Original Article

Tumor cell-derived exosomal microRNA-146a promotes non-small cell lung cancer cell invasion and proliferation by inhibiting M1 macrophage polarization

Zhi Yan[1,2#], Jian-Xun Wen[3#], Xi-Shan Cao[1], Wen Zhao[1], Yu-Ling Han[1,2], Xu-Hui Wen[1,2], Li Yan[4], Man Zhang[5], Ya-Fei Wang[1], Ling Hai[6,7], Ying-Jun Wang[1], Wen-Qi Zheng[1,2], Zhi-De Hu[1]^

[1]Department of Laboratory Medicine, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China; [2]Department of Parasitology, the Basic Medical Sciences College of Inner Mongolia Medical University, Hohhot, China; [3]Department of Medical Experiment Center, the Basic Medical Sciences College of Inner Mongolia Medical University, Hohhot, China; [4]Department of Respiratory and Critical Care Medicine, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China; [5]Department of Thoracic Surgery, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China; [6]Department of Pathology, the College of Basic Medical, Inner Mongolia Medical University, Hohhot, China; [7]Department of Pathology, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

Contributions: (I) Conception and design: ZD Hu, WQ Zheng; (II) Administrative support: L Hai, YF Wang, YJ Wang; (III) Provision of study materials or patients: L Yan, M Zhang, JX Wen; (IV) Collection and assembly of data: Z Yan, JX Wen, W Zhao, XS Cao, YL Han, XH Wen; (V) Data analysis and interpretation: Z Yan, JX Wen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Zhi-De Hu, MD. Department of Laboratory Medicine, the Affiliated Hospital of Inner Mongolia Medical University, Tongdao North Street 1, Hohhot 010050, China. Email: hzdlj81@163.com.

Background: Tumor-associated macrophages (TAMs) affects the outcomes of non-small cell lung cancer (NSCLC). NSCLC cells released exosomes to suppress the antitumor activity of TAMs. MiR-146a is a critical regulator in TAM polarization. We hypothesized that NSCLC cells released exosomal miR-146a to regulate TAM polarization and thus affected its antitumor activity.

Methods: We used H1299 cells-derived exosomes to stimulate THP-1 cells that was pretreated with phorbol 12-myristate 13-acetate (M0 macrophage). Flow cytometry and reverse transcription-quantitative polymerase chain reaction (PCR) were used to determine the polarization of macrophages. The conditioned medium of exosome-treated M0 cells was used to culture H1299 cells, and the Cell Counting Kit-8, Ki67, transwell and scratch wound assays were used to determine the biological behavior of H1299 cells. To investigate whether exosomal miR-146a regulates TAM macrophages through targeting tumor necrosis factor receptor-associated factor 6 (TRAF-6) and interleukin-1 receptor-associated kinase 1 (IRAK-1), we used small interfering RNA to knockdown the expressions of them.

Results: Upregulation of miR-146a inhibited M1 polarization and thus impaired the antitumor activity of TAMs. Exosomes released by H1299 cells can be taken by M0 macrophage, and they upregulated the expression of miR-146a in M0 macrophage. The exosome suppresses M1 polarization by exosomal miR-146a. TRAF-6 and IRAK-1 mediated the inhibitive effects of exosomal miR-146a on M1 polarization.

Conclusions: NSCLC cells released exosomal miR-146a to inhibit the expressions of TRAF-6 and IRAK-1 in TAMs, resulting in the impaired antitumor activity of TAMs. NSCLC cell-derived exosomal miR-146a represents a novel therapeutic target for NSCLC treatment.

Keywords: Non-small cell lung cancer (NSCLC); exosome; miR-146a; macrophage

^ ORCID: 0000-0003-3679-4992.

Submitted Nov 08, 2022. Accepted for publication Dec 21, 2022.
doi: 10.21037/atm-22-5565
View this article at: https://dx.doi.org/10.21037/atm-22-5565

Introduction

Lung cancer is a leading cause of mortality worldwide (1). About 80% of lung cancer are non-small cell lung cancer (NSCLC) (2). The prognosis of NSCLC is poor, with a 2-year survival of only 42% (2). The interaction between the tumor cells and stromal cells in the tumor microenvironment (TME) is crucial for the development and metastasis of NSCLC, and thus largely determines the prognosis of NSCLC (3). Macrophages are a type of stromal cell that affects the growth and invasion of NSCLC by secreting various cytokines, chemokines and growth factors (4). These macrophages are termed tumor-associated macrophages (TAMs) (4). TAMs can be categorized into the classical subtype of activated macrophage (M1) and the alternative subtype of activated macrophages (M2) (5). The M1 macrophages are characterized by producing high levels of interleukin (IL)-23, IL-6, tumor necrosis factor (TNF)-α, IL-12, and IL-1β, while the M2 macrophages produce IL-4, IL-10 and transforming growth factor-β (TGF-β) (6). M1 macrophage acts as a tumor suppressor, while M2 macrophage acts as a tumor promotor (5). Increased M1 macrophage density in the tumor islets and stroma is associated with an improved prognosis in NSCLC (7,8), while increased M2 macrophage is associated with a poor prognosis (9). Therefore, the M1/M2 balance is a potential therapeutic target in NSCLC (5).

Exosomes are a subset of extracellular vesicles with diameters between 30 and 200 nm (10). They are composed of a lipid bilayer and contain a substantial number of bioactive molecules such as proteins, DNAs, and RNAs [e.g., microRNAs (miRNAs), mRNAs and long non-coding RNAs (lncRNAs)]. By transmitting these bioactive molecules, exosomes can regulate the function of recipient cells. Previous studies indicated that exosomes were critically involved in the development of lung cancer (11,12). The immune cells (e.g., macrophages, T helpers, and dendritic cells) can take exosomes released by lung cancer cells (11). Among these immune cells, the critical role of TAM in the development of cancer has attracted much attention. By releasing the bioactive molecules, the exosomes can modulate the function of immune cells in the TME and thus affect the progression of NSCLC (12,13).

MiRNAs are a class of short and single-stranded RNAs that regulate the expressions of various target genes at a post-transcriptional level (14). They are essential modulators in the immune response against lung cancer (15). Previous studies indicated that miRNAs are critical regulators of TAM polarization (16,17). In addition, lung cancer cells can regulate the polarization of TAMs by releasing miRNAs in exosomes, termed exosomal miRNAs (18). By targeting some signaling proteins of innate immune responses, such as TNF receptor-associated factor 6 (TRAF-6) and IL-1 receptor-associated kinase 1 (IRAK-1), miR-146a tightly regulates the strength of the inflammation induced by lipopolysaccharide (19,20). Furthermore, miR-146a promotes M2 macrophage polarization in mouse and human cell lines (21-23). MiR-146a is a critical regulator in TME. For example, in breast cancer, miR-146a promotes M2 TAM polarization and suppresses tumor growth in mice (24). In addition, exosomal miR-146a released by hepatocellular carcinoma (HCC) can promote the M2 TAM polarization and the educated TAM inhibits T cell response (25). Here, we found that lung cancer cells released exosomal miR-146a to inhibit M1 macrophage polarization, which in turn impaired

Highlight box

Key findings

- Lung cancer cells released exosomal miR-146a to decrease the anti-tumor activity of tumor-associated macrophages by inhibiting M1 polarization *in vitro*;
- Lung cancer cell-derived exosomal miR-146a inhibited M1 macrophage polarization by targeting TRAF-6 and IRAK-1.

What is known and what is new?

- MiR-146a regulates the strength of innate immune response and the polarization of macrophages, but its role in lung cancer remains unclear;
- Lung cancer cells released exosomes could be taken by macrophages *in vitro*. The exosome released miR-146a and thus upregulated the expression of miR-146a in the lung cancer cell. By targeting IRAK-1 and TRAF-6, miR-146a inhibited M1 macrophage polarization and decreased its anticancer activity.

What is the implication, and what should change now?

- Lung cancer cell-derived exosomal miR-146a represents a novel therapeutic target for lung cancer.

the antitumor activity of macrophages. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5565/rc).

Methods

Cell culture

H1299 and THP-1 cell lines were purchased from BNCC (Henan, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Shanghai, China) containing 10% fetal bovine serum (04-001, BIOIND, Israel), 1% streptomycin and penicillin (Gibco, Shanghai, China) in a constant temperature incubator at 37 ℃ with 5% CO_2. THP-1 cells (2×10^6) were treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (A606759, Sangon Biotech, Shanghai, China) for 24 h to induce M0 macrophages (26). The H1299 cells were cultured at a concentration of 5×10^5.

Cell transfection

M0 macrophages and H1299 cells were transfected with miR-146a mimic, inhibitor and their controls (GenePharma, Shanghai, China). In addition, small interfering RNA (siRNA) of IRAK-1 (5'-GAGCCACCGCAGATTATCA-3') or TRAF-6 (5'-AGGGTCGCCTTGTAAGACA-3') (Ribio, Guangzhou, China) and their controls were also transfected into M0 macrophages. We used lipofectamine 3000 (88-8005, Invitrogen by Thermo Fisher Scientific, Lithuania) for transfecting miR-146a mimic, inhibitor and all siRNAs. The transfection was performed following the instructions provided by the manufacturer.

Isolation and characterization of exosomes

We used ultracentrifugation to isolate exosomes in the medium of H1299 cells according to a previously published protocol (27). The isolated exosomes were resuspension in 50 μL PBS and stored at −80 ℃ for further analysis. The size and structure of the isolated exosomes were measured by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), respectively. In addition, exosome markers, including CD9, CD63, HSP70 and TSG101, were detected by Western blot. The exosomes were labeled with 100 μM PKH67 fluorescent (UR52303, Umibio, Shanghai, China) and isolated again with the Cell Culture Media Exosome Purification Mini kit (60400, NORGEN, Canada). The labeled exosomes were used to treat M0 macrophages for 8 h. Then, a fluorescent microscope was used to observe the exosome ingestion of macrophages. Before the fluorescent microscope observation, 10 μg/mL DAPI (C0065, Solarbio, Beijing, China) was used to stain the nuclear of the cell.

Reverse transcription-quantitative PCR (RT-qPCR)

The total RNA and miRNA in M0 macrophages and H1299 cells were extracted using the RNAsimple Total RNA Kit (DP419, TIANGEN, Beijing, China) and the miRcute miRNA Isolation Kit (DP501, TIANGEN), respectively. Total RNA in exosomes was extracted using the Exosomal RNA Isolation Kit (58000, NORGEN). RNA was quantified using Microvolume Spectrometer (Titertek Berthold, Germany). The PrimeScrpt™ RT reagent Kit with gDNA Eraser (RR047A, TaKaRa, Dalian, China) and TransScript miRNA First-Strand cDNA Synthesis SuperMix (AT351-01, TransGen Biotech, Beijing, China) were used for total RNA and miRNA reverse transcription, respectively. The TB Green Premix Ex Taq™ II (RR820A, TaKaRa, Dalian, China) and PerfectStart Green qPCR SuperMix (TG-AQ601-02, TransGen Biotech, Beijing, China) were used for amplification, respectively. The primers (*Table 1*) used in this study were synthesized by General Biosystems (Durham, NC, USA), and the expressions of mRNA and miRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6, respectively. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method (28).

Western blot analysis

The Mixed RIPA buffer (R0010, Solarbio, Beijing, China) with phenylmethanesulfonyl fluoride (PMSF) (P0100, Solarbio) at 100:1 was used to lysis cells and exosomes. The extracted proteins were quantified using the BCA Protein Assay Kit (C503021, Sangon Biotech, Shanghai, China). The antibodies used in Western blot includes rabbit anti-human CD9 (ab263019, 1:1,000, Abcam), CD63 (ab134045, 1:2,000, Abcam), HSP70 (ab181606, 1:1,000, Abcam), TSG101 (ab125011, 1:2,000, Abcam), IRAK-1 (ab180747, 1:1,000, Abcam), TRAF-6 (ab33915, 1:2,000, Abcam), GAPDH (D110016, 1:5,000, Sangon Biotech), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (D110058, Sangon Biotech). The relative expressions of the target proteins were normalized to GAPDH.

Table 1 Primer sequences for RT-qPCR

Gene	Forward primer	Reverse primer
miR-146a	TGAGAACTGAATTCCATGGGTT	GATCGCCCTTCTACGTCGTAT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
TNF-α	TGAGCACTGAAAGCATGATC	TTATCTCTCAGCTCCACGCC
iNOS	ACAGGAGGGGTTAAAGCTGC	TTGTCTCCAAGGGACCAGG
IL-10	GAGGCTACGGCGCTGTCA	TCGACGGCCTTGCTCTTG
TGF-β	AGGTCACCCGCGTGCTAAT	GCTTCTCGGAGCTCTGATGTGT
IRAK-1	TGAAGAGGCTGAAGGAGAA	CACAATGTTTGGGTGACGAA
TRAF-6	GTCCCTTCCAAAAATTCCAT	CACAAGAAACCTGTCTCCTT
GAPDH	GTCATCCCTGAGCTGAACGG	GGGTCTTACTCCTTGGAGGC

RT-qPCR, reverse transcription-quantitative PCR; miR-146a, microRNA-146a-5p; TNF-α, tumor necrosis factor α; iNOS, inducible nitric oxide synthase; IL-10, interleukin 10; TGF-β, transforming growth factor β; IRAK-1, interleukin-1 receptor-associated kinase 1; TRAF-6, tumor necrosis factor receptor-associated factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Flow cytometry

M0 macrophages were collected and resuspended using 500 μL PBS. Cells were treated with 20 μL fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD86 (555657, BD Biosciences, NJ, USA) and allophycocyanin (APC)-labeled mouse anti-human CD206 (550889, BD Biosciences) in the dark at room temperature for 15 minutes before analysis. The data were analyzed by Flow Cytometry (BD Biosciences) and the CellQuest software. In apoptosis assay, H1299 cells cultured in the conditioned medium were tested by Annexin V (BD Biosciences) and 5 μL Propidium Iodide assay.

Cell Counting Kit-8 (CCK-8) assay

The H1299 cells (3×10^3 cells/well) were cultured in 96-well plates for 4 h. Subsequently, the cells were washed and cultured in the conditioned medium from M0 macrophages. The proliferation of the cells was tested by CCK-8 (BS350B, Biosharp, Beijing, China) at 24, 48, 72 and 96 h, respectively, according to the manufacturer's manual. A multimode plate reader was used to measure the absorbance at 450 nm (PerkinElmer, Shanghai, China).

Scratch wound assay

H1299 cells were cultured in a 6-well plate at 10^6 cells/well for 4 h and scratched with a 200 μL sterile pipette spearhead. After twice gently PBS washing to remove cell debris, H1299 cells were cultured in the conditioned medium from M0 macrophages. After 24 h, the cells were observed and imaged under a microscope (Olympus, Tokyo, Japan).

Transwell assay

Matrigengel (082704, ABW, Shanghai, China) was diluted at 300 μg/mL with serum-free RPMI-1640 medium. Then 100 μL solution was incubated in the upper chamber for 30 minutes (the migration assay did not have the Matrigengel). The pore size of the upper chamber was 8 μm. H1299 cells were suspended in serum-free RPMI-1640 medium and then inoculated in the upper chamber at 2×10^4 cells/300 μL. Meanwhile, 700 μL of conditioned medium from M0 macrophage was added to the lower chamber. After 24 h, the cells in the lower chamber were stained with 0.1% crystal violet and photographed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Ki67 assay

H1299 cells were cultured with the conditioned medium of M0 macrophages transfected with tumor necrosis factor receptor-associated factor 6 (TRAF-6) or interleukin-1 receptor-associated kinase 1 (IRAK-1) siRNA. After 24 h, a Ki67 assay was used to determine the proliferation of H1299 cells. We performed the Ki67 assay according to the manual provided by the manufacturer.

Statistical analyses

Data analyses were performed using SPSS 26.0 statistical software (IBM, Armonk, NY, USA). Continuous data are expressed as mean ± standard deviation. Means between two different groups were compared using the independent samples *t*-test. One-way analysis of variance (ANOVA) was used to compare the means of multiple groups. $P<0.05$ was considered statistically significant.

Results

MiR-146a promoted M2 polarization and inhibited M1 polarization in macrophages

To study the roles of miR-146a on macrophage polarization, we treated THP-1 cells with 100 ng/mL PMA for 24 h to generate M0 cells. MiR-146a mimic and inhibitor were transfected into M0 cells. Flow cytometry and RT-qPCR were used to detect the polarization of the M0 macrophage. *Figure 1A* shows the high efficacy of transfection, tested by fluorescence staining and RT-qPCR. Upregulation of miR-146a reduced CD86 expression, while its downregulation did not affect CD86 expression (*Figure 1B*). In addition, the upregulation of miR-146a increased CD206 expression, and its downregulation decreased it (*Figure 1B*). MiR-146a mimic significantly reduced the expressions of M1 markers [TNF-α and inducible nitric oxide synthase (iNOS)] and increased the expression of M2 markers (IL-10 and TGF-β), while the miR-146a inhibitor had the opposite effect (*Figure 1C*). These results suggest that miR-146a promotes macrophage polarization toward the M2 phenotype.

Upregulation of miR-146a in macrophages promoted H1299 cell proliferation, migration, and invasion

We investigated the functional impact of miR-146a in macrophages on the proliferation, migration, and invasion of H1299. MiR-146a mimic or inhibitor was transfected into M0 cells. After 24 h, its conditioned medium was used to culture H1299 cells. As shown in *Figure 2A*, miR-146a mimic inhibited the apoptosis of H1299 cells, and the miR-146a inhibitor promoted apoptosis. *Figure 2B* shows that miR-146a mimic promoted the proliferation, and the miR-146a inhibitor decreased the proliferation of H1299 cells. *Figures 2C,2D* show that conditioned medium from miR-146a mimic-transfected macrophage significantly promoted the migration and invasion of H1299 cells, while miR-146a inhibitor transfection had opposite effects. Taken together, miR-146a impairs the antitumor activities of macrophages *in vitro*.

Exosomes derived from H1299 cells could be taken by M0 macrophage and upregulate miR-146a expression

With ultracentrifugation, we isolated exosomes from the culture medium of H1299 cells. The exosomes were further determined by TEM (*Figure 3A*), NTA (*Figure 3B*) and Western blotting (*Figure 3C*). We transfected miR-146a mimic into H1299 cells, and the transfection efficiency was tested by fluorescence microscope (*Figure 3D*) and RT-qPCR (*Figure 3E*). We noted that the transfection of miR-146a mimic significantly increased the miR-146a level in exosomes from H1299 cells (*Figure 3F*). The exosomes released by H1299 cells could be taken by M0 macrophages (*Figure 3G*) and increased the expression of miR-146a in M0 macrophages (*Figure 3H*). Furthermore, exosomes released by H1299 cells transfected with miR-146a mimic could significantly upregulate the expression of miR-146a in M0 macrophages (*Figure 3I*). Taken together, these results indicate that H1299 cell-released exosomal miR-146a can upregulate the expression of miR-146a in M0 macrophages.

Exosomal miR-146a inhibited M1 macrophage polarization, H1299 cells migration and invasion

To detect the effects of exosomal miR-146a on the polarization and antitumor activity of macrophages, we used the H1299 cells-released exosome to treat M0 macrophages. As shown in *Figure 4A*, TNF-α and iNOS on macrophages were significantly decreased by exosomes released by H1299 cells, while the expressions of IL-10 and TGF-β were not affected. In addition, exosomes from H1299 cells that transfected with miR-146a mimic significantly decreased the expression of CD86, but failed to increase the expression of CD206 (*Figure 4B*). Furthermore, exosomes from H1299 cells transfected with miR-146a mimics significantly decreased the mRNA levels of TNF-α and iNOS, increased the expression of IL-10, but not TGF-β (*Figure 4C*).

To investigate the antitumor activity of M0 macrophage treated with H1299-released exosomes, we used the conditioned medium of exosome-treated M0 macrophage to culture H1299 cell, and a transwell assay was used to test the migration and invasion abilities of H1299 cells. As shown in *Figure 4D*, transfection of miR-146a mimic promoted the migration and invasion abilities of H1299 cells. Taken together, exosomal miR-146a could inhibit the

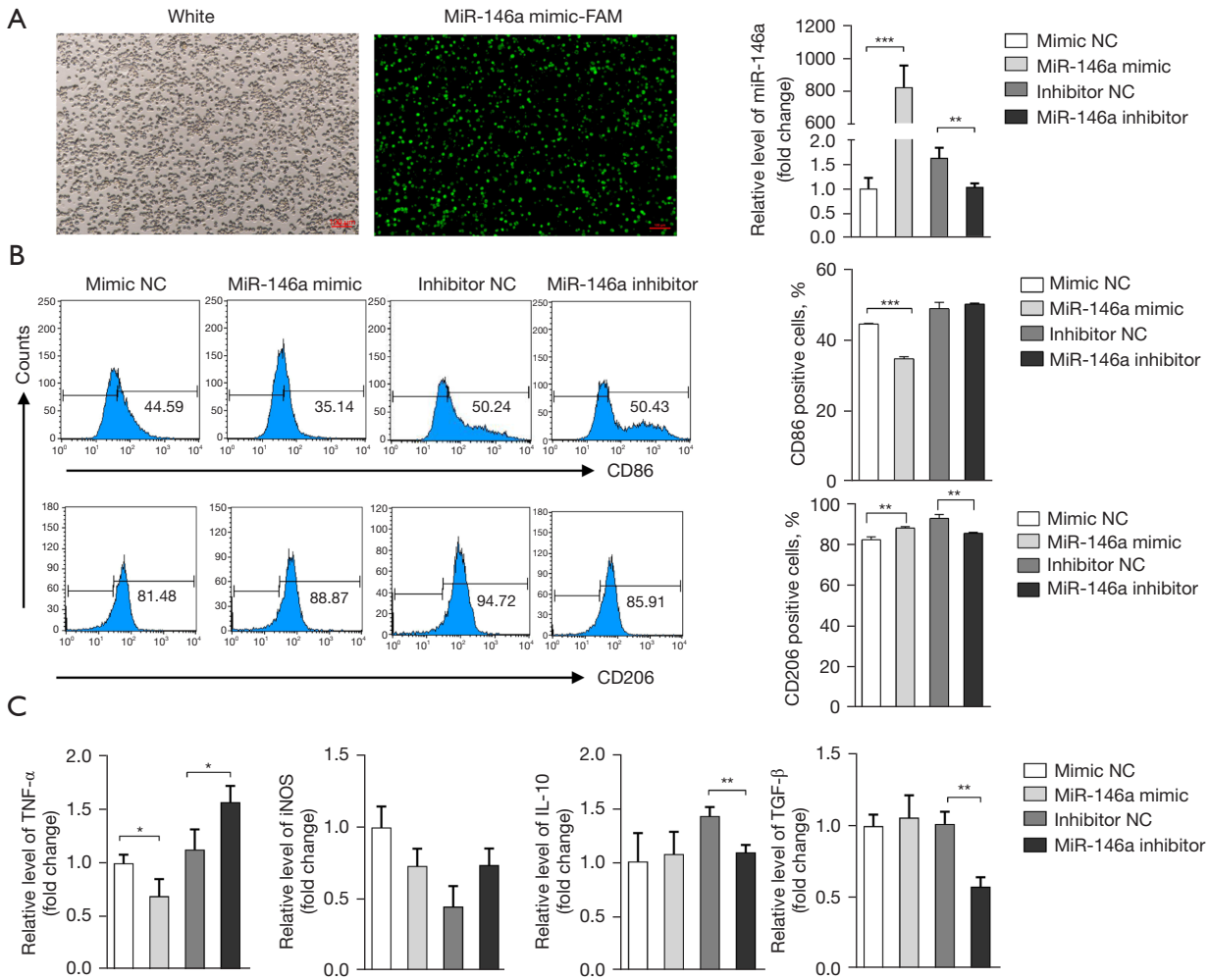

Figure 1 MiR-146a inhibited M1 macrophage polarization and promoted M2 polarization. (A) Fluorescence staining analysis of FAM-labeled miR-146a mimic in M0 macrophages (scale bar =100 μm). FAM is a fluorophore used for the labeling of DNA as a fluorescent marker. The expression level of miR-146a in M0 macrophages transfected with miR-146a mimic or miR-146a inhibitor was tested by RT-qPCR. (B) The expressions of CD86 or CD206 in M0 macrophages transfected with miR-146a mimic or miR-146a inhibitor were tested by flow cytometry. (C) The mRNA levels of TNF-α, iNOS, IL-10, and TGF-β in M0 macrophages transfected with miR-146a mimic or miR-146a inhibitor were tested by RT-qPCR. All data are presented as mean ± standard deviation (n=3). *, P<0.05; **, P<0.01; ***, P<0.001. NC, negative control; FAM, carboxyfluorescein; RT-qPCR, reverse transcription-quantitative PCR; TNF-α, tumor necrosis factor α; iNOS, inducible nitric oxide synthase; IL-10, interleukin 10; TGF-β, transforming growth factor β.

antitumor activity of macrophages *in vitro*.

MiR-146a inhibited M1 macrophages polarization by downregulating IRAK-1 and TRAF-6

As previous studies indicated that IRAK-1 and TRAF-6 are targets of miR-146a (19,20), we tested whether the activity of exosomal miR-146a was mediated by IRAK-1 and TRAF-6. The expressions of IRAK-1 and TRAF-6 in M0 macrophages were significantly decreased by miR-146a mimic and increased by miR-146a inhibitor, as indicated by RT-qPCR and Western blot (*Figures 5A*,*5B*). We knocked down the expressions of IRAK-1 and TRAF-6 with siRNA (*Figures 5C*,*5D*). IRAK-1 or TRAF-6 siRNAs significantly inhibited CD86 expression in M0 macrophages, but they showed no significant effect on CD206 expression (*Figure 5E*). Taken together, these results indicate that miR-146a inhibits M1 macrophage polarization by

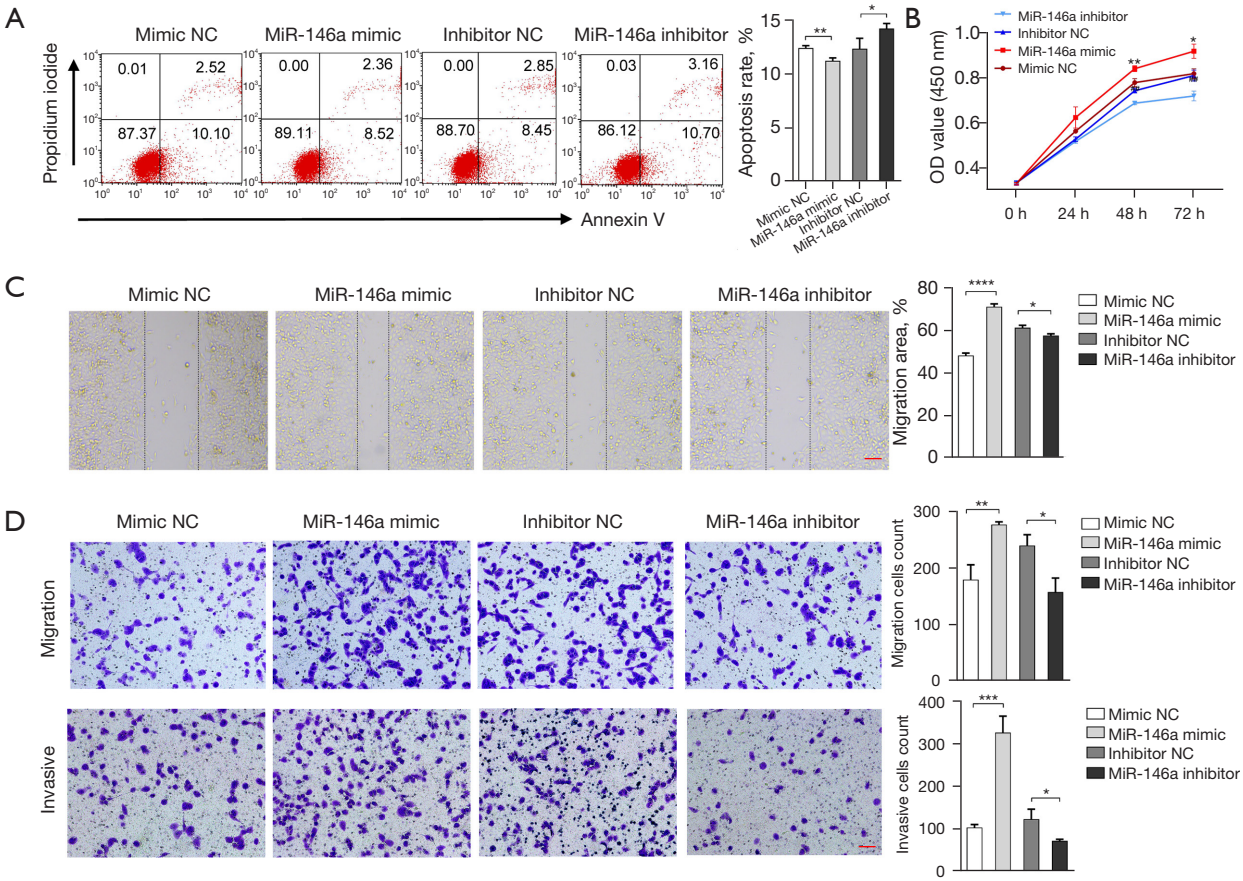

Figure 2 Conditioned medium from M0 macrophage transfected with miR-146a mimic promoted H1299 cell viability, migration, and invasion. The M0 macrophages were transfected with miR-146a mimic or inhibitor. After 24 h, the conditioned medium was used to culture H1299 cells. (A) The apoptosis of H1299 cells was tested by annexin V/propidium iodide apoptosis assay. (B) The viability of H1299 cells was tested by CCK-8 assay. (C) The migration of H1299 cells was tested by scratch wound assay (scale bar =100 μm). (D) The migration and invasion of H1299 cells were tested by a transwell assay (scale bar =100 μm). Cells were stained with 0.1% crystal violet. All data are presented as mean ± standard deviation (n=3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. NC, negative control; CCK-8, Cell Counting Kit-8.

downregulating IRAK-1 and TRAF-6.

Downregulation of IRAK-1 or TRAF-6 in M0 macrophages impaired its ability to promote H1299 proliferation, migration, and invasion

Next, we tested whether the effects of exosomal miR-146a derived from H1299 cells were mediated by IRAK-1 and TRAF-6. M0 macrophages were transfected with IRAK-1 and TRAF-6 siRNAs, and their conditioned medium was used to culture H1299 cells. As shown in *Figure 6A*, the proliferation of H1299 cells was significantly promoted by IRAK-1 and TRAF-6 siRNAs transfection. In addition, IRAK-1 and TRAF-6 siRNAs also promoted the migration and invasion of H1299 cells (*Figure 6B*). Therefore, we concluded that miR-146a in M0 macrophages regulates its antitumor activity by targeting IRAK-1 or TRAF-6.

Discussion

TEM is crucial for the progression of cancer. Macrophages are one of the most abundant cells within TME. They have noteworthy plasticity in TME and can polarize to M1 and M2 macrophages, depending on the cytokines and chemokines in TME. The balance between M1 and M2 macrophages largely determines the prognosis of NSCLC.

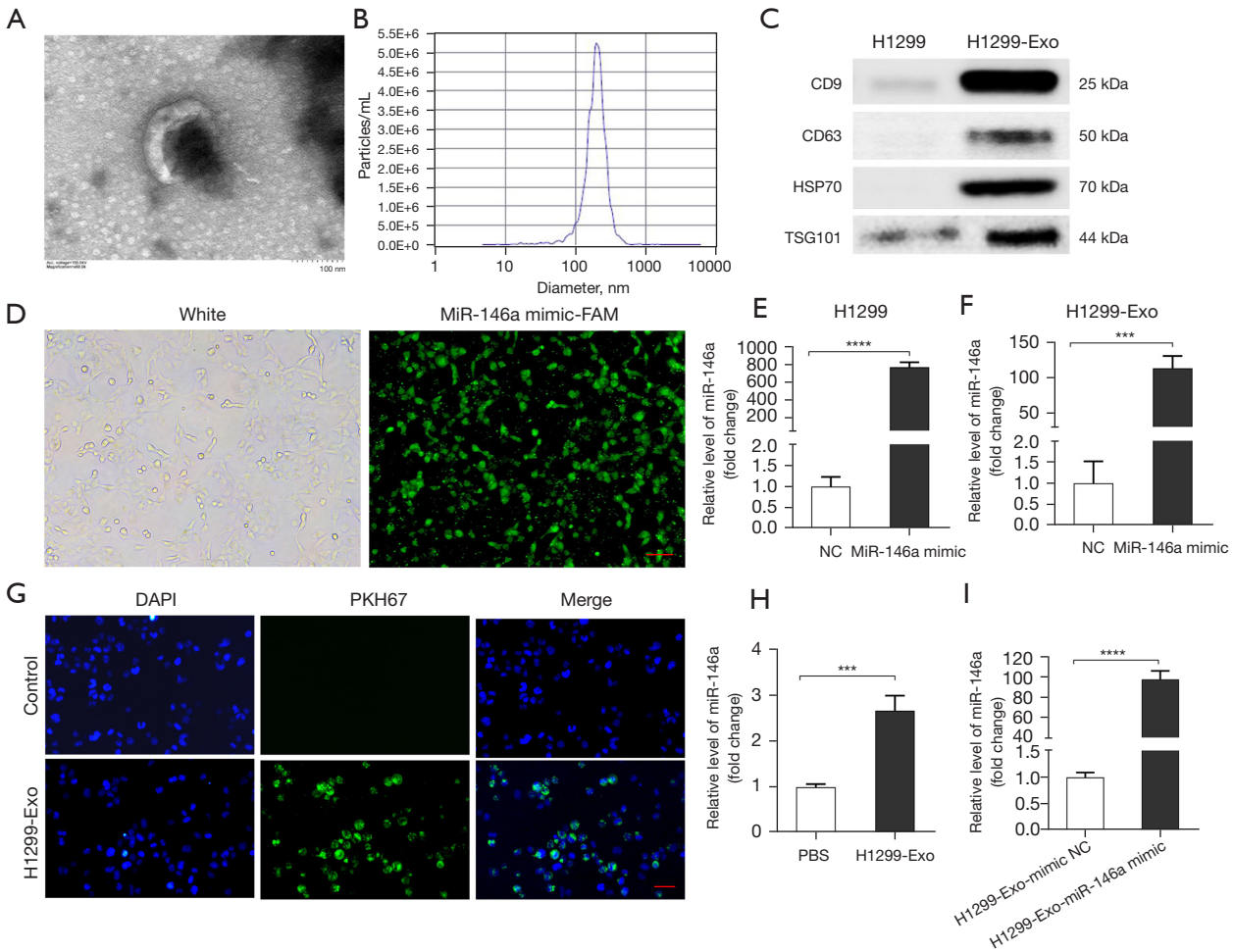

Figure 3 Exosomal miR-146a could be transferred from H1299 cells to macrophages and upregulated miR-146a expression. (A) H1299 cells-derived exosomes were examined by TEM (scale bar =100 nm). (B) The particle sizes of exosomes were measured by NTA. (C) The expressions of CD9, CD63, HSP70, and TSG101 in exosomes were detected by Western blot. (D) Fluorescence staining analysis of FAM-labeled miR-146a mimic in H1299 cells (scale bar =100 μm). (E) RT-qPCR tested the miR-146a expression in H1299 cells transfected with miR-146a mimic. (F) The expression of miR-146a in exosomes derived from H1299 cells transfected with miR-146a mimic was tested by RT-qPCR. (G) PKH67-labeled exosomes derived from H1299 cells were co-cultured with M0 macrophages for 8 h and were observed by fluorescence microscope (scale bar =100 μm). PKH67 is a lipophilic dye used to label exosomes. (H,I) The expression of miR-146a in M0 macrophages treated with exosomes derived from H1299 without (H) or with (I) miR-146a mimic was tested by RT-qPCR. All data are presented as mean ± standard deviation (n=3). ***, P<0.001; ****, P<0.0001. NC, negative control; Exo, exosomes; FAM, carboxyfluorescein; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription-quantitative PCR; TEM, transmission electron microscopy.

M1 macrophage in TME improves the outcomes of NSCLC, while M2 macrophage has the opposite effect. In this study, we found that NSCLC cells released exosomes to inhibit M1 macrophage polarization, resulting in the impairment of its antitumor activity. The inhibitive activity of tumor cell-released exosome was mediated by miR-146a, which inhibits M1 macrophage polarization by targeting TRAF-6 and IRAK-1. Although the findings in this study was not validated by animal model, this is the first study investigating the role of exosomal miR-146a in regulating TAM polarization in lung cancer.

Some studies have revealed that NSCLC cells can release

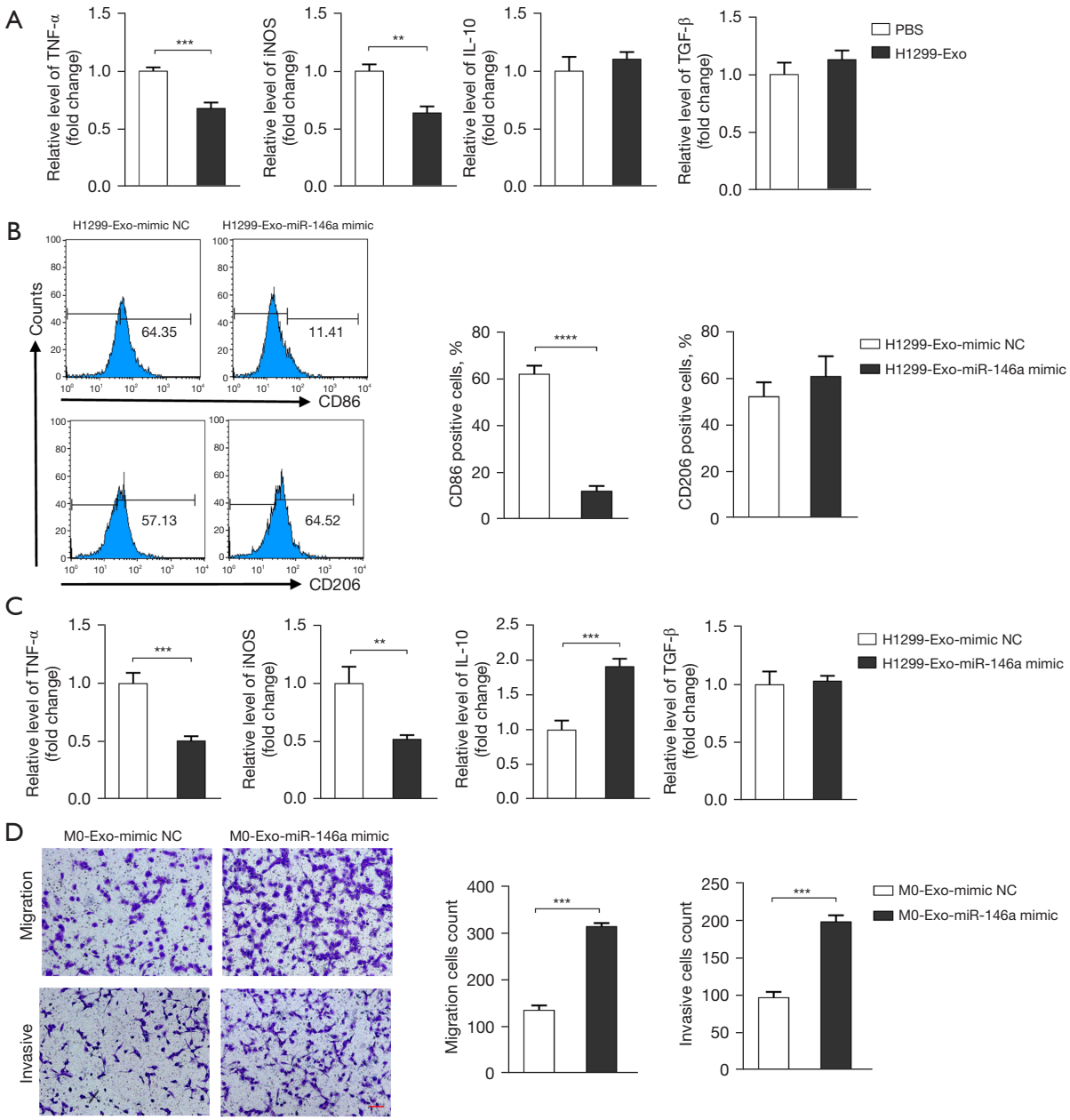

Figure 4 Exosomal miR-146a promoted migration and invasion of H1299 cells by inhibiting the polarization of M1 macrophages. (A) M0 macrophages were co-cultured with exosomes derived from H1299 cells for 24 h. The expression of M1 markers (TNF-α and iNOS) and M2 markers (IL-10 and TGF-β) were tested by RT-qPCR. (B) H1299 cells were transfected with miR-146a mimic and the exosomes were isolated to treat M0 cell. The expressions of CD86 and CD206 were detected by flow cytometry. (C) The mRNA levels of TNF-α, iNOS, IL-10, and TGF-β were tested by RT-qPCR. (D) A conditioned medium of M0 macrophages was used to culture H1299 cells. The migration and invasion of H1299 cells were tested by a transwell assay (scale bar =100 μm). Cells were stained with 0.1% crystal violet. All data are presented as mean ± standard deviation (n=3). **, P<0.01; ***, P<0.001; ****, P<0.0001. TNF-α, tumor necrosis factor α; iNOS, inducible nitric oxide synthase; IL-10, interleukin 10; TGF-β, transforming growth factor β; PBS, phosphate-buffered saline; Exo, exosomes; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

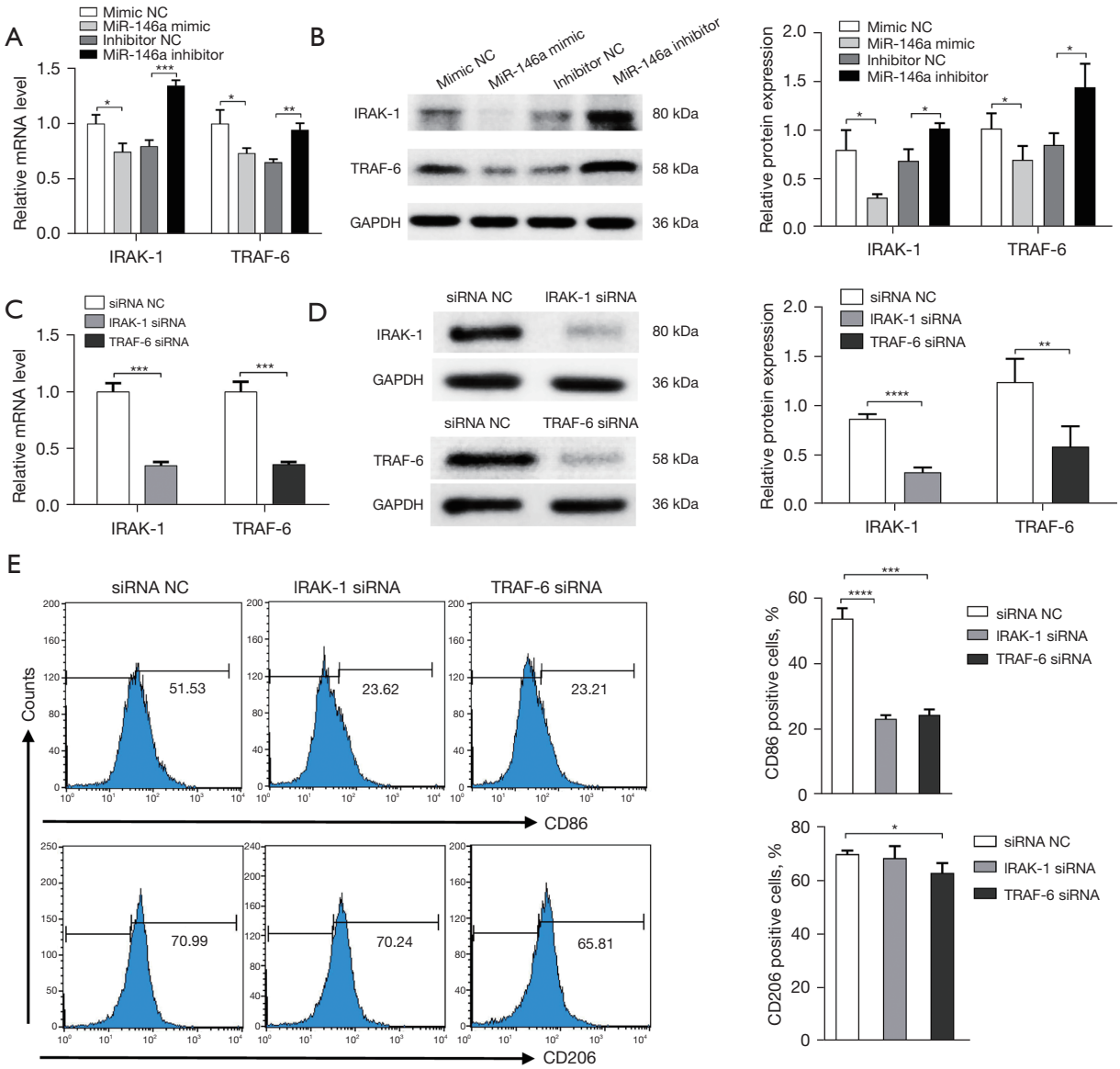

Figure 5 MiR-146a inhibited M1 macrophage polarization by downregulating IRAK-1 and TRAF-6. M0 macrophages transfected with miR-146a mimic or inhibitor, and the expressions of IRAK-1 and TRAF-6 were tested by RT-qPCR (A) and Western blot (B). M0 macrophages transfected with IRAK-1 or TRAF-6 siRNA, and the expressions of IRAK-1 and TRAF-6 were tested by RT-qPCR (C) and Western blot (D). (E) The expressions of CD86 or CD206 in M0 macrophages were tested by flow cytometry. All data are presented as mean ± standard deviation (n=3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. NC, negative control; IRAK-1, interleukin-1 receptor-associated kinase 1; TRAF-6, tumor necrosis factor receptor-associated factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR.

exosomes to disturb the macrophage polarization and thus promote the prognosis of NSCLC in turn. For example, under hypoxic conditions, NSCLC cell-derived exosomes could enhance M2 macrophage polarization and NSCLC progression by regulating the PKM2/AMPK pathway (29). In addition, NSCLC cells can also promote M2 macrophage polarization by enhancing the oxygen consumption rate of macrophages (30) or releasing circRNA (31), lncRNA (32) or miRNA (18,33,34) in it. A previous study indicated that the exosomes released by hypoxic NSCLC cells have a higher

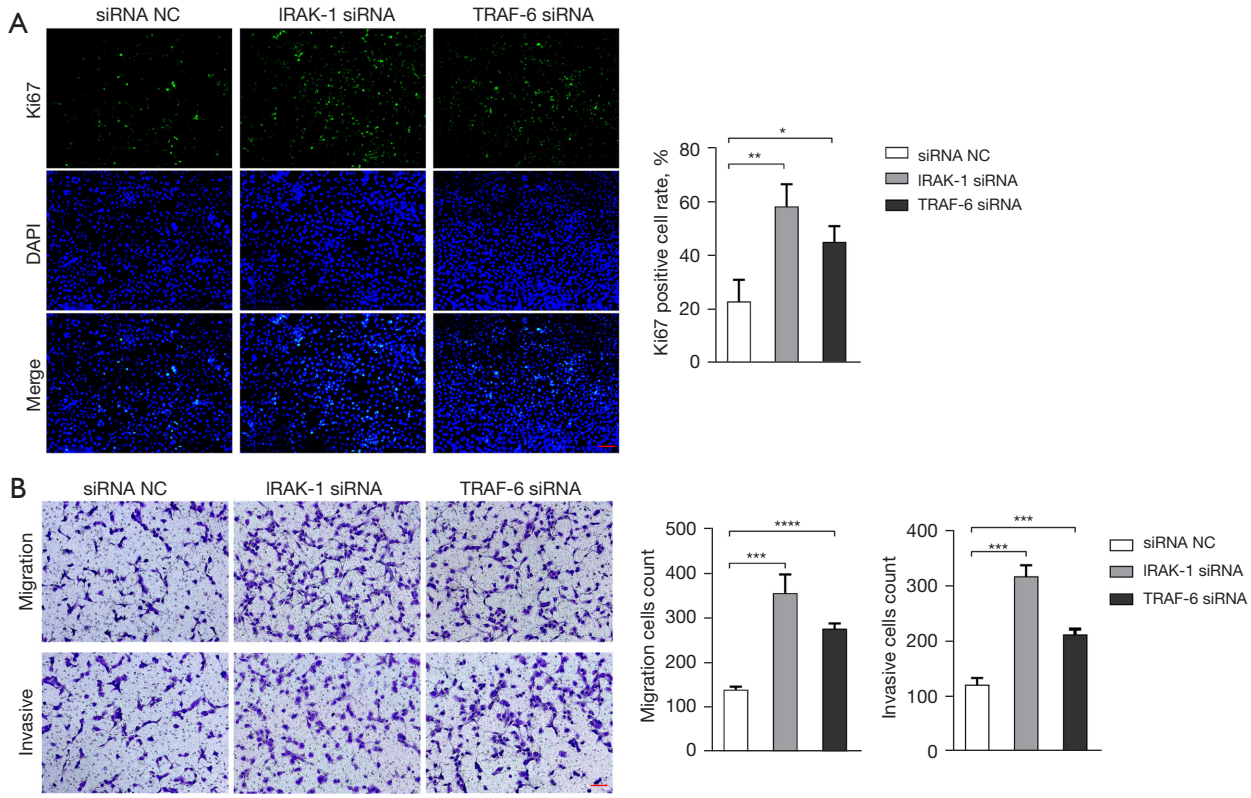

Figure 6 Downregulation of IRAK-1 or TRAF-6 promotes H1299 proliferation, migration, and invasion by inhibiting M1 macrophage polarization. (A) A conditioned medium of M0 macrophages after 24 h of IRAK-1 siRNA or TRAF-6 siRNA treatment was used to culture H1299 cells. Then, the proliferation of H1299 cells was investigated by Ki67 staining (scale bar =100 μm). (B) The migration and invasion of H1299 cells were tested by a transwell assay (scale bar =100 μm). Cells were stained with 0.1% crystal violet. n=3. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

level of miR-21, which can be transmitted to macrophages and induce M2 macrophage polarization by targeting interferon regulatory factor 1 (IRF1) (33). Another study shows that NSCLC cells released exosomal miR-181b to promote M2 polarization and suppress M1 polarization (34). Our study added new evidence that exosomal miRNAs released by NSCLC cells can regulate the polarization of TAM. In addition to promoting M2 macrophage polarization, exosomes derived from NSCLC cells can also inhibit M1 macrophage polarization. This finding is also supported by two previous studies (32,34).

Accumulating evidence has revealed that miR-146a was critically involved in the development of NSCLC (35). It acts as an antiproliferative, anti-inflammatory and proapoptotic agent, as well as a metastatic modulator in NSCLC cells (35). In addition, miR-146a rs2910164 polymorphism is associated with lung cancer risk (36). However, no study has revealed that NSCLC cells released exosomal miR-146a to modulate TAM's function and polarization. MiR-146a was firstly reported to be a negative regulator in innate immune response triggered by lipopolysaccharides (19) or vesicular stomatitis virus (37). Previous studies also revealed that the expression level of miR-146a is higher in M2 macrophages than that in M1 macrophages (22,38). Increased miR-146a promoted M2 polarization and suppressed M1 macrophage polarization by targeting Notch1 (21), TRAF-6 (39) and inhibin-β (22). Nevertheless, no study has investigated the effects of miR-146a on the macrophage's antitumor activity to NSCLC. Our study revealed that miR-146a inhibited M1 polarization and impaired its antitumor activity. Our work is also supported by some clinical observational studies. For example, a previous study indicated that exosomal miR-146a in serum of lung cancer patients was significantly higher

than that in healthy individuals (40), and the NSCLC patients with increased M1 macrophage density in the tumor islets and stroma had a better prognosis (7,8).

We used TRAF-6 and IRAK-1 siRNAs to knockdown their expression and found that downregulations of TRAF-6 and IRAK-1 reproduced the effects of miR-146a mimic on M0 macrophage polarization and its antitumor activity. This result indicates that TRAF-6 and IRAK-1 partially mediated the biological effects of miR-146a in macrophage polarization. TRAF-6 and IRAK-1 are two signaling proteins of innate immune responses. They mediated the signals from Toll-like receptor 4 (TLR4) and its adaptor MyD88, and modulated the activation of NF-κB, resulting in the production of inflammatory cytokines (e.g., IL-6, TNF-α) (41). Because the secretion of inflammatory cytokines is the typical characteristic of M1 macrophage, it is reasonable to hypothesize that inhibiting the production of inflammatory cytokines would inhibit M1 polarization. Our work was supported by a previous study that revealed that IRAK-1 and TRAF-6 were upregulated in M1 macrophage but downregulated in M2 macrophage (38). Furthermore, downregulating of TLR4 (42,43) or TRAF-6 (39) would inhibit M1 polarization or promote M2 polarization.

Conclusions

Taken together, our study indicated that NSCLC cells could release exosomal miR-146a to suppress M1 macrophage polarization. Suppressed M1 macrophage polarization impairs its antitumor activity and thus promotes the development of NSCLC. Therefore, exosomal miR-146a represents a potential therapeutic target for NSCLC.

Acknowledgments

Funding: This work is supported by the National Natural Science Foundation of China (No. 81860501); and the Natural and Science Foundation of Inner Mongolia Autonomous Region for Distinguished Young Scholars (No. 2020JQ07).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5565/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5565/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5565/coif). ZDH serves as an unpaid Executive Editor of *Annals of Translational Medicine* from April 2020 to March 2025. He received fundings from the National Natural Science Foundation of China (No. 81860501); and the Natural and Science Foundation of Inner Mongolia Autonomous Region for Distinguished Young Scholars (No. 2020JQ07). The funders provided no role in performing experiment, data analysis, manuscript preparing and submission. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Siegel RL, Miller KD, Fuchs HE, et al. Cancer statistics, 2022. CA Cancer J Clin 2022;72:7-33.
2. Siegel RL, Miller KD, Fuchs HE, et al. Cancer Statistics, 2021. CA Cancer J Clin 2021;71:7-33.
3. Anderson NM, Simon MC. The tumor microenvironment. Curr Biol 2020;30:R921-5.
4. Pathria P, Louis TL, Varner JA. Targeting Tumor-Associated Macrophages in Cancer. Trends Immunol 2019;40:310-27.
5. Sedighzadeh SS, Khoshbin AP, Razi S, et al. A narrative review of tumor-associated macrophages in lung cancer: regulation of macrophage polarization and therapeutic implications. Transl Lung Cancer Res 2021;10:1889-916.
6. Tamura R, Tanaka T, Yamamoto Y, et al. Dual role of macrophage in tumor immunity. Immunotherapy 2018;10:899-909.

7. Ma J, Liu L, Che G, et al. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer 2010;10:112.
8. Jackute J, Zemaitis M, Pranys D, et al. Distribution of M1 and M2 macrophages in tumor islets and stroma in relation to prognosis of non-small cell lung cancer. BMC Immunol 2018;19:3.
9. Sumitomo R, Hirai T, Fujita M, et al. M2 tumor-associated macrophages promote tumor progression in non-small-cell lung cancer. Exp Ther Med 2019;18:4490-8.
10. Pegtel DM, Gould SJ. Exosomes. Annu Rev Biochem 2019;88:487-514.
11. Zhou L, Lv T, Zhang Q, et al. The biology, function and clinical implications of exosomes in lung cancer. Cancer Lett 2017;407:84-92.
12. Duréndez-Sáez E, Torres-Martinez S, Calabuig-Fariñas S, et al. Exosomal microRNAs in non-small cell lung cancer. Transl Cancer Res 2021;10:3128-39.
13. Khan FH, Reza MJ, Shao YF, et al. Role of exosomes in lung cancer: A comprehensive insight from immunomodulation to theragnostic applications. Biochim Biophys Acta Rev Cancer 2022;1877:188776.
14. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215-33.
15. Rusek AM, Abba M, Eljaszewicz A, et al. MicroRNA modulators of epigenetic regulation, the tumor microenvironment and the immune system in lung cancer. Mol Cancer 2015;14:34.
16. Ahmad A. Epigenetic regulation of immunosuppressive tumor-associated macrophages through dysregulated microRNAs. Semin Cell Dev Biol 2022;124:26-33.
17. Self-Fordham JB, Naqvi AR, Uttamani JR, et al. MicroRNA: Dynamic Regulators of Macrophage Polarization and Plasticity. Front Immunol 2017;8:1062.
18. Liu J, Luo R, Wang J, et al. Tumor Cell-Derived Exosomal miR-770 Inhibits M2 Macrophage Polarization via Targeting MAP3K1 to Inhibit the Invasion of Non-small Cell Lung Cancer Cells. Front Cell Dev Biol 2021;9:679658.
19. Taganov KD, Boldin MP, Chang KJ, et al. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 2006;103:12481-6.
20. Nahid MA, Pauley KM, Satoh M, et al. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY. J Biol Chem 2009;284:34590-9.
21. Huang C, Liu XJ, QunZhou, et al. MiR-146a modulates macrophage polarization by inhibiting Notch1 pathway in RAW264.7 macrophages. Int Immunopharmacol 2016;32:46-54.
22. Li D, Duan M, Feng Y, et al. MiR-146a modulates macrophage polarization in systemic juvenile idiopathic arthritis by targeting INHBA. Mol Immunol 2016;77:205-12.
23. Essandoh K, Li Y, Huo J, et al. MiRNA-Mediated Macrophage Polarization and its Potential Role in the Regulation of Inflammatory Response. Shock 2016;46:122-31.
24. Li Y, Zhao L, Shi B, et al. Functions of miR-146a and miR-222 in Tumor-associated Macrophages in Breast Cancer. Sci Rep 2015;5:18648.
25. Yin C, Han Q, Xu D, et al. SALL4-mediated upregulation of exosomal miR-146a-5p drives T-cell exhaustion by M2 tumor-associated macrophages in HCC. Oncoimmunology 2019;8:1601479.
26. MacKinnon AC, Farnworth SL, Hodkinson PS, et al. Regulation of alternative macrophage activation by galectin-3. J Immunol 2008;180:2650-8.
27. Chen BY, Sung CW, Chen C, et al. Advances in exosomes technology. Clin Chim Acta 2019;493:14-9.
28. Ayuk SM, Abrahamse H, Houreld NN. The role of photobiomodulation on gene expression of cell adhesion molecules in diabetic wounded fibroblasts in vitro. J Photochem Photobiol B 2016;161:368-74.
29. Zhou S, Lan Y, Li Y, et al. Hypoxic Tumor-Derived Exosomes Induce M2 Macrophage Polarization via PKM2/AMPK to Promote Lung Cancer Progression. Cell Transplant 2022;31:9636897221106998.
30. Pritchard A, Tousif S, Wang Y, et al. Lung Tumor Cell-Derived Exosomes Promote M2 Macrophage Polarization. Cells 2020;9:1303.
31. Liu Y, Li L, Song X. Exosomal circPVT1 derived from lung cancer promotes the progression of lung cancer by targeting miR-124-3p/EZH2 axis and regulating macrophage polarization. Cell Cycle 2022;21:514-30.
32. Kong W, Zhang L, Chen Y, et al. Cancer cell-derived exosomal LINC00313 induces M2 macrophage differentiation in non-small cell lung cancer. Clin Transl Oncol 2022;24:2395-408.
33. Jin J, Yu G. Hypoxic lung cancer cell-derived exosomal miR-21 mediates macrophage M2 polarization and promotes cancer cell proliferation through targeting IRF1. World J Surg Oncol 2022;20:241.
34. Ma J, Chen S, Liu Y, et al. The role of exosomal miR-181b

in the crosstalk between NSCLC cells and tumor-associated macrophages. Genes Genomics 2022;44:1243-58.
35. Wani JA, Majid S, Khan A, et al. Clinico-Pathological Importance of miR-146a in Lung Cancer. Diagnostics (Basel) 2021.
36. Zhang J, Shao W, Zhang Z, et al. The rate of miR-146a rs2910164 mutations in patients with lung cancer: a meta-analytic review. Transl Cancer Res 2020;9:6792-800.
37. Hou J, Wang P, Lin L, et al. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol 2009;183:2150-8.
38. Ye HX, Liao GN, Dong YJ, et al. miR-146a-5p enhances embryo survival in unexplained recurrent spontaneous abortion by promoting M2 polarization of decidual macrophages. Int Immunopharmacol 2022;110:108930.
39. Ren W, Xi G, Li X, et al. Long non-coding RNA HCG18 promotes M1 macrophage polarization through regulating the miR-146a/TRAF6 axis, facilitating the progression of diabetic peripheral neuropathy. Mol Cell Biochem 2021;476:471-82.
40. Wu Q, Yu L, Lin X, et al. Combination of Serum miRNAs with Serum Exosomal miRNAs in Early Diagnosis for Non-Small-Cell Lung Cancer. Cancer Manag Res 2020;12:485-95.
41. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783-801.
42. Zhao Q, Wang X, Hu Q, et al. Suppression of TLR4 by miR-448 is involved in Diabetic development via regulating Macrophage polarization. J Pharm Pharmacol 2019;71:806-15.
43. Zhao J, Li X, Hu J, et al. Mesenchymal stromal cell-derived exosomes attenuate myocardial ischaemia-reperfusion injury through miR-182-regulated macrophage polarization. Cardiovasc Res 2019;115:1205-16.

Cite this article as: Yan Z, Wen JX, Cao XS, Zhao W, Han YL, Wen XH, Yan L, Zhang M, Wang YF, Hai L, Wang YJ, Zheng WQ, Hu ZD. Tumor cell-derived exosomal microRNA-146a promotes non-small cell lung cancer cell invasion and proliferation by inhibiting M1 macrophage polarization. Ann Transl Med 2022;10(24):1307. doi: 10.21037/atm-22-5565

