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Isolation and Characterization of T Lymphocyte-Exosomes Using Mass Spectrometry

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Abstract

Exosomes are cell-derived vesicles that have been implicated in the pathogenesis of many inflammatory diseases. In the immune system, it has been shown that T lymphocyte-derived exosomes are able to induce diverse cellular responses. There are several methods to isolate and to characterize exosomes, each with their own advantages and disadvantages. Here, we describe a centrifugation approach, combined with mass spectrometry characterization, as a means to study exosomes derived from primary human T lymphocytes. This method is sensitive and therefore can be applied when a limited amount of sample is available.

Keywords

Centrifugation; Exosomes; Mass spectrometry; Signaling; T cell

1. Introduction

Similar to microvesicles and apoptotic bodies, exosomes are a type of Extra-cellular vesicle. The nomenclature of the different vesicle types depends on their size, morphology, function, and cell of origin. Exosomes show standard round morphology in cryo-electron microscopy, ranging in size between 30 and 100 nm [1]. They are secreted by nearly all types of cells and are widely distributed in various body fluids [2]. Unlike the larger microvesicles (size above 100 nm), exosomes are either released from the cells when multivesicular bodies fuse with the plasma membrane or discharged directly from the plasma membrane [3]. Since their discovery over 50 years ago, exosomes have been found to play a vital role in many biological processes, including intercellular communication, viral replication, development and differentiation of stem cells, cell signaling, and tissue regeneration [4]. It is well established that exosomes released by immune cells can modulate responses through antigen presentation and transfer of pro- or anti-inflammatory mediators. Exosomes also play a key role in the pathogenesis of asthma and rheumatoid arthritis, and have a potential role in cancer immuno-editing [5].

Exosomes contain a variety of bioactive molecules derived from their parent cells. These include nucleic acids involving DNA and RNA, proteins such as growth factors and cytokines, and lipid mediators. The functional impact of exosomes is conveyed by the molecular components that they carry [6]. The cargo of exosomes is not a result of a random process, but rather involves a complex sorting mechanism that favors specific biomolecules

over others [7, 8]. Although structural proteins are commonly found in large numbers in these vesicles regardless of their cellular origin [9], additional knowledge about the specific cargo of T cell-derived exosomes is crucial to better understand the function of the acquired immune responses. Thus, we have taken advantage of a proteomic approach to more accurately characterize the unique set of proteins that are found in T cell-derived exosomes. Our findings support a mechanistic role for exosomes in immune cell activation, and also imply that exosomes should be considered as a biomarker for the activity of inflammatory diseases [10].

There are various methods to isolate exosomes from biological fluids, including centrifugation, immunological separation, polymer-based precipitation, and filtration [11]. These methods have made the isolation process faster and easier; however, each method has its own strengths and weaknesses [11]. Differential centrifugation utilizes several centrifugation cycles of different centrifugal forces and durations to isolate exosomes based on their density and size differences from other components in a sample of cells, large vesicles, and debris. This method requires little technical expertise, is affordable over time (i.e., one ultracentrifuge machine for long-term use), and can be used to isolate exosomes from biological fluids and large volumes of media faster than other methods. For these reasons, ultracentrifugation-based techniques have become a rather popular option among researchers in exosome research. The main weakness of the method is its low efficiency when isolating exosomes from viscous fluids such as serums. Density gradient centrifugation combines ultracentrifugation with a sucrose density gradient to allow for the separation of low-density exosomes based on their size, mass, and density [12]. Immunoaffinity separation incorporates magnetic beads bound to specific antibodies that recognize exosome-specific surface markers [13]. This allows for the isolation of selective subsets of exosomes, but is limited by a small sample volume and high cost of reagents. Polymer-based precipitation involves mixing the samples with polymer-containing precipitation solutions, incubation, and centrifugation at low speeds [14–16]. The advantage of this method is the minimal harming effect of the process on the isolated exosomes. Difficulties include the precipitation of non-vesicular contaminants and a limited ability to perform downstream functional analysis. Finally, a filtration membrane is used to separate exosomes from other macromolecules while concentrating the exosomes into a small volume [17]. Limitations vary from the stickiness of the exosomes to the filtration membranes and subsequent loss of material. In addition, the use of force may result in the deformation and breakup of large vesicles, which may potentially skew the results of the downstream analysis [16].

The various technologies applied for exosome characterization include biophysical, molecular, and microfluidic methods. Biophysical methods use size range to characterize exosomes. These include optical particle tracking [18], photon correlation spectroscopy [19], resistive pulse sensing [20], atomic force microscopy [21], transmission electron method [22], and cryo-electron microscopy [23]. Molecular approaches, such as flow cytometry, are used for the characterization of exosome surface proteins. Moreover, flow cytometry allows for the measurement of both the size and the structure of the exosomes [24]. The microfluidic-based methodologies are used for the characterization of exosomes by binding exosomes to specific antibodies on microfluidic channels followed by the elution of bound

vesicles [25]. Several methods have been developed to analyze exosome RNA content, including microarray analysis, next-generation sequencing, and digital droplet PCR [26]. Exosome protein analysis can be done by western blotting and fluorescence-based cell sorting [27, 28].

In this chapter, we describe an alternative method of collecting and preparing exosomes from primary human T cells for proteomic analysis using high-resolution mass spectrometry. This approach can be easily modified to study vesicles derived from other type of cells.

2. Materials

Prepare all solutions using distilled water unless mentioned otherwise. Filter and sterilize all solutions through a 0.45 μm filter. Prepare all reagents in a sterile hood. Store all reagents at 4 °C unless mentioned otherwise. Keep all solutions and reagents sterile and diligently follow waste disposal regulation.

2.1 Human T-Cell *Isolation*

1. A whole-blood sample (e.g., Leukopacks).
2. Sterile phosphate-buffered saline (PBS).
3. Human CD3 T-cell enrichment cocktail.
4. Multiple 50 mL polypropylene tubes.
5. Lymphoprep.
6. Roswell Park Memorial Institute (RPMI) enriched cell culture medium 1640, containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 1% HEPES with 100 IU IL-2.
7. T75 tissue culture flasks.

2.2 Preparation of Magnetic Beads for Cell Stimulation

1. Dynabeads M-270 Epoxy antibody coupling kit.
2. DynaMag-2 magnet.
3. Analytic weight.
4. Mixer allowing rotation or tilting of tubes.
5. Anti-CD3 antibody.
6. Anti-CD28 antibody.
7. Anti-human IgG isotype antibody.

2.3 T-Cell Activation

1. Bovine serum albumin (BSA).
2. PBS with 0.1% BSA.

3. Exosome-free RPMI enriched media 1640, containing 10%exosome-free fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 1% HEPES with 100 IU IL-2

2.4 Isolation of Exosomes

1. Refrigerated centrifuge running at 10,000 × g.
2. Ultracentrifuge.
3. Beckman Ti84 rotor.
4. Beckman centrifuge tubes.
5. Sterile PBS.
6. Mass spectrometry collection buffer (8 M urea in 100 mM Tris–HCl at pH 8.5).
7. Mass spectrometry sample vials with snap lid.

2.5 Mass Spectrometry In-Solution Digestion

1. 1 mM Dithiothreitol (DTT) in 100 mM ammonium bicarbonate.
2. 50 mM Iodoacetamide (IAA) in 100 mM ammonium bicarbonate (pH 8).
3. 50 mM Ammonium bicarbonate.
4. Trypsin/Lys-C mix (MS grade).
5. Sequencing-grade modified trypsin.
6. 0.1% Trifluoroacetic acid (TFA) (v/v) in water (LC–MS grade).
7. 0.1% Formic acid (v/v) in water (LC–MS grade).
8. Buffer A (0.1% formic acid).
9. Buffer B (80% acetonitrile, LC–MS grade/0.1% formic acid).
10. C18-StageTip column (3 M).
11. 75 μm i.d. × 50 cm long EASY-spray PepMap columns.
12. Speed-vac vacuum concentrators.
13. Acetonitrile solution (2% acetonitrile/0.1% TFA).

2.6 Liquid Chromatography (LC)–Mass Spectrometry (MS) Analysis

1. EASY-nLC1000 HPLC coupled to the Q-Exactive (QE).
2. 75 μm i.d. × 50 cm long EASY-spray PepMap columns.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. All procedures should be done under sterile conditions unless otherwise specified.

3.1 Isolation of Human Peripheral Blood T Lymphocytes

1. Obtain healthy donor human blood (Leukopacks). Allow the blood to reach room temperature before proceeding to the next step.
2. Split the blood into four sterile tubes (50 mL tubes), 25 mL of blood in each tube.
3. Add RosetteSep human CD3 T-cell enrichment cocktail to the tubes. To each tube of 25 mL of blood, add 1250 μL of RosetteSep cocktail (50 μL of RosetteSep cocktail per 1 mL of blood).
4. Mix the blood using a 25 mL sterile pipette and let it rest for20 min at room temperature allowing the antibodies in the cocktail to bind to the cells.
5. Dilute the blood samples with an equal volume of PBS at room temperature. Add 25 mL of sterile PBS to each of the tubes containing 25 mL blood.
6. Prepare eight 50 mL tubes and add 20 mL of a density gradient medium (Lymphoprep) to each tube. Procedure is done at room temperature in a sterile hood. The volume of the density gradient medium can be changed based on the specific requirements. Every 20 mL of density gradient medium can be layered by up to 25 mL of diluted blood. Calculate the number of tubes required based on the amount of blood needed for the T-cell isolation.
7. Layer the diluted blood samples on the density gradient medium, being careful to minimize their mixing. For each density gradient medium tube, layer not more than 25 mL diluted blood.
8. Centrifuge the tubes at a speed of 1200 × *g*, at room temperature, for 20 min with the “brake off.”
9. Harvest the enriched cell layer with a plastic pipette and transfer it to a clean sterile 50 mL collection tube. Add all the cells harvested into the same 50 mL collection tube.
10. Wash the enriched cells. Top up the tube with sterile PBS.
11. Centrifuge at 400 × *g*, room temperature, for 10 min, “brake off” low.
12. Discard the supernatant by gentle aspiration. Be careful not to disrupt the cell pellet.
13. Repeat the wash one more time (steps 10–12).
14. Resuspend cells in 10 mL of sterile PBS.
15. Count cells using a hemocytometer. Add 15 μL of cell suspension to the hemocytometer chamber and cover the glass. Count the number of cells in all four outer squares and divide this number by four (the mean number of cells/square). The number of cells per square 10^4 = the number of cells in 1 mL of suspension (*see* Note 1).

16. Primary Tcells can be maintained at a concentration of 1×10^6 cells per 1 mL of RPMI enriched media at 5% CO_2 at 37 °C with 100 IU of IL-2 for up to a period of 1 week.

3.2 Preparation of Magnetic Beads

Coat the magnetic beads with anti-CD3 and anti-CD28 antibodies. Use 2 μg of the anti-CD3 (0.5 mg/mL) and 2 μg of the anti-CD28 (0.5 mg/mL) antibodies to couple 1 mg of magnetic beads. As a negative control, coat another set of beads (1 mg) with 4 μg of an anti-human IgG isotype antibody (10 mg/mL) (*see* Notes 2 and 3) Disinfect the magnets to prevent sample contamination.

1. Weigh out 3 mg of magnetic beads. Weight can be adjusted based on the total amount of desired reagents.
2. Wash the beads with 1 mL of C1 buffer (provided with the kit) and mix by pipetting up and down.
3. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Gently remove the supernatant.
4. Add the appropriate volume of antibody to the C1 buffer to reach a volume of 150 μL.
5. For example, to couple 3 mg of beads, the required quantity of antibodies is 12 μL of anti-CD3 and 12 μL of anti-CD28 to be added to 126 μL of C1 buffer. For the control beads, the anti-human IgG isotype should be diluted 1:10 (final concetntration 1.0 mg/mL) before coupling 3 mg of beads with 12 μL of diluted anti-human IgG isotype and 138 μL of C1 buffer.
6. Add 150 μL of C2 buffer (provided with the kit) and mix by gently pipetting up and down.
7. Incubate on a roller at 37 °C overnight. Adjust the speed of the roller to ensure thorough mixing and to avoid the beads from settling.
8. Place the tubes on the magnet for 1 min and allow the beads to collect on the tube wall. Remove the supernatant.
9. Add 800 μL of HB wash buffer (provided with the kit) and mix by pipetting up and down.
10. Place the tubes on a magnet for 1 min and allow the beads to collect on the tube wall. Remove the supernatant.

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1. For an accurate determination of the total number of cells, the cell count should be between 20 and 50 cells per one single square of the hemocytometer. If the cell density is over 100 cells per square, the cell suspension should be diluted.
2. The choice of antibody or ligand is the most important factor for successful target capture. Not all antibodies are suitable for functional assays. The coupling of antibodies or other proteins stabilized in glycerol is not recommended. The presence of antibody aggregates in the antibody stock used for coupling can result in antibody leakage during the downstream assay. To help reduce this, we recommend removing the antibody aggregates from the antibody stock by centrifugation at 16,000 × g for 10 min at 4 C. The moisture on the unused beads deactivates the reactive groups necessary for covalent antibody coupling. To avoid condensation on unused beads, ensure that the beads are at room temperature prior to opening the bottle.
3. Dynabeads-coupled antibodies can be stored for up to 2 weeks at 4 °C or may be made fresh a day before usage.

11. Add the 800 μL of LB wash buffer (provided with the kit) and mix by pipetting up and down.
12. Place the tubes on the magnet for 1 min and allow the beads to collect on the tube wall. Remove the supernatant.
13. Add the 800 μL of SB wash buffer (provided with the kit) and mix by pipetting up and down.
14. Place the tube on a magnet for 1 min and allow the beads to collect on the tube wall. Remove the supernatant.
15. Repeat the wash described in step 13 once more.
16. Add the 800 μL of SB wash buffer and mix by pipetting up and down.
17. Incubate on a roller at room temperature for 15 min.
18. Place the tube on a magnet for 1 min and allow the beads to collect on the tube wall. Remove the supernatant.
19. Resuspend antibody-coupled beads in 300 μL SB buffer and store at 4 °C until use. The final bead concentration is 10 mg/ mL.

3.3 The Activation of Human Peripheral Blood T Lymphocytes (See Note 4)

1. Wash coated beads for 5 min in PBS with 0.1% BSA.
2. Prepare an exosome-free enriched RPMI medium (see Note 5).
3. Count primary human T cells using a hemocytometer, as described above. Use 80 106 T cells per condition.
4. Cells should be pelleted by centrifugation at 400 × g, at room temperature for 5 min. Replace the media with 20 mL of fresh exosome-free enriched RPMI medium.
5. Add anti-CD3/CD28 or IgG control-coated magnetic beads to the cells in a 1:2 ratio (number of beads to number of cells). Mix the cells with the beads by pipetting up and down.
6. Spin down the cells with the antibody-coupled beads, at a speed of 400 × g, at room temperature for 1 min.
7. Transfer the cells with the beads using 10 mL sterile pipette to aT75 flask.
8. Incubate the cells with the beads for 18 h at 5% CO2 at 37 C.

[4]It is preferred to perform T-cell activation as soon as possible after the isolations, and no more than a day after T-cell isolation.
[5]The enriched RPMI medium should be warmed up to 37 °C before use.

3.4 Isolation of Exosomes (See Note 6)

1. Collect media from 80 × 10^6 resting, stimulated (with beads coated with anti-CD3/CD28 antibodies), and controlled (beads coated with IgG isotype control antibody) primary human T cells (*see* Note 7)
2. Centrifuge supernatants at 800 × *g* at 4 °C for 5 min.
3. Collect supernatants and recentrifuge at 3200 × *g* at 4 °C for 10 min to discard dead cells.
4. Collect supernatants and recentrifuge at 10,000 × *g* at 4 °C for 30 min to discard cell debris.
5. Supernatants should be ultracentrifuged at 100,000 × *g* at 4 °C for 60 min (*see* Notes 8 and 9).
6. Discard supernatant by using 1 mL pipette tips (*see* Notes 8 and 9 below) and wash with 1 mL sterile PBS (*see* Notes 10 and 11).
7. Ultracentrifuge at 100,000 × *g* at 4 °C for 60 min.
8. Discard supernatant by pouring the supernatant or use 1 mL pipette tips (*see* Note 12).
9. Resuspend and collect exosomes using 50 μL MS collection buffer (*see* Note 13). Transfer the samples to MS sample vials (*see* Note 14). Let samples rest at room temperature for 30 min.

3.5 In-Solution Digestion

More details about this section are found at doi.org/10.1074/mcp.RA119.001362 [29]

1. Reduce the samples with 1 mM dithiothreitol (DTT) at room temperature for 30 min.
2. Alkylate samples with 5 mM iodoacetamide (IAA) for 30 min in the dark (*see* Note 15).

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6. All centrifugations for exosome isolation and purification should be performed at 4 C. Ensure that none of the pellet is collected and contaminates the supernatant. Use a pipette (rather than pouring off the supernatant), and leave behind half a centimeter of liquid above the pellet.
7. Overall, the contamination of isolated exosomes with other particles should be avoided. Exosomes are to be isolated from cultured media; an important consideration is to use either serum-free media or exosome-free fetal bovine serum.
8. Tubes should be weighed with samples to ensure that weight distribution is equal during centrifugation. It is crucial to properly balance ultracentrifuge tubes.
9. For ultracentrifugation, mark each ultracentrifuge tube with a waterproof marker and orient the tube in the rotor with the mark facing up. The mark is a reference for the location of a pellet following centrifugation.
10. After the first ultracentrifugation the exosome pellet will not be visible. For swinging-bucket rotors, the pellet is at the bottom of the tube. For fixed-angle rotors, the pellet is on the side of the tube near the base. When removing the supernatant with the pipette, hold the tube at an angle, so that the pellet is always covered with supernatant, and leave 2 mm of supernatant above the pellet.
11. Exosome sterility is not necessary in cases of biochemical analyses. The use of filtered and sterile PBS is recommended to ensure that no contaminants are included during preparation and solubilization.
12. For a complete supernatant removal following an ultracentrifugation wash, for fixed-angle rotors, pour the supernatant rather than using a pipette. For swinging-bucket rotors, remove the supernatant with the pipette.
13. To characterize Extra-cellular vesicles as exosomes, it is important to demonstrate the expression of common exosome proteins using immunoblotting. The commonly used markers are CD63 (tetraspanin CD63), CD81 (tetraspanin CD81), TSG101 (tumor susceptibility gene 101), Alix (PDCD6IP, programmed cell death 6 interacting protein), and HSP70 (heat-shock protein 70).
14. MS sample vials should be low binding.

3. Dilute the samples fourfold with 50 mM ammonium bicarbonate.
4. Digest lysates with trypsin/Lys-C mix (1:100 for the enzyme-to-protein ratio) and sequencing-grade modified trypsin (1:50 enzyme-to-protein ratio) at 37 °C overnight.
5. Acidified resulting peptides with trifluoroacetic acid (TFA) and force the acidified peptide sample through the C18-StageTip column (3 M Empore). Separate peptides on 75 μm i. d. 50 cm long EASY-spray PepMap columns packed with 2 μm C18 material with 100 A pore size.
6. Wash the column with buffer A. Elute the peptides from theC18 material using 20–30 μL buffer with a gradient of 7–28% buffer B, at a flow rate of 300 nL/min, over a gradient of 210 min. Elute directly into a microfuge tube or autosampler plate.
7. Carefully dry samples in the speed-vac without heating, until all acetonitrile has evaporated (2–3 μL of final volume) (*see* Note 16).
8. Resuspend the dried peptides in 2% acetonitrile/0.1% TFA.
9. Samples can be stored for a short term at 4 °C in a refrigerator, or for a long term at 80 °C in a freezer.

3.6 Liquid Chromatography (LC), Mass Spectrometry (MS), and Mass Spectrometry (MS) Analysis (See Note 17)

1. Analyze peptides by LC using the EASY-nLC1000 HPLC-coupled to the Q-Exactive (QE) Plus or Q-Exactive HF mass spectrometers.
2. Perform MS acquisition in a data-dependent manner, with a positive-ion mode and selection of the top ten peptides from each MS spectrum for fragmentation and MS/MS analysis.
3. Acquire the full MS spectra at a resolution of 70,000 (QE-Plus) or 60,000 (QE-HF), *m/z* range of 300–1800 Th, with AGC target of 3E+06 ions and maximal injection time of 20 ms (QE-Plus) or 100 ms (QE-HF).
4. Peptides were isolated for fragmentation with an isolation window of 1.6 *m/z*. Perform higher energy collisional dissociation (HCD) fragmentation with normalized collisional energy of (NCE) 25 (QE-Plus) or 27 (QE-HF).
5. Acquire the MS/MS spectra at a resolution of 17,500 (QE-Plus) or 30,000 (QE-HF), with AGC target of 1E+05 (QE-Plus) or 5E+04 (QE-HF) and a maximal injection time of 60 ms (QE-Plus) or 50 ms (QE-HF). Set the dynamic exclusion to 30 s.

15. IAA solution pH should be exactly 8.0.
16. When drying samples in the speed-vac to evaporate acetonitrile, do not completely dehydrate/overdry the peptide sample.
17. The experiment should be carried out with at least three biological replicates of each treatment. Biological replicates are essential and can be easily obtained by different blood donors.

3.7 Database Searching and Protein Identification

1. Analyze the raw MS files in the MaxQuant software (version 1.5.2.18) and the Andromeda search engine.
2. MS/MS spectra are searched against the UniProt database(UniProt, http://www.uniprot.org) [30], a decoy, reverse database of the same size, and a list of common contaminants.
3. Searching parameters include fixed modification: carbamidomethyl-cysteine, and variable modifications: N-terminal acetylation and methionine oxidation.
4. MaxQuant search parameters for the initial mass recalibration of the precursors were 20 ppm, and in the main search, the mass tolerance for precursor and fragment ions was 4.5 ppm and 20 ppm, respectively.

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