BMC Pulmonary Medicine

RESEARCH

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β-elemene inhibits tumor-promoting in small cell lung cancer by affecting M2 macrophages and TGF-β

Wenhui Huang[1†], Bing Fu[1†] and Haoran Xu[1*]

Abstract

Objective M2 macrophages have been implicated in promoting tumor growth and metastasis in various cancers, including small cell lung cancer (SCLC). This study investigated the role of M2 macrophages in SCLC progression and explored the therapeutic potential of β-elemene, a natural compound, in modulating M2 macrophage-mediated tumor promotion.

Methods We differentiated THP-1 monocytes into M2 macrophages using PMA (phorbol 12-myristate 13-acetate), IL-4 (interleukin-4), and IL-13 (interleukin-13). M2 macrophages were co-cultured with the SCLC cell line NCI-H209, and CCK-8, Transwell, and flow cytometry assays were performed. TGF-β expression levels were detected by ELISA. M2 macrophages and NCI-H209 co-cultured cells were treated with β-elemene, or M2 macrophages were transfected with TGF-β shRNA lentivirus, and then co-cultured with NCI-H209 cells. Flow cytometry was used to analyze cell apoptosis. Immunofluorescence staining was performed to assess TGF-β expression.

Results Our findings demonstrate that M2 macrophages significantly enhance the viability, proliferation, and migration of SCLC cells, and this effect is associated with increased TGF-β expression in SCLC cells co-cultured with M2 macrophages. Furthermore, β-elemene treatment significantly reduced the migration and viability of SCLC cells co-cultured with M2 macrophages. Silencing TGF-β expression in M2 macrophages also suppressed SCLC cell proliferation and migration, suggesting that β-elemene may inhibit the pro-tumorigenic effects of M2 macrophages in SCLC by modulating TGF-β signaling. Immunofluorescence staining revealed that β-elemene treatment significantly reduced TGF-β levels in SCLC cells co-cultured with M2 macrophages, supporting the hypothesis that β-elemene exerts its antitumor activity by modulating the TGF-β pathway.

Conclusions Our results suggest that β-elemene has the potential to suppress SCLC development by modulating M2 macrophages and the TGF-β, offering a new therapeutic avenue and potential drug candidate for SCLC treatment.

Keywords B-elemene, Small cell lung cancer, Immune microenvironment, M2 macrophages, TGF-β

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Background

Lung cancer cases and deaths are rising globally. The rapid and alarming increase in lung cancer incidence in both men and women in China has made it the most prevalent form of cancer and the leading cause of cancer-related mortality, prompting urgent and comprehensive public health efforts to address its devastating impact [1, 2].

Macrophages, as vital components of the innate immune system, play a pivotal role in orchestrating inflammatory responses and defending the body against invading microorganisms, thus serving as the primary line of defense and contributing significantly to the maintenance of overall immune surveillance and homeostasis. They have two phenotypes, M1 or M2, producing different effects on tumor cells [3]. Studies shown that TAMs (tumor-associated macrophages) often display a predominantly M2 macrophage phenotype, characterized by their ability to promote angiogenesis and facilitate tumor invasion, while concurrently exerting immunosuppressive effects that hinder the development of effective anti-tumor immune responses [4]. Furthermore, M2-related TGF-β has been reported to be an essential factor under the context of tumor inflammation and immune system, exerting a significant influence on the establishment of a tumor-promoting microenvironment within lung cancer tissue [5]. Therefore, we speculate that the high expression of TGF-β in M2 macrophages suppresses inflammatory responses and reduces the body's immune responses, thereby promoting the proliferation of cancer cells.

Chinese medicine, curcuma wenyujin, has been renowned for its multifunctional properties with a history over thousands of years. In vivo and in vitro experiments have indicate that the extract or active compounds derives from wenyujin have anti-tumor, anti-inflammatory, antioxidant activities [6]. β-elemene, an active compound extracted from wenyujin, has been proven to have many advantages, especially in cancer treatment. Prior research has indicated that β-elemene exhibits significant anticancer efficacy in both in vitro and in vivo experimental models by inducing cell cycle arrest and triggering apoptotic cell death [7]. It also changes the tumor inflammatory environment in lung cancer [8]. Studies have demonstrated that β-elemene is capable of activating the process of autophagy in a variety of cancer types, including lung, gastric, and renal cancer. This effect is achieved by inhibiting the activity of the PI3K/AKT/mTOR signaling pathway, which plays a critical role in regulating autophagy and is frequently dysregulated in cancer cells [9]. Studies have shown that β-elemene can also inhibit lung cancer by regulating the polarization of macrophages from M2 to M1, or by inhibiting TGF-β to suppress M2 polarization, thereby achieving anti-cancer effects [8, 10–12]. β-elemene has been introduced in clinical practice in China for more than 20 years. Its efficacy and safety have been confirmed [13, 14]. Unluckily, scarce studies focus on the effect of β-elemene on inflammation and immune response nor the M2 macrophage in SCLC.

This research has proved the evidence of the antitumor effect of β-elemene, as it has been demonstrated to change tumor inflammatory microenvironment, increase the immune response by M2 macrophages on small cell lung cancer cells, Furthermore, we explored these changes were related to TGF-β.

Materials and methods

Cell culture

THP-1 (CL-0233) cells were purchased from Procell Life Technology Co., Ltd. (Wuhan, China), while NCI-H209 (BNCC352112) cells were obtained from BeNa Biological. Cells were cultured in complete culture solution containing 10% FBS (AC03L055, Life-iLab Biotechnology, Shanghai, China), 1% penicillin-streptomycin (C0009, Beyotime Biotechnology, Shanghai, China) and 89% DMEM (C11995500BT, Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged at a ratio of 1:2 and incubated at 37 °C in a cell culture incubator. Logarithmically growing THP-1 cells were treated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) (HY-18739, MCE, Shanghai, China) for 24 h to induce macrophage differentiation. Subsequently, the cells were incubated with THP-1 cell-specific culture medium (CM-0233, Procell, Wuhan, China) containing IL-4 (20 ng/mL) (HY-P700130AF, MCE, Shanghai, China) and IL-13 (5 ng/mL) (HY-P70568, MCE, Shanghai, China) for 48 h to polarize them into M2 macrophages.

Construction of TGF-β interference vector

The interference targets designed based on the TGF-β transcript are shown in Table 1, and primers were arranged for synthesis. Single-stranded oligos were annealed to form double-stranded DNA and then ligated into the linearized pLVX-shRNA2 vector (BamHI-EcoR1). Competent E. coli cells (DH5α strain) (TSV-A07, TsingKe Biotech, Beijing, China) were transformed, and positive transformants were selected and sent to a sequencing company for sequencing. Positive clones verified by sequencing were selected for high-purity plasmid extraction.

Cell transfection

Successfully induced M2 macrophages were seeded at a density of $5 \times 10^{\wedge 5}$ per 10 cm culture dish. Dilute the sh-TGF-β and sh-NC plasmids separately by 50 times with OPTI-MEM (31985070, Gibco, MA, USA). Add the transfection reagent (24765-1, BioMedicine, Chongqing, China) to the tubes containing the sh-TGF-β and sh-NC

Table 1 Interference target site sequence

Target site	Sequence
TGF-β-oligo-1	GCAACAATTCCTGGCGATACC
Top Strand-1	5′-GATCCGGCAACAATTCCTGGCGATACCCTCGAGGGTATCGCCAGGAATTGTTGCTTTTTTG-3′
Bottom Strand-1	5′-AATTCAAAAAAGCAACAATTCCTGGCGATACCCTCGAGGGTATCGCCAGGAATTGTTGCCG-3′
TGF-β-oligo-2	GCAGAGTACACACAGCATATA
Top Strand-2	5′-GATCCGGCAGAGTACACACAGCATATACTCGAGTATATGCTGTGTGTACTCTGCTTTTTTG-3′
Bottom Strand-2	5′-AATTCAAAAAAGCAGAGTACACACAGCATATACTCGAGTATATGCTGTGTGTACTCTGCCG-3′
TGF-β-oligo-3	GGACATCAACGGGTTCACTAC
Top Strand-3	5′-GATCCGGGACATCAACGGGTTCACTACCTCGAGGTAGTGAACCCGTTGATGTCCTTTTTTG-3′
Bottom Strand-3	5′-AATTCAAAAAAGGACATCAACGGGTTCACTACCTCGAGGTAGTGAACCCGTTGATGTCCCG-3′

Note: Loop Sequence in shRNA is: CTCGAG

Table 2 Primer information

Gene	Primer-F	Primer-*R*
Arg-1	CCTGCCCTTTGCTGACATCC	TCTTCTTGACTTCTGCCACCTTG
CD68	AGTCATGGAAATGCCACGGT	TGGGCAGAACTGGTGAATCC
CD71	GCTGCCAGCTTTACTGGAGA	CGTCACCAGAGAGGGCATTT
CD14	GAACCTTGTGAGCTGGACGA	CAGACACACACTGGAAGGCT
iNOS	CGCATGACCTTGGTGTTTGG	CATAGACCTTGGGCTTGCCA
TGF-β	GAGAAGCGGTACCTGAACCC	TGAACCCGTTGATGTCCACT
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA

plasmids, mix thoroughly, and let it stand at room temperature for 15 min. Then, add the mixture evenly to the culture dishes, gently swirl in a crosshatch pattern to ensure even distribution, and return the cells to the incubator for continued culture.

Cell grouping

Cells were divided into five groups: NCI-H209 group, NCI-H209 + M2 group, NCI-H209 + M2 + β-elemene group, NCI-H209 + M2 + sh-NC group and NCI-H209 + M2 + sh-TGF-β group. The cell suspension was collected into centrifuge tubes, centrifuged at 1000 rpm for 5 min, the supernatant was discarded, the cells were resuspended and counted, and the cell density was adjusted to 2×10^6 cells/mL. Cells were seeded according to the experimental requirements. NCI-H209 group: NCI-H209 cells were cultured normally. NCI-H209 + M2 group: M2 macrophages were seeded into the upper chamber of a Transwell, and NCI-H209 cells were seeded into the lower chamber. After co-culturing for 12 h, the upper chamber containing M2 macrophages was removed, and the NCI-H209 cells were cultured alone. NCI-H209 + M2 + β-elemene group: M2 macrophages were seeded into the upper chamber of a Transwell, and NCI-H209 cells were seeded into the lower chamber. After co-culturing for 12 h, the cells were treated with 20 μg/mL β-elemene (HY-107324, MCE, Shanghai, China), and then the upper chamber containing M2 macrophages was removed, and the NCI-H209 cells were cultured alone. NCI-H209 + M2 + sh-NC group/NCI-H209 + M2 + sh-TGF-β group: M2 macrophages transfected with empty vector or TGF-β interfering vector (pLVX-shRNA2) were seeded into the upper chamber of a Transwell, and NCI-H209 cells were seeded into the lower chamber. After co-culturing for 12 h, the upper chamber containing M2 macrophages was removed, and the NCI-H209 cells were cultured alone. The cells in each group were further cultured for 48 h before the experiments.

qPCR

Total RNA from THP-1 cells, M0 macrophages, and M2 macrophages was extracted using TRIzol® (15596026, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was then synthesized using the Goldenstar™ RT6 cDNA Synthesis Kit Ver.2 (TSK302M, Tsingke Biotechnology, Beijing, China). The relative expression of Arg-1 was detected using the 2 × T5 Fast qPCR Mix (SYBR Green I) kit (TSE002, Tsingke Biotechnology, Beijing, China). Real-time PCR was performed using prepared cDNA templates, SYBR Green MasterMix, primer sets, and Dye2. The thermal cycling conditions were: 95 °C for 60 s, 95 °C for 10 s, and 60 °C for 30 s for 45 cycles. The relative expression level of Arg-1 was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal control. Specific primers for qPCR are listed in Table 2.

CCK-8

A standard curve was prepared to determine the cell number. After seeding cells into culture plates, the culture medium was diluted proportionally in a cell density

gradient. It is recommended to establish 3–5 cell density gradients with 3–6 replicates for each concentration. The seeded cells were incubated for 2–4 h to allow them to adhere to the culture plate. Measure the optical density (OD) value and plot a standard curve. After grouping and treating the cells, collect the cell suspension (100 μL/well) and seed it into a 96-well plate. Incubate the plate in a CO_2 incubator (37 °C, 5% CO_2). Subsequently, add 10 μL CCK-8 solution (C0038, Beyotime, Shanghai, China) to each well and incubate the plate in the incubator for 1–4 h. Measure the absorbance of the cells at a wavelength of 450 nm using a microplate reader.

Cell viability (%) = [A (treated) - A (blank)] / [A (untreated) - A (blank)] × 100.

A (treated): Absorbance of the well containing cells, CCK-8 solution, and drug solution.

A (blank): Absorbance of the well containing culture medium and CCK-8 solution (without cells).

A (untreated): Absorbance of the well containing cells, CCK-8 solution, and no drug. *Cell viability: Cell proliferation or cytotoxicity activity.

Cell cycle analysis by flow cytometry

Cells were seeded in 60 mm cell culture plates at a density of 2×10^6 cells/well. After 24 h of incubation, the cells were washed with PBS and resuspended in 1 mL DNA staining solution and 10 μL permeabilization solution (Multisciences, Hangzhou, China). The cells were incubated in the dark at room temperature for 30 min. Cell cycle distribution was analyzed by flow cytometry using a FACS Canto TM II instrument (BD Biosciences, San Jose, CA, USA). Each treatment group was subjected to three independent replicates. When cells reached approximately 80% confluence, the culture medium was discarded, cells were collected, digested with trypsin, and 3 replicate wells were used for each group. After the reaction was terminated, the cells were collected. To ensure sufficient cells for analysis, the cell count should be ≥ 1,000,000 cells/treatment group. The cells were then centrifuged at 1200 rpm for 5 min, the supernatant was discarded, and the cell pellet was washed with pre-chilled PBS at 4 °C by centrifuging at 1500 rpm for 5 min. The cells were fixed in pre-chilled 70% ethanol at 4 °C for at least 1 h. After centrifuging at 1500 rpm for 5 min, the fixation solution was discarded, and the cell pellet was washed with PBS. A cell staining solution was prepared: 40× PI stock solution (2 mg/ml): 100× RNase stock solution (10 mg/ml): 1× PBS = 25:10:1000. An appropriate volume of cell staining solution (1-1.5 ml) was added based on the cell count, and the cells were resuspended. The cell flow rate was adjusted to be between 200 and 350 cells/s for analysis. The cells were filtered through a 300-mesh sieve into a flow cytometry tube and analyzed by flow cytometry.

Cells were collected by centrifugation at 2000 rpm for 5 min. The cell pellet was washed twice with ice-cold PBS (G0002, Servicebio Biotechnology, Wuhan, China) at 2000 rpm for 5 min, and the supernatant was carefully aspirated leaving approximately 50 μL to avoid loss of cells. The cells were fixed by adding 1 mL of ice-cold 70% ethanol (64-17-5, Biochem safebuy, Wuhan, China), gently vortexed, and incubated at 4 °C for 12 h. Fixed cells were washed twice with ice-cold PBS at 1000 rpm for 5 min, and the supernatant was carefully aspirated leaving approximately 50 μL of PBS. The cell pellet was gently resuspended to prevent cell clumping. For staining, 0.5 mL of propidium iodide staining solution (AC12L544, Life-iLab Biotechnology, Shanghai, China) was added to each tube, and cells were gently resuspended and incubated in the dark at room temperature for 30 min. Samples were then stored in the dark at 4 °C. Flow cytometric analysis was performed within 24 h of staining. Red fluorescence was detected using a flow cytometer with a 488 nm excitation wavelength (CytoFLEX, Beckman, Brea, CA, USA), and light scattering characteristics were simultaneously recorded.

Transwell

The cell suspension was collected into centrifuge tubes, centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the cells were washed with PBS. The cells were resuspended in serum-free culture medium, and the cell density was adjusted to 5×10^5 cells/mL. 100 μL of cell suspension was added to the upper chamber of a Transwell (3422, Corning, Corning, NY, USA), and 500 μL of RPMI-1640 culture medium containing 10% FBS (L210KJ, Basalmedia, Shanghai, China) was added to the lower chamber. The cells were incubated in a CO_2 incubator (37 °C, 5% CO_2) for 24 h. After incubation, the Transwell was removed, and the cells in the lower chamber were photographed under a microscope at 100× magnification. Cells were counted in random fields of view, and the average value was recorded.

ELISA detection of TGF-β expression

The TGF-β content in the NCI-H209 cell line and M2 macrophages supernatant was determined using an ELISA kit (ab100647, ABcam, Shanghai, China). The prepared ELISA plate included standard wells and sample wells. The experimental procedure was followed according to the kit manual. The OD value of the blank well was set to zero using a spectrophotometer (NanoDrop One/One C, Thermofisher, Waltham, MA, USA) at a wavelength of 450 nm. Absorbance at 450 nm was then measured to determine the results.

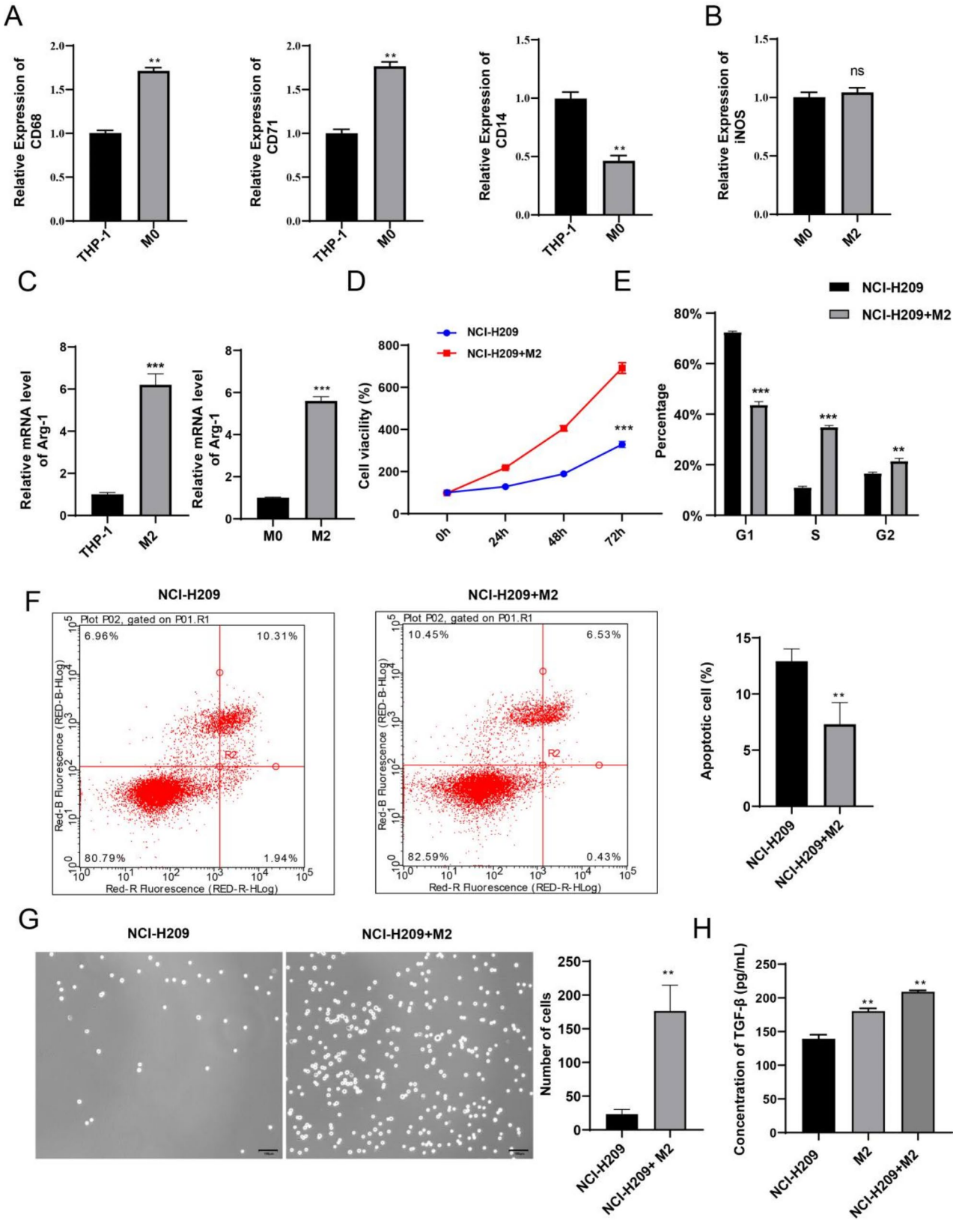

Fig. 1 (See legend on next page.)

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Fig. 1 The Effect of M2 Macrophages on NCI-H209 Cells. (**A**) PMA-induced differentiation of THP-1 into M0 macrophages. (**B** and **C**) IL-4 and IL-13 induce the differentiation of M0 macrophages into M2 macrophages. (**D**) CCK8 assay to detect the effect of M2 macrophages on the viability of NCI-H209 cells. (**E**) Flow cytometry to analyze the cell cycle distribution of NCI-H209 cells treated with M2 macrophages. (**F**) Flow cytometry to analyze the cell apoptosis of NCI-H209 cells treated with M2 macrophages. (**G**) Transwell assays to examine the effect of M2 macrophages on the migration of NCI-H209 cells. Scale bar = 100 μm. (**H**) ELISA assay to detect the effect of M2 macrophages on TGF-β expression. $^{**}p < 0.01$, $^{***}p < 0.001$, ns" stands for "no significant difference

Cell apoptosis analysis by flow cytometry

The culture medium from each group was aspirated into centrifuge tubes, centrifuged at 1000 rpm for 3 min, the supernatant was discarded, and the cells were collected. The cells were gently resuspended in PBS and counted. 1×10^6 resuspended cells were centrifuged at 1000 rpm for 3 min, the supernatant was discarded, and the cells were gently resuspended in 195 μL Annexin V-FITC binding solution (40302ES60, Yeasen, Shanghai, China). 5 μL Annexin V-FITC was added and gently mixed. The cells were incubated in the dark at room temperature (20–25 °C) for 10 min. Aluminum foil can be used to block light. The cells were centrifuged at 1000 g for 5 min, the supernatant was discarded, and the cells were gently resuspended in 190 μL Annexin V-FITC binding solution. 10 μL PI staining solution was added, gently mixed, and incubated in the dark on ice. The cells were immediately analyzed using a CytoFLEX flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA, USA).

Immunofluorescence staining

Fixed cells were washed with PBS 3 times, 5 min each. Cells were blocked with goat serum at room temperature for 30 min. After drying the blocking solution, TGF Beta 1 Polyclonal antibody (21898-1-AP, proteintech, Wuhan, China) diluted 1:200 was added and incubated overnight at 4 °C in the dark. The cells were washed with PBS 3 times, 5 min each. FITC-conjugated Goat anti-Rabbit IgG (H + L) (AS011, ABclona Technology, Wuhan, China) diluted 1:200 was added and incubated in the dark at room temperature for 1.5 h. Cells were washed with PBS 3 times, 5 min each. DAPI staining solution (C1005, Beyotime, Shanghai, China) was added and incubated in the dark for 5 min, followed by washing with PBS 3 times, 5 min each, to remove excess DAPI. The cells were mounted with an anti-fluorescence quenching agent (P0126, Beyotime, Shanghai, China). DAPI staining resulted in blue-stained nuclei, while positive regions exhibited green fluorescence.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean ± standard deviation. Each experiment was repeated at least three times. For comparisons between two groups, an independent-sample t-test was used. For comparisons between three or more groups, a one-way analysis of variance (ANOVA) followed by a Turkey's multiple comparison test was used. $p < 0.05$ was considered statistically significant.

Results

M2 macrophages significantly increased the viability, proliferation, migration, and TGF-β expression in SCLC

To investigate the role of M2 macrophages in SCLC, we treated THP-1 cells with PMA for 24 h, followed by IL-4 and IL-13 to induce differentiation. The expression of M0 macrophage biomarkers CD67 and CD71 significantly increased, while the monocyte biomarker CD14 expression significantly decreased ($p < 0.01$), indicating that THP-1 cells have successfully differentiated into M0 macrophages after PMA treatment (Fig. 1A). After inducing M0 macrophages with IL-4 and IL-13, there was no significant difference in the expression of the M1 macrophage biomarker iNOS. (Fig. 1B), and the expression of the M2 macrophage biomarker Arg-1 (Arginase-1) significantly increased ($p < 0.001$) (Fig. 1C), indicating that M0 macrophages have successfully induced into M2 macrophages. Subsequently, M2 macrophages were co-cultured with NCI-H209 cells. The CCK-8 assay showed that M2 macrophages significantly increased the cell viability of NCI-H209 compared with the control group ($p < 0.001$) (Fig. 1D). Consistently, the results of flow cytometry showed that the proportion of cells in S and G2 phase in the NCI-H209 + M2 group was significantly increased, indicating that M2 macrophages significantly promoted the proliferation of NCI-H209 cells ($p < 0.01$) (Fig. 1E). Furthermore, M2 macrophages significantly suppressed NCI-H209 cell apoptosis ($p < 0.01$) (Fig. 1F). Transwell assays were performed to detect the effect of M2 macrophages on the migration of NCI-H209 cells. The results showed that within 24 h, the number of migrated cells in the NCI-H209 + M2 group was significantly higher than that in the NCI-H209 group, suggesting that M2 macrophages promoted the migration of NCI-H209 cells ($p < 0.01$) (Fig. 1G). Furthermore, ELISA was used to measure the effect of M2 macrophages on TGF-β expression. Compared to NCI-H209 group, the expression of TGF-β was significantly increased in the NCI-H209 + M2 group, and M2 macrophage supernatant also contains high levels of TGF-β ($p < 0.001$) (Fig. 1H). These findings indicate that M2 macrophages can enhance the proliferation, viability, and migration of NCI-H209 cells, potentially through the upregulation of TGF-β.

β-elemene and TGF-β inhibition suppress M2 macrophage-induced SCLC proliferation and migration

To further investigate the effects of β-elemene and TGF-β on SCLC, we treated M2 macrophages and NCI-H209 co-cultured cells with β-elemene, and co-cultured NCI-H209 cells with M2 macrophages transfected with TGF-β silencing vectors. Transwell and CCK-8 assays revealed that the number of migrated NCI-H209 cells and cell viability in the NCI-H209 + M2 + β-elemene group were significantly lower than those in the NCI-H209 + M2 group ($p < 0.001$) (Fig. 2A and B). The number of migrated cells and cell viability in the NCI-H209 + M2 + sh-TGF-β group were significantly lower than those in the NCI-H209 + M2 + sh-NC group ($p < 0.05$), indicating that both β-elemene and TGF-β inhibition suppressed the increased migration and viability of NCI-H209 cells induced by M2 macrophages (Fig. 2A and B). qPCR was used to detect the expression levels of TGF-β in M2 macrophages. The results showed that the TGF-β expression in the M2 + sh-TGF-β group was significantly lower than that in the M2 group ($p < 0.01$) (Fig. 2C). This indicates that the transfection of TGF-β-interfering shRNA was successful.

Additionally, flow cytometry analysis of cell cycle distribution in NCI-H209 cells revealed that the proportion of S phase cells in the NCI-H209 + M2 + β-elemene group was significantly reduced compared to the NCI-H209 + M2 group ($p < 0.01$). While a reduction in the proportion of S phase cells was observed in the NCI-H209 + M2 + sh-TGF-β group compared to the

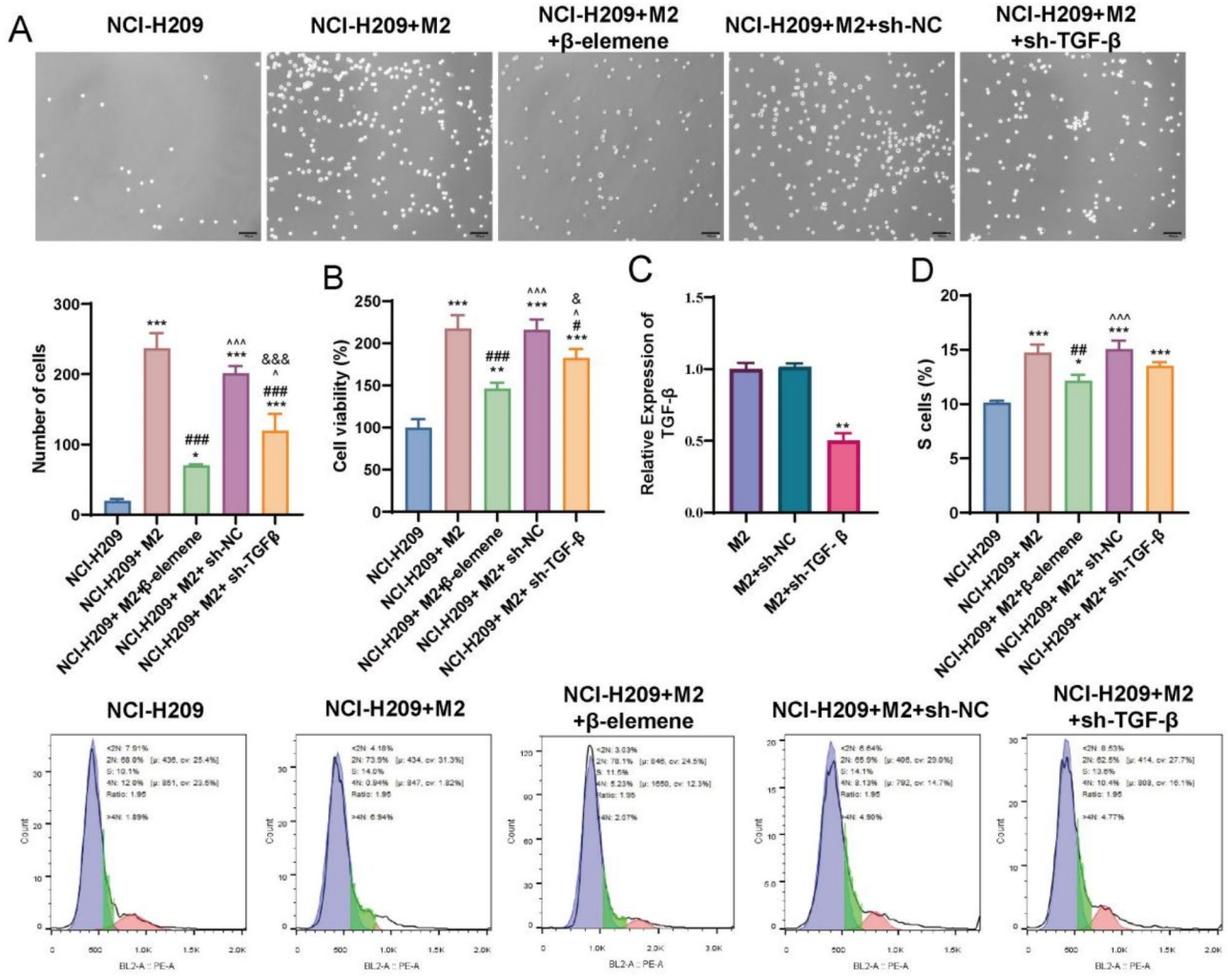

Fig. 2 Effects of β-elemene treatment in M2 and NCI-H209 Co-cultured cells, and interference with TGF-β in M2 macrophages on SCLC migration, viability, and cell cycle. (**A**) Transwell assay for NCI-H209 cell migration. Scale bar = 100 μm. (**B**) CCK8 assay to detect NCI-H209 cell viability. (**C**) qPCR was used to verify the interference effect. (**D**) Flow cytometry analysis of the cell cycle in NCI-H209 cells. 2 N: Proportion of G0/G1 phase cells to the total cell population; S: Proportion of S phase cells to the total cell population. 4 N: Proportion of G2/M phase cells to the total cell population. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: compared to the NCI-H209 group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$: compared to the NCI-H209 + M2 group. ^$p < 0.05$, ^^^ $p < 0.001$: compared to the NCI-H209 + M2 + β-elemene group. & $p < 0.05$ and &&& $p < 0.001$: compared to the NCI-H209 + M2 + sh-NC group

NCI-H209 + M2 + sh-NC group, but no statistical significance was observed ($p > 0.05$) (Fig. 2D). Apoptosis analysis in NCI-H209 cells revealed a significant increase in the apoptosis rate in the NCI-H209 + M2 + β-elemene group compared to the NCI-H209 + M2 group ($p < 0.001$), and in the NCI-H209 + M2 + sh-TGF-β group compared to the NCI-H209 + M2 + sh-NC group ($p < 0.001$) (Fig. 3). These findings indicate that both β-elemene and TGF-β inhibition suppress M2 macrophage-induced SCLC proliferation and migration, and promote cell apoptosis.

β-elemene may affect SCLC through TGF-β

Immunofluorescence staining (Fig. 4A) revealed a significant decrease in TGF-β levels in NCI-H209 cells in the NCI-H209 + M2 + β-elemene group compared to the NCI-H209 + M2 group ($p < 0.01$). Similarly, TGF-β levels were significantly reduced in the NCI-H209 + M2 + sh-TGF-β group compared to the NCI-H209 + M2 + sh-NC group ($p < 0.001$). TGF-β levels in all groups were measured by ELISA, and the trends were consistent with the results from immunofluorescence detection (Fig. 4B). These findings suggest that β-elemene may inhibit the pro-tumorigenic effects of M2 macrophages on NCI-H209 cells, potentially through the suppression of TGF-β.

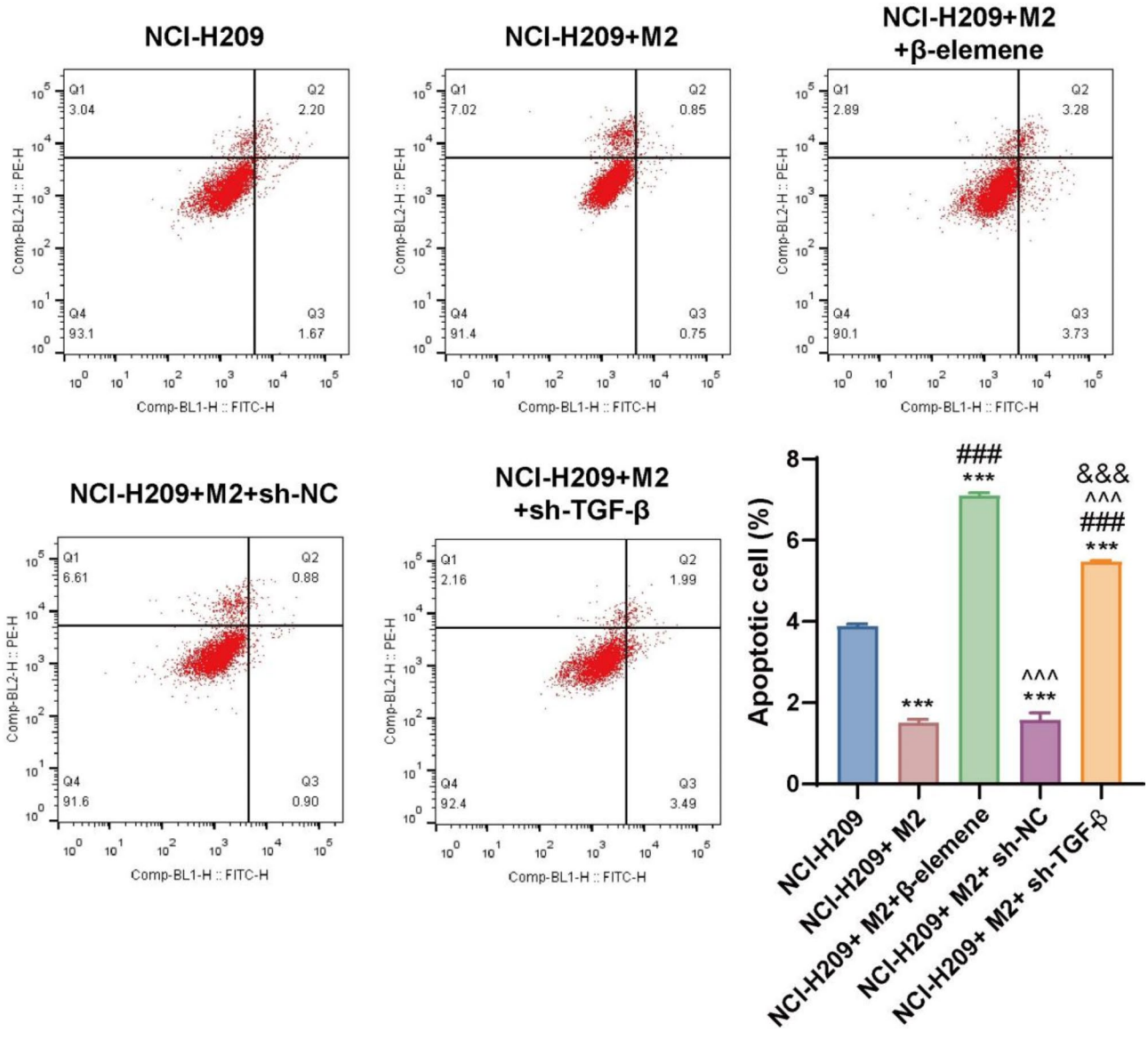

Fig. 3 Flow Cytometry Analysis of Apoptosis in NCI-H209 Cells Treated with β-elemene or TGF-β Inhibition. *** $p < 0.001$: compared to the NCI-H209 group. ### $p < 0.001$: compared to the NCI-H209 + M2 group. ^^^ $p < 0.001$: compared to the NCI-H209 + M2 + β-elemene group. &&& $p < 0.001$: compared to the NCI-H209 + M2 + sh-NC group

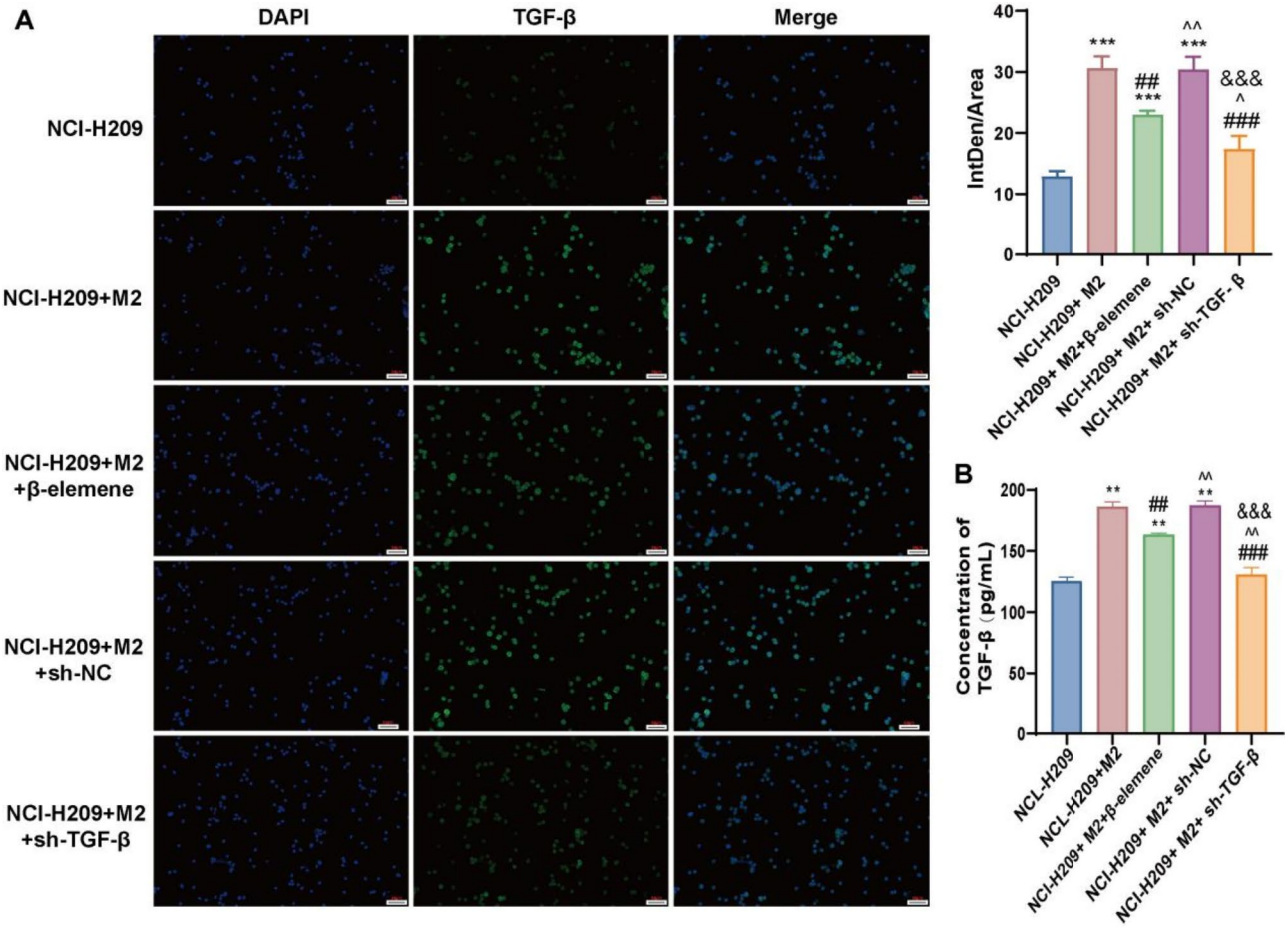

Fig. 4 TGF-β Expression in NCI-H209 Cells Treated with β-elemene or TGF-β Silencing. (**A**) Immunofluorescence detection of TGF-β Expression. (**B**) The levels of TGF-β were determined by ELISA. ** $p < 0.01$ and *** $p < 0.001$: compared to the NCI-H209 group. ## $p < 0.01$ and ### $p < 0.001$: compared to the NCI-H209 + M2 group. ^ $p < 0.05$ and ^^ $p < 0.01$: compared to the NCI-H209 + M2 + β-elemene group. && $p < 0.001$: compared to the NCI-H209 + M2 + sh-NC group

Discussion

From a histological perspective, lung cancer is the most prevalent cancer form globally and can be broadly classified into two major subtypes, namely, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), each possessing distinct clinical and pathological characteristics, warranting optimized management with tailored therapeutic approaches [15]. SCLC, also known as oat cell carcinoma, is a highly aggressive malignancy, prone to early relapse and metastasis [16]. Tumor-associated macrophages (TAMs) constitute a prominent component of the tumor microenvironment (TME), often implicated in tumor progression by adopting an M2-like phenotype [17]. M2 macrophages have been demonstrated to promote tumor growth, metastasis, and immune suppression in various cancers, including SCLC [18]. While previous studies have emphasized the significance of targeting the tumor microenvironment as a therapeutic strategy, such targeted agents may elicit adverse effects, and prolonged treatment can lead to the development of multi-drug resistance in a subset of patients [19]. The extract of the Chinese medicinal herb Wenyujin, β-elemene, has been shown to exert antitumor effects on NSCLC without apparent side effects [20, 21]. Comprehensively investigating and elucidating the underlying mechanisms of β-elemene-mediated antitumor activity in SCLC are crucial for successful drug discovery. In the present study, we explored the influence of M2 macrophages on SCLC cells and investigated the potential therapeutic role of β-elemene in modulating these effects.

Our results demonstrated that induction of THP-1 cells into M2 macrophages using PMA, IL-4, and IL-13 resulted in elevated Arg-1 levels, confirming successful differentiation into the M2 phenotype. Co-culturing NCI-H209 cells with M2 macrophages led to a significant increase in cell viability, proliferation, and migration, as evidenced by CCK-8 and Transwell assays, and flow cytometry analysis. In the flow cytometry results, we also found that after co-culturing NCI-H209 and M2,

the proportion of Q1 increased. but it decreased when β-elemene or M2 with knocked down TGF-β was added. Research has shown that TGF-β can induce the death of melanoma cells, and TGF-β plays multiple roles in the tumor microenvironment [22]. Therefore, we speculate that in our experiment, the increase of TGF-β may lead to an increase in necrotic cell death in cancer cells, and we will be committed to investigating this finding in the future. Furthermore, the enhanced expression of TGF-β in the co-cultured cells suggested a possible mechanism underlying the tumor-promoting role of M2 macrophages. These findings are in line with the established tumor-promoting functions of M2 macrophages [23] and indicate that TGF-β could be a critical mediator in this process. TGF-β is a pivotal factor regulating diverse biological processes, including cell proliferation, differentiation, apoptosis, ECM synthesis, and stem/progenitor cell fate [24]. Numerous studies have implicated TGF-β overexpression in tumorigenesis, contributing to immune evasion and tumor progression [25]. TGF-β can also induce apoptosis in various cancer cells through different mechanisms [26]. SCLC is an aggressive form of lung cancer, characterized by rapid growth and propensity to metastasize, accounting for approximately 10–15% of all diagnosed lung cancers, and poses a significant challenge in effective treatment due to its invasive and recurrent nature [27]. TGF-β has been shown to modulate the survival of SCLC cells in vitro and in vivo [28]. TGF-β is also known to regulate the function and phenotype of macrophages [25].

Importantly, we found that β-elemene treatment significantly inhibited the viability and migration of NCI-H209 cells co-cultured with M2-type macrophages. Immunohistochemical results showed that silencing the expression of TGF-β in M2 macrophages reduces the TGF-β level in NCI-H209 cells co-cultured with them. In the co-culture system with β-elemene, the TGF-β level in NCI-H209 cells was also significantly reduced. This suggests that β-elemene can lower the TGF-β level in NCI-H209 cells by inhibiting the expression of TGF-β in the tumor microenvironment, which is created by M2 macrophages. Studies have shown that TGF-β is an effective inducer of epithelial-mesenchymal transition (EMT), a key cellular process in cancer metastasis [29]. Therefore, inhibiting TGF-β signaling has become a promising therapeutic approach for cancer. Flow cytometry analysis further revealed that β-elemene reduced the percentage of cells in the S phase, implying cell cycle arrest, and increased the apoptotic cell population, highlighting its potential as an effective therapeutic agent. Our results are consistent with previously reported anticancer properties of β-elemene in various cancer types [30–32], underscoring its ability to induce apoptosis and suppress proliferation. The novelty of our study lies in demonstrating that the antitumor activity of β-elemene in SCLC is likely mediated by modulating M2-type macrophages and TGF-β signaling in the tumor microenvironment.

Nevertheless, our study has certain limitations. Firstly, the in vitro nature of our experiments warrants further validation in in vivo models to confirm the therapeutic potential of β-elemene in a more complex biological system. Additionally, while we have identified TGF-β as a key mediator, the involvement of other cytokines and signaling pathways cannot be entirely ruled out and requires further investigation. Future studies should focus on deciphering the detailed molecular mechanisms underlying the effects of β-elemene on M2 macrophages and SCLC cells and explore its combinatorial effects with other therapeutic agents. Moreover, clinical studies are essential to determine the safety and efficacy of β-elemene in patients with SCLC.

In summary, our study demonstrates that M2-type macrophages significantly promote the viability, proliferation, migration, and TGF-β expression of SCLC cells, and β-elemene effectively counteracts these tumor-promoting effects. These findings suggest that β-elemene could be a promising therapeutic agent for the treatment of SCLC by targeting the tumor microenvironment, specifically by modulating TGF-β signaling. This research contributes to our understanding of the tumor microenvironment in SCLC and underscores the potential of β-elemene in cancer therapy.

Acknowledgements
Not applicable.

Author contributions
WH, BF and HX conceived and designed the research, drafted the manuscript, revised the manuscript and provided support. All authors contributed to the article and read and approved the final version of the manuscript.

Funding
Not applicable.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 12 November 2024 / Accepted: 28 January 2025
Published online: 28 February 2025

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