



Isolation and Functional Aspects of Eosinophil-Derived Exosomes

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Abstract

Eosinophils are granulocytes involved in inflammatory processes related to type 2 immune responses as allergy and parasitic infestation. Eosinophils are scarce in homeostatic situations, comprising 3–6% of total granulocytes. In peripheral blood, they have a life span of 18–25 h. These cells are characterized by the presence of several types of granules that are very important because of their functions. Recently, we have described that these immune cells are able to release exosomes, which are nanovesicles involved in intercellular communication and implicated in development of several diseases such as cancer, cardiovascular diseases, and asthma. Here, we describe a technique for isolation of eosinophil-derived exosomes from human peripheral blood and for their utilization in cell functional assays.

Key words Eosinophils, Exosomes, Isolation, Cell functional assays

1 Introduction

Eosinophils are bone marrow-derived leukocytes characterized by having multiple granules inside them. They are in a low percentage in peripheral blood (1–5%) and comprise 3–6% from total granulocytes [1]. Traditionally they have been associated with allergy and defense processes against helminthic parasites, but they can be involved in other functions [2]. These cells are generated in the bone marrow and their maturation is mediated, essentially, by three cytokines: interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Eosinophil recruitment to peripheral blood and tissues is mediated by several chemokines like eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26). The eosinophil contains a great number of receptors such as adhesion molecules, integrins, and chemokine receptors. Some of these surface markers are useful for eosinophil identification; thus CCR3, SIGLEC8, CD49d, and CD9 are usually used for eosinophil characterization by flow cytometry [3].

In healthy states eosinophil life span in peripheral blood is 18–25 h, but in pathological processes eosinophils can augment their survival and they can be activated releasing their molecular arsenal stored inside of their cytoplasmic granules (cytokines, chemokines, growth factors, inflammatory lipid mediators and cytotoxic cationic proteins such as eosinophil cationic protein [ECP], major basic protein [MBP], eosinophil peroxidase [EPO], and eosinophil-derived neurotoxin [EDN]) [4]. Among the multiple cytoplasmic granules of eosinophils are the multivesicular bodies (MVB), which are exosome precursors [5].

Exosomes are small extracellular lipid vesicles (nanovesicles), with a diameter of 30–100 nm, containing several molecules (proteins, lipids, and nucleic acids) and playing an important role in intercellular communication. They are released to the extracellular microenvironment by several cell types (lymphocytes, dendritic cells, mast cells, epithelial cells, eosinophils, and others) and they can be taken up by both neighbor and remote cells, modifying their functions. Exosome biogenesis starts with early endosome formation that matures toward late endosome. In this process multiple intraluminal vesicles (ILV) are formed and late endosome is now called MVB. This MVB goes to the cellular membrane, fusing with it and releasing exosomes to the extracellular medium [6]. Exosomes contain specific proteins that characterize them such as tetraspanins (CD63 and CD9), ALIX, and Tsg101, which are useful as exosome markers [7]. Exosomes from eosinophils have been demonstrated to have functional effects on several cell types: eosinophils themselves, bronchial smooth muscle cells, and small airway epithelial cells [8, 9].

The aim of this chapter is to present a method to isolate eosinophil-derived exosomes by ultracentrifugation without commercial kit utilization. Also, we show how to quantify them and their use in functional experiments.

2 Materials

All material, reagents, and equipment must be germ free and a sterile manipulation is mandatory for the complete protocol. All reagents must be stored at 4 °C and brought to room temperature (RT) at least 30 min before starting the protocol. All reagents should be diluted or reconstituted with bi-distilled water or 1× phosphate-buffered saline (PBS), according to each reagent's requirements.

2.1 Eosinophil Purification from Human Peripheral Blood

1. Ethylenediaminetetraacetic acid (EDTA)-anticoagulant tubes.
2. 0.9% Sodium chloride solution.
3. 100 mL Sterile urine container.
4. 10 mL and 25 mL serological sterile pipettes.

5. Lymphoprep™ (lymphocyte isolation solution).
6. Erythrocyte lysing solution: 155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.5.
7. Sterile exosome-free 1× PBS/2% heat-inactivated fetal bovine serum (iFBS)/1 mM EDTA.
8. EasySep™ Human Eosinophil Isolation Kit (STEMCELL Technologies Inc., Vancouver, Canada): Negative selection antibody cocktail (isolation cocktail) and dextran magnetic beads (RapidSpheres™).
9. EasySep™ magnet (STEMCELL Technologies Inc.).
10. Polystyrene round-bottom tubes of 5 mL (12 × 75 mm style).
11. Sterile 15 mL and 50 mL centrifuge conical bottom tubes.
12. Neubauer and Fuchs-Rosenthal cell-counting chambers.
13. Trypan blue staining reagent.
14. Basophil staining reagent: 0.05% Toluidine blue, 0.85% sodium chloride, 95% ethanol, and saturated saponin.
15. Optional: Antibodies anti-human SIGLEC8, CD16 and CD45 conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein complex (PerCP) fluorochromes, respectively.

2.2 Eosinophil Culture

1. Sterile 24-well cell culture plate.
2. iFBS-exosome free: iFBS must be ultracentrifuged at $100,000 \times g$ for 1 h at 4 °C. Supernatant is collected and filtered through 0.22 µm pore size filters in sterile conditions.
3. Exosome-free RPMI-1640 complete medium: RPMI-1640 without phenol red supplemented with 10% iFBS-exosome free, 0.1 nmol/L nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 nmol/L L-glutamine.
4. Human recombinant IL-5 (5 ng/mL), human recombinant GM-CSF (10 ng/mL), and human recombinant interferon gamma (IFN-γ) (20 ng/mL).
5. Cell incubator.

2.3 Exosome Isolation

1. Centrifuge.
2. Ultracentrifuge and ultracentrifuge tubes.
3. 1.5 mL Centrifuge tubes.
4. 1× PBS filtered.
5. Exosome solution: Filtered 1× PBS, 5 mM EDTA.
6. NanoSight LM10 (Malvern Instruments Ltd., Malvern, UK).

2.4 Functional Assays

1. Exosomes purified and suspended in $1\times$ PBS filtered, 5 mM EDTA.
2. Syringe with 29-gauge 1 cc 1/2-inch features.
3. Desired cell types: For example, eosinophils, small airway epithelial cells, and bronchial smooth muscle cells.
4. Appropriated sterile 6-well, 24-well cell culture plates.
5. PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).
6. Exosome Spin Column (MW 3000) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

3 Methods

A summary of complete protocol is shown as a flowchart in Fig. 1. All procedures must be performed in a sterile culture room or in a flow laminar hood.

3.1 Eosinophil Isolation

Eosinophil purification will be performed according to two-step procedure described by Lopez et al. [10]:

1. Collect 50 mL of human peripheral in EDTA-anticoagulant tubes and invert tubes with blood.
2. Measure total blood volume, put it in a 100 mL sterile urine container, and add 0.9% sodium chloride solution in a ratio of 1:1 (e.g., adding 50 mL of 0.9% sodium chloride to 50 mL of peripheral blood).
3. In a 50 mL centrifuge conical bottom tube, 15 mL of Lymphoprep™ will be added and above them 25 mL of diluted blood with a 10 mL serological sterile pipette will be added slowly, ensuring that both fluids do not mix (blood:Lymphoprep™ ratio must be 5:3).
4. Centrifuge at 22 °C, $350 \times g$, for 30 min (without brake or acceleration).
5. After centrifugation, remove plasma, mononuclear cells, and Lymphoprep™ layers. Only the bottom layer (granulocyte and erythrocyte layer) is required. Aspirate the granulocyte layer with a clean 10 mL serological pipette and transfer it to a new clean 50 mL centrifuge conical bottom tube.
6. Cells are washed with 0.9% sodium chloride. Centrifuge at 22 °C, $600 \times g$, for 10 min.
7. Remove the supernatant with a clean 25 mL serological pipette and add 40 mL of erythrocyte lysing solution. Keep it on ice for 10 min and then centrifuge at 22 °C, $600 \times g$, for 10 min.

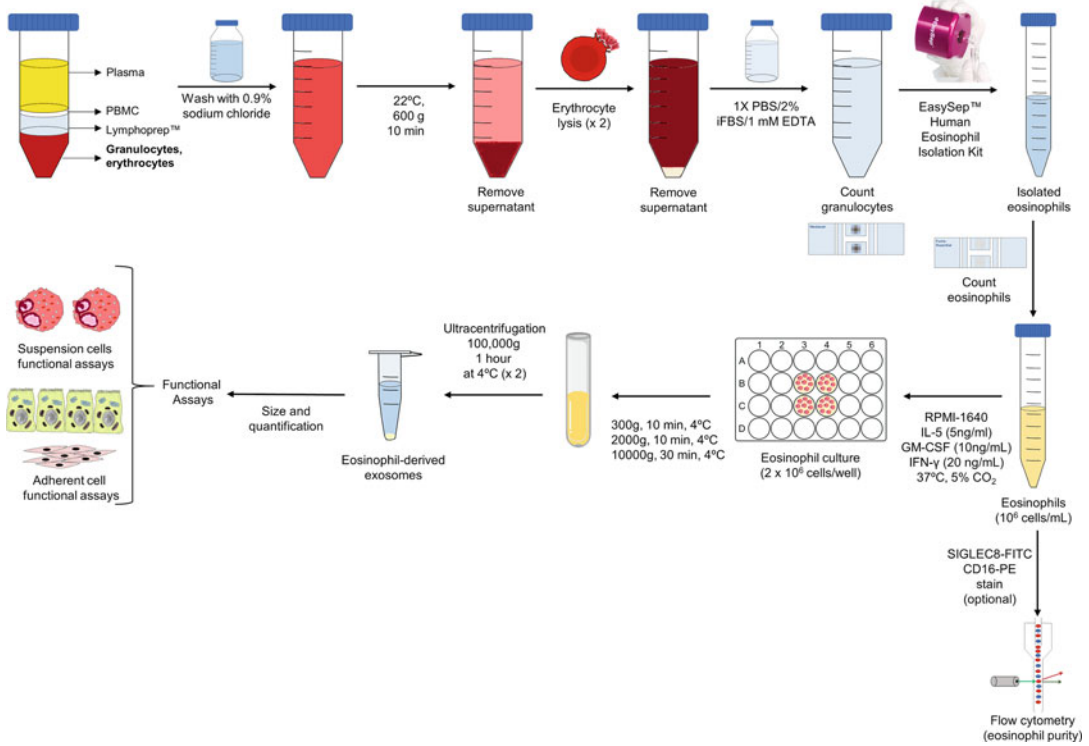


Fig. 1 Flowchart of the optimal protocol to isolate eosinophil-derived exosomes from human peripheral blood. PBMC, peripheral blood mononuclear cells

8. Discard the supernatant and repeat step 7.
9. After removing the supernatant, cell pellet will be homogenized in 50 mL of sterile exosome-free 1× PBS/2% iFBS/1 mM EDTA.
10. Granulocyte counting is performed using a Neubauer cell-counting chamber after trypan blue stain (trypan blue:cell suspension ratio should be 1:1). Determine the total number of living cells.
11. Centrifuge at 22 °C, 600 × *g*, for 10 min. Remove and discard the supernatant.
16. Suspend the granulocyte pellet in 1× PBS/2% iFBS/1 mM EDTA, according to 1 mL of 1× PBS/2% iFBS/1 mM EDTA per 5 × 10⁷ granulocytes. Transfer the suspension to a sterile 5 mL (12 × 75 mm) polystyrene round-bottom tube (maximum 2 mL of cell suspension per tube).
12. Add 50 µL of isolation cocktail (from EasySep™ Human Eosinophil Isolation Kit) per 1 mL cell suspension. Mix and incubate at RT for 5 min.

13. Add 50 μL of RapiDspheres™ (previously they must be mixed by vortexing for 30 s) to the sample and mix by pipetting up and down 4–5 times. No incubation is required; immediately proceed to the next step.
14. Add 1 $\times$ PBS/2% iFBS/1 mM EDTA to top up the sample to 2.5 mL. Mix gently by pipetting up and down 4–5 times.
15. Place the tube into the EasySep™ magnet and incubate at RT for 3 min.
16. Invert the magnet and tube, in one continuous motion, in a new polystyrene tube. Caution: Leave the magnet and tube inverted for 2–3 s, and then return upright. Do not shake or blot off any drops.
17. Repeat steps 14 and 15.
18. Again, invert the magnet and tube, in one continuous motion, in a new sterile 15 mL centrifuge conical bottom tube. Caution: Leave the magnet and tube inverted for 2–3 s, and then return upright. Do not shake or blot off any drops.
19. Count eosinophils in a Fuchs-Rosenthal cell counting chamber, by previous staining with basophil stain reagent (eosinophil suspension:basophil staining reagent ratio should be 1:10).
20. Add 1 $\times$ PBS/2% iFBS/1 mM EDTA to top up the sample to 15 mL. Centrifuge at 22 °C, 600 $\times g$, for 10 min. Remove and discard the supernatant.
21. Optional: Eosinophil purity can be confirmed through flow cytometry, staining 1×10^5 cells with SIGLEC8-FITC, CD16-PE, and CD45-PerCP (eosinophils will be shown as FSC-A^{high}/SSC^{high} and SIGLEC8⁺/CD16⁻/CD45⁺) (see **Note 1**).

3.2 Eosinophil Culture

1. Suspend the eosinophil pellet in exosome-free RPMI-1640 complete medium (without phenol red), according to 1 mL medium per 1×10^6 eosinophils.
2. Plate 2×10^6 eosinophils per well and add recombinant human IL-5 and GM-CSF (final concentration 5 ng/mL and 10 ng/mL, respectively)
3. Place eosinophil culture in a humidified 5% CO₂ incubator at 37 °C and incubate overnight (16–24 h).
4. After culture time, add to cells recombinant human IFN- γ (final concentration 20 ng/mL) for 10 min to stimulate exosome secretion.

3.3 Exosome Purification

1. Collect the cell culture supernatant and add 1 mL per 1.5 mL tube.
2. Centrifuge at $300 \times g$ for 10 min at 4 °C to pellet cells and bulky debris. Transfer supernatant to a new 1.5 mL tube.
3. Centrifuge at $2000 \times g$ for 10 min at 4 °C to pellet cell debris (e.g., apoptotic bodies). Transfer supernatant to a new 1.5 mL tube.
4. Centrifuge at $10,000 \times g$ for 30 min at 4 °C to pellet both large extracellular vesicle and protein aggregates. Transfer supernatant to an ultracentrifuge tube (maximum 2 mL of supernatant per tube).
5. Ultracentrifuge the supernatant at $100,000 \times g$ for 1 h at 4 °C.
6. Carefully discard the supernatant by aspiration with a pipette tip and homogenize the pellet in 1 mL of exosome-free $1 \times$ PBS to wash exosomes.
7. Ultracentrifuge the resuspended exosome pellet at $100,000 \times g$ for 1 h at 4 °C.
8. Carefully discard supernatant by aspirating with a pipette tip and dilute pellet in 100 μ L of sterile exosome-free $1 \times$ PBS/5 mM EDTA to collect exosomes. Exosome pellet from several tubes can be put together to obtain in the same volume (100 μ L) all exosomes released by 4×10^6 eosinophils.
9. Exosomes are ready for use (*see Note 2*).

3.4 Exosome Analyses

3.4.1 Exosome Size Determination and Quantification

Determination of vesicle size distribution is crucial to confirm that these extracellular vesicles are exosomes. Also, quantification is necessary to determine optimal concentration to use in functional assays.

1. Measurements will be performed in a Nanosight LM10 instrument (Nanosight Ltd), based on a nanoparticle tracking analysis.
2. Dilute an exosome aliquot with filtered exosome-free $1 \times$ PBS 1:100 in a final volume of 500 μ L (5 μ L exosomes to 495 μ L exosome-free filtered $1 \times$ PBS).
3. With a 29-gauge 1 cc 1/2-inch syringe mix up and down 4–5 times to disperse possible exosome aggregates.
4. Place the exosome dilution in a quartz chamber with a syringe. Take caution of not introducing any bubbles.
5. Measure the samples. This technology is based on light scattering and Brownian movement properties to obtain concentration and size distribution measurements. Several repetitions of the same samples will be performed to obtain a precise size and concentration measurement.

3.4.2 Eosinophil-Derived Exosome Staining

Exosomes can be stained with a fluorescent lipophilic probe PKH67, to be used in cellular uptake experiments (using PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling, Sigma-Aldrich, Merck KGaA):

1. Add to an exosome aliquot (20 μ L) 1 $\times$ PBS/EDTA 5 mM until it reaches 100 μ L of final volume (80 μ L).
2. Prepare PKH67 probe at 4 μ M final concentration (dilute 4 μ L of PKH67 probe in 1 mL of diluent C).
3. Add 100 μ L of PKH67 (4 μ M final concentration) to 100 μ L of exosome dilution. Homogenate with a pipette tip (mix up and down several times).
4. Incubate for 5 min at RT, mixing carefully each minute.
5. Add 200 μ L sterile 1 $\times$ PBS/5% bovine serum albumin (BSA) to stop the reaction. Incubate for 1 min at RT.
6. Add 100 μ L of marked PKH67 exosome solution into an Exosome Spin Column (MW 3000) (Invitrogen, Thermo Fisher Scientific), to remove BSA excess. Centrifuge at $750 \times g$ for 2 min at RT. Repeat this step the necessary times to seep complete sample volume.
7. Stained exosomes will be collected in the eluted fraction and they can be stored at 4 °C in darkness up to utilization.

3.4.3 Suspension Cell Functional Assays

Eosinophil-derived exosomes can be used over suspension cell culture, e.g., eosinophils, to determine the functional effects on these cells (*see Note 3*):

1. After cell suspension culture (each experiment requires specific conditions), plate $1\text{--}5 \times 10^5$ cells/well in 24-well plates.
2. Add different quantities of eosinophil-derived exosomes. It is a strongly recommended step, because it is necessary to perform a dose-effect curve to know the optimal exosome concentration. The following concentrations are recommended: $1 \times 10^6\text{--}10^7$ exosomes/mL.
3. Exosomes will be maintained with cells during different times: 90 min, 2 h, or 24 h is the recommended time. However, each assay is different, so it is necessary to determine in each experiment the optimal time of cell and exosome co-culture.
4. For flow cytometry experiments, collect the cells from culture and centrifuge them at $300 \times g$ for 2 min at RT. Discard the supernatant and wash the cells with exosome-free 1 $\times$ PBS 500 μ L and centrifuge them at $300 \times g$ for 2 min at RT. Discard the supernatant and proceed to stain protocol (flow cytometry).

5. Also, cells and supernatant can be collected from wells and centrifuge them at maximum speed for 2 min at RT. Aspirate the supernatant and store it at -80°C . The cells can be used for RNA and protein isolation adding 1 mL of Trizol and storing them at -80°C .
6. For other experiments (adhesion, chemotaxis) cells can be used directly from cell culture.

3.4.4 Adherent Cell Functional Assays

Eosinophil-derived exosomes can be used over adherent cell culture, e.g., structural lung cells such as bronchial smooth muscle cells and small airway epithelial cells, to determine exosome functional effects on these cells (*see Note 4*):

1. Plate structural cells in an appropriate concentration ($2500\text{--}3500\text{ cells/cm}^2$) in 6-well or 24-well plates (depending on the experiment) and allow them to reach 80–90% of confluency. Then, they are ready for experiments.
2. Add different quantities of eosinophil-derived exosomes. It is a strongly recommended step, because it is necessary to perform a dose-effect curve to know the optimal exosome concentration. The following exosome concentrations are recommended: $1 \times 10^6\text{--}10^7$ exosomes/mL.
3. Exosomes will be maintained with cells during different times: 2 h, 24 h, or 48 h is the recommended time. However, each assay is different, so it is necessary to validate in each experiment the optimal time to maintain cells with exosomes.
4. If the supernatant is required to measure molecules released by cells (cytokines, chemokines, and others), collect it by aspirating directly from wells and centrifuge it at a maximum speed for 2 min at 4°C to remove any cells or cellular debris. Store at -80°C until use.
5. For other experiments (apoptosis, surface marker determination by flow cytometry, RNA and protein extraction, etc.), remove supernatant and proceed to cell dissociation using trypsin. Centrifuge cells at $300 \times g$ for 5 min at RT and discard supernatant. Wash cells with 1 mL $1 \times \text{PBS}$ and centrifuge them at $300 \times g$ for 5 min at RT. Cells are then ready for the staining procedure prior to analysis by flow cytometry. Also, Trizol or RIPA buffer can be added to cells in order to extract RNA and proteins.
6. For wound-healing assays, TUNEL assays, proliferation assays (using Cell Proliferation Biotrak ELISA, Amersham, GE Healthcare, Chicago, IL, USA), and exosome uptake, cells can be used directly after co-culture with exosomes.

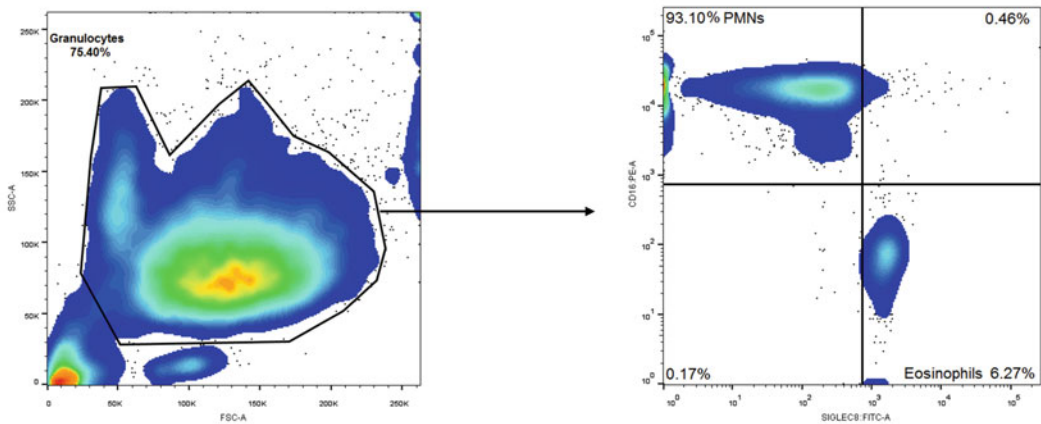
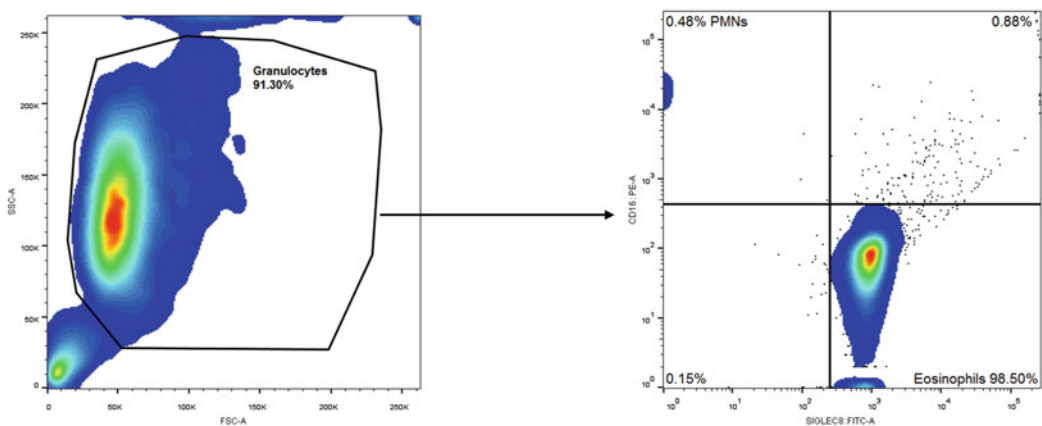
A**B**

Fig. 2 Gating strategy of flow cytometry for eosinophil visualization. **(a)** Cell populations before negative immunoselection of eosinophils. Granulocytes are gated according to unique forward scatter (FSC) and side scatter (SSC) characteristics for distinguishing them from other cell populations (remaining mononuclear cells and red blood cells). Eosinophil population can be differentiated by SIGLEC8-FITC and CD16-PE staining. Polymorphonuclear cells (PMNs), mainly neutrophils, are SIGLEC8-FITC⁻/CD16-PE⁺ (93.10%) and eosinophils are SIGLEC8-FITC⁺/CD16-PE⁻ (6.27%). **(b)** Cell populations after negative immunoselection. Eosinophils are identified as SIGLEC8-FITC⁺/CD16-PE⁻. High purity of eosinophils (98.50%) was achieved after purification with EasySep™ Human Eosinophil Isolation Kit

4 Notes

1. Isolation kit provides up to 99% eosinophil purity; however it is strongly recommended to confirm real purity and efficiency of the isolation method. Figure 2 shows values routinely obtained with this method.
2. Exosomes could be used directly or they could be stored at 4 °C for a short time or frozen at -80 °C for long-term storage. However, freezing can affect size, structure, integrity,

and molecular composition of exosomes [11]. Aliquoting exosomes (20 μ L) to avoid repeated freeze-thaw cycles is strongly recommended.

3. Eosinophil functional assays can be consulted in Subheading 3 of Cañas et al. [8].
4. Structural lung cell functional assays can be consulted in Subheading 3 of Cañas et al. [9].

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