Preparation, Antituberculosis Function and Mechanism of Macrophage-Targeted circTRAPPC6B and Rifampicin Exosome Delivery System

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Abstract: Objective: To construct a composite exosome delivery system with macrophage-targeting ability, combining circular RNA circTRAPPC6B and the antituberculosis drug rifampicin (RIF) for the efficient treatment of pulmonary tuberculosis. The aim is to enhance the enrichment efficiency of drugs in infected macrophages and strengthen the clearance of intracellular Mycobacterium tuberculosis (MTB) by regulating the host immune response. Methods: Exosomes (Exo) were extracted from human dendritic cells by ultra-centrifugation. circTRAPPC6B was encapsulated into exosomes by electroporation. Rifampicin was embedded into the lipid bilayer of exosomes by hydrophobic insertion to obtain the RIF@ Exo-circTRAPPC6B composite system. Subsequently, the surface of exosomes was modified with mannose to construct the M-Exo-circTRAPPC6B-RIF targeted delivery system. Transmission electron microscopy (TEM), nanoparticle size analyzer and Western blot were used to characterize the morphology, particle size, Zeta potential and marker proteins of exosomes. In vitro experiments used the J774A.1 macrophage model infected with H37Rv to evaluate the cell uptake rate, minimum inhibitory concentration (MIC) and the expression level of autophagy-related protein LC3-II/I of the delivery system. Results: The M-Exo-circTRAPPC6B-RIF delivery system with good stability and targeting was successfully constructed. This system significantly enhanced the uptake of drug-loaded exosomes by macrophages, effectively inhibited the proliferation of intracellular MTB, and activated the cell autophagy pathway. Conclusion: The mannose-modified exosome co-delivery system developed in this study can achieve the synergistic effect of circTRAPPC6B and rifampicin. While improving drug targeting, it also regulates the host immune response, providing a new strategy for the precise treatment of pulmonary tuberculosis.

***Keywords*:** macrophage-targeting; circTRAPPC6B; rifampicin; exosome delivery system; antituberculosis function

1. Introduction

Pulmonary tuberculosis is a chronic respiratory infectious disease caused by Mycobacterium tuberculosis (MTB), and it remains one of the top ten causes of death worldwide. According to the World Health Organization, there are more than ten million new cases every year. The problem of drug resistance is becoming increasingly severe, and traditional chemotherapy regimens face bottlenecks such as insufficient efficacy, large side effects, and long treatment courses. MTB mainly parasitizes in alveolar macrophages, evading host immune clearance and leading to long-term latency or recurrence of the pathogen [1]. Therefore, how to efficiently deliver drugs to infected macrophages and activate their intrinsic defense mechanisms has become a key research direction. In recent years, due to their natural biocompatibility, low immunogenicity and transmembrane transport ability, exosomes have been regarded as promising nanocarriers [2]. Based on this background, this study designed a new targeted exosome delivery system, combining non-coding RNA circTRAPPC6B and the first-line antituberculosis drug rifampicin, to explore its function and mechanism of action in antituberculosis treatment.

2. Materials and Methods

2.1 Extraction and Identification of Exosomes

In this study, human dendritic cells (DCs) from healthy donors were selected as exosome-producing cells. The cells were routinely cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) until they reached 80% confluence, and then the medium was replaced with exosome-free pre-treated FBS medium and cultured for another 48 hours. The supernatant was collected and processed as follows: First, centrifuge at 2000×g for 20 minutes to remove cell debris, then centrifuge at 10,000×g for 30 minutes to remove large vesicles, and finally ultra-centrifuge at 120,000×g for 70 minutes at 4℃ to precipitate exosome particles. The obtained precipitate was resuspended in pre-cooled PBS, filtered through a 0.22μm filter, and then aliquoted and stored at-80℃ for later use.

The extracted exosomes were identified by various methods. Transmission electron microscopy (TEM) was used to

observe their typical "cup-shaped" morphology: 5 μL of the sample was dropped onto a copper grid, allowed to stand at room temperature for 2 minutes, then negatively stained with 2% phosphotungstic acid for 5 minutes, and air-dried naturally before observation under a JEOL JEM-1400Plus transmission electron microscope. Nanoparticle size and Zeta potential analysis were measured using a Malvern Zetasizer Nano ZS instrument. The temperature was set at 25°C, the scattering angle was 173°, and each sample was measured three times and the average value was taken. Western blot was used to detect the expression of exosome-specific marker proteins CD63, TSG101 and Alix to verify their purity.

2.2 Encapsulation of circTRAPPC6B and Loading of Rifampicin

The circTRAPPC6B sequence was customized by a synthesis company, with a length of 412 nt. High-purity RNA products were obtained by in vitro transcription. It was loaded into exosomes by electroporation: 100 μg of exosomes were mixed with 20 μg of circTRAPPC6B in electroporation buffer (containing 20 mM HEPES pH7.2, 135 mM NaCl, 2.5 mM $CaCl_2$), placed in a 0.4 cm electroporation cuvette, and electroporation was performed under the parameter settings of a voltage of 1200 V, a pulse width of 30 ms, and one pulse. After the reaction, an equal volume of complete medium pre-warmed to 37°C was immediately added to terminate the reaction, and the mixture was incubated at 37°C for 30 minutes for the membrane structure to self-repair. Unencapsulated free RNA was removed by ultra-filtration centrifugation (100 kDa cut-off molecular weight), and finally the Exo-circTRAPPC6B complex was obtained.

For the loading of rifampicin, the hydrophobic insertion method was used: 5 mg of rifampicin was dissolved in 1 mL of DMSO to prepare a mother liquor, and added to the above Exo-circTRAPPC6B suspension at a final concentration of 50 μg/mL. The mixture was incubated at 37°C in the dark with shaking for 2 hours to allow the drug to spontaneously embed into the exosome lipid bilayer. Then, the unbound drug was removed by ultra-filtration and washing three times to obtain RIF@Exo- circTRAPPC6B. The drug encapsulation efficiency was determined by high- performance liquid chromatography (HPLC). The mobile phase was acetonitrile: water (75:25), the flow rate was 1.0 mL/min, the detection wavelength was 254 nm, and the encapsulation efficiency was calculated by the standard curve method.

2.3 Construction of Mannose-Modified Exosomes

In order to achieve active targeting to macrophages, a chemical coupling method was used to introduce mannose ligands on the surface of exosomes. The specific operation was as follows: 1 mg of N-hydroxysuccinimide-activated esterified mannose (Man-NHS) was dissolved in DMSO, and slowly added to the PBS solution (pH7.4) containing 100 μg of RIF@Exo-circTRAPPC6B. The reaction was stirred at room temperature in the dark for 4 hours. After the reaction was completed, an ultra-filtration tube (100 kDa) was used to wash five times to remove unreacted mannose derivatives, and the final product M-Exo-circTRAPPC6B-RIF was obtained. The modification efficiency was detected by lectin ELISA: concanavalin A (ConA) was used as the capture antibody, and HRP-labeled goat anti-rabbit IgG was used as the secondary antibody. The OD450 value reflected the degree of mannose exposure.

2.4 Physicochemical Property Characterization

The finally constructed M-Exo-circTRAPPC6B-RIF was comprehensively physically characterized. The TEM sample preparation was the same as before to observe its morphological integrity. Dynamic light scattering (DLS) was used to measure its average particle size and polydispersity index (PDI), and the Zeta potential reflected the surface charge stability. Fourier-transform infrared spectroscopy (FTIR) was scanned in the range of 4,000-400 cm^{-1} with a resolution of 4 cm^{-1} to confirm whether the characteristic peak of mannose (C-O stretching vibration at about 1030 cm^{-1}) was present. In addition, the total protein content was determined by the BCA method to estimate the amount of exosomes.

2.5 Establishment of Cell Experimental Model and Functional Evaluation

The mouse mononuclear macrophage cell line J774A.1 was selected as the in vitro model. The cells were cultured in DMEM + 10% FBS and seeded in 96-well plates or 6-well plates for use. The tuberculosis strain H37Rv was resuscitated and adjusted to a concentration of MOI = 5 to infect macrophages. After adsorption for 2 hours, the medium was changed to remove free bacteria, and a stable intracellular infection model was established.

(1) Cell uptake experiment: DiO-fluorescently labeled M-Exo-circTRAPPC6B-RIF was added to the infected cells. At the time points of 1h, 4h, 8h and 12h, the cells were collected, washed twice with PBS, digested with trypsin to make a single-cell suspension, and the positive rate of green fluorescence was detected by flow cytometry, representing the proportion of exosomes taken up by cells. At the same time, the unmodified group (Exo-circTRAPPC6B-RIF) was set as a control.(2) Minimum inhibitory concentration (MIC) determination: The MIC values of different treatment groups were determined by the modified broth dilution method. The H37Rv bacterial solution was diluted to 1×10^5 CFU/mL, and serial concentration gradients of M -Exo-circTRAPPC6B-RIF (0.1-10 μg/mL), free RIF, M-Exo-RIF, M -Exo-circTRAPPC6B and

other treatment groups were added. After static culture at 37°C for 7 days, the turbidity change of the bacterial solution was observed, and the lowest drug concentration without obvious growth was recorded as the MIC.(3) Detection of autophagy pathway protein expression: After treating the cells for 48 hours, the total protein was extracted, quantified by the BCA method, and then subjected to SDS-PAGE electrophoresis. After transfer, the membrane was blocked for 1 hour, incubated overnight with primary antibodies (LC3-I/II antibody 1:1000, P62 antibody 1:800, β-actin internal reference 1:5000). The next day, after washing the membrane, HRP-labeled secondary antibody was added, developed by ECL, and the band gray value ratio was analyzed by ImageJ software to calculate the relative expression level of LC3-II/LC3-I to evaluate the autophagy activation state.

2.6 Statistical Methods

All experiments were independently repeated three times. The data were expressed as mean ± standard deviation. One-way analysis of variance was performed using SPSS26.0. A $P < 0.05$ was considered to have a statistically significant difference.

3. Results

3.1 Cell Uptake Rate Index

Flow cytometry analysis showed that at the 12-hour time point, the macrophage uptake rate of the M-Exo-circTRAPPC6B-RIF group reached (86.7 ± 3.2)%, which was significantly higher than that of the unmodified group (Exo-circTRAPPC6B-RIF) of (54.1 ± 4.5)% ($P < 0.01$), indicating that mannose modification significantly improved the targeting recognition and endocytosis efficiency of exosomes to macrophages.

Table 1. Comparison of macrophage uptake rates of different exosome groups (12-hour time point)

Group	Macrophage uptake rate (%, x±s)
M- Exo-circTRAPPC6B-RIF group	86.7 ± 3.2
Unmodified group (Exo-circTRAPPC6B-RIF)	54.1 ± 4.5

3.2 Minimum Inhibitory Concentration (MIC) Index

In the H37Rv infection model, the MIC of M-Exo-circTRAPPC6B-RIF was (0.78 ± 0.06) μg/mL, which was significantly lower than that of the free rifampicin group of (1.56 ± 0.11) μg/mL and the simple M-Exo-RIF group of (1.25 ± 0.09) μg/mL ($P < 0.05$), suggesting that the combination of circTRAPPC6B and rifampicin could synergistically enhance the antibacterial effect.

Table 2. Comparison of minimum inhibitory concentrations (MIC) of different groups against H37Rv

Group	Minimum inhibitory concentration (MIC, μg/mL, x±s)
M-Exo-circTRAPPC6B-RIF group	0.78 ± 0.06
Free rifampicin group	1.56 ± 0.11
Simple M-Exo-RIF group	1.25 ± 0.09

3.3 Autophagy-Related Protein Expression Index

Western blot results showed that after treatment with M-Exo-circTRAPPC6B-RIF, the LC3-II/LC3-I protein expression ratio in macrophages reached (3.21 ± 0.24), which was significantly higher than that of the control group (1.05 ± 0.12) and the drug-only group (M-Exo-RIF, 1.89 ± 0.17) ($P < 0.01$), indicating that this system could effectively activate the cell autophagy pathway and promote the clearance of intracellular MTB.

Table 3. Comparison of autophagy-related protein (LC3-II/LC3-I) expression in macrophages of different groups

Group	LC3-II/LC3-I protein expression ratio (x±s)
M-Exo-circTRAPPC6B-RIF group	3.21 ± 0.24
Control group	1.05 ± 0.12
Drug-only group (M-Exo-RIF)	1.89 ± 0.17

4. Discussion

As a natural carrier for intercellular communication, exosomes have good biocompatibility, low toxicity and the ability to cross biological barriers, and have been widely used in the treatment exploration of tumors, inflammation and infectious

diseases. However, the research on using exosomes for tuberculosis treatment is still in its infancy, especially the lack of a multi-functional platform that integrates gene regulatory elements and chemical drugs at the same time [3].

This study put forward an innovative idea in this context: using exosomes as a dual carrier, carrying both the emerging non-coding RNA molecule circTRAPPC6B and the classic antibiotic rifampicin, forming a "drug + immunomodulation" dual-track intervention mode. circTRAPPC6B is a newly discovered circular RNA. Research shows that it can up-regulate the expression of autophagy-related genes by sponging miR-142-3p and other ways, thereby enhancing the ability of macrophages to clear MTB. Rifampicin is a core bactericidal agent that blocks the transcription process of MTB by inhibiting RNA polymerase. The synergistic effect of the two is expected to break the immune escape mechanism of MTB [4]. The data of this study show that the proportion of mannose-modified exosomes taken up by macrophages is as high as 86.7%, far exceeding that of the unmodified group. This phenomenon can be explained at two levels: first, the high-affinity binding between mannose and C-type lectin receptors (such as MR, DC-SIGN) on the surface of macrophages triggers receptor-mediated endocytosis; second, the exosomes themselves are derived from human cells, and their membrane proteins (such as ICAM-1) may also participate in the cell adhesion process, further promoting uptake. The significant decrease in the MIC value reflects the strong antibacterial efficacy of this delivery system. The traditional view is that the antibacterial effect mainly depends on the drug concentration and its penetration ability [5]. However, this experiment found that even at the same dose of rifampicin, the MIC of M-Exo- circTRAPPC6B-RIF was still significantly lower than that of other groups, indicating that circTRAPPC6B played an important auxiliary role. The possible mechanisms include: circTRAPPC6B regulates the autophagy pathway, leading to more MTB being exposed to the lysosomal environment, increasing the chances for rifampicin to act. Autophagy activation is accompanied by the release of inflammatory factors (such as IL-1β and TNF-α), creating a micro-environment unfavorable for the survival of MTB. The exosome-encapsulated form reduces the distribution of the drug in non-target tissues and increases the local effective concentration. These factors work together to form an ideal pattern of "enhancing efficacy and reducing toxicity". The significant increase in the LC3-II/I ratio directly proves the activation of the autophagy pathway. LC3 is a marker protein for autophagosome formation, and the process of its conversion from cytoplasmic LC3-I to membrane-bound LC3-II is a key step in the initiation of autophagy. P62, as an autophagy substrate adaptor protein, usually degrades with the enhancement of autophagy. Although the specific value of P62 was not listed in this study, the significant increase in LC3-II/I allows us to infer the overall enhancement of the autophagic flux. More importantly, this effect is not solely caused by the drug because the use of rifampicin alone cannot significantly up-regulate the expression of LC3-II. This suggests that circTRAPPC6B is a core factor driving autophagy, and its mechanism of action may involve the regulation of downstream signaling pathways such as AMPK/mTOR or ULK1 [6].

Compared with traditional liposomes or polymer nanoparticles, the exosome system used in this study has several unique advantages. First, exosomes are derived from endogenous cells, avoiding the common problems of immune recognition and clearance of synthetic materials, and having a longer half-life in the body. Second, their natural membrane structure contains a variety of homing molecules, which have a certain degree of autonomous targeting ability. When combined with mannose modification, the targeting efficiency is further amplified. Third, exosomes can accommodate both water-soluble (RNA) and lipid-soluble (rifampicin) substances simultaneously, achieving true multimodal co-delivery, which is difficult for most single carriers. In addition, compared with viral vectors, exosomes have no risk of insertional mutagenesis, are safer, and are suitable for long-term or repeated administration. Although the large-scale production of exosomes still faces challenges, with the development of cell-free culture technology and engineering modification methods, their industrialization prospects are gradually becoming clearer. For example, some companies have developed an exosome mass-production platform based on CHO cells, with a yield of up to milligrams per liter, which is sufficient to support the needs of pre-clinical research.

5. Conclusion

The M-Exo-circTRAPPC6B-RIF system constructed in this study not only achieves an innovative breakthrough in the technical route but also demonstrates the dual antituberculosis potential of "chemical killing + immune remodeling" at the functional level, providing a solid foundation for the development of a new generation of precise anti-infectious therapies.

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