

# Mechanism of Sanguinarine in Inhibiting Macrophages to Promote Metastasis and Proliferation of Lung Cancer via Modulating the Exosomes in A549 Cells

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**Objective:** Sanguinarine (SNG) is a benzophenanthridine alkaloid obtained from the roots of *Sanguinaria canadensis* and has an anticancer effect. The aim of this study was to explore the mechanism of SNG in inhibiting macrophages via regulating the exosomes derived from lung carcinoma cells to reduce metastasis and proliferation of lung carcinoma.

**Methods:** Human lung cancer cells (A549 cells) were treated with 4μM of SNG. Exosomes of A549 cells were extracted from A549 cells supernatant, and THP-1 cells were cultured with exosomes. Then, the supernatant of THP-1 cells was collected and cultured with A549 cells. Cell proliferation was measured via plate clone formation and CCK-8 assays. Migration was assessed by using Transwell assay and scratch test. Cellular invasion was detected by Transwell assay. Apoptosis was determined using flow cytometry. Moreover, the protein expressions of GAPDH, P65 and P-P65 in THP-1 cells were measured by Western blot. Levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and chemotactic cytokines ligand 2 (CCL-2) extracted from THP-1 cells were determined by reverse transcription-polymerase chain reaction (RT-PCR).

**Results:** Compared to the control group, exosomes could activate THP-1 cells, and the invasion, migration, and proliferation of A549 cells were consequently enhanced. Exosomes could increase the protein expression of p-p65 and the RNA expression levels of TNF-α, IL-6, and CCL-2 in THP-1 cells. Compared with the exosome group, SNG-treated exosomes inhibited THP-1 cells so that the invasion, proliferation, and migration of A549 cells were attenuated and apoptosis was promoted. In THP-1 cells, SNG-treated exosomes inhibited P-P65 expression and the RNA expression levels of TNF-α, IL-6, and CCL-2.

**Conclusion:** Exosomes treated by SNG inhibited THP-1 cells so that the invasion, proliferation, and migration of A549 cells were inhibited, and the apoptosis was promoted. The mechanism is possibly associated with the inhibition of NF-κB pathway in THP-1 cells.

**Keywords:** SNG, exosomes, THP-1 cells, NF-κB signaling pathway

## Introduction

Exosome, a kind of extracellular vesicles (EV), has a lipid-bilayer spheroid structure without replicating capacity and contains proteins, nucleic acids, lipids, etc. The sizes of the exosomes range from 30 nm to 100nm.<sup>1</sup> Exosomes are regarded as key mediators in intercellular communications because their outer membrane structure can stably preserve the contents and deliver them to the

receptor cells. It has been shown that exosomes from human lung cancer cells could promote the proliferation and ameliorate the apoptosis of A549 cells, thus enhancing development of malignancy.<sup>2</sup> Furthermore, it is confirmed that the exosomes derived from tumor cells could change the microenvironment surrounding the tumors and establish a pro-cancer environment.<sup>3</sup> There are various components in the tumor microenvironment, including stromal cells, endothelial cells, and immune cells. The interaction between these cells contributes to inflammatory responses in the microenvironment, which promotes development of the tumors, angiogenesis, and tumor metastasis.<sup>4,5</sup> Macrophages respond to different signals in the microenvironment and have functional plasticity.<sup>6</sup> In the microenvironment of tumors, macrophages can induce the development, metastasis, development and drug resistance of lung carcinoma.<sup>7</sup> Therefore, inhibiting the influence of exosomes in tumor cells can reduce the connection between tumor cells and macrophages, thus inhibiting tumor cell metastasis and proliferation.

As a fatal malignancy, lung cancer owns the highest morbidity and mortality among all cancers across the world. Non-small cell lung cancer (NSCLC) accounts for about 85% among all kinds of lung cancers and can be treated by surgical resection, radiotherapy, chemotherapy, molecular targeted therapy and immunotherapy.<sup>8</sup> NF- $\kappa$ B, a pro-inflammatory transcription factor, is a key activator in inflammation and decisively promotes tumor cell survival, proliferation, invasion, angiogenesis and metastasis. NF- $\kappa$ B can regulate expressions of inflammatory genes,<sup>9</sup> including TNF- $\alpha$ , IL-6, CCL-2 and other inflammatory factors. Inflammatory factors in tumor microenvironment play an important role in the development, invasion and metastasis of tumors. TNF- $\alpha$  enhances tumor-producing inflammatory pathways such as NF- $\kappa$ B signaling pathway, which in turn stimulates the proliferation of malignant cells.<sup>10</sup> IL-6 promotes the progression of colorectal cancer, tumor development and metastasis by regulating local and systemic inflammation and angiogenesis.<sup>11</sup> CCL2 promotes tumor invasion and metastasis by recruiting mononuclear macrophages.<sup>12</sup> In recent years, great attention has been paid to the natural products derived from plants to develop candidate anticancer drugs. SNG is a benzophenanthridine alkaloid obtained from the roots of *Sanguinaria canadensis*.<sup>13</sup> A number of studies have shown that SNG had a therapeutic

potential in a variety of human diseases, including lung cancer,<sup>14</sup> cervical cancer,<sup>15</sup> gastric cancer,<sup>16</sup> hepatocellular carcinoma.<sup>17</sup> However, the effect of SNG on exosomes in tumor microenvironment and the mechanism of its regulation on immunity are still unclear. It was found that SNG suppressed invasion, migration and proliferation of A549 cells and promoted cell apoptosis via regulating the exosomes which inhibited NF- $\kappa$ B signaling pathway in THP-1 cells. This study aimed to explore the mechanisms of SNG in ameliorating metastasis and proliferation of lung carcinoma cells through inhibiting macrophages via regulating the exosomes derived from lung carcinoma cells.

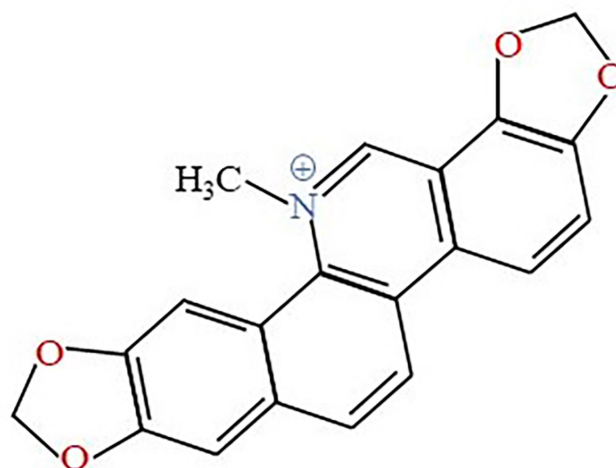
## Materials and Methods

### Reagents and Drugs

SNG (Figure 1) was obtained from Selleck (Shanghai, China). Dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich (St. Louis, MO, USA), was used to dissolve the agent (stock concentration: 10 mM). Annexin V-FITC/PI and CCK-8 assay kit was purchased from SigmaAldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was provided by GIBCO/BRL (Grand Island, NY, USA) and RPMI-1640 cell culture medium was purchased from HyClone (Logan, Utah, USA). The flow cytometry antibodies CD206, CD68, CD14 were purchased from Peprotech (New Jersey, NJ, USA). Primary antibodies of human p-NF- $\kappa$ B (1:1000), NF- $\kappa$ B (1:1000), and GAPDH (1:2000) were bought from Cell Signaling Technology (Boston, Massachusetts, USA). Primary antibodies of CD63 (dilution), Alix (dilution), and TSG101 (dilution) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody of Calnexin (1:1000) was bought from Proteintech company (Chicago, Illinois, USA). Total exosome isolation reagent and exosome-depleted FBS media supplement were purchased from SBI (Johnston, Pennsylvania, USA).

### Cell Culture

Human acute monocytic leukemia (THP-1) and human lung cancer (A549) cell lines were purchased from the cell bank of Shanghai institute of life sciences (Shanghai, China). THP-1 and A549 cell lines were cultured by using Roswell Park Memorial Institute 1640 (RPMI-1640) medium added with 10% exosomes-depleted FBS (SBI, Johnston, Pennsylvania, USA).<sup>18</sup> All



**Figure 1** Chemical structure of sanguinarine.

cells were cultured in an incubator (37 °C) with humidified environment and CO<sub>2</sub> (5%).

## Exosome Separation and Its Characterization

Exosomes were obtained from the supernatant of A549 cells, which was described previously. A549 cell line was cultured in the medium without exosomes and SNG was added 24 hours later. Then, the supernatant was obtained at 48 h and centrifuged for 15 minutes at 3000g to eliminate cell debris (optimal-90K; BeckmanCoulter, Brea, California, USA). After centrifugation, the solution was filtrated by using filters (0.22-μm pore)(Millipore, St. Louis, MO, USA), and ExoQuick Exosome Precipitation Solution was added for incubation overnight. Afterwards, the solution was centrifuged for 30 minutes at 1500g. Sterile phosphate buffered saline (PBS) was used to dissolve the precipitation and the solution was kept in a -80 °C refrigerator. The concentrations of the proteins from exosomes were measured by BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Concentrations and sizes of the exosomes were detected by Nanoparticle Tracking Analysis (NTA). The exosome morphology was observed under a transmission electron microscope (Tecnai 12; Philips, Amsterdam, Netherlands).<sup>18,19</sup> The marker proteins were detected by Western Blot.

## NTA

Exosomes were diluted with PBS to a concentration of 1x10<sup>7</sup>/mL ~1x10<sup>9</sup>/mL and carefully injected into the particle point titration and particle size analyzer without bubbles. Particle matrix ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) was used to determine the concentration of the exosomes isolated at 405nm. The size and mass were also determined. Meanwhile, the particle trajectory of the exosomes could be analyzed.

## Fluorescence Labeling of Exosomes

For staining, 1μM 3, 3'-dioctadecyloxacarbocyanine perchlorate (Dio) was added to the purified exosomes for 10min. Then stained exosomes were centrifuged at 1 x 10<sup>5</sup>g for 40 min at 4 °C for twice. The labeled exosomes in the precipitation were kept and suspended with Dulbecco's Modified Eagle Medium (DMEM). THP-1 cells were cultured with the labeled exosomes for 24h, and a fluorescence microscope was used to observe exosomes entering the cells. After the culture solution was discarded, the cells were rinsed for 3 times with PBS, fixed for 10min with paraformaldehyde (4%), and then rinsed with PBS for 3 times. The cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) for 5min in room temperature. After staining, the cells were rinsed with PBS, and confocal microscopy was used to observe whether the Dio labeled exosomes were internalized by THP-1 cells.

## THP-1 Cells Treated by Exosomes

THP-1 cells ( $5 \times 10^5$ ) were inoculated in 6-well culture plates for 12 hours followed by exosomes treatment. Then, the exosomes (200  $\mu$ g/mL) were added into THP-1 cells. In order to prepare for Western Blot and RT-PCR, THP-1 cells were treated with exosomes for 24h and 12h, respectively.

## Preparation of Supernatant

THP-1 cells ( $5 \times 10^5$ ) were cultured for 12 h in 6-well culture plates followed by exosome treatment. PBS was used to suspend the exosomes, and the final concentration was 200  $\mu$ g/mL. After 24h treatment, culture medium with the exosomes was replaced. The supernatant was filtered, and then stored in a  $-80^\circ\text{C}$  refrigerator. In the NF- $\kappa$ B inhibition group, 10  $\mu$ M 4,6-Quinazoline diamine (QNZ) (Selleck, Shanghai, China) was added and cultured with the cells for 2 h. In the function experiments, the supernatant was mixed with exosome-free culture medium in equal volume.

## Flow Cytometry

THP-1 cells ( $5 \times 10^5$ ) were cultured for 12 h in 6-well culture plates followed by exosome treatment. PBS was used to suspend the exosomes, and the final concentration was 200  $\mu$ g/mL. After 24h treatment, differentiated cells were collected and identified by flow cytometry with specific markers (CD68, CD14 for M1, and CD206, CD14 for M2 macrophages). Unstained cells, as negative control, were used to determine the background autofluorescence, which can visualize the cells stained with anti-CD68 (or anti-CD14 or anti-CD206) antibodies.

## CCK-8 Assay

After supernatant treatment for 48h, A549 cell line was cultured in 96-well culture plates (5000/200  $\mu$ L) for 24 hours. Then, the supernatant was replaced with the conditioned medium prepared before, and the cells were cultured for 24 hours. Afterwards, the supernatant was replaced with 200  $\mu$ L serum-free medium added with CCK-8 (10%) and incubated for 2 hours. Absorbance was detected at a wavelength of 490 nm by using a microplate reader (Thermo scientific, Waltham, Massachusetts, USA). Three wells were set for repeated experiments in every group.

## Cell Colony Formation Assay

After supernatant treatment for 48h, A549 cells were cultured in 6-well culture plates (600/well) for 12 days. Paraformaldehyde (4%) was used for cell fixing. After 30 minutes, the cells were stained with crystal violet for 15 minutes and then photographed. Every experiment was repeated for three times.

## Wound Healing Assay

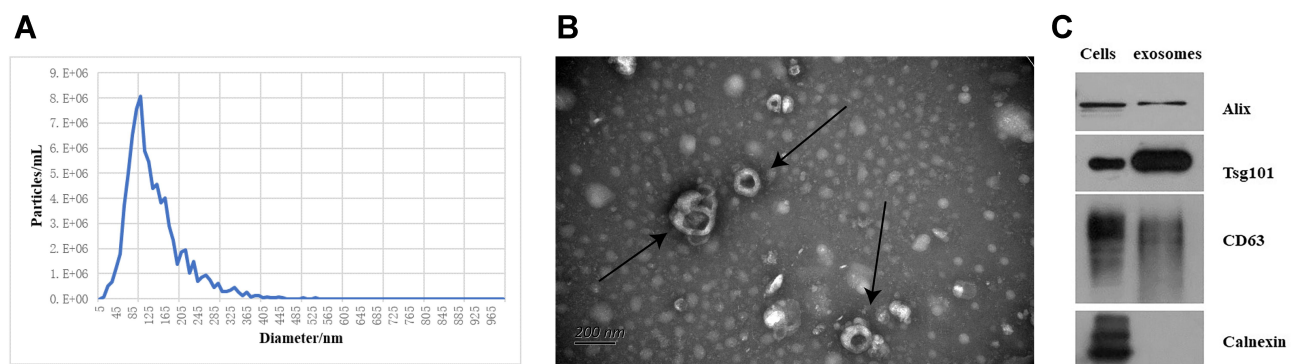
After supernatant treatment for 48h, A549 cells were cultured in 6-well culture plates ( $8 \times 10^5$ /well) for 24 h. When the confluence of the cells was 80%, vertical scratches on the cell layer were made by using a 20  $\mu$ L sterile tip. PBS was used to wash the cells once and the picture was taken under the microscope. The cells were cultured for another 24 h and photographed.

## Transwell Migration/Invasion Assay

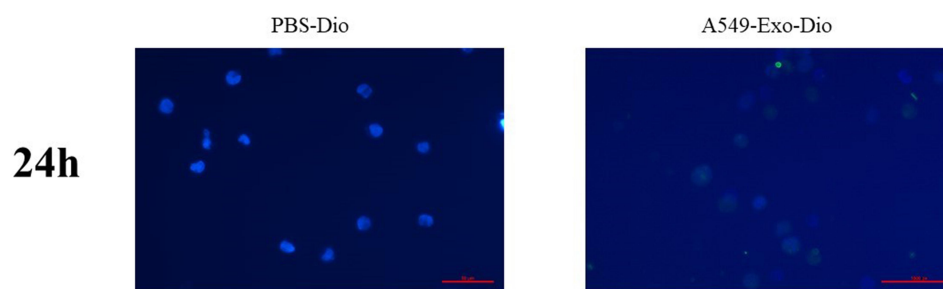
After supernatant treatment for 48h, A549 cells were inoculated in 6-well culture plates ( $8 \times 10^5$ /well) for 24 h. Culture medium without serum was used to suspend the cells and the concentration was adjusted to  $5 \times 10^5$ /mL. In the invasion experiment, the matrigel basement membrane (provided by BD Biosciences, Franklin Lake, New Jersey, USA) was diluted to 300  $\mu$ L/mL by using the pre-cooled culture medium without serum. Diluted matrigel (100  $\mu$ L) was placed in each chamber 30 min before the incubation (if Transwell migration assay was performed, this step was skipped). Afterwards, 200  $\mu$ L cells were added in the upper compartment and 500  $\mu$ L culture medium supplemented with 10% serum was added in the lower compartment for culture at  $37^\circ\text{C}$  for 24h. The cells on the bottom surface of the upper compartment were fixed by using paraformaldehyde (4%) for 30 minutes. The chambers were photographed after stained with crystal violet for 15 minutes.

## Apoptosis Assay

After supernatant treatment for 48h, A549 cells were cultured with DMEM medium in 6-well plates ( $2 \times 10^5$  cells/mL) for 24 hours. A549 cells were cultured with the prepared conditioned medium for 36 hours. Afterwards, staining buffer (200  $\mu$ L) was added into A549 cells ( $1 \times 10^6$  cells/mL). Annexin V (2  $\mu$ L) was incubated with the cells at  $2\sim 8^\circ\text{C}$  in darkness for 15 minutes. Afterwards, PI



**Figure 2** (A) The particle size range of exosomes was determined by NTA, and the size of the particles varied from 50nm to 200nm. (B) The typical cup-shaped structure of exosomes (the bar indicated 200nm) was shown by using the transmission electron microscopy, and the significance of the black arrows in the figure is to point to exosomes. (C) Three positive protein markers and one negative protein marker of exosomes were present in Western blot.



**Figure 3** The uptake of Dio-labeled exosomes (green fluorescence) in THP-1 cells was recorded. DAPI (blue fluorescence) was used for nuclei staining. PBS was control group. The bar indicated 50 μm.

solution (4 μL) was added for incubation at 2~8°C in darkness for 5 minutes. A549 cell apoptosis was detected by a flow cytometer (BD, Franklin Lake, New Jersey, USA).

## Western Blot Assay

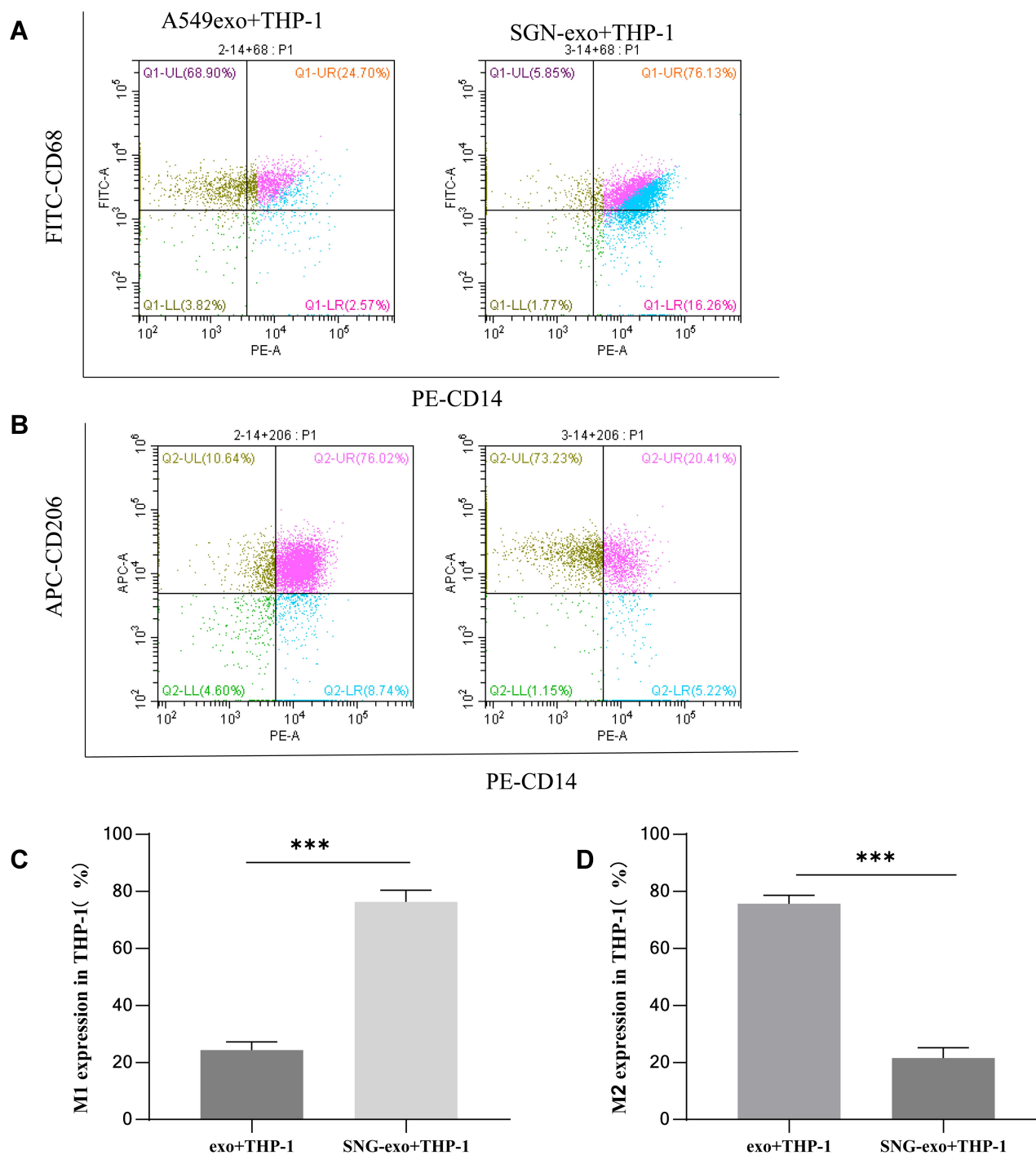
Exosomes or cells were lysed by using RIPA buffer supplemented with protease inhibitor (Beyotime Biotechnology, Shanghai, China). Protein concentrations were detected by using BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Proteins were subjected to electrophoresis by using 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto Polyvinylidene Fluoride (PVDF) membranes. Afterwards, primary antibodies (human p-NF-κB, NF-κB, Calnexin, CD63, Alix, TSG101), GAPDH and secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody) were incubated with the membranes. The protein bands were scanned by using ChemDoc™ XRS+ and image Lab™ software.

## Extraction of RNA and Real-Time PCR

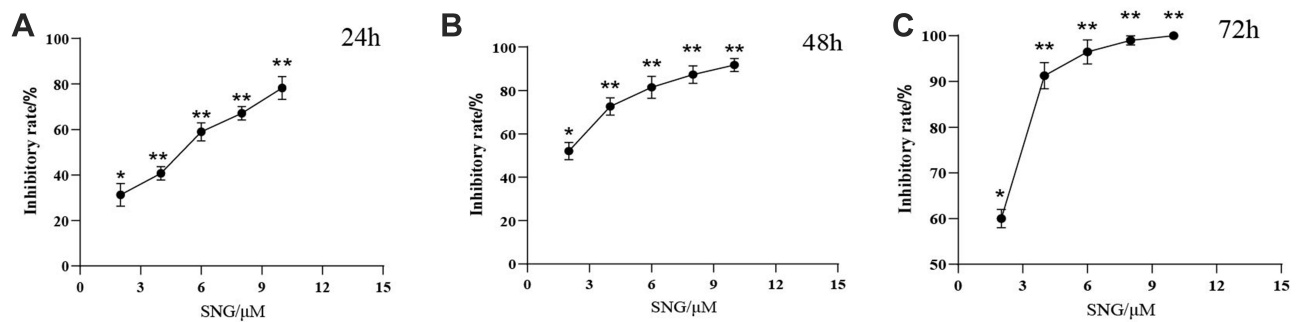
Trizol (Thermo scientific, Waltham, Massachusetts, USA) was used to obtain total RNA. Reverse transcription was conducted by using 1μL RNA and reverse transcriptase (TaKaRa, Japan). SYBR Green I real-time detection kit (TaKaRa, Japan) was used to conduct real-time PCR by using QuantStudio 6 Flex. GAPDH was used for normalization. Specific primers used in this experiment were listed as follows: IL-6 forward, GGTGTTGCCTGCTGCCTTCC, reverse, GCACAGCTCTGGCTTGTTCTCTC; TNF-α forward, CCACGCTCTTCTGCCTGCTG, reverse, GACGG CGATGCGGCTGATG; CCL-2 forward, AGTCTCTGCC GCCCTTCTGTG, reverse, GGGTTTGCTTGTCCT GGTGGTC.

## Statistics

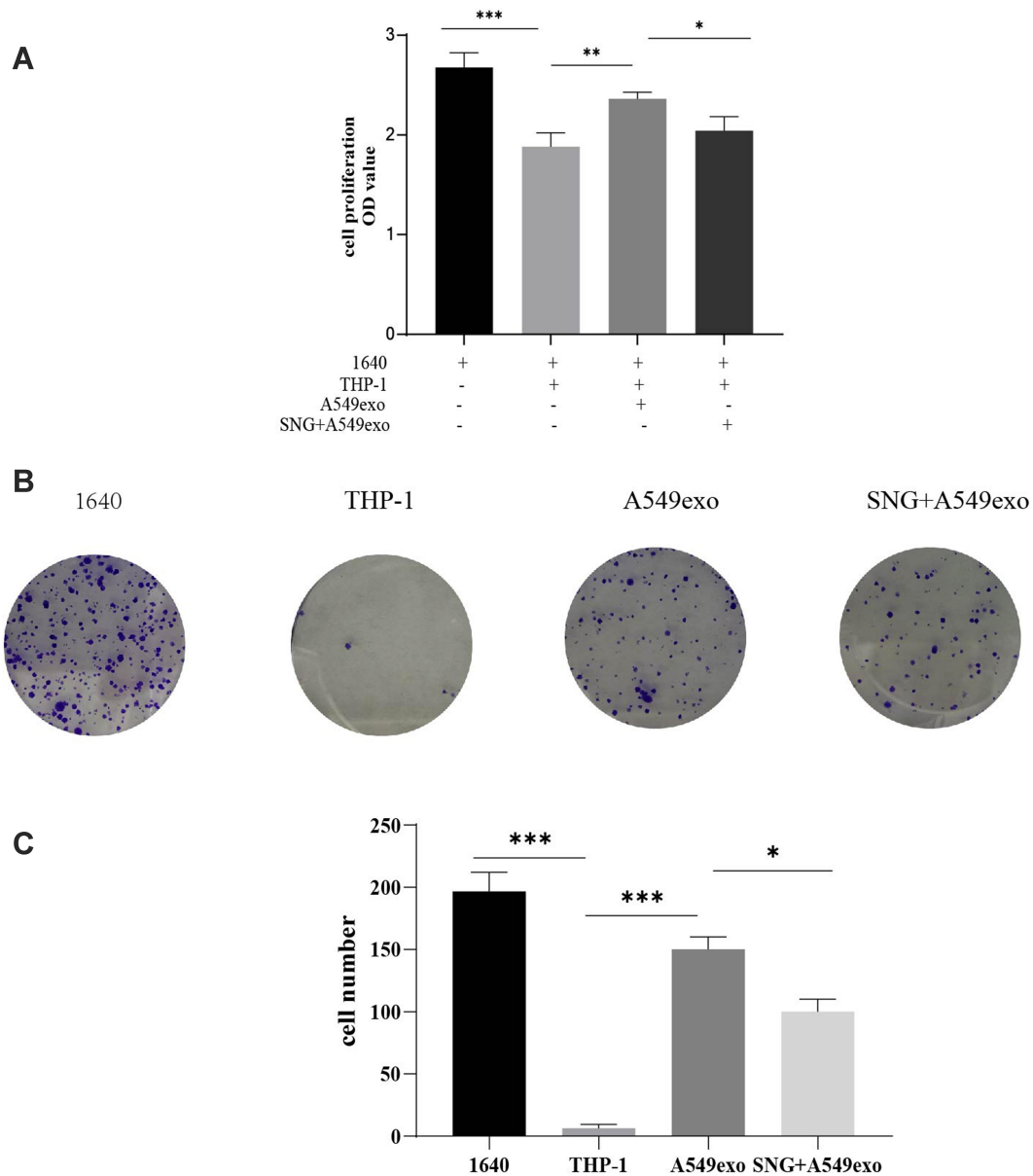
All results were obtained from three repeated experiments. Experimental results were presented as average value ± standard deviation. One-way ANOVA was used



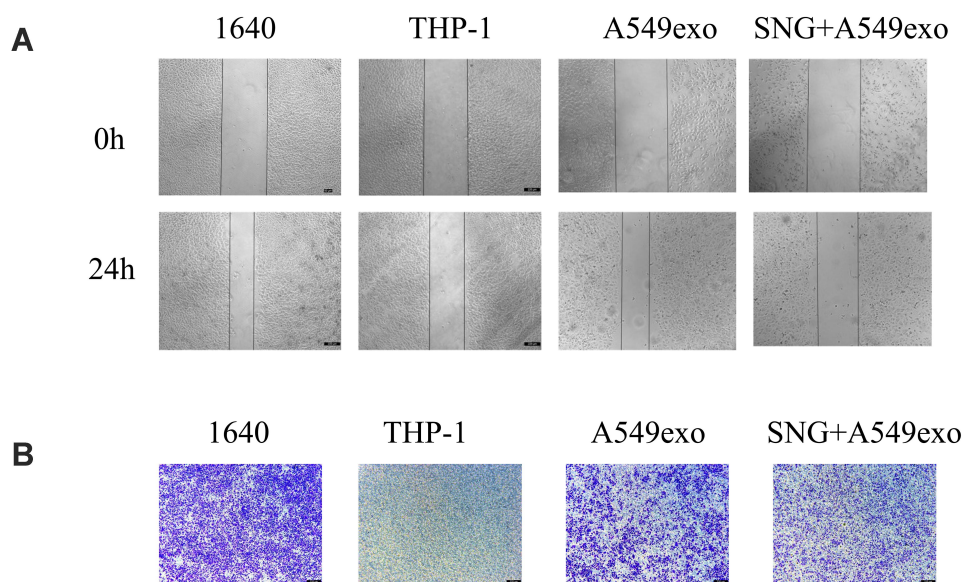
**Figure 4** Expression of macrophage markers in THP-1 cells was assessed by flow cytometry. Positive expressions of CD68 and CD14 after treatment of exosomes (**A**) and statistical results (**C**); Positive expressions of CD206 and CD14 after treatment of exosomes (**B**) and statistical results (**D**). Data were presented as average value  $\pm$  standard deviation (n=3). \*\*\*P<0.001.



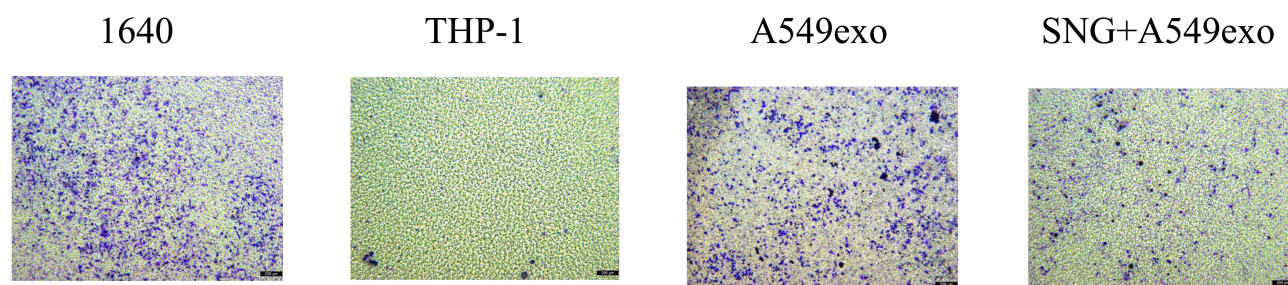
**Figure 5** Proliferation was inhibited when A549 cells were treated by SNG which was detected by using CCK-8 assay. As shown in the figure, cells were treated by various concentrations of SNG for **(A)** twenty-four hours, **(B)** forty-eight hours, and **(C)** seventy-two hours. The inhibition rates were compared with control group (0μM SNG). Data were demonstrated as average value  $\pm$  standard deviation ( $n=3$ ). \*\* $p<0.01$ , \* $p<0.05$ , in comparison with the control.



**Figure 6** **(A)** CCK-8 assay was utilized to detect A549 cells growth **(B)**. **(C)** cell colony formation experiment was performed to detect A549 cells growth, and the growth differences between groups was analyzed. Representative graphs and the count of the A549 cells are shown. Magnification,  $\times 100$ . \*\*\* $P<0.001$ , \*\* $p<0.01$ , \* $p<0.05$ .



**Figure 7** (A) Wound healing test was employed to assess migration ability of A549 cells, and migration differences between groups were analyzed. Magnification,  $\times 100$ . (B) Representative graphs are shown. Magnification,  $\times 100$ .



**Figure 8** Transwell assay was employed to assess invasion ability of A549 cells, and invasion differences between groups were analyzed. Representative graphs of invasion are shown. Magnification,  $\times 100$ .

to compare the difference between groups, and  $P < 0.05$  indicated significant difference. All statistical analyses were performed using SPSS23.0 or Graphpad 8.0 software.

## Results

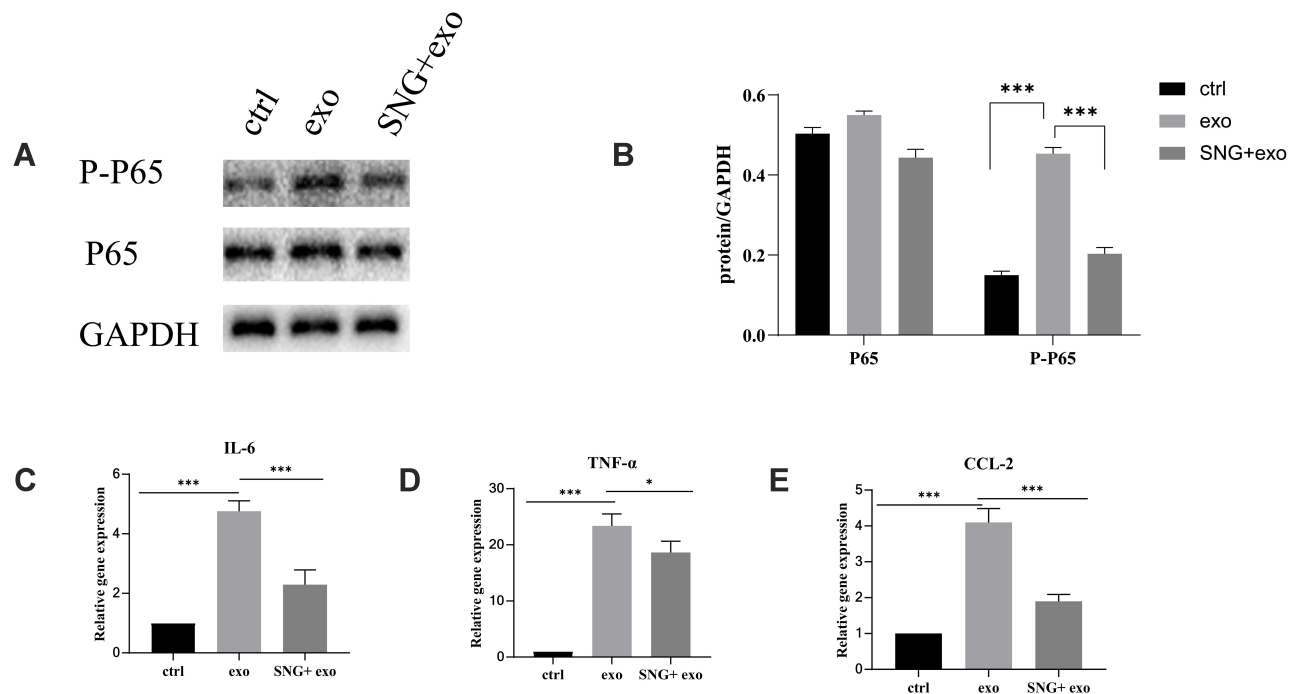
### The Features of the Exosomes in the Lung Cancer Cells

Exosomes were extracted from the cell supernatant by using ExoQuick Exosome Precipitation Solution. The Nanoparticle Tracking Analysis demonstrated that size of the particles varied from 50nm to 200nm. The average particle size and concentration were 138.4nm and  $8.3 \times 10^{10}$  particles/mL, respectively (Figure 2A). It was

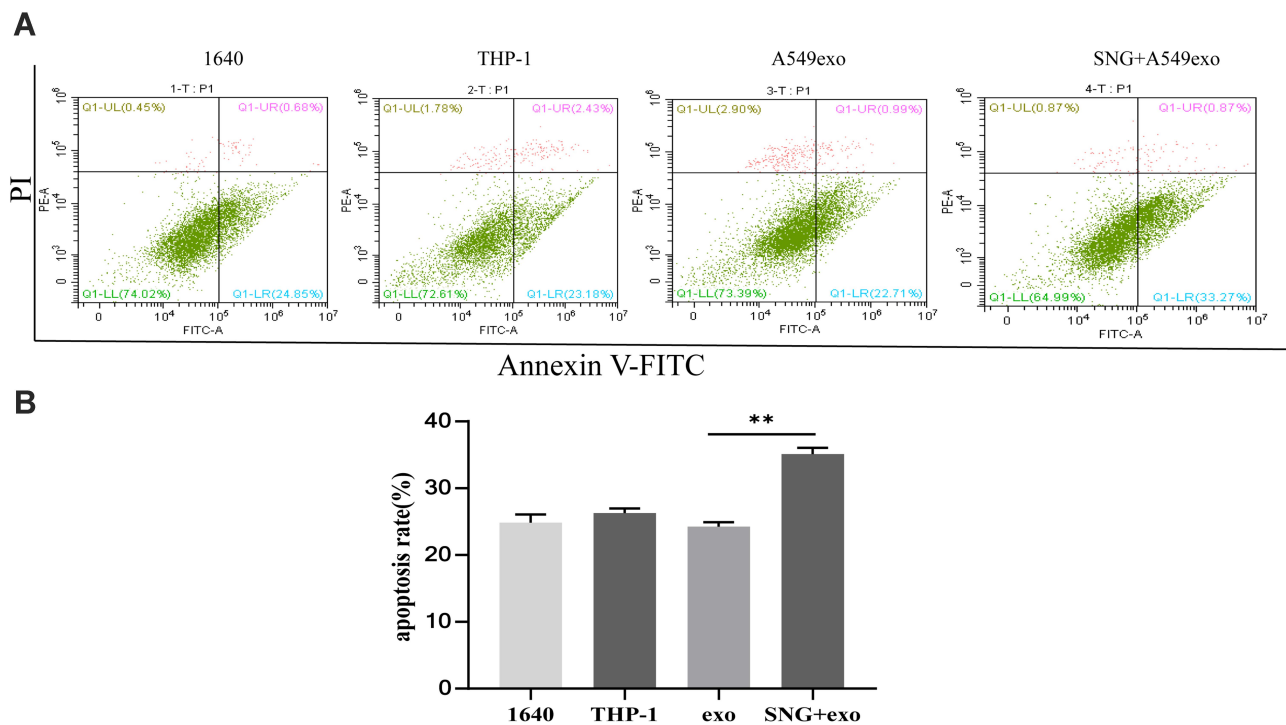
proved by transmission electron microscopy that the particle has a typical cup-shaped structure (Figure 2B). Exosome positive marker proteins, CD63, Alix and TSG101 were expressed, while negative marker proteins, calnexin were not (Figure 2C). As a kind of extracellular vesicles, exosome does not contain mitochondrial proteins.

### The Internalization of Exosomes in THP-1 Cells

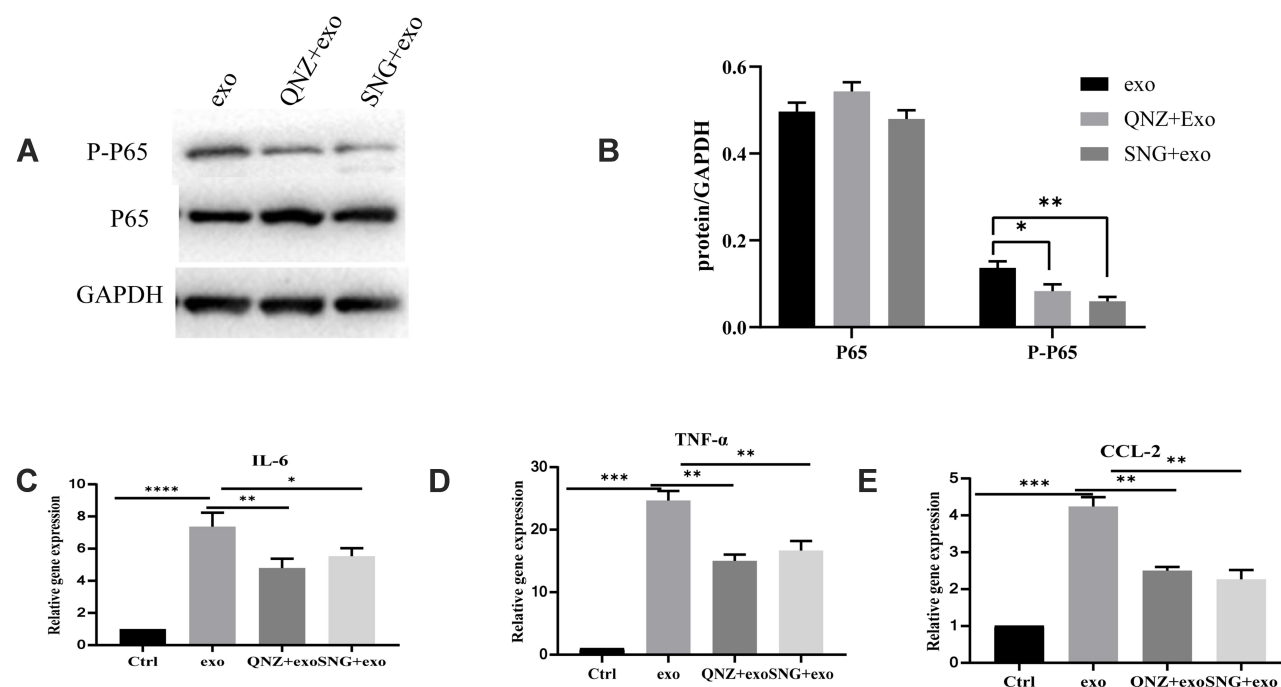
To figure out whether the exosomes of lung cancer cells can be internalized into THP-1 cells, the exosomes were stained by Dio dye and co-cultured with THP-1 cells for 24h. Following co-culture with THP-1 cells for 24h, laser confocal microscope photography showed that the labeled exosomes were



**Figure 10** (A) Expressions of p65 and p-p65 in THP-1 cells following exosome treatment. (B) P65 and p-p65 expressions (normalized to GAPDH) in THP-1 cells induced by exosomes. (C–E) The levels of CCL2, interleukin 6 and tumor necrosis factor alpha in THP-1 cells treated by exosomes were determined by real-time polymerase chain reaction assay. Data were presented as average value  $\pm$  standard deviation (n=3). \*\*\* $P < 0.001$ , \* $P < 0.05$ .



**Figure 9** (A) A549 cell apoptosis was assessed by flow cytometry, and apoptosis differences between groups were analyzed. (B) The percentage of apoptotic cells (apoptosis %) from 3 independent experiments was presented as average value  $\pm$  STD, and ANOVA test was conducted. \*\* $p < 0.01$ .



**Figure 11** (A) p-p65 and P65 protein expressions in THP-1 cells induced by QNZ (10  $\mu$ M) for two hours before exosome treatment for 24 h. (B) P65 and p-p65 protein expressions (normalized to GAPDH) in the THP-1 cells treated by exosomes. (C–E) Quantitative RT-PCR assay was employed to determine the levels of tumor necrosis factor alpha, CCL2, and interleukin 6 in the THP-1 cells pretreated by 10  $\mu$ M QNZ for 2 hours before exosomes treatment for 12 hours. \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

absorbed by THP-1 cells (Figure 3), indicating that exosomes can be internalized by THP-1 cells and affect THP-1 cells.

## THP-1 Cell Polarization Towards M2 Macrophages Induced by Exosomes

Macrophages can be divided into the classical subtype of activated macrophage (M1) and the alternative subtype of activated macrophages (M2).<sup>20</sup> M1 macrophages secrete pro-inflammatory cytokines to play an anti-tumor role, while M2 macrophages express immunosuppressive signaling molecules to promote tumor growth, invasion and metastasis.<sup>21</sup> The role of M1 and M2 macrophages in the tumor microenvironment is completely different. We examined the morphological changes of THP-1 cells and macrophage markers after treatment of exosomes. No obvious morphological changes were observed. CD14, CD68 and CD206 are the surface markers of human macrophages, M1 macrophages and M2 macrophages, respectively. Therefore, we used CD14 and CD68 double staining for M1-type macrophages, and CD14 and CD206 for M2-type macrophages. Flow cytometry (Figure 4A–D) results showed that most exosomes-treated THP-1 cells can be polarized to M2 macrophages while after SNG-treated

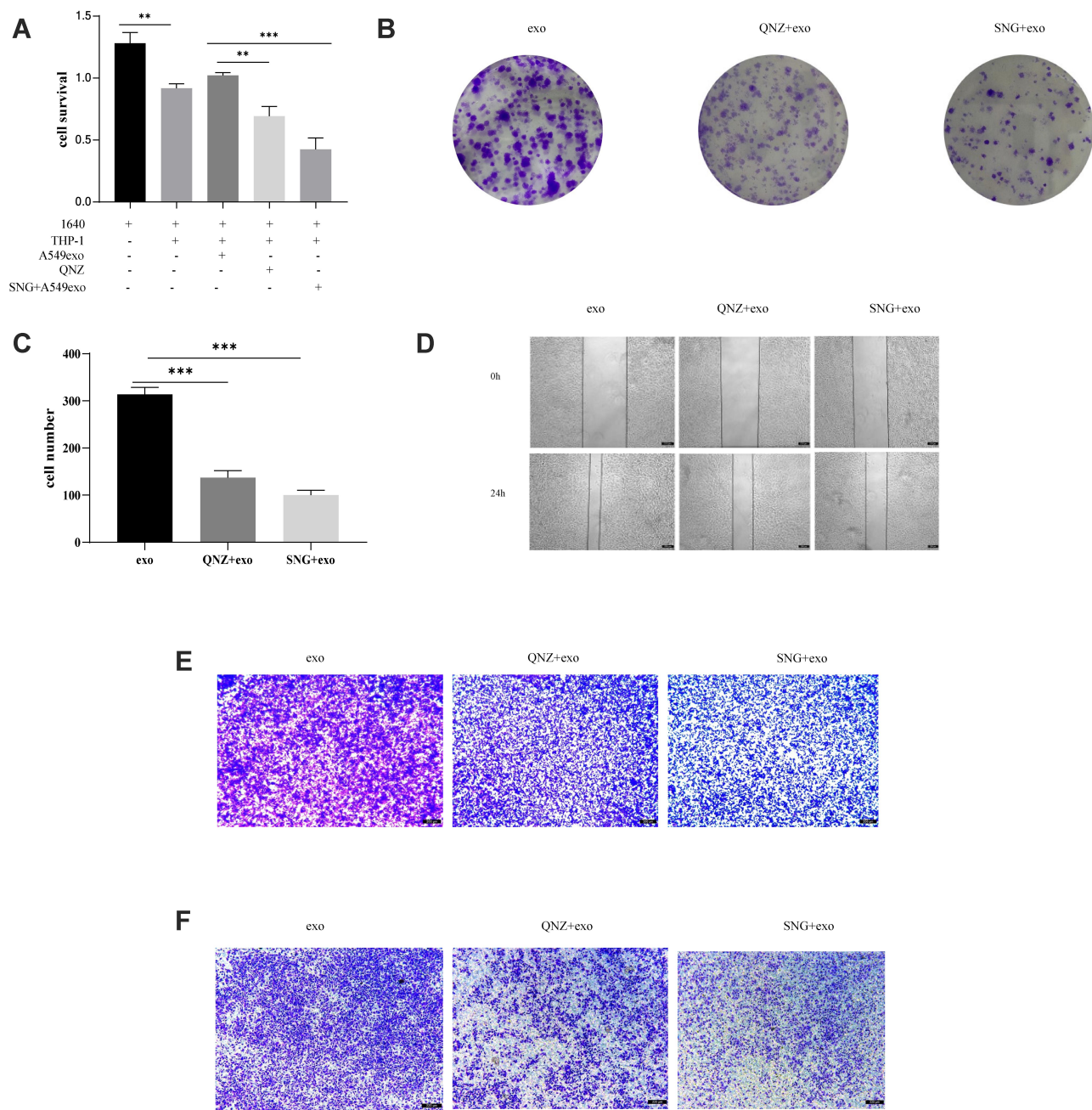
exosomes treatment, most THP-1 cells can be polarized to M1 macrophages.

## SNG Inhibited the Proliferation of A549 Cells

A549 cells were induced by multiple concentrations of SNG for 24, 48, and 72 hours, respectively. CCK-8 assay was applied to measure the proliferation of A549 cells. In Figure 5, proliferation was remarkably decreased when A549 cells were treated with SNG in a concentration- and time-dependent pattern compared to the control (0  $\mu$ M SNG).

## The Exosomes Induced THP-1 Cells Promote Migration, Development and Invasion of Lung Cancer Cells, Which Could Be Inhibited by SNG

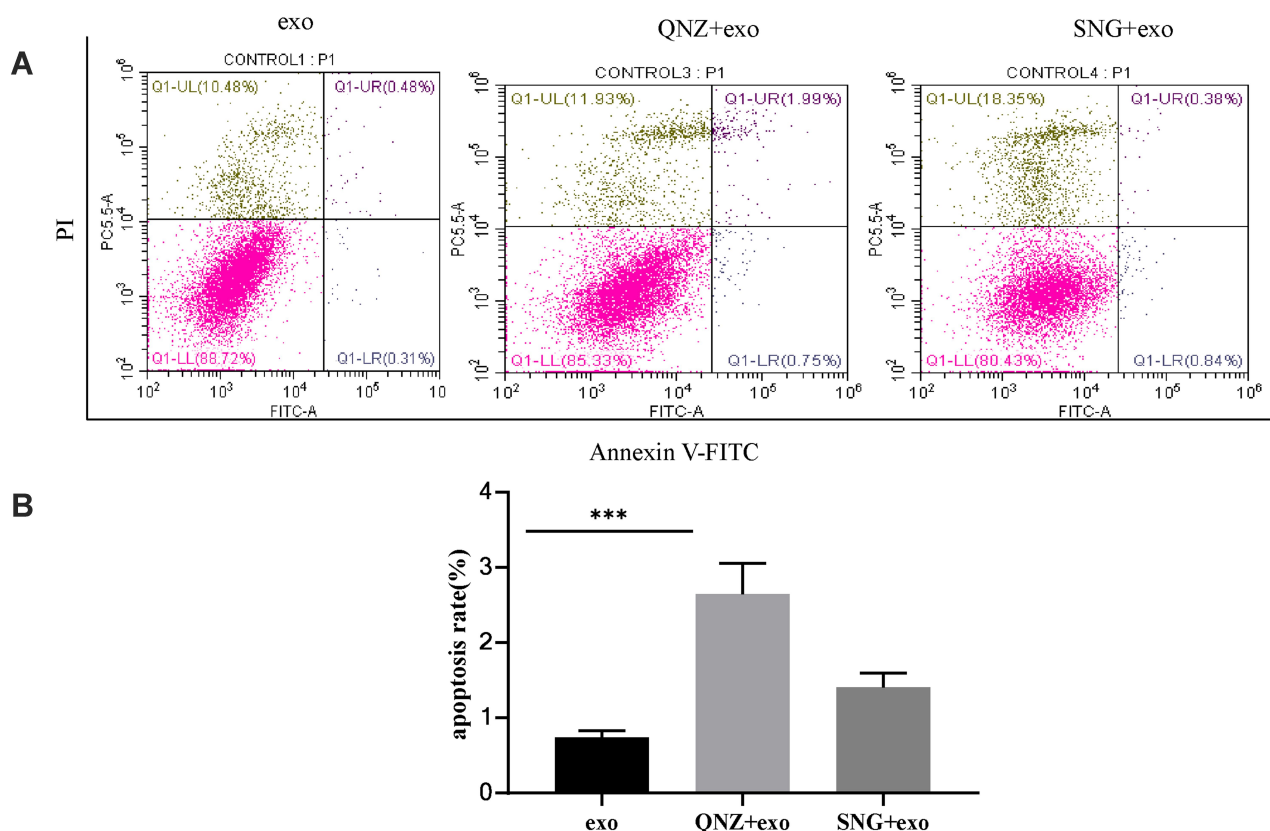
We further investigated the effects of THP-1 cells on lung cancer cells after treatment with exosomes or SNG-treated exosomes. Two groups were set as controls. The conditioned medium was used to stimulate lung cancer cells. After incubation with the supernatant of the THP-1 cells treated by the exosomes, the



**Figure 12** (A) CCK-8 test was used to detect A549 cell growth. (B and C) cell colony formation test was employed to detect A549 cells growth. Representative images and the count of the A549 cells are shown. Magnification,  $\times 100$ . (D) Wound healing assay was used to evaluate the migration of A549 cells. Magnification,  $\times 100$ . (E) Representative graphs of migrated A549 cells. Magnification,  $\times 100$ . (F) Representative graphs of A549 cells invasion. Magnification,  $\times 100$ . \*\*\* $P < 0.001$ , \*\* $p < 0.01$ .

proliferation (Figure 6A–C), migration (Figure 7A and B) and invasion of A549 cells were remarkably enhanced. After incubation with the supernatant of the THP-1 cells induced by SNG-treated exosomes, the proliferation, migration and invasion (Figure 8) of A549 cells were dramatically reduced. There was no

obvious change in the apoptosis after the cells were incubated with SOT induced by the exosomes in lung cancer cells. But the apoptosis of A549 cells was increased after incubated with SOT induced by SNG-treated exosomes in comparison with the exosomes without SNG treatment (Figure 9A and B). In



**Figure 13** (A) Apoptosis of A549 cells was evaluated by applying flow cytometry. (B) The percentage of apoptotic cells (apoptosis %) measured in the 3 independent experiments was presented as average value  $\pm$  STD. ANOVA was used in statistical analysis. \*\*\* $P < 0.001$ .

summary, lung cancer cells were activated by SOT so that the development, invasion, and migration of A549 cells were promoted, while SNG inhibited these effects.

### Exosomes of Lung Cancer Cells Could Activate NF- $\kappa$ B Pathway and Increase Levels of Pro-Inflammation Cytokines in THP-1 Cells, Which Could Be Reversed by SNG Treatment

To investigate the possible mechanisms in the inhibitory effects of SNG-treated exosomes on THP-1 cells, the expression level of NF- $\kappa$ B, a common signal transducer related to cancer development and inflammation, was measured. It was demonstrated that there was no obvious change in P65 levels in the THP-1 cells treated by the exosomes in lung carcinoma cells (group A) or

by exosomes from lung cancer cells induced by SNG (group B) for 24h. However, the protein level of P-P65 was remarkably elevated in group A and decreased in group B (Figure 10A and B). The mRNA levels of TNF- $\alpha$ , CCL2, and IL-6 in THP-1 cells were detected by RT-PCR. It was demonstrated that the mRNA levels of CCL2, TNF- $\alpha$ , and IL-6 in THP-1 cells were remarkably elevated in group A and decreased in group B (Figure 10C–E). We further investigated whether activated NF- $\kappa$ B contributes to the increase of pro-inflammation cytokines in THP-1 cells after exosomes treatment. After exosome treatment, NF- $\kappa$ B phosphorylation was blocked by QNZ, an inhibitor for NF- $\kappa$ B pathway. In Western blot, the level of p-NF- $\kappa$ B was remarkably decreased by QNZ (Figure 11A and B). In RT-PCR, the levels of TNF- $\alpha$ , CCL2, and IL-6 were also decreased (Figure 11C–E). In summary, the expression levels of pro-inflammation cytokines in

THP-1 cells after exosomes treatment relied on the NF- $\kappa$ B activation, and SNG could reverse the changes in the expression.

## Inhibited NF- $\kappa$ B Could Reverse the Effects of Exosomes on Development, Migration, Invasion, and Apoptosis of Lung Carcinoma Cells

The role of NF- $\kappa$ B inhibitors in the effects of exosomes on development, invasion, migration, and apoptosis of A549 cells was investigated. THP-1 cells were pretreated with NF- $\kappa$ B inhibitor before exosome treatment. CCK-8 and cell colony formation assays demonstrated that proliferation (Figure 12A–C), migration (Figure 12D and E) and invasion (Figure 12F) of A549 cells were remarkably enhanced and apoptosis (Figure 13A and B) reduced after incubation with the supernatant of the THP-1 cells treated by the exosomes in lung cancer cells, which was remarkably ameliorated by using NF- $\kappa$ B inhibitor. In summary, it was indicated that exosomes in cancer cells could activate THP-1 cells, which could subsequently promote tumor growth, invasion and migration of lung carcinoma cells and inhibit the apoptosis via NF- $\kappa$ B pathway.

## Discussion

Cancer progression is related to microenvironment.<sup>22</sup> Exosomes are the key substance of intercellular communication.<sup>19</sup> Exosomes derived from cancer cells consist of mRNAs, noncoding RNAs, proteins, and DNA. There were specific mechanisms in inserting chosen molecules into the exosomes.<sup>23</sup> In this study, THP-1 cells simulated the human mononuclear macrophage system. The basic activities of cancer cells include development, differentiation, division, migration, invasion and death. A series of complex signal transmission and biological activities can increase the expression levels of the associated genes, regulate the biological activities of cells and determine the progress of cancer. In the present research, it was demonstrated that there were communications between macrophages and exosomes, and macrophages treated by exosomes affected tumor development. It has been discovered that exosomes in lung carcinoma cells were effectively absorbed into macrophages. It was also illustrated that macrophages activated by lung cancer exosomes could promote the development, invasion, and migration, and SNG could reverse these effects. In addition, the apoptosis was enhanced after the cells were incubated with the supernatant of the THP-1 cells induced by SNG-

treated exosomes compared with the supernatant of the THP-1 cells induced by exosomes.

Macrophages are important drivers of inflammation in tumors.<sup>24</sup> The functions of macrophage are diverse and the phenotypes are different in various tumors or different parts of the same tumor.<sup>25</sup> Macrophages can polarize to two types under different stimulation: M1 type (pro-inflammation) or M2 (anti-inflammation).<sup>26</sup> Advances have been achieved in identifying molecular pathways in macrophages related to tumors, including NF- $\kappa$ B signaling pathway.<sup>24</sup>

The study showed that NF- $\kappa$ B pathway could regulate the expression levels of inflammation-related genes.<sup>27</sup> NF- $\kappa$ B plays a critical part in modulating the immediate reactions to pathogens, cellular proliferation, and survival.<sup>28</sup> The study showed that NF- $\kappa$ B could regulate levels of inflammation-related genes.<sup>9</sup> NF- $\kappa$ B p65, as the most common activated form in NF- $\kappa$ B family, is an important component in NF- $\kappa$ B pathway.<sup>29</sup> In our research, we investigated the mechanisms of NF- $\kappa$ B pathway in the activated macrophages induced by exosomes. It was indicated that exosomes in lung carcinoma cells could facilitate NF- $\kappa$ B pathway activation in macrophages. Furthermore, the exosomes could not deliver the activated molecules via NF- $\kappa$ B pathway to the macrophages after NF- $\kappa$ B pathway was sufficiently suppressed in the macrophages pretreated by NF- $\kappa$ B inhibitor. SNG can inhibit the exosomes in lung carcinoma cells so as to inhibit NF- $\kappa$ B pathway activation in macrophages. SNG is a benzophenanthridine alkaloid obtained from the roots of *Sanguinaria canadensis* and has anticancer effect.<sup>13,30</sup> It was reported that SNG could act on the Shh-Gli-Nanog pathway and inhibit the proliferation of pancreatic cancer stem cells.<sup>30</sup> It was reported that SNG inhibited proliferation of non-small cell lung cancer cells by upregulating Fas-associated factor 1.<sup>14</sup> Therefore, the discovery of new anticancer drugs from Chinese herbal medicine resources is promising. Our study suggested that SNG could suppress macrophages, and hence inhibit metastasis and proliferation of lung cancer through modulating the exosomes from the lung cancer cells. However, changes in exosomes were not explored in this study. Therefore, the morphology, quantity and substance of exosomes under the treatment of SNG and how do exosomes affect THP-1 cells remain to be further explored. This research will help to seek effective anticancer drugs from traditional Chinese medicine and find more therapeutic targets.

## Conclusions

To conclude, our research implied that exosomes could activate THP-1 cells to promote the proliferation, invasion and migration and inhibit the apoptosis of A549 cells. SNG-treated exosomes could inhibit THP-1 cells so as to ameliorate cancer progression. The mechanism may have relationships with suppressed NF- $\kappa$ B pathway in macrophages.

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## Disclosure

The authors declare no conflicts of interest.

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