells were then collected and differentiated into M1 by LPS and IFN γ . With the help of IL-4 and IL-13, M0 cells were differentiated into M2. In addition, the secretion of IL-6 and IL-1 β was increased after LPS and IFN γ induction. After the induction with IL-4 and IL-13, the secretion of IL-10 and TGF- β was increased. The results showed that macrophage differentiation was successful.

CD11b/CD18 is one of the main adhesion molecules involved in immune response in tumor microenvironment. The abnormal microenvironment mediated by adhesion molecules is not only a good soil for tumor cells to grow during tumor formation, but also a refuge for tumor cells to escape apoptosis and immune recognition, as well as a breakthrough for tumor cells to infiltrate and metastasize.^{23,24} It has shown that different types of complement have different effects on macrophage polarization, among which CD11b/CD18, as the receptor of C3b, mainly induces macrophage to differentiate into M2 type.²⁵ Through allosteric reduction of CD11b expression, the proliferation of lung cancer cells can be

significantly reduced, and the proportion of M2 macrophages decreases, while the proportion of M1 macrophages increases.²⁶ Therefore, it is reasonable to speculate that targeting CD11b/CD18 to regulate the polarization type of tumor macrophages can eventually regulate the malignant progression of tumor. In our experiment, it was found that induced polarization of macrophages resulted in significantly increased expressions of CD11b and CD18.

Traditional Chinese medicine AB23A has many pharmacological activities, such as anti-hepatitis virus, anti-bacterial, anti-allergic and so on.²⁷ In recent years, the antitumor activity of AB23A has also been gradually concerned. It was showed that AB23A could induce apoptosis of gastric cancer cells through cell cycle arrest and mitochondrial pathways, with cascade of Caspase and MAPK signals.⁸ AB23A could inhibit the development of liver cancer by promoting the apoptosis of liver cancer cells as well as inhibiting cell migration and invasion.¹⁵ These results indicated that AB23A may be a novel anticancer drug for cancer treatment. At present, it has shown that AB23A could induce apoptosis of human lung cancer cells through mitochondrial pathway.²⁸ AB23A inhibited cell viability and induced apoptosis of NSCLC cells through PI3K/AKT/mTOR signaling pathway.¹⁰ However, the association and regulatory mechanism among AB23A, CD11b/CD18 and tumor microenvironment of lung cancer have not been reported so far. BATMAN database predicted that AB23A could target CD18 (ITGB2). In our experiment, it was found that AB23A inhibited the polarization of macrophage to M2, but promoted partial polarization of macrophages to M1, presenting a certain form of pro-inflammatory response. In addition, tumor cell culture medium induced M0 macrophages to M2 and inhibited M0 macrophages to M1, while AB23A reversed this effect. Meanwhile, AB23A could significantly inhibit the expression of CD18 in M2-type macrophages, thereby improving the polarization of tumor-associated macrophages and affecting tumor invasion, migration and apoptosis.

In the experiment, we found that AB23A could still significantly inhibit tumor growth in lung cancer mice after CD18 was knocked down. There are two possibilities for this phenomenon. One is that AB23A acts on signaling pathways other than CD18 cascade, thus affecting tumor cell growth. Another possibility is that the combination of AB23A and CD18 knockout causes tumor cells to grow slowly. We will further explore two possibilities in future experiments.

Conclusion

In this study, we concluded that AB23A affected the polarization of tumor-associated macrophages through the targeted regulation of CD11b/CD18, thus inhibiting the development of lung cancer.

Data Sharing Statement

The analyzed data sets generated during the present study are available from the corresponding author Yingna Chen and Jieya Lu on reasonable request.

Ethics Approval and Consent to Participate

All animal procedures were approved by Ethics Committee of Changzhou University (approval number: CCZU-BM2021R001), and were conducted in accordance with the ARRIVE guidelines.

Consent for Publication

All the authors agreed to be published.

Funding

This study was supported by Changzhou University Talent Introduction Research Fund (ZMF19020381).

Disclosure

There is no conflict of interest.

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