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Tumor-Infiltrating T Cells and Their Exosomes: Functional Roles and Microenvironmental Impact

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Abstract: Exosomes are nanosized phospholipid bilayer vesicles secreted by various cells, rich in bioactive molecules such as proteins, nucleic acids and lipids, which play important roles in intercellular communication and microenvironmental regulation. In recent years, T cell-derived exosomes have become a hotspot in tumor immunity research due to their immune specificity and functional diversity. The aim of this study was to systematically analyze the physicochemical properties of T cell-derived exosomes and their functional effects on tumor cells. T cell-derived exosomes were activated and isolated in vitro, and their particle size distribution, marker protein expression and cellular uptake ability were verified by nanoparticle tracking analysis, Western blot and confocal imaging. Functional experiments showed that T-cell exosomes can effectively inhibit tumor cell proliferation and induce apoptosis, suggesting that they have potential anti-tumor activity. This study provides experimental basis and theoretical support for the application of T-cell exosomes in tumor immunotherapy and expands its research horizons in the field of precision medicine.

1. Introduction

As an important leukocyte, T cells are a key component of the immune system. According to their functional differentiation status, T cells can be divided into subpopulations such as cytotoxic T lymphocytes, helper T cells, regulatory T cells and memory T cells. Among them, cytotoxic T cells directly induce the apoptosis of target cells by recognizing foreign antigens presented on the surface of target cells and releasing cytotoxic molecules such as Perforin and Granzyme B. Helper T cells regulate the activity of other immune cells through the secretion of cytokines (e.g., IL-2, IFN-γ) and play a coordinating role in the immune response[1].

Exosomes are small extracellular vesicles with lipid bilayer membranes in the size range of 30-150 nanometers released by cells[2], which are enriched with proteins, lipids, DNA, and RNA and other bioactive molecules[3]. Exosomes enable precise delivery of contents to target cells and, as an emerging messenger, are changing our perception of intercellular messaging[4]. In recent years,

the study of the functional regulation of T cells in the tumor microenvironment and their derivatives has gradually become an important research direction in tumor immunology.

In recent years, scientists have found that exosomes secreted by T cells also play an important and complex role in tumor therapy. Akansha's team found that exosomes isolated from regulatory T cells (Tregs) and other T cell subpopulations have been demonstrated to have immunosuppressive ability[5].Xiang-Jun Tang's team found that chimeric antigen receptor (CAR)-based T cell derived exosomes can replace CAR-T cells as the ultimate attacker, overcoming limitations and becoming a highly promising approach for cancer therapy[6]. However, there are still many unanswered questions about the isolation and purification method of T-cell exosomes, their functional mechanisms, and their behaviors in the tumor microenvironment, which urgently need to be verified and expanded by systematic experiments.

This paper focuses on exosomes secreted by T cells. After T cells are activated by the TCR signaling pathway, the exosomes released by them not only carry membrane proteins such as CD3 and TCR, but are also enriched with a variety of immunomodulatory factors, which have the potential to regulate the immune status of target cells[7]. IL-12-stimulated $CD8^+$ cytotoxic T cells can release active EVs, which can induce resting T cells to produce functional responses in the absence of antigenic stimulation, suggesting that they have a certain degree of immune activation ability. In addition, exosomes are naturally of low immunogenicity and good biocompatibility due to their origin in host cells, and they are not easy to trigger immune rejection in vivo, so they have the potential to become drug delivery carriers. Compared with traditional tumor therapies such as surgery, radiotherapy and chemotherapy, exosome-mediated immunomodulation strategies have the advantages of strong targeting and low side effects. Especially in the face of challenges such as tumor immune escape and immunosuppression, T-cell exosomes provide an extracellular and controllable immune intervention, which opens up a new research direction for tumor therapy.

2. Experimental materials and Method

2.1 Experiment name: cell resuscitation and culture

2.1.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 1, Table 2 and Table 3 below.

Table 1 Reagents for cell resuscitation and culture

Reagent	Manufacturer	Item No.
RPMI 1640 medium	GIBCO	C11875500BT
DMEM medium	GIBCO	C11995500BT
Fetal Bovine Serum (FBS)	GIBCO	10270106
Penicillin/streptomycin antibiotic	GIBCO	15140163
Recombinant mouse IL-2 protein (mIL-2)	Soluble	P00198

Table 2 Consumables for cell resuscitation and culture

Material	Manufacturer	Item No.
Pipette gun	Thermo Scientific FINNPIPETTE F3	
Pipette tips in 10 μL cartridge	KIRGEN	KG1031
Pipette tips in 200 μL cartridge	KIRGEN	KG1232
1000 μL cartridge tip with extended cartridge	KIRGEN	KG5333
1.5 μm Aluminum Foil Tinfoil	Jellyfish	
Ultracentrifuge tubes	BECKMAN	355618
5mL single-use sterile syringe with needle	Hongda	
1.5mL Microcentrifuge Tube	biosharp	BS-15-M
5mL Microcentrifuge Tube	biosharp	BS-50-M
15mL Centrifuge Tube	KIRGEN	KG2614
50mL centrifuge tube	KIRGEN	
90mm×15mm round petri dish	NEST	704001

Table 3 Instruments for cell resuscitation and culture

Instrument	Manufacturer	Item No.
Carbon Dioxide Incubator	Thermo Scientific	FORMA STERI-CYCLE i160
Bio-safety Cabinet - Ultra-clean bench	Airstream	AC2-5S1
Thermostatic baths and water baths	bluepard	BWS-10
Inverted phase contrast system microscope	OLYMPUS	CKX53
Centrifuge	cence Xiangqi	TDZ5-WS
Waste liquid suction pump	Haimen Kylin-Bell Kylin-Bell	GL-80

2.1.2 Experimental procedure

We first pre-warm the complete medium in a 37 ℃ water bath or incubator. We then remove the cells from liquid nitrogen and quickly thaw the frozen tubes in a 37 ℃ water bath. After thawing, we sterilize the outside of the tubes with ethanol and transfer the cell suspension into a centrifuge tube containing 10 mL of pre-warmed complete medium to dilute the DMSO. We place the tube symmetrically in a centrifuge and spin it at 1000×g for 5 minutes. We aspirate the supernatant, retain it until the white precipitate is processed, and resuspend the cells in fresh culture medium. We gently blow the suspension 20 times to mix thoroughly before inoculating the cells into culture flasks or petri dishes. Finally, we shake the vessels crosswise to distribute the cells evenly and place them into a 37 ℃ incubator with 5% CO_2.

2.2 Experiment name: Fluorescence staining of T cells

2.2.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 4 and Table 5 below.

Table 4 Reagents for T cell fluorescence staining

Reagent	Manufacturer	Item No.
DAPI		
Actin-Tracker Green-488	Beyotime	
1×PBS buffer	biosharp	BL302A

Table 5 Instruments for T cell fluorescence staining

Instrument	Manufacturer	Item No.
Fully Automated Cellular Fluorescence Microscopy	Keyence Corporation	KEYEN CEBZ-X800LE

2.2.2 Experimental procedure

CTLL-2 cells were rinsed 1-2 times using PBS buffer, and the cells were transferred to culture dishes at a density of about 60%. DAPI stain was added to the nucleus of the cells in a volume of 1:200, after which the cell membrane was stained according to the addition of AF488 stain in a volume of 1:1000, and the cells were incubated at room temperature and protected from light for 10 min, and the fluorescence of the cells was observed under a fully automated cytofluorescence microimaging microscope.

2.3 Experiment name: isolation, potential and particle size detection of exosomes

2.3.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 6 below.

Table 6 Instrument for exosome isolation, zeta potential, and size distribution analysis

Instrument	Manufacturer	Item No.
Benchtop high speed and large capacity freezing centrifuge	cence Xiang Yi	H1850R
Ultra-microbalance	METTLER TOLEDO	ME204
Pocket Centrifuge	SCILOGEX	D1008
Floor standing ultra-high speed centrifuge	BECKMAN COULTER	Optima XE-100
Nano Particle Size and Zeta Analyzer	Malvern	Zetasizer Nano Zen

2.3.2 Experimental procedure

We first add the DMEM medium containing T cells in batches into multiple 50 ml centrifuge tubes. We then place these tubes into the centrifuge, ensure they are balanced and tightly sealed, and centrifuge at 2000×g for 15 minutes at 4 °C.

After centrifugation, we remove the tubes and transfer the supernatant to new centrifuge tubes. We place these new tubes into the centrifuge and centrifuge again at 3000 ×g for 30 minutes at 4 °C.

We then remove the tubes, transfer the supernatant once more to new tubes, and centrifuge at 13000×g for another 30 minutes at 4 °C.

Next, we transfer the supernatant to ultra-high-speed centrifuge tubes. Using an analytical balance, we carefully balance the mass of liquid in each tube, ensuring the difference between them is within 0.0001 g. We take the rotor and caps from the refrigerator, place the tubes symmetrically into the rotor, tighten the caps, and load them into the ultra-high-speed centrifuge. We set the centrifuge to 100,000×g for 1.5 hours, wait for the vacuum to drop below 200 μL, and start the run.

After completion, we release the vacuum, remove the rotor, and return both the rotor and caps to the refrigerator. We pour off the supernatant and resuspend the exosome pellet in PBS before transferring it to an EP tube.

For exosome particle size measurement, we prepare three 15 ml EP tubes per sample, labeling them #1, #2, and #3. We add 4 ml of purified water and 3 μL of the sample to EP tube #1. Using a syringe and filter membrane, we filter the solution from tube #1 into tube #2 and then discard tube #1.

We repeat this filtering process, alternating between tube #2 and tube #3 ten times. Finally, we pipette 1 ml of the filtered sample along the edge of the measuring dish, place it into the nanoparticle analyzer, and record the particle size data.

For exosome potential measurement, we pipette the sample into the measuring dish, ensuring it covers the electrode plate. We then place the dish into the nanoparticle analyzer and record the zeta potential data.

2.4 Experiment name: Western Blotting

2.4.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 7 and Table 8 below.

Table 7 Reagents for Western blotting

Reagent	Manufacturer	Item No.
One-step gel preparation reagent (concentrated gel)	Fdbio science	FD346
10% One Step Gel Preparation Reagent	Fdbio science	FD341
Tween-20	Fdbio science	FD0020
Tetramethylethylenediamine (TEMED)	Aladdin	
RIPA lysate	Biotin	P0013C
Methanol	Xihua XIHUA	B2305261
Anti-CD63 antibody	Abcam	ab217345

Table 8 Instruments for Western blotting

Instrument	Manufacturer	Item No.
Transfer Plate	DFRL	1703931
Glass plate (with 1mm spacers)	BIO-RAD	1653311
PVDF transfer film	Millipore	ISEQ00010

2.4.2 Experimental procedure

(1) Sample Preparation

The blank control NC cells and experimental group cells were centrifuged at 800 × g for 5 min to remove the culture medium, and washed twice with PBS. At the end of the washing, cells were collected by centrifugation, and RIPA lysate (containing protease inhibitor) was added, and cell fragmentation was performed by using an ultrasonic cell crusher with a setting of 25% power, and the sonication was performed for 3 s followed by a pause of 3 s, and repeated for 4 times, and the whole operation was performed on ice. After sonication, centrifugation was performed at 12,000×g for 10 min at 4 ℃, and the supernatant was removed. Add SDS-PAGE Protein Sampling Buffer, vortex and mix well, then cook the sample in a metal bath, heated at 100 ℃ for 10 min. The sample preparation was completed.

(2) Gel preparation

Table 9 Composition of separated gel and concentrated gel

Composition	Separation gel (10ml)	Concentrated gel (4 ml)
TEMED	3μl	3μl
10%APS	80μl	30μl

We first fix the glass plate in place. We then prepare the separation gel by taking 10 ml of it in a 50 ml centrifuge tube, adding 80 μl of 10% ammonium persulfate and mixing well, followed by the addition of 3 μl TEMED and thorough mixing. Next, we prepare the stacking gel by taking 4 ml of it in another 50 ml centrifuge tube, adding 30 μl of 10% ammonium persulfate and mixing well, then adding 3 μl TEMED and mixing again.

We immediately pour an appropriate amount of the mixed separation gel into the glass plate. Without waiting for the separation gel to solidify, we use a pipette to slowly add the stacking gel, moving from one side of the glass plate to the other to ensure even distribution over the separation gel. We then slowly insert the comb and allow the gel to solidify for 50 minutes. (Table 9)

(3) Protein electrophoresis

1) 30 μg of protein sample was added to each well of the gel and electrophoresis was performed using a voltage of 60 V at first, increasing to 100 V after 30 minutes.

2) The PVDF membrane was pre-activated with methanol and covered with the SDS-PAGE gel. Proteins were transferred to the PVDF membrane using the wet transfer method by placing the membrane transfer tank in ice water at 250 mA for 2.5 h at constant flow.

3) The PVDF membrane was closed at room temperature for 1 h using 5% skim milk powder.

4) TSG101 antibody (1:1000) and CD63 antibody (1:1000) were prepared using 5% BSA and co-incubated with PVDF membrane on a slow shaker at 4 ℃ with the shaking speed set to 80 rpm.

5) The membranes were washed three times with TBST on the shaker for 5 min each time, and the shaking speed was set to 120 rpm.

6) The secondary antibody was incubated at room temperature for 1 h with the shaking speed set at 80 rpm.

7) We washed the membrane three times with TBST on a shaker for 5 min each time with the shaking speed set to 120 rpm.

8) We configured ECL luminescent solution, add drops to cover the membrane, and machine exposure.

2.5 Experiment name: Co-incubation of tumor cells with exosomes

2.5.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 10 and Table 11 below.

Table 10 Reagents for the co-incubation of tumor cells with exosomes

Reagent	Manufacturer	Item No.
4% PFA fixative (general-purpose tissue fixative)	Biosharp	BL539A
Anti-fluorescence quenching peak slice solution (with DAPI)	Beyotime	P0131-25ml
Cy5.5-NHS ester	Genuine Leaf Bio	S27624

Table 11 Instruments for the co-incubation of tumor cells with exosomes

Instrument	Manufacturer	Item No.
Research Inverted Microscope	Nikon	Eclipse Ti2-E

2.5.2 Experimental procedure

(1) Cell spreading: The B16F10 tumor cell line was counted and inoculated in a 3cm dish to complete cell spreading.

(2) Cy5.5 labeled T-cell exosomes: We add 300 μL NK/T-cell-derived exosome samples and 1 μL cy5.5 stain into EP tubes, blow and mix to ensure the dye is in full contact with the exosome membrane, and wait for 30 minutes to complete the staining. The stained EP tubes were flattened into a centrifuge and set at 20,000xg for 10 min. The supernatant was discarded and resuspended by adding 300 μL of PBS.

(3) Co-incubation and binding reaction: We add exosomes into cell culture dish, incubate for 15 min away from light, discard supernatant and wash with PBS.

(4) Cell fixation and staining treatment: We add 4% concentration of fixative PFA to fix the cell morphology and surface antigen, incubate for 10 min away from light. We discard the PFA and wash it with PBS twice. We add 50-100 μL of anti-fluorescence quenching blocking solution.

(5) Microimaging observation: Observation and photographing were performed with a Nikon inverted microscope.

2.6 Experiment name: Exosome-induced tumor cell apoptosis experiment

2.6.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 12 and Table 13 below.

Table 12 Reagents for the apoptosis assay

Reagent	Manufacturer	Item No.
CCK-8	Selleckchem	B34302

Table 13 Instruments for the apoptosis assay

Instrument	Manufacturer	Item No.
Biological Safety Cabinet	Thermo Scientific	1374
Inverted phase contrast system microscope	OLYMPUS	CKX53
Waste liquid suction pumps	Haimen Kylin-Bell	GL-80
Multi-functional Microplate Reader	Molecular Devices	SpectraMaxiD5

2.6.2 Experimental procedure

We first seed B16F10 cells into a 96-well plate and incubate them for 6 hours to allow cell attachment. Then, we add T cell-derived exosomes at concentrations of 10 μg/mL, 50 μg/mL, and 100 μg/mL to the sixth, seventh, and eighth columns, respectively, with six replicate wells for each concentration. After mixing gently, we place the plate back into the incubator for 24 hours.

Following incubation, we discard the supernatant and add 100 μL of fresh DMEM medium to each well. Next, we add 10 μL of CCK8 reagent to each well of the three experimental groups, mix thoroughly, and incubate the plate for 4 hours in the dark. Finally, we measure the absorbance using a microplate reader.

2.7 Experiment name: Mouse experiment

2.7.1 Experimental materials

The instruments and materials used in the experiment are listed in the Table 14 below.

Table 14 Instruments for the in vivo mouse study

Instrument	Manufacturer	Item No.
Tail vein injector		
Ear Marker	Tongxin	DX092
Mouse fixator		

2.7.2 Experimental procedure

(1) Subcutaneous injection in mice: We correctly grasp the mice, identify the mice, dehair the abdomen of the mice, insert the needle flatly for about 1 cm, and judge whether the drug is injected by whether there is a small mound on the chest and abdomen.

(2) Mouse tail vein injection: We grab the mouse correctly and put it into the fixator, put the fixator into the mouse intravenous injection visualizer, wipe the tail with alcohol cotton, insert the needle parallel to the tail vein of the mouse.

(3) Steps of intraperitoneal injection in mice: We grasp the mice correctly, enter the needle at 45 degrees and inject at the root of the thigh, slowly push out the liquid and then rotate the needle.

3. Experimental results and analysis

3.1 Experiment name: cell resuscitation and culture

3.1.1 Experimental results

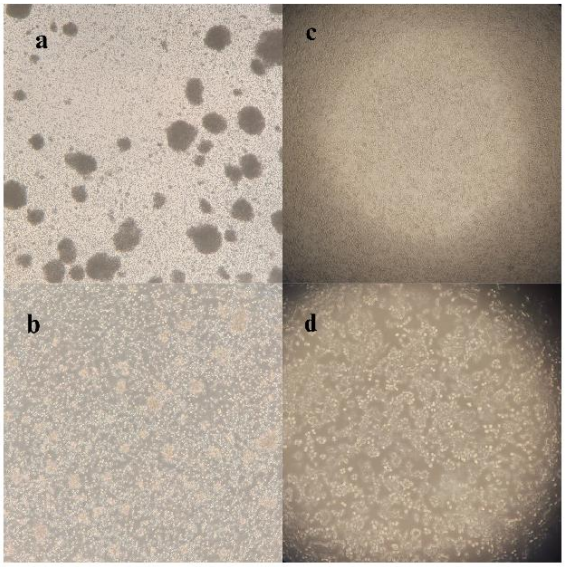

Figure 1 Micrograph of CTLL-2 cells and 293T cells

Note: Figure 1a shows T cells under 4Xx objective, Figure 1b shows T cells under 10Xx objective, Figure 1c shows 293T cells under 4Xx objective, and Figure 1d shows 293T cells under 10Xx objective.

As shown in Figure 1a and 1b, CTLL-2 cells, as suspension cells, showed smooth and full

spherical shape with clear edges, translucent cytoplasm, single or clustered distribution, and the spacing of cells in the field of view was 1-2 times the diameter. Cells in good condition were smooth in shape and evenly suspended; cells in bad condition showed degeneration, depression, apoptotic vesicles, tightly packed clusters or large amounts of fragments, overcrowding or sparseness. As shown in Figure 1c and 1d, 293T cells, as adherent cells, showed a typical shuttle or polygonal shape with the extended cytoplasm and large centered nuclei. The edge was clear, the nucleolus was clear, the cytoplasm was translucent, uniformly dispersed or locally loosely grown in clusters, the spacing of cells in the field of view was 1-2 times the diameter, and the spacing of cells within the clusters was homogeneous. The state of good cells with clear nucleoli, neat edges, cell spacing is uniform; the state of particles deposited, stacked, overlap or gap is too large.

3.1.2 Reflection and Discussion

(1) Why is it necessary to add specific cytokines or stimulating factors in the process of culturing immune cells?

When immune cells are cultured in vitro, specific cytokines or stimulating factors need to be added to activate their function. Isolated immune cells are usually in a resting state and lack stimulation by antigen or inflammatory signals in vivo. If not activated, the cells will show low function, limited proliferation, or even apoptosis. Commonly used activation factors include IL-2, IL-15, CD3/CD28 antibodies, etc. In this experiment, we chose IL-2 as a cytokine for T cell culture to induce T cells into an activated state and enhance their killing ability and secretion function. The activated immune cells can be expanded in a medium containing cytokines and serum for subsequent functional validation or therapeutic studies.

(2) 37℃, 5% CO_2 saturated humidity culture environment for the maintenance of cell physiological function of the significance of?

37℃, 5% CO_2, saturated humidity culture environment is designed to simulate physiological conditions in mammals to maintain the normal metabolism and function of cells. 37℃ is the body temperature of mammals. The enzyme activity is the strongest at this temperature, which is conducive to DNA replication, protein synthesis and signaling; the temperature is too high can lead to protein denaturation, and too low is to inhibit metabolism. 5% CO_2 and the medium of the formation of carbonate - bicarbonate buffer system, to maintain the function of cells, and to maintain the function of cells. Bicarbonate buffer system to maintain pH stability and prevent acidosis or alkalization due to acid production by cell metabolism. Saturated humidity prevents evaporation of the culture fluid and maintains osmotic pressure and volume stability. The three synergistic effects provide a stable and controllable environment for cell growth in vitro.

3.2 Experiment name: fluorescence staining experiment of T cells

3.2.1 Experimental results

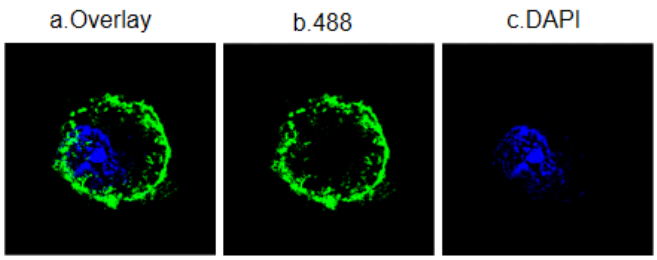

Figure 2 Fluorescence microscope image of CTLL-2 cells

Figure 2b shows the results of cell membrane labeled with 488 staining, and Figure 2c shows the results of cell nucleus labeled with DAPI. Figure 2a shows the localization of the cell membrane (green) in relation to the nucleus (blue), which demonstrates a better staining result, with the range of green fluorescence encircling the blue fluorescence, nicely demonstrating the localization of the nucleus in the cells. This provides cell-level visualization results for subsequent experiments on NK cell structure and function.

3.2.2 Reflection and Discussion

What are some of the reasons for possible causes if results are not found to be as expected during T-cell exosome isolation or staining?

(1) Sample quality: Check whether T cell activity is normal and whether there is a large amount of apoptosis or necrosis, so as not to affect the yield and quality of exosomes.

(2) Separation process: Confirm whether the centrifugation parameters are accurate and whether the ultracentrifugation step is strictly executed; exclude protein aggregates or apoptotic body contamination, and recommend using ultracentrifugation or SEC instead of precipitation to improve purity.

(3) Staining and detection conditions: optimize the concentration and time of antibody incubation, confirm whether the antibody recognizes exosome surface markers (e.g., CD63, CD81); avoid excessive freeze-thawing resulting in membrane structure damage; assess whether the detection platform (e.g., nano-flow, NTA) has sufficient sensitivity. Through the above troubleshooting, we can quickly locate the source of the problem and adjust the experimental plan.

3.3 Experiment name: isolation, potential and particle size detection of exosomes

3.3.1 Experimental results

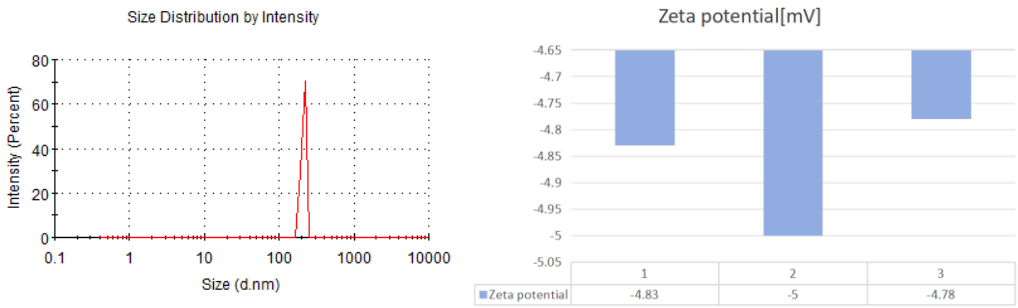

Figure 3 Particle size distribution map and zeta potential of T cell-derived exosomes

We used dynamic light scattering and zeta potential to analyze the particle size and surface charge properties of T cell-derived exosomes. Figure 3a Particle size distribution plot shows that the exosomes are mainly concentrated between 150-300 nm, presenting a narrow single-peak distribution, which is consistent with the range of exosomes. Figure 3b Zeta potential analysis plot shows that the potential results of three repetitions of the sample are -4.83 mV, -5.00 mV and -4.78 mV, with an average value of about -4.87 mV. The negative value indicates that the exosome surface carries a slight negative charge and these ions may play an important role in maintaining colloid stability.

3.3.2 Reflection and Discussion

Table 15 compares the method of differential separation of T-cell exosomes with other common Method (e.g., SEC, immunomagnetic beads).

Table 15 Comparison of differential separation with other common methods for T-cell exosome isolation

Method	Advantages	Limitations	Applicable scenarios
Differential centrifugation	Proven operation, suitable for large sample sizes	Time consuming, limited purity	Routine laboratory separations
SEC (gel filtration)	High purity of separation, maintains exosome activity	No targeting, limited sample size	High purity analysis or functional validation
Immunomagnetic bead assay	Targets specific markers, high selectivity	High cost, suitable for small sample volume	Enrichment of specific subpopulations

3.4 Experiment name: Western Blotting

3.4.1 Experimental results

We used Western Blot to verify whether T-cell-derived exosomes were raised by detecting the expression of the exosome marker protein CD63. CD63, as an exosome-specific marker protein, can effectively validate that exosomes have been successfully isolated if CD63 bands appear in Western Blot, providing a reliable basis for subsequent studies. The result shows that obvious CD63-specific bands were detected in exosome samples, proving that the vesicles we extracted were exosomes.

3.4.2 Reflection and Discussion

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a classical method for separating proteins based on molecular weight differences.

1) SDS effect: SDS is an anionic surfactant that disrupts the secondary and tertiary structure of proteins, denaturing and unfolding them, and at the same time uniformly encapsulates a negative charge on the protein surface, so that different proteins have similar charge densities, thus eliminating the effect of the difference in original charge on migration.

2) Gel sieving effect: The polyacrylamide gel forms a cross-linked mesh structure, which produces a sieving effect on proteins of different molecular weights. Small molecule proteins are more likely to cross the pores and migrate faster; large molecule proteins are resisted and migrate slower.

3) Electrophoretic driving force: Under the action of an electric field, the negatively charged SDS-protein complex migrates from negative pole to positive pole. The electric field force is the main driving force for migration, and the migration rate is inversely proportional to the molecular weight of the protein. Eventually, the proteins are separated in the gel according to the molecular weight and form clear bands.

In Western Blot, internal reference proteins are used to calibrate sample loading, transmembrane efficiency and experimental errors to ensure comparable quantitative analysis of target protein expression. The following factors should be considered when selecting internal reference:

1) Expression stability: the internal reference should maintain stable expression under different treatment conditions.

2) Cell/tissue type: different samples are suitable for different internal reference, e.g. GAPDH is suitable for most cell lines, β-actin is suitable for cell lysate, Histone H3 is suitable for nuclear protein analysis.

3) Experimental purpose: if the research treatment has an effect on metabolism or the cytoskeleton,

the use of related pathway proteins as internal references should be avoided.

3.5 Experiment name: Co-incubation of tumor cells with exosomes

3.5.1 Experimental results

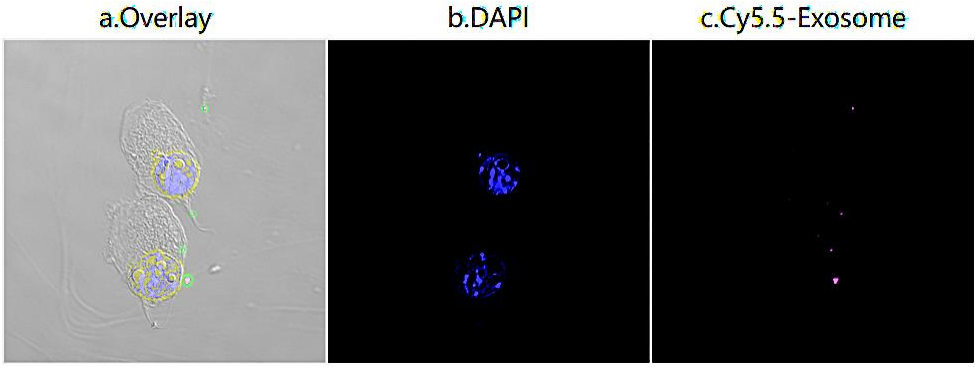

Figure 4 Co-incubation of B16F10 tumor cells with T-cell exosomes to develop the image

We performed imaging analysis using fluorescence microscopy to observe the co-localization of T cell-derived exosomes with B16F10 cells. Figure 4a shows the superimposed effect of DAPI staining with Cy5.5 fluorescence signals, demonstrating the spatial distribution relationship between exosomes and nuclei; Figure 4b shows the staining results of nuclei, in which blue fluorescence is the spatial location of nuclei; Figure 5c shows the localization of Cy5.5-labeled exosomes, and four clear magenta fluorescent dots in the figure are the locations of exosomes. The results of this image suggest that T-cell-derived exosomes can effectively bind to or enter tumor cells, with potential tumor-regulating ability.

3.5.2 Reflection and Discussion

The interaction between T-cell-derived exosomes and tumor cells includes three main stages:

1) Recognition and binding: exosomes express integrins (e.g., α4β7, αLβ2), glycoproteins, PD-1 and other molecules on the surface of the exosomes, which can specifically bind to receptors such as ICAM-1, PD-L1 and others on the surface of the tumor cells to complete the initial recognition and adhesion.

2) Uptake mechanism: after binding, exosomes can release their contents directly to the cytoplasm via membrane fusion or enter the cell via receptor-mediated endocytosis (e.g., the Clathrin/Caveolin pathway), or cytosolization (e.g., microtubule-associated endocytosis). Different pathways determine their localization to endosomes, lysosomes or cytoplasm, affecting delivery efficiency and function.

3) Functional delivery: exosomes carry functional molecules such as proteins, miRNAs, mRNAs, cytokines, etc., which can regulate tumor cell proliferation, apoptosis, immune escape, and other processes. For example, we detected that T cell exosomes express α4β7 integrins on the surface, which can bind to ICAM-1 to promote uptake; meanwhile, some exosomes carry PD-1, which may be involved in the immunosuppressive mechanism in the tumor microenvironment.

3.6 Experiment name: Exosome-induced tumor cell apoptosis experiment

3.6.1 Experimental results

In order to study the correlation between T cell-derived exosomes and tumor cell activity, as

shown in Figure 5, we used the CCK-8 method to detect changes in the activity of tumor cells after adding different concentrations of exosomes. The results showed that exosomes at 10 μg/mL had no significant effect on cell activity, while medium and high concentrations of exosomes at 50 μg/mL and 100 μg/mL significantly reduced tumor cell activity. It can be concluded that T-cell-derived exosomes can effectively inhibit the proliferation of tumor cells at a certain concentration and have potential anti-tumor functions.

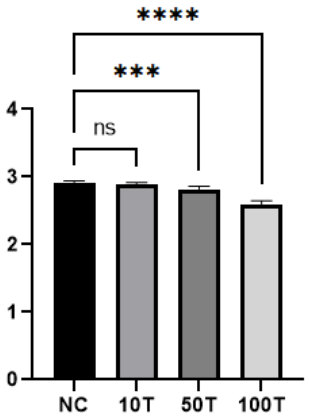

Figure 5 CCK8 experimental results

3.6.2 Reflection and discussion

(1) Do T-cell-derived exosomes play a killing role by regulating apoptosis-related signaling pathways of tumor cells?

T cell-derived exosomes may play a killing role by regulating the apoptosis-related signaling pathway of tumor cells. Specific mechanisms include: exosomes carry functional molecules such as perforin, granzyme B, miRNA, etc., which can activate the death receptor pathway (e.g., Fas/FasL) or the mitochondrial pathway, inducing caspase cascade reactions and inhibiting anti-apoptotic proteins such as BCL-2, thus promoting apoptosis of tumor cells. Some studies have observed tumor growth inhibition in vitro and in mouse models. However, the delivery efficiency and targeting of exosomes and their potential to carry immunosuppressive molecules (e.g., PD-L1) still need to be further optimized. In the future, their anti-tumor efficacy can be enhanced by engineering modifications or combination therapy strategies.

(2) Do T-cell exosome have an immune-activating role in the tumor microenvironment?

T-cell exosomes may play an immune-activating role in the tumor microenvironment, but they may also have immunosuppressive potential, depending on their molecular composition and target cell type. On the one hand, exosomes may carry pro-inflammatory cytokines (e.g., IFN-γ, IL-2) or tumor-associated antigens, which activate dendritic cells, macrophages, and other T cells to enhance anti-tumor immune responses. On the other hand, some exosomes may also carry immunosuppressive molecules such as PD-L1, which bind to PD-1 on the surface of T cells and inhibit their activity, leading to immune escape. Therefore, the role of T-cell exosomes in TME is bi-directional and needs to be functionally verified with specific experimental data.

(3) In this experiment, were there abnormal fluctuations in the MTT results of the T-cell exosome-treated group? Is it possible that the high concentration of exosomes or the long incubation time led to cellular stress response, thus affecting the apoptosis judgment?

In this experiment, the MTT results of the T-cell exosome-treated group showed an overall decreasing trend, suggesting suppressed cell activity. A small number of unstained black dots and purple areas appear in the images, which may represent dividing cells or apoptotic vesicles. Stress response is usually manifested as cell swelling, vacuolization, loss of membrane integrity and other

morphologies, while no obvious stress features were observed in this experiment, suggesting that the exosome concentration or incubation time did not trigger a significant stress response. However, it is still recommended to set concentration gradients and time points in subsequent experiments to exclude potential nonspecific toxic effects.

3.7 Experiment name: Mouse experiment

3.7.1 Experimental results

Figure 6 shows the standard mouse grasping posture, using the thumb and forefinger to grasp the back of the neck of the mouse, and the middle finger to fix the tail of the mouse, so that it is fixed and cannot turn back, to ensure the safety of animal experiments, and to facilitate the smooth operation of subsequent experiments.

Figure 6 Standard grasping mouse posture

3.7.2 Reflection and Discussion

In mouse experiments, why do some studies choose subcutaneous injection of tumor cells and others choose in situ injection or tail vein injection?

In the construction of mouse tumor models, researchers usually choose different tumor cell injection methods according to the experimental purpose, technical feasibility and the requirements for the tumor microenvironment simulation. Subcutaneous injection is one of the most commonly used methods. Injecting tumor cells subcutaneously into the back or abdomen of mice is easy and less traumatic, and has a high tumorigenicity rate, which facilitates external measurement of tumor volume and is suitable for drug screening and growth curve analysis. However, its microenvironment differs from the organ of origin, which may affect the biological behavior of the tumor. In situ injection, on the other hand, injects tumor cells directly into the organ of origin, such as breast cancer into the breast fat pad, which more realistically simulates the growth and microenvironment of the tumor at the site of origin, and is suitable for the study of tumor infiltration, angiogenesis, and natural metastasis, but technically demanding, with the help of imaging or anatomical localization. Tail vein injection, on the other hand, is used to construct a hematogenous metastasis model, in which tumor cells are injected into the tail vein and then reach the distal organs through the circulatory system. It is commonly used to study the metastatic mechanism of the lungs

or liver, but it does not form the primary tumor foci and is not suitable for the study of the tumor initiation stage. Each of the three methods has its own advantages and should be chosen reasonably according to the research focus.

4. Summary and Prospect

In this study, we systematically investigated the isolation and identification of T cell-derived exosomes and their functional effects on tumor cells, covering a variety of aspects such as cell culture, molecular labeling, functional validation and animal model construction. Firstly, the resuscitation and culture operation of tumor cells and immune cells was completed. Subsequently, exosomes were isolated by ultracentrifugation and analyzed to detect their particle size and potential. The results showed that the average particle size of exosomes was 150-300 nm, and the zeta potential was about -4.87 mV, which was consistent with the physical characteristics of typical exosomes. To further prove that the T cells successfully secreted exosomes, we performed Western Blotting experiments to detect the expression of the transmembrane protein CD63, which serves as one of the exosome markers, and the presence of a clear band of CD63 suggests successful isolation. For exosome function verification, we used cy5.5 to label the exosome membrane, co-incubated with tumor cells, and observed its localization by confocal microscopy. The images showed that the red fluorescent signals were distributed in close proximity to the DAPI-labeled nuclei, and some of the exosome signals were located in the cytoplasm, suggesting that T-cell-derived exosomes can efficiently bind to or enter tumor cells with potential tumor-regulating ability. In the apoptosis function experiment, we used the CCK8 method to detect tumor cell viability, and the results showed that cell proliferation in the exosome-treated group was significantly decreased. Especially when the concentration of exosomes was 50 μg/mL or more, the cell viability decreased by more than 30%.

For the animal experiment part, we constructed a variety of injection models, including subcutaneous injection, tail vein injection and intraperitoneal injection, and combined them with animal ethical training to ensure that the experiments complied with the 3R principles. There were some deficiencies in our experimental process, the lack of a control group in the experiment of Western blot could not visually compare the difference of CD63 protein expression between immune cells and other non-immune cells, in addition, in the CCK8 experiment, there was a problem of high cell density, which led to the phenomenon of contact inhibition and nutrient competition, which affected the significance of the difference between the groups, and in order to optimize the experimental conditions, we suggested that control the inoculation density of 96-well plate at 1×10^{3}-5×10^{3} cells/well, and ensure that the cells are evenly distributed with adequate coverage of culture medium.

This study focuses on whether T cell-derived exosomes can cause killing effects on tumor cells, which provides a new idea for tumor immunotherapy. In the future, the signaling pathway of tumor cell apoptosis induced by exosomes will be explored in depth; the targeting and therapeutic efficiency will be improved by surface modification or loading siRNA/drugs; and the distribution, uptake, and clearance pathways of exosomes in animals will be tracked by combining them with fluorescent or radiolabeling. This will make T cell exosomes an important part of precision medicine and bring new hope for tumor treatment.

References

[1] Ohnmacht, C., & Eyerich, S. (2022). Diversity of T Helper and Regulatory T Cells and Their Contribution to the Pathogenesis of Allergic Diseases. Handbook of experimental pharmacology, 268, 265–296.

[2] Théry, C., Ostrowski, M., & Segura, E. (2009). Membrane vesicles as conveyors of immune responses. Nature

reviews. Immunology, 9(8), 581–593.
[3] Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., & Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature cell biology, 9(6), 654–659.
[4] Zhang, L., & Yu, D. (2019). Exosomes in cancer development, metastasis, and immunity. Biochimica et biophysica acta. Reviews on cancer, 1871(2), 455–468.
[5] Agarwal, A., Fanelli, G., Letizia, M., Tung, S. L., Boardman, D., Lechler, R., Lombardi, G., & Smyth, L. A. (2014). Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation. Frontiers in immunology, 5, 555.
[6] Tang, X. J., Sun, X. Y., Huang, K. M., Zhang, L., Yang, Z. S., Zou, D. D., Wang, B., Warnock, G. L., Dai, L. J., & Luo, J. (2015). Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy. Oncotarget, 6(42), 44179–44190.
[7] Li, L., Jay, S. M., Wang, Y., Wu, S. W., & Xiao, Z. (2017). IL-12 stimulates CTLs to secrete exosomes capable of activating bystander CD8+ T cells. Scientific reports, 7(1), 13365.