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## Differentiation of Monocytes into Phenotypically Distinct Macrophages After Treatment with Human Cord Blood Stem Cell (CB-SC)-Derived Exosomes --Manuscript Draft--

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**1 TITLE:**

2 Differentiation of Monocytes into Phenotypically Distinct Macrophages After Treatment with  
3 Human Cord Blood Stem Cell (CB-SC)-Derived Exosomes  
4

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**21 KEYWORDS:**

22 CB-SC, Stem Cell Educator (SCE) therapy, exosomes, monocyte, type 2 macrophage,  
23 differentiation, immune modulation  
24

**25 SUMMARY:**

26 Exosome application is an emerging tool for drug development and regenerative medicine. We  
27 establish an exosome isolation protocol with high purity to isolate exosomes from novel  
28 identified stem cells called CB-SC for mechanistic studies. We also coculture CB-SC-derived  
29 exosomes with human monocytes, leading to their differentiation into phenotypically distinct  
30 macrophages.  
31

**32 ABSTRACT:**

33 Stem Cell Educator (SCE) therapy is a novel clinical approach for the treatment of type 1 diabetes  
34 and other autoimmune diseases. SCE therapy circulates the isolated patient's blood mononuclear  
35 cells (e.g., lymphocytes and monocytes) through an apheresis machine, co-cultures the patient's  
36 blood mononuclear cells with adherent cord blood-derived stem cells (CB-SC) in the SCE device,  
37 and then returns these "educated" immune cells to the patient's blood. Exosomes are nano-sized  
38 extracellular vesicles between 30–150 nm existing in all biofluid and cell culture media. To further  
39 explore molecular mechanisms underlying SCE therapy and determine the actions of exosomes  
40 released from CB-SC, we investigate which cells phagocytize these exosomes during the  
41 treatment with CB-SC. By co-culturing Dio-labeled CB-SC-derived exosomes with human  
42 peripheral blood mononuclear cells (PBMC), we found that CB-SC-derived exosomes were  
43 predominantly taken up by human CD14-positive monocytes, leading to the differentiation of  
44 monocytes into type 2 macrophages (M2), with spindle-like morphology and expression of M2-

45 associated surface molecular markers. Here, we present a protocol for the isolation and  
46 characterization of CB-SC-derived exosomes and the protocol for the co-culture of CB-SC-derived  
47 exosomes with human monocytes and the monitoring of M2 differentiation.

48

#### 49 **INTRODUCTION:**

50 Cord blood stem cells (CB-SC) are unique type of stem cells identified from human cord blood  
51 and are distinguished from other known types of stem cells such as mesenchymal stem cells  
52 (MSC) and hematopoietic stem cells (HSC)<sup>1</sup>. Based on their unique properties of immune  
53 modulation and their ability to tightly adhere to the surface of Petri dishes, we developed a new  
54 technology designated as Stem Cell Educator (SCE) therapy in clinical trials<sup>2,3</sup>. During SCE therapy,  
55 a patient's peripheral blood mononuclear cells (PBMC) are collected and circulated through a cell  
56 separator and co-cultured with adherent CB-SC in vitro. These "educated" cells (CB-SC-treated  
57 PBMC) are then returned to the patient's circulation in a closed-loop system. Clinical trials have  
58 already demonstrated the clinical safety and efficacy of SCE therapy for the treatment of  
59 autoimmune diseases including type 1 diabetes (T1D)<sup>2,4</sup> and alopecia areata (AA)<sup>5</sup>.

60

61 Exosomes are a family of nanoparticles with diameters ranging 30–150 nm and exist in all biofluid  
62 and cell culture media<sup>6</sup>. Exosomes are enriched with many bioactive molecules including lipids,  
63 mRNAs, proteins, and microRNAs (miRNA), and play an important role in cell-to-cell  
64 communications. Of late, exosomes have become more attractive for researchers and  
65 pharmaceutical companies due to their therapeutic potentials in clinics<sup>7–9</sup>. Recently, our  
66 mechanistic studies demonstrated that CB-SC-released exosomes contribute to the immune  
67 modulation of SCE therapy<sup>10</sup>.

68

69 Here, we describe the protocol to explore the mechanism of SCE therapy targeting monocytes by  
70 CB-SC-released exosomes. First, CB-SC-released exosomes were isolated from CB-SC-derived  
71 conditioned media using ultracentrifugation methods and validated by flow cytometry, western  
72 blot (WB) and dynamic light scattering (DLS). Second, CB-SC-derived exosomes were labeled with  
73 a green fluorescent lipophilic dye: Dio. Third, they were co-cultured with PBMC to examine the  
74 positive percentages of Dio-labeled CB-SC-derived exosomes at the different subpopulations of  
75 PBMC by flow cytometry. This protocol provides guidance to study the action of exosomes  
76 underlying the immune modulation of stem cells.

77

#### 78 **PROTOCOL:**

79

80 The protocol follows the guidelines of institutional human research ethics committee at Center  
81 for Discovery and Innovation, Hackensack Meridian Health. Human buffy coat blood units were  
82 purchased from the New York Blood Center (New York, NY). Human umbilical cord blood units  
83 were collected from healthy donors and purchased from Cryo-Cell International blood bank  
84 (Oldsmar, FL). Both New York Blood Center and Cryo-Cell have received all accreditations for  
85 blood collections and distributions, with IRB approval and signed Consent Forms from donors.

86

#### 87 **1. Cell culture and preparation of CB-SC-derived conditioned medium**

88

89 1.1. Transfer 25 mL of cord blood (**Table of Materials**) over 20 mL of density gradient medium ( $\gamma$   
90 = 1.077) into a 50 mL conical tube.

91

92 1.2. Centrifuge at 1,690 x *g* for 20 min at 20 °C in a swinging-bucket rotor without brake.

93

94 1.3. Carefully transfer the mononuclear cell layer (buffy coat) to a new 50 mL conical tube. Fill  
95 the conical tube with phosphate buffered saline (PBS) to 40 mL. Mix and centrifuge to pellet cells  
96 at 751 x *g* for 10 min at 20 °C.

97

98 1.4. Discard the supernatant and add 15 mL of ACK lysis buffer (**Table of Materials**) to the cell  
99 pellet. Re-suspend cells through pipetting. Then incubate for 10 min at room temperature.

100

101 NOTE: This step removes the red blood cells.

102

103 1.5. Fill the conical tube with 25 mL of PBS. Centrifuge at 751 x *g* for 5 min and discard  
104 supernatant to obtain pelleted mononuclear cells.

105

106 1.6. Wash 2x with 40 mL of PBS to remove the remaining lysis buffer.

107

108 1.7. Centrifuge at 751 x *g* for 5 min to pellet the cells.

109

110 1.8. Discard the supernatant and re-suspend cord blood mononuclear cells with 10 mL of  
111 chemical-defined serum-free medium (**Table of Materials**) per tube.

112

113 1.9. Combine cord blood mononuclear cells to one tube.

114

115 1.10. Take 20  $\mu$ L cell suspension and mix with 20  $\mu$ L of 0.4% trypan blue solution (**Table of**  
116 **Materials**) in a 1.5 mL tube.

117

118 1.11. Load into the chamber slide and quantify the cell number and cell viability with an  
119 automated cell counter.

120

121 NOTE: Cell suspension is diluted at 1:10 if cell concentration is above  $1 \times 10^7$  cells/mL.

122

123 1.12. Seed mononuclear cells in 150 mm x 15 mm Petri dishes at  $1 \times 10^6$  cells/mL, 25 mL/dish in  
124 chemical-defined serum-free cell culture medium.

125

126 1.13. Incubate at 37 °C under 8% CO<sub>2</sub> conditions for 10–14 days until CB-SC reach more than 80%  
127 confluence.

128

129 1.14. Discard the supernatant and wash with 15 mL of PBS per Petri dish; then, remove the PBS.

130

131 NOTE: CB-SC are attached to Petri dishes tightly.

132

133 1.15. Repeat step 1.14 two times.

134

135 1.16. Add 25 mL of chemical-defined serum free medium per Petri dish.

136

137 1.17. Incubate at 37 °C under 8% CO<sub>2</sub> conditions for 3–4 days.

138

139 1.18. Collect the CB-SC-derived conditioned medium into 50 mL conical tubes.

140

## 141 **2. Characterization of CB-SC**

142

143 2.1. Detach CB-SC by pipetting 10 mL of PBS-based cell dissociation buffer up and down with a 5  
144 mL pipette tip (Table of Materials).

145

146 2.2. Centrifuge at 1,690 x *g* for 5 min to pellet cells and resuspend in 200 µL of PBS.

147

148 2.3. Fix and permeabilize cells for intracellular staining via staining preparation kit (**Table of**  
149 **Materials**).

150

151 2.4. Add 5 µL of Fc blocker (**Table of Materials**) per sample and incubate for 15 min at room  
152 temperature.

153

154 2.5. Add fluorescence-conjugated mouse anti-human monoclonal antibodies including CD34,  
155 CD45, SOX2, OCT3/4, CD270, and Galectin 9 at 25 µg/mL (**Table of Materials**) to 100 µL volume  
156 of cells. Incubate for 30 min at room temperature with light protection.

157

158 2.6. After staining, wash cells with 1 mL of PBS and centrifuge at 751 x *g* for 10 min to pellet cells.

159

160 2.7. Re-suspend cells with 200 µL of PBS and transfer into a 5 mL tube.

161

162 2.8. Perform flow cytometry to validate the expression of CB-SC-associated above specific  
163 markers.

164

## 165 **3. Isolation of CB-SC-derived exosomes**

166

167 3.1. Centrifuge the conditioned medium collected from step 1.18 at 300 x *g* for 10 min at 4 °C.  
168 Transfer the supernatant to a new 50 mL conical tube.

169

170 3.2. Centrifuge the supernatant collected from step 3.1 at 2,000 x *g* for 20 min at 4 °C. Transfer  
171 the supernatant to a new 50 mL conical tube.

172

173 3.3. Centrifuge the supernatant collected from step 3.2 at 10,000 x *g* for 30 min at 4 °C. Transfer  
174 the supernatant to a new 50 mL conical tube.

175

176 NOTE: The fixed angle rotor is used so that the cell pellets are precipitated to the side of the tube.  
177 Mark the side of the cap and draw a circle on the side of the tube where the pellet is expected.

178  
179 3.4. Filter supernatants collected from step 3.3 with a 0.22  $\mu\text{m}$  filter (**Table of Materials**).

180  
181 3.5. Transfer 15 mL of media to each 10 kDa centrifugal filter unit (**Table of Materials**).

182  
183 3.6. Centrifuge at 4,000  $\times g$  for 30 min to isolate the concentrated exosome media.

184  
185 3.7. Transfer the concentrated exosomes to an ultracentrifuge tube. Then, pellet exosomes at  
186 100,000  $\times g$  for 80 min at 4  $^{\circ}\text{C}$ .

187  
188 3.8. Discard the supernatant and re-suspend the pellet exosomes in 10 mL of PBS.

189  
190 3.9. Centrifuge at 100,000  $\times g$  for 80 min at 4  $^{\circ}\text{C}$  to collect the exosomes pellet.

191  
192 3.10. Re-suspend the exosomes pellet in 200  $\mu\text{L}$  of PBS by pipetting up and down.

#### 193 194 4. Characterization of CB-SC-derived exosomes

195  
196 4.1. Quantifying total protein concentration of exosome preparation by bicinchoninic acid assay  
197 (BCA) kit

198  
199 4.1.1. Pipette 10  $\mu\text{L}$  of each albumin standard and isolated exosome sample prepared in step 3.10  
200 into a 96-well plate in duplicate.

201  
202 4.1.2. Add 200  $\mu\text{L}$  of the working reagent from the BCA kit to each well. Mix contents of the plate  
203 thoroughly on the plate shaker for 10 s.

204  
205 NOTE: Working reagent (WR): 50-part reagent A with 1-part reagent B.

206  
207 4.1.3. Cover the plates with foil and incubate them at 37  $^{\circ}\text{C}$  for 30 min.

208  
209 4.1.4. Cool the plates to room temperature (RT).

210  
211 4.1.5. Measure sample absorbance at 562 nm via a plate reader.

212  
213 4.2. Preparation and staining of exosomes for flow cytometry

214  
215 4.2.1. Capture exosomes by adding 20  $\mu\text{L}$  of anti-human CD63 magnetic beads (4.5  $\mu\text{m}$  size)  
216 (**Table of Materials**) into 25  $\mu\text{g}$  of CB-SC-derived exosomes prepared in step 3.10 in total 100  $\mu\text{L}$   
217 volume of PBS.

218  
219 4.2.2. Incubate the tube overnight (18–22 h) at 4  $^{\circ}\text{C}$  on the shaker at 800 rpm.

220  
221 4.2.3. Centrifuge the tube at 300 x *g* for 30 s to collect the sample at the bottom of the tube.  
222  
223 4.2.4. Add 300  $\mu$ L of isolation buffer (0.1% bovine serum albumin (BSA) in PBS) and mix gently by  
224 pipetting.  
225  
226 NOTE: This step washes the bead-bound exosomes.  
227  
228 4.2.5. Place the tube on a magnet stand for 1 min (Table of Materials) and discard the  
229 supernatant.  
230  
231 4.2.6. Repeat steps 4.2.4–4.2.5.  
232  
233 4.2.7. Re-suspend the bead-bound exosomes with 400  $\mu$ L of isolation buffer.  
234  
235 4.2.8. Aliquot 100  $\mu$ L of bead-bound exosomes to each tube.  
236  
237 4.2.9. Add fluorescence-conjugated antibodies (CD9-FITC, CD81-PE, and CD63-FITC at 25  $\mu$ g/mL,  
238 respectively) to each flow tube with CD63 bead-captured exosomes.  
239  
240 NOTE: Isotype-matched IgGs serve as negative control.  
241  
242 4.2.10. Incubate for 45 min at room temperature with light protection on the shaker at 800 rpm.  
243  
244 4.2.11. Repeat steps 4.2.4–4.2.5.  
245  
246 4.2.13. Re-suspend the bead-bound exosomes in 200  $\mu$ L of isolation buffer and transfer to 5 mL  
247 flow tubes.  
248  
249 4.2.15. Place the tubes in the sample carousel of the flow cytometer.  
250  
251 4.2.16. Open the protocol for exosome testing.  
252  
253 4.2.17. Run the sample automatically by flow cytometer.  
254  
255 **4.3. Exosome detection by western blot**  
256  
257 NOTE: Western blot is a well-established method and we will not go into details of the method  
258 itself.  
259  
260 4.3.1. Lyse the pellets of CB-SC-derived exosomes from step 3.9 with 100  $\mu$ L of RIPA buffer,  
261 pipette 20x, then place on ice for 5 min.  
262

263 4.3.2. Quantify the protein concentration of exosome lysate by BCA kit and load 25 µg of protein  
264 per well.

265  
266 4.3.3. Separate the proteins by gel electrophoresis for 40 min at 150 V.  
267

268 4.3.4. Transfer the protein to polyvinylidene fluoride (PVDF) membrane using semi-dry  
269 transferring method<sup>11</sup>.

270  
271 4.3.5. Block the membrane with 5% non-fat milk for 30 min.  
272

273 4.3.6. Incubate with 2 µg/mL anti-human ALIX (Table of Materials) and 1 µg/mL anti-human  
274 CALNEXIN antibodies (Table of Materials).

275  
276 4.3.7. Detect the protein by chemiluminescence with a digital imaging system.  
277

#### 278 4.4. Exosome validation by dynamic light scattering (DLS)

279  
280 4.4.1. Dilute 10 µg of CB-SC-derived exosome samples in 1 mL of PBS.  
281

282 4.4.2. Transfer the 1 mL of diluted sample into disposable semi-micro cuvette (Table of  
283 Materials).

284  
285 4.4.3. Place the cuvette in the DLS instrument. Set the refractive index (RI) as 1.39 for all the  
286 sample monitor.

287  
288 4.4.4. Run samples at 25 °C and acquire three measurements per fraction to get an average size  
289 distribution.

#### 290 291 4.5. Exosome validation by transmission electron microscopy (TEM)

292  
293 4.5.1. Coat formvar on 300 mesh copper grids<sup>12</sup> (Table of Materials).  
294

295 4.5.2. Strengthen the formvar with the additional layer of evaporated carbon on copper grids<sup>12</sup>.

296  
297 NOTE: Such a coating approach is excellent for specimen support.  
298

299 4.5.3. Load 10 µL of exosome samples onto grids and leave to air-dry.  
300

301 4.5.4. Negatively stain samples with uranyl acetate for 5 min.  
302

303 4.5.5. Wash three times with DI water and leave to air-dry.  
304

305 4.5.6. Observe and photograph the samples under TEM. Set the accelerating voltage at 200 kV  
306 and spot size at 2.



307  
308 **5. Measure Dio-labeled CB-SC-derived exosomes up taken by different subpopulation of PBMC**

309  
310 **5.1. Label CB-SC-derived exosomes with green fluorescent lipophilic dye Dio**

311  
312 5.1.1. Transfer 100 µg of CB-SC-derived exosomes (prepared in step 3.10) into a 15 mL centrifuge  
313 tube.

314  
315 5.1.2. Dilute sample with PBS to 5 mL.

316  
317 5.1.3. Add green fluorescent lipophilic dye Dio (Table of Materials) until working concentration  
318 reaches 5 µM.

319  
320 5.1.4. Incubate for 15 min at room temperature protected from light.

321  
322 5.1.5. Transfer the sample into an ultracentrifuge tube.

323  
324 5.1.6. Centrifuge at 100,000 x *g* for 80 min to pellet Dio-labeled CB-SC-derived exosomes.

325  
326 5.1.7. Re-suspend the labeled exosomes in 200 µL of PBS.

327  
328 **5.2. Preparation of human PBMC**

329  
330 5.2.1. Transfer 25 mL of human buffy coat (see **Table of Materials**) over 20 mL of density gradient  
331 medium ( $\gamma = 1.077$ ) into a 50 mL conical tube.

332  
333 5.2.2. Repeat steps 1.2 to 1.11.

334  
335 5.2.3. Transfer  $1 \times 10^6$  PBMC into a non-tissue-treated hydrophobic 24-well plate (1 mL/well).

336  
337 NOTE: A non-tissue-treated plate was used to avoid adhering of monocytes.

338  
339 **5.3. Co-culture Dio-labeled exosomes with PBMC**

340  
341 5.3.1. Transfer 40 µL Dio-labeled CB-SC-derived exosomes prepared in step 5.1.7 to each PBMC-  
342 containing well in a 24-well plate using a 200 µL pipette. Add the same volume of PBS to control  
343 wells.

344  
345 5.3.2. Mix by pipetting 10x. Incubate for 4 h.

346  
347 5.3.3. Collect 200 µL exosome-treated PBMC and label with Hoechst 33342 for 10 min at room  
348 temperature.

349

350 5.3.4. Centrifuge at 300 x *g* for 10 min at room temperature. Discard the supernatant and re-  
351 suspend the cell pellet in 100 µL of PBS.

352  
353 5.3.5. Mount cells onto microscope slides.

354  
355 5.3.6. Observe and photograph the interaction of Dio-labeled CB-SC-derived exosomes with  
356 Hoechst 33342-labeled PBMC using a microscope.

357  
358 5.3.7. Transfer the remaining cells from step 5.3.3 into a 1.5 mL tube.

359  
360 5.3.8. Centrifuge at 300 x *g* for 10 min at 4 °C. Discard the supernatant and re-suspend the cell  
361 pellet in 200 µL of PBS.

362  
363 5.3.9. Add 5 µL of Fc blocker (Table of Materials) per sample. Incubate for 15 min at room  
364 temperature.

365  
366 NOTE: Fc blocker inhibits non-specific binding when staining with antibodies.

367  
368 5.3.10. Add antibodies (CD3, CD4, CD8, CD11c, CD14, CD19, and CD56 at 25 µg/mL) (Table of  
369 Materials) to stain PBMC.

370  
371 NOTE: Isotype-matched IgGs serve as negative control.

372  
373 5.3.11. Incubate for 30 min at room temperature with light protection.

374  
375 5.3.12. Add 1 mL of PBS and centrifuge at 300 x *g* for 10 min at 4 °C to pellet the cells.

376  
377 5.3.13. Re-suspend the cells with 200 µL PBS. Add 5 µL of propidium iodide.

378  
379 5.3.14. Use flow cytometry to evaluate the level of Dio-labeled exosome uptake in different  
380 subpopulation of PBMC.

381

## 382 **6. Examine the action of CB-SC-derived exosomes on monocytes**

383

### 384 **6.1. Isolation human CD14-positive monocytes**

385

386 6.1.1. Transfer 3 x 10<sup>7</sup> human PBMC into a 15 mL tube.

387

388 6.1.2. Centrifuge at 300 x *g* for 10 min at 4 °C.

389

390 6.1.3. Place the separation column (Table of Materials) in the magnet separator (Table of  
391 Materials).

392

393 6.1.4. Wash separation columns three times with 2 mL of cold running buffer (Table of Materials).

394  
395 6.1.5. Re-suspend the cells in 300  $\mu$ L of cold PBS. Add 60  $\mu$ L of CD14 microbeads. Mix well and  
396 incubate on ice for 15 min.

397  
398 6.1.6. Add 6 mL of cold PBS. Centrifuge at 300 x *g* for 10 min at 4 °C.

399  
400 6.1.7. Re-suspend the pelleted cells in 500  $\mu$ L of cold running buffer.

401  
402 6.1.8. Transfer cells into the separation column (prepared in step 6.14) and let them pass  
403 through.

404  
405 6.1.9. Wash the separation column three times with 2 mL of running buffer per wash. Lift the  
406 column from the magnet separator and place it in a 15 mL centrifuge tube.

407  
408 NOTE: The 15 mL tube should be placed on ice due to the adherence of CD14-positive monocytes  
409 to the tube at room temperature.

410  
411 6.1.10. Transfer 2 mL of cold running buffer to the top of the column and isolate the CD14-  
412 positive cells into the 15 mL tube.

413  
414 6.1.11. Centrifuge at 300 x *g* for 10 min at 4 °C to pellet the CD14-positive cells.

415  
416 6.1.12. Re-suspend the cells with 2 mL of cold chemical-defined serum free medium (Table of  
417 Materials).

418  
419 6.1.13. Transfer 50  $\mu$ L of cells into a 1.5 mL tube.

420  
421 6.1.14. Stain with 10  $\mu$ L of Krome Orange-conjugated anti-human CD14 mAb (Table of Materials)  
422 for 20 min.

423  
424 NOTE: Isotype-matched IgGs serve as negative controls.

425  
426 6.1.15. Add 1 mL PBS to the cells. Centrifuge at 300 x *g* for 10 min to pellet cells.

427  
428 6.1.16. Re-suspend cells in 200  $\mu$ L of PBS and transfer it to a 5 mL tube. Determine the purity of  
429 CD14-positive monocytes by flow cytometry.

430  
431 **6.2. Treatment of monocytes with CB-SC-derived exosomes**

432  
433 6.2.1. Seed 1 x 10<sup>6</sup> purified monocytes with chemical-defined serum-free culture medium (Table  
434 of Materials) in tissue culture-treated 6-well plate (2 mL/well).

435  
436 6.2.2. Incubate for 2 h at 37 °C under 5% CO<sub>2</sub>.

437

438 6.2.3. Discard the supernatant with 1 mL pipette. Add 2 mL of 37 °C pre-warmed chemical-  
439 defined serum-free culture medium (Table of Materials) gently.

440  
441 NOTE: Monocytes were adhered to the plate within 2 h. Floating cells were identified as dead or  
442 other cell contaminations.

443  
444 6.2.4. Add 80 µg CB-SC-derived exosomes isolated from step 3.10 to monocyte cultures in a 6-  
445 well plate with total volume of 2 mL.

446  
447 NOTE: The same volume of PBS was added to control wells.

448  
449 6.2.5. Incubate at 37 °C under 5% CO<sub>2</sub> for 3–4 days.

450  
451 6.2.6. Photograph the cell morphology using an inverted microscope at 200× magnification  
452 (Table of Materials).

453  
454 6.2.7. Detach cells by pipetting up and down in 1 mL of a PBS-based cell dissociation buffer with  
455 1 mL pipette tip.

456  
457 6.2.8. Harvest the remaining attached cells via a cell scraper.

458  
459 NOTE: Since primary monocytes or differentiated macrophages attach tightly, some cells remain  
460 adhered to the bottom after the treatment with dissociation buffer. Therefore, these cells are  
461 harvested with a cell scraper.

462  
463 6.2.9. Collect cells at 1,690 x g for 5 min. Re-suspend cells in 200 µL of PBS.

464  
465 6.2.10. Add 5 µL of Fc blocker (25 µg/mL) to block non-specific binding.

466  
467 6.2.11. Add antibodies (CD14, CD80, CD86, CD163, CD206, and CD209 at 25 µg/mL, Table of  
468 Materials) to cells. Incubate for 30 min at room temperature.

469  
470 NOTE: Isotype-matched IgGs serve as negative control

471  
472 6.2.12. Add 1 mL of PBS to cells and centrifuge at 300 x g for 10 min. Discard the supernatant and  
473 re-suspend with 200 µL of PBS.

474  
475 6.2.13. Add 5 µL of propidium iodide per sample (200 µL) and transfer cells to a new 5 mL flow  
476 tube.

477  
478 6.2.14. Perform the flow cytometry and evaluate the levels of CD14, CD80, CD86, CD163, CD206,  
479 and CD209 expressions.

480  
481 **REPRESENTATIVE RESULTS:**

482 Initially, the phenotype and purity of CB-SC were examined by flow cytometry with CB-SC-  
483 associated markers such as leukocyte common antigen CD45, ES cell-specific transcription factors  
484 OCT3/4, and SOX2. CB-SC display high levels of CD45, OCT3/4, SOX2, CD270, and galectin 9  
485 expression, but no expression of CD34 (**Figure 1A**). Flow cytometry analysis confirmed the  
486 expression of exosome-specific markers including CD9, CD81, and CD63 were on CB-SC-derived  
487 exosomes (**Figure 1B**). Morphology and size distribution of exosomes were characterized by TEM  
488 and DLS (**Figure 1C,D**), with the size of  $85.95 \pm 22.57$  nm. Western blot further proved the  
489 expression of the exosome-associated marker Alix, without expression of the ER-associated  
490 marker Calnexin (**Figure 1E**).

491  
492 PBMC were treated with Dio-labeled CB-SC-Exo. The microscopy observation demonstrated the  
493 direct interaction of Dio-labeled CB-SC-Exo with PBMC (**Figure 2A**). To better define which cell  
494 population interacted with the Dio-labeled CB-SC-Exo, different cell compartments were gated  
495 with cell-specific markers such as CD3 for T cells, CD11c for myeloid dendritic cells (DC), CD14 for  
496 monocytes, CD19 for B cells, and CD56 for NK cells (**Figure 2B**). After an incubation for 4 hr, flow  
497 cytometry demonstrated that different blood cell compartments displayed at different median  
498 fluorescence intensity (MFI) of Dio-positive exosomes (**Figure 2C**). Notably, monocytes exhibited  
499 higher median fluorescence intensity of Dio-positive CB-SC-Exo than those of other immune cells  
500 (**Figure 2C**), highlighting that monocytes were primarily targeted by the CB-SC-derived exosomes.

501  
502 To explore the direct effects of CB-SC-derived exosomes on monocytes, the purified CD14<sup>+</sup>  
503 monocytes were co-cultured with CB-SC-derived exosomes for 3 days. The exosome-treated  
504 monocyte successfully differentiated into spindle-like morphologies (**Figure 3A**). Next,  
505 phenotypes of the CB-SC-Exo treated or untreated monocytes were tested, revealing the  
506 expressions of M2-associated markers including CD163, CD206, CD209 were markedly increased  
507 among the exosome-treated group (**Figure 3B**, red histogram). Comparing with the conventional  
508 M2 macrophages generated by M-CSF + IL-4, CB-SC-Exo-treated monocytes expressed similar  
509 levels of M2-associated markers such as CD163, CD206, CD209, with no significant differences  
510 (**Figure 3C**). Therefore, the data indicates that monocytes differentiate into macrophages with  
511 M2 phenotype after the treatment with CB-SC-derived exosomes.

## 512 513 **FIGURE LEGENDS:**

514  
515 **Figure 1: Characterization of CB-SC-derived exosomes.** (A) Phenotypic characterization of CB-SC,  
516 high expression of CD45, OCT3/4, SOX2, CD270 and Galectin, no expression of CD34. (B)  
517 Expressions of exosome-associated markers (CD63, CD9, CD81) on CB-SC-derived exosomes.  
518 Isotype-matched IgGs served as control for flow cytometry (gray histogram). (C) Transmission  
519 Electron Microscopy (TEM) image of the CB-SC-derived exosomes. (D) Size distribution of CB-SC-  
520 derived exosomes using Dynamic Light Scattering (DLS). (E) Western blots show that CB-SC-  
521 derived exosomes display the exosome-specific marker Alix, but negative for endoplasmic  
522 reticulum (ER)-associated marker Calnexin.

523  
524 **Figure 2: Interaction of CB-SC-derived exosomes with different population of PBMC.** (A) The  
525 interaction of Dio-labeled CB-SC-Exo (green) with PBMC (blue, nuclear staining with Hoechst

526 33342) was photographed with Nikon Eclipse Ti2 microscope with NIS-Elements software version  
527 5.11.02, with a high magnification showing the distribution of Dio-labeled exosomes (green) in  
528 the PBMC cells after the co-incubation for 4 h 5% CO<sub>2</sub> in the non-tissue culture-treated 24-well  
529 plate. n = 2. (B) Gating strategy for flow cytometry analysis with cell-specific surface markers for  
530 different subpopulation in PBMC, including CD3 for T cells, CD14 for monocytes, CD19 for B cells,  
531 CD56 for NK cells, and CD11c for DCs. (C) Display different median fluorescence intensity (MFI)  
532 of Dio-labeled exosome among different PBMC subpopulations (e.g., T cells, Monocytes, B cells,  
533 NK cells, DCs).

534  
535 **Figure 3: Effects of CB-SC-derived exosomes on monocytes.** (A) Morphological change of  
536 monocytes into the spindle-like cells after treatment with CB-SC-derived exosomes. (B) Up-  
537 regulated the level of M2-associate markers' expression after the treatment with CB-SC-derived  
538 exosomes, such as CD163, CD206, and CD209 (red line). Untreated monocytes (green line) served  
539 as control. Isotype-matched IgG served as negative control (gray line). (C) Phenotypic comparison  
540 between conventional M2 macrophages and the CB-SC-Exo-induced M2 macrophages. To  
541 generate the conventional M2 macrophages, the purified CD14<sup>+</sup> monocytes were treated with  
542 50 ng/mL macrophage colony-stimulating factor (M-CSF) at 37 °C, 5% CO<sub>2</sub> conditions for 7 days,  
543 and followed by the overnight treatment with 10 ng/mL IL-4. M2-associated markers including  
544 CD14, CD80, CD86, CD163, CD206, and CD209 were evaluated by flow cytometry. Isotype-  
545 matched immunoglobulin G (IgG) serve as control. The data is presented as mean + SD; N = 3.

#### 546 547 **DISCUSSION:**

548 Application of exosomes is an emerging field for clinical diagnosis, drug developments and  
549 regenerative medicine. Here, we present a detailed protocol regarding the preparation of CB-SC-  
550 derived exosomes and the functional study of exosomes on the differentiation of human  
551 monocytes. The current protocol demonstrated that functional CB-SC-derived exosomes are  
552 isolated by sequential centrifugation and ultracentrifugation with high purity and exhibiting the  
553 immune modulation on monocytes.

554  
555 As compared with other conventional protocols, ultrafiltration is an established approach for the  
556 isolation and purification of exosomes from different cells or media, based upon the molecular  
557 weight and exclusion sizes that are different from other extracellular vesicles (EVs). While  
558 ultrafiltration isolation is more time-saving than the ultracentrifugation-based separation, it may  
559 cause structural damage to vesicles at large sizes. Exosomes can also be collected by polyethylene  
560 glycol (PEG)-mediated precipitation at low cost, though this method risks the exosome purity due  
561 to the protein contaminations<sup>13,14</sup>. Therefore, the current protocol was cost-effective to produce  
562 exosomes at high purity. Based on the immune modulations of CB-SC-derived exosomes<sup>10</sup>,  
563 characterization of CB-SC-derived exosomes may offer a valuable biomarker to evaluate the  
564 potency of Stem Cell Educator with CB-SC before clinical applications.

565  
566 Macrophages are professional antigen-presenting cells against viral and bacterial infections, with  
567 varied biological functions and heterogeneities. Based on their differences in surface markers  
568 and immune function, macrophages are categorized with two sub-populations: type 1

569 macrophages (M1, conventional macrophages causing inflammation) and type 2 macrophages  
570 (M2, displaying anti-inflammation)<sup>15</sup>. This study established that purified human monocytes were  
571 differentiated into type 2 macrophages after the treatment with CB-SC-derived exosomes,  
572 displaying an anti-inflammation phenotype<sup>10</sup>. CB-SC-derived exosome-treated monocytes  
573 exhibited the elongated morphology and expressed the M2-associated surface markers (e.g.,  
574 CD163, CD206, and CD209), with the similar phenotype as the conventional M2 macrophages  
575 generated using cytokines M-CSF + IL-4. Such phenotypic changes of monocytes highlight the  
576 new mechanism underlying the immune modulation of CB-SC for the treatment of type 1  
577 diabetes and other autoimmune diseases. During the SCE therapy, patients' immune cells were  
578 co-cultured with CB-SC for around 8–9 h. The SCE-treated monocytes carried the CB-SC-derived  
579 exosomes back into the body, which contributed to the M2 differentiation and the expansion of  
580 the induction of immune tolerance, leading to the improvement of clinical outcomes after the  
581 treatment with SCE therapy.

582

#### 583 **ACKNOWLEDGMENTS:**

584 We are grateful to Mr. Poddar and Mr. Ludwig for generous funding support via Hackensack UMC  
585 Foundation. We appreciate Laura Zhao for English editing.

586

#### 587 **DISCLOSURES:**

588 Dr. Zhao is a founder of Tianhe Stem Cell Biotechnology Inc. Dr. Zhao is an inventor of Stem Cell  
589 Educator technology. All other authors have no financial interests that may be relevant to the  
590 submitted work.

591

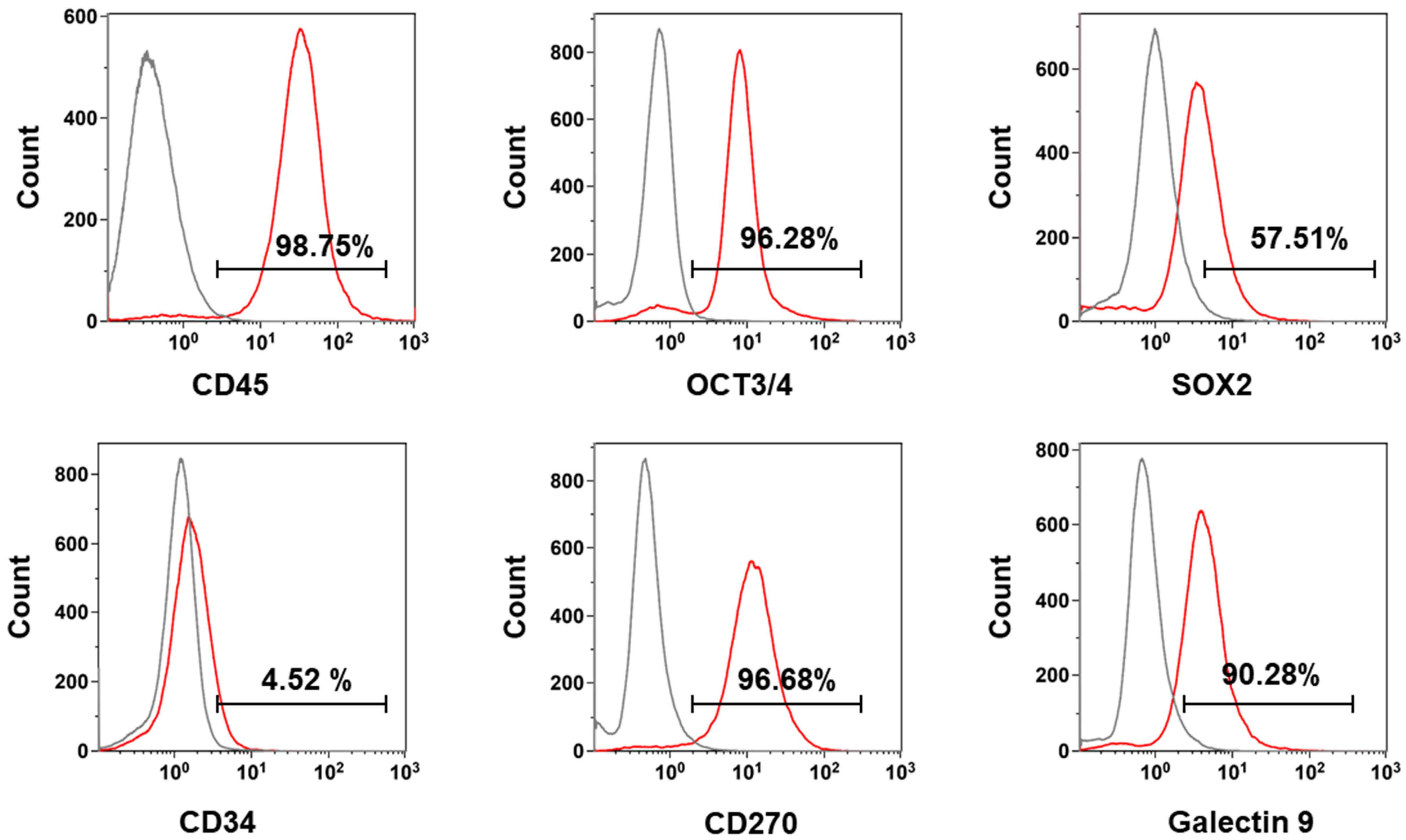
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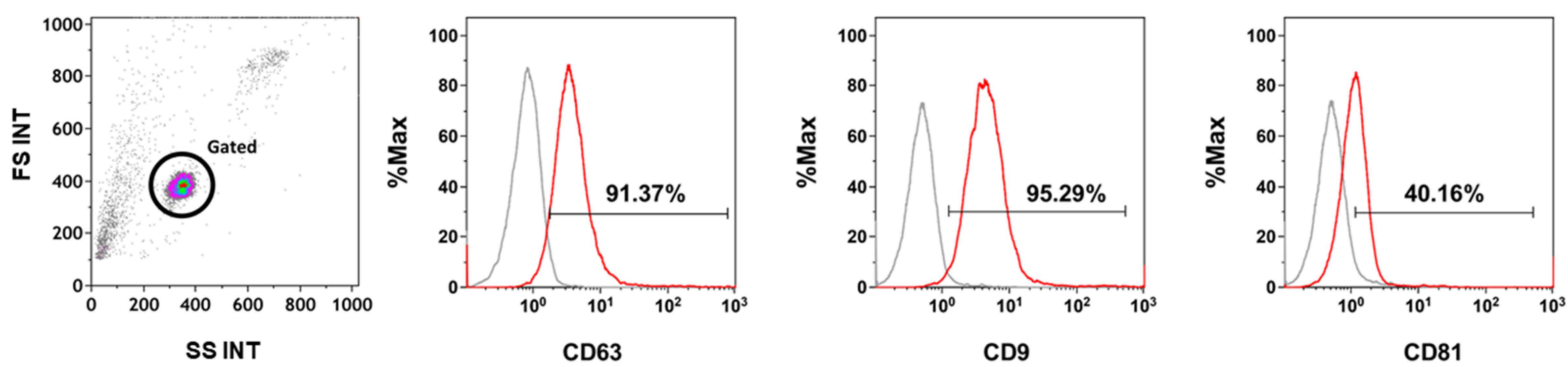
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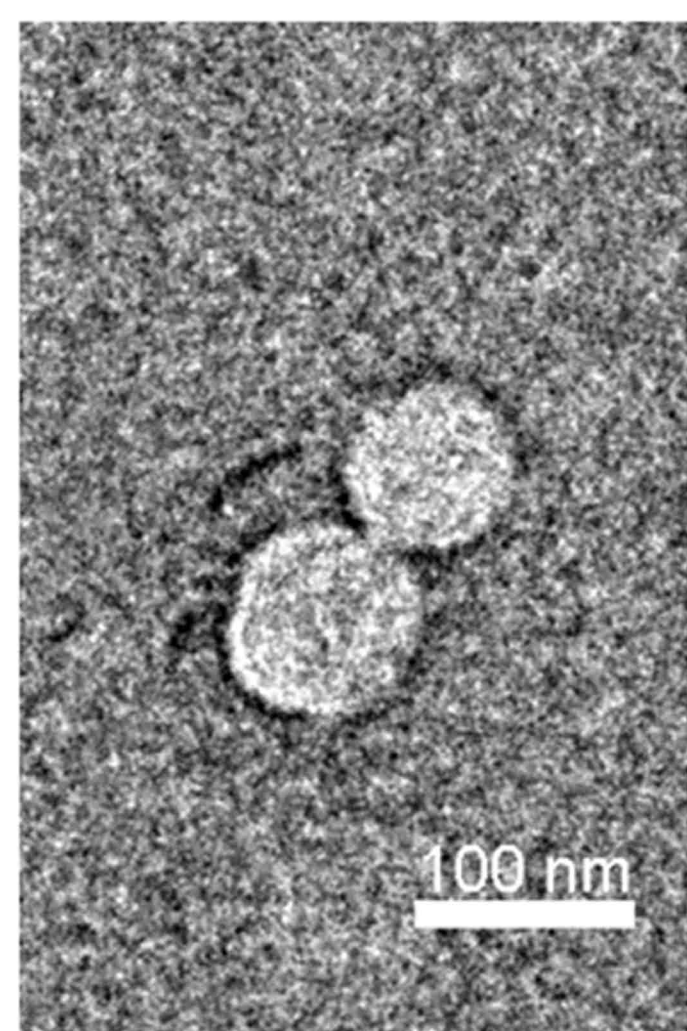
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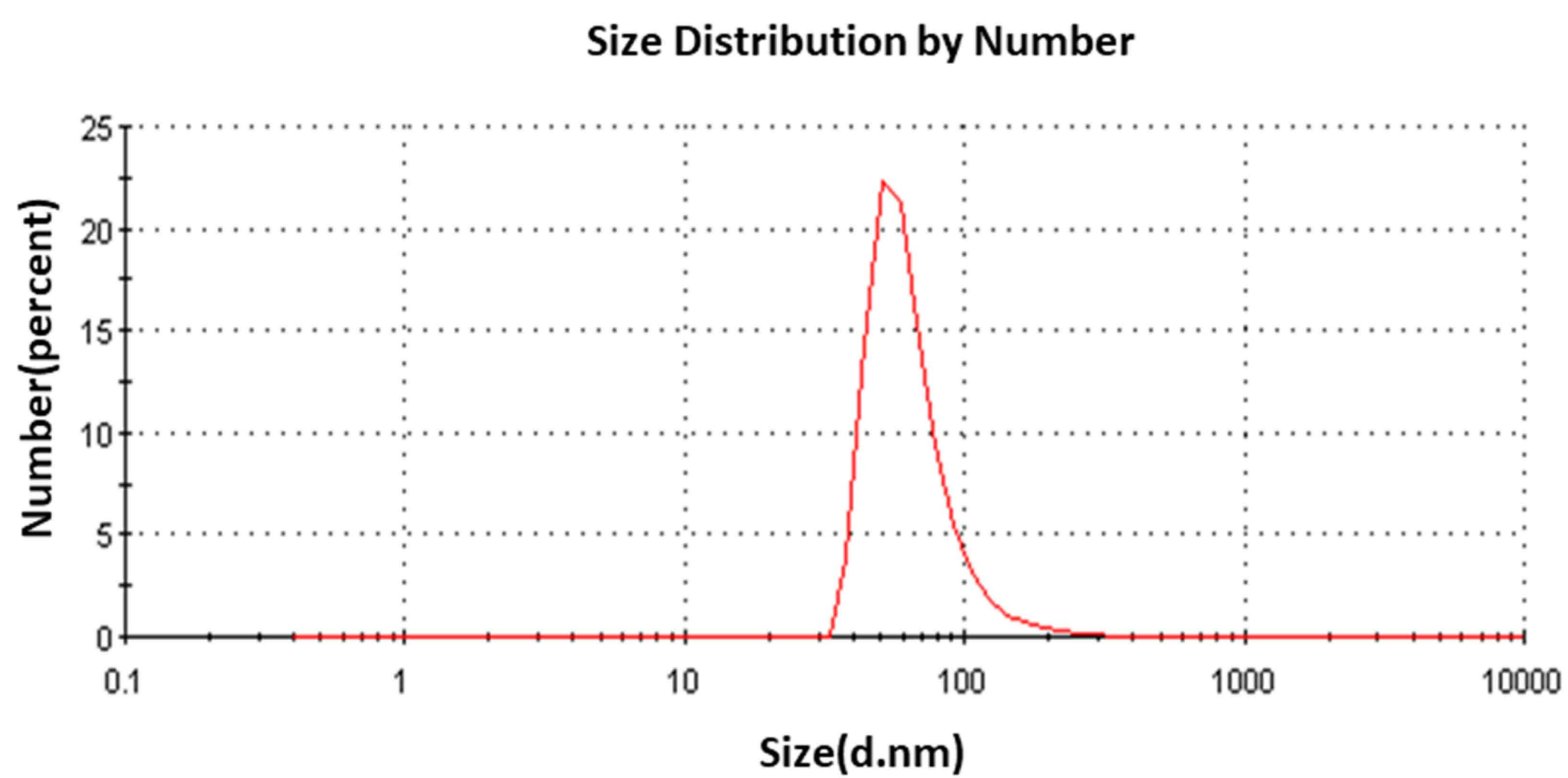
**B**



**C**



**D**



**E**

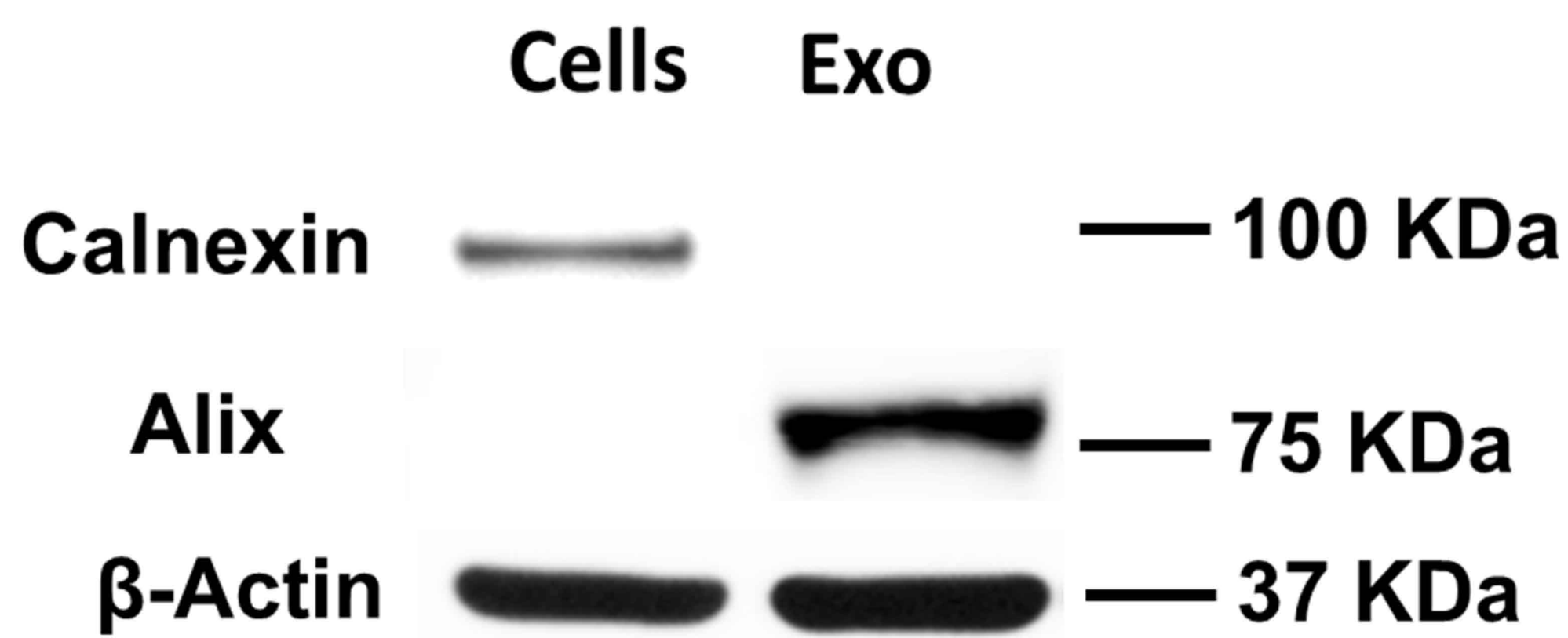
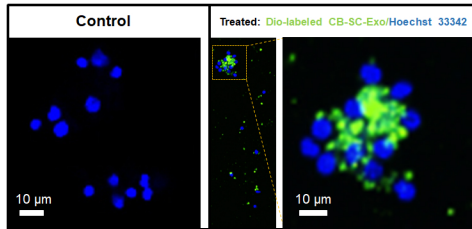
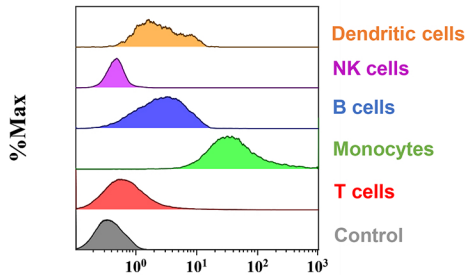
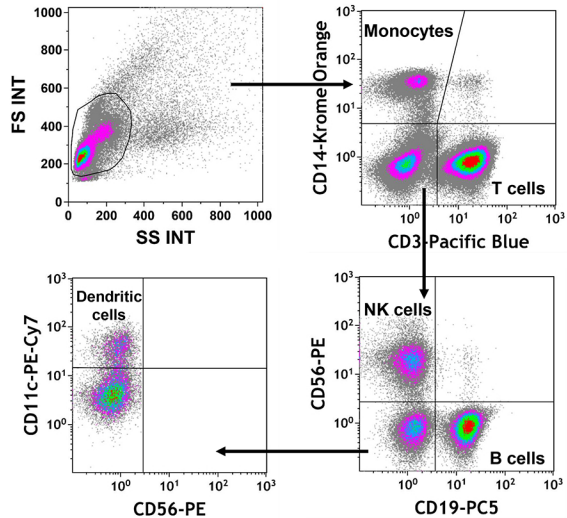
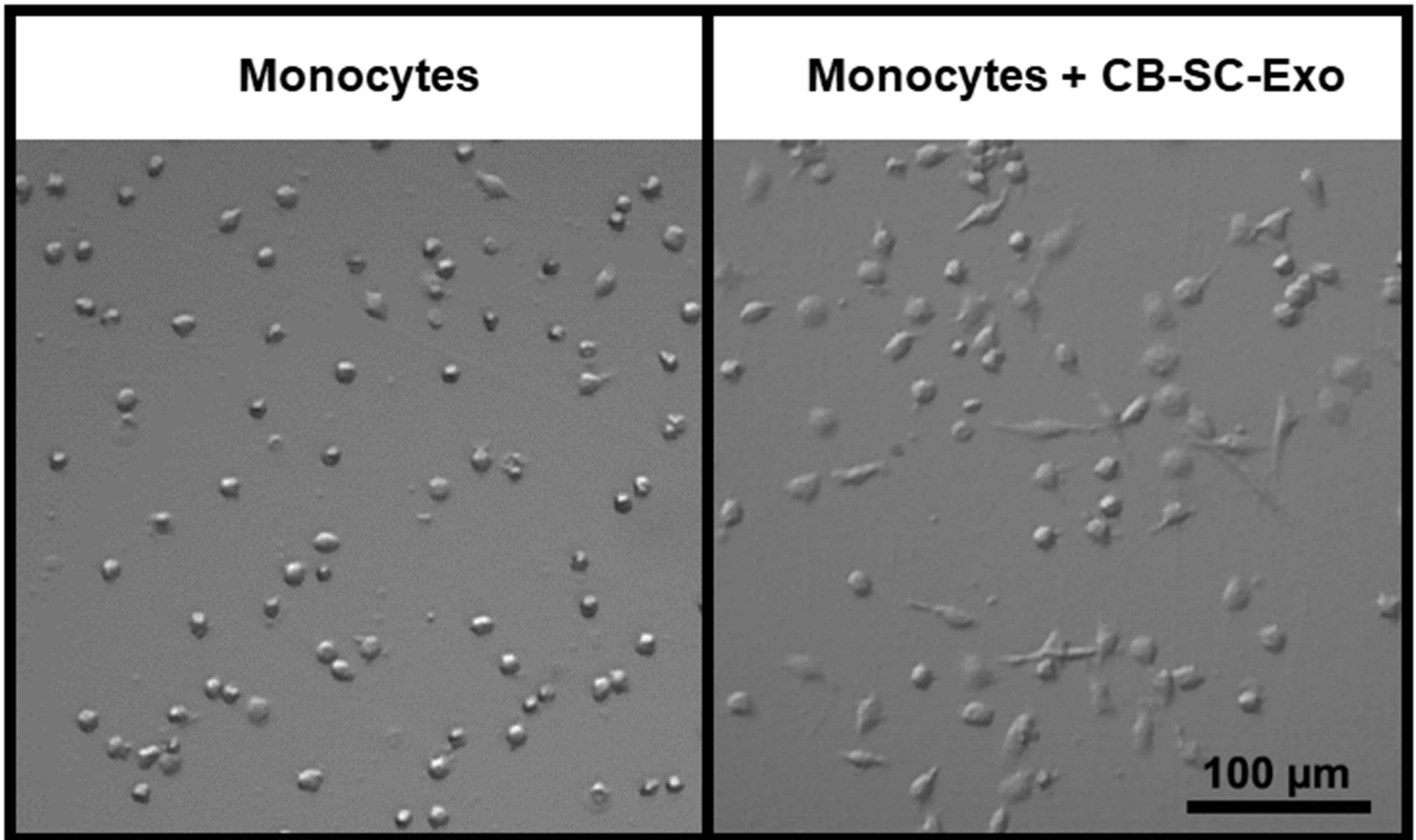
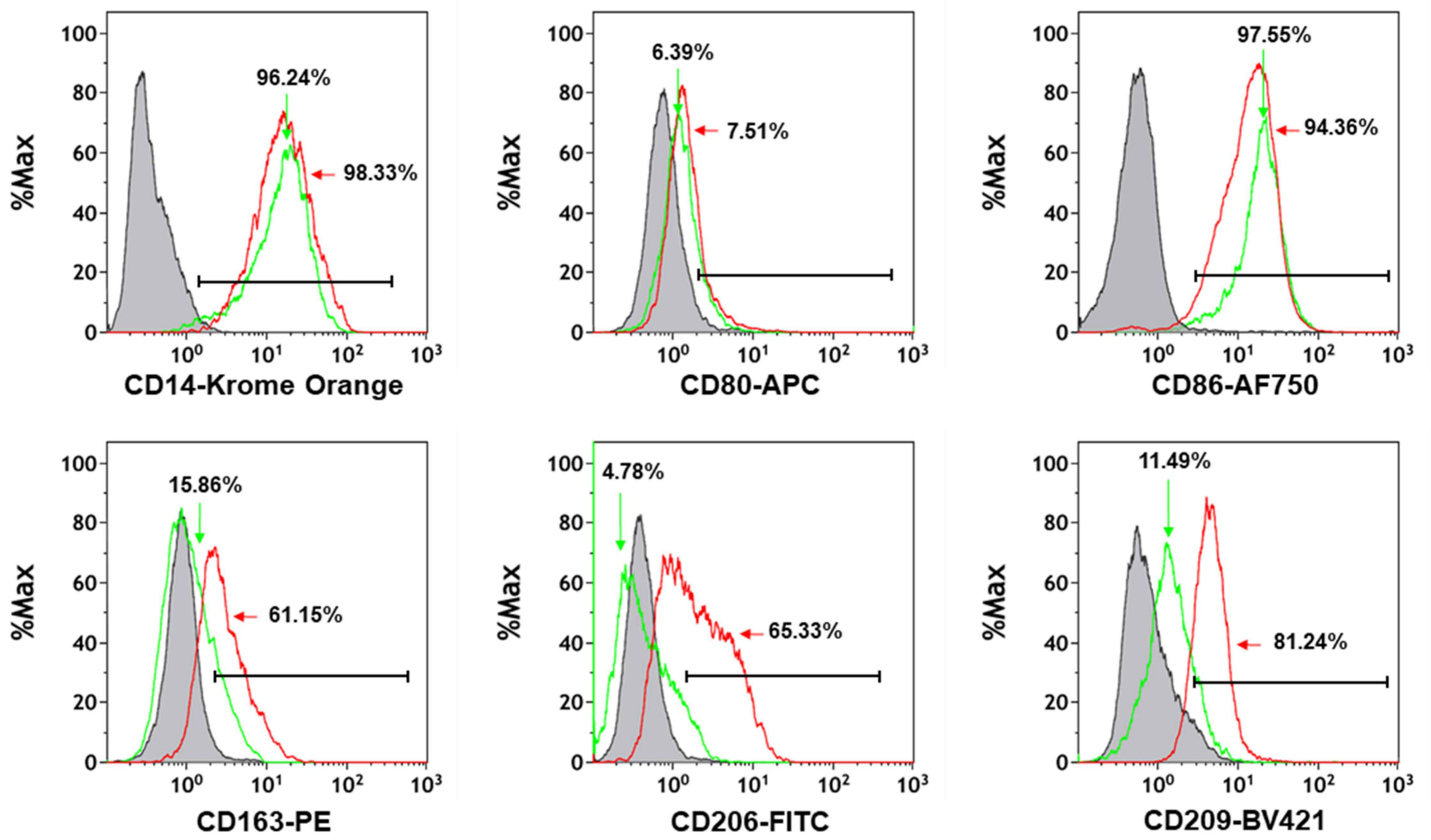
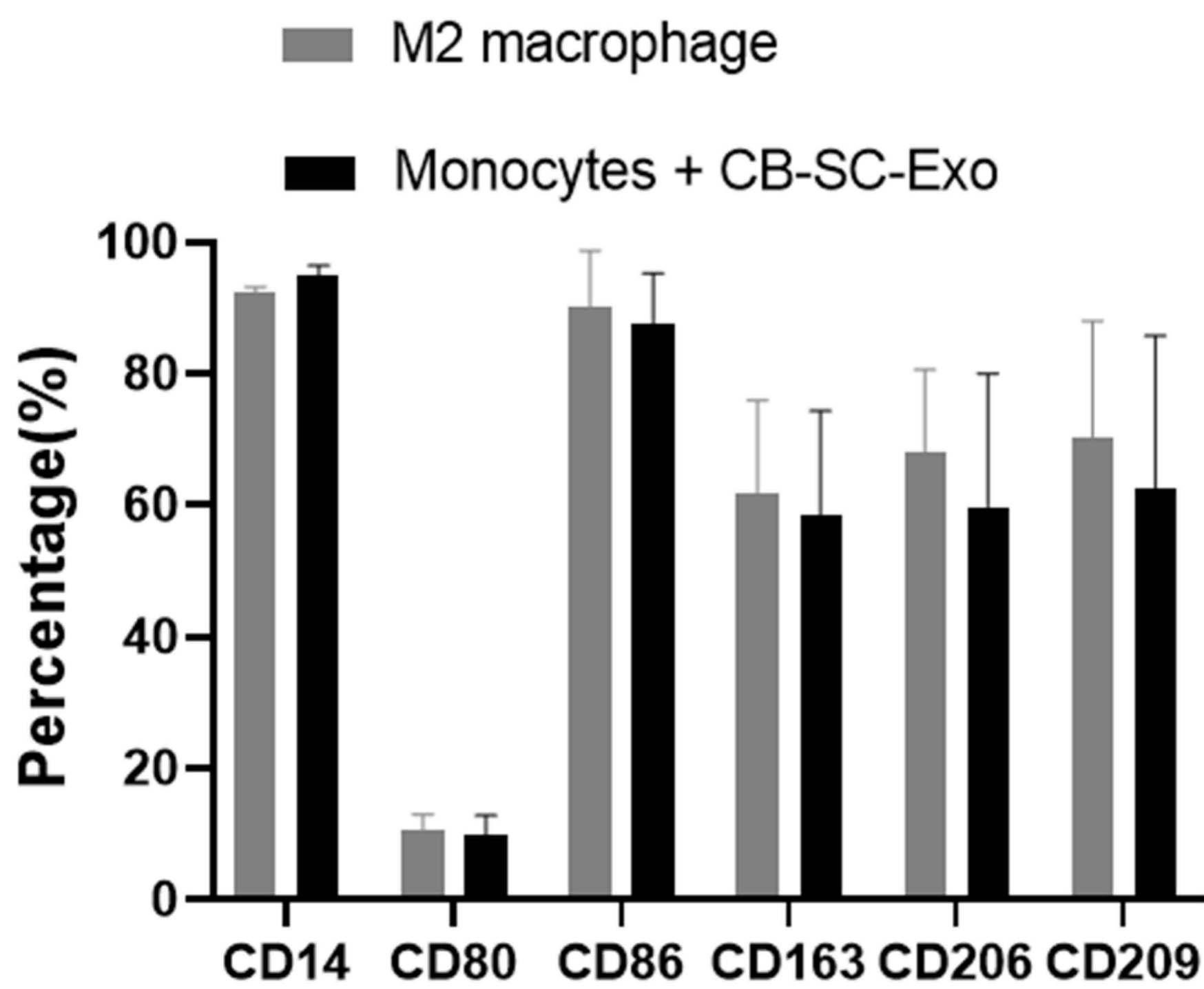


Figure 2

**A****C****B**

[Click here to access/download;Figure;Figure 2.pdf](#) 

**A****B****C**

<b>Name</b>	<b>Company</b>	<b>Catlog Number</b>
1.5 ml Microcentrifuge tube	Fisher Scientific	05-408-129
15 ml conical tube	Falcon	352196
24-well plate	Falcon	351147
3,3'-Diostadecyloxacarbocyanine perchlorate(Dio)	Millipore sigma	D4292-20MG
300 Mesh Grids	Ted Pella	1GC300
50 mL conical tube	Falcon	352070
6-well plate	Falcon	353046
96-well plate	Falcon	353072
ACK Lysis Buffer	Lonza	10-548E
Amicon-15 10kDa Centrifuge Fliter Unit	Millipore sigma	UFC901024
Anti-Human Alix	Biologend	634501
Anti-Human Calnexin	Biologend	699401
Anti-Human CD11c antibody, Pe-Cy7	BD Bioscience	561356
Anti-Human CD14 antibody, Karma Orange	Beckman Coulter	B36294
Anti-Human CD163 antibody, PE	BD Bioscience	556018
Anti-Human CD19 antibody, PC5	Beckman Coulter	IM2643U
Anti-Human CD206 antibody, FITC	BD Bioscience	551135
Anti-Human CD209 antibody, Brilliant Violet 421	BD Bioscience	564127
Anti-Human CD270 antibody, PE	Biologend	318806
Anti-Human CD3 antibody, Pacific Blue	Biologend	300431
Anti-Human CD34 antibody, APC	Beckman Coulter	IM2427U
Anti-Human CD4 antibody, APC	BD Bioscience	555349
Anti-Human CD45 antibody, Pe-Cy7	Beckman Coulter	IM3548U
Anti-Human CD56 antibody, PE	Beckman Coulter	IM2073U
Anti-Human CD63 antibody,PE	BD Bioscience	561925
Anti-Human CD8 antibody, APC-Alexa Fluor 750	Beckman Coulter	A94686
Anti-Human CD80 antibody, APC	Beckman Coulter	B30642
Anti-Human CD81 antibody, FITC	BD Bioscience	561956

Anti-Human CD86 antibody, APC-Alexa Fluor 750	Beckman Coulter	B30646
Anti-Human CD9 antibody, FITC	ThermoScientific	MA5-16860
Anti-Human Galectin 9 antibody, Brilliant Violet 421	Biolegend	348919
Anti-Human OCT3/4 antibody, eFluor660	ThermoScientific	50-5841-82
Anti-Human SOX2 antibody, Alexa Fluor 488	ThermoScientific	53-9811-82
BCA Protein Assay Kit	ThermoFisher Scientific	23227
Bovine Serum Albumin	Millipore Sigma	A1933
Buffy coat	New York Blood Center	
Cell scraper	Falcon	353085
Disposable semi-micro cuvette	VWR	97000590
Dissociation buffer	Gibco	131510014
Dual-Chamber cell counting slides	Bio-Rad	1450015
Exosome-Human CD63 Detection reagent	ThermoFisher Scientific	10606D
Ficoll-Paque PLUS density gradient media	GE Health	17-1440-03
Fixed-Angle Rotor(25°)	Thermo Scientific	75003698
Gallios flow cytometer	Beckman Coulter	
Human cord blood	Cryo-Cell International	
Human Fc Block	BD Bioscience	564220
Immun-Blot PVDF membrane	Bio-Rad	1620177
Millex-GP Syringe Filter Unit, 0.22 µm	Millipore Sigma	SLGP033RS
Optima XE-90 Ultracentrifuge	Beckman Coulter	
Orbital Shaker MP4	BioExpress	S-3500-1
PBS	ThermoFisher Scientific	10010049
Propidium Iodide	BD Bioscience	56-66211E
Nikon Eclipse Ti2 microscope	Nikon instruments Inc	Eclipse Ti2
Hoechst 33342	Thermo Scientific	62249
Revert microscopy	Fisher scientific	12563518
Rotor 41 Ti	Beckman Coulter	331362
Sorvall St 16R Centrifuge	Thermo Scientific	75004381
Swinging Bucket Rotor	Thermo Scientific	75003655
TC-20 cell counter	Bio-Rad	

ThermoScientific Forma 380 Steri Cycle CO2 Incubator

ThermoFisher Scientific

Transmission electron microscopy

JEOL

JEM-2100 PLUS

Ultracentrifuge tube

Beckman Coulter

331372

X'VIVO 15 Serum-free medium

Lonza

BEBP04-744Q

## Comments

Non-tissue culture  
plate  
Store at 4 °C

Tissue culture plate  
Tissue culture plate  
100 ml

store at 4 °C, RRID: AB\_2268110  
store at 4 °C, RRID: AB\_2728519  
store at 4 °C, RRID: AB\_10611859  
store at 4 °C, RRID: AB\_2728099  
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store at 4 °C, RRID: AB\_394065  
store at 4 °C, RRID: AB\_2738610  
store at 4 °C, RRID: AB\_2203703  
store at 4 °C, RRID: AB\_1595437  
store at 4 °C, RRID: N/A  
store at 4 °C, RRID: AB\_398593  
store at 4 °C, RRID: AB\_1575969  
store at 4 °C, RRID: AB\_131195  
store at 4 °C, RRID: AB\_10896821  
store at 4 °C, RRID: N/A  
store at 4 °C, RRID: N/A  
store at 4 °C, RRID: AB\_394049

store at 4 °C, RRID: N/A

store at 4 °C, RRID: AB\_2538339

store at 4 °C, RRID: AB\_2716134

store at 4 °C, RRID: AB\_11218882

store at 4 °C, RRID: AB\_2574479

40-60 ml/unit

100 ml

store at 4 °C

500 ml

Maxium 24,700 x g

3 lasers

10 Color Max

40-100 ml/unit

store at 4 °C

500 ml

store at 4 °C

NIS-Elements software version 5.11.02

5 ml

Maxium 41,000 rpm

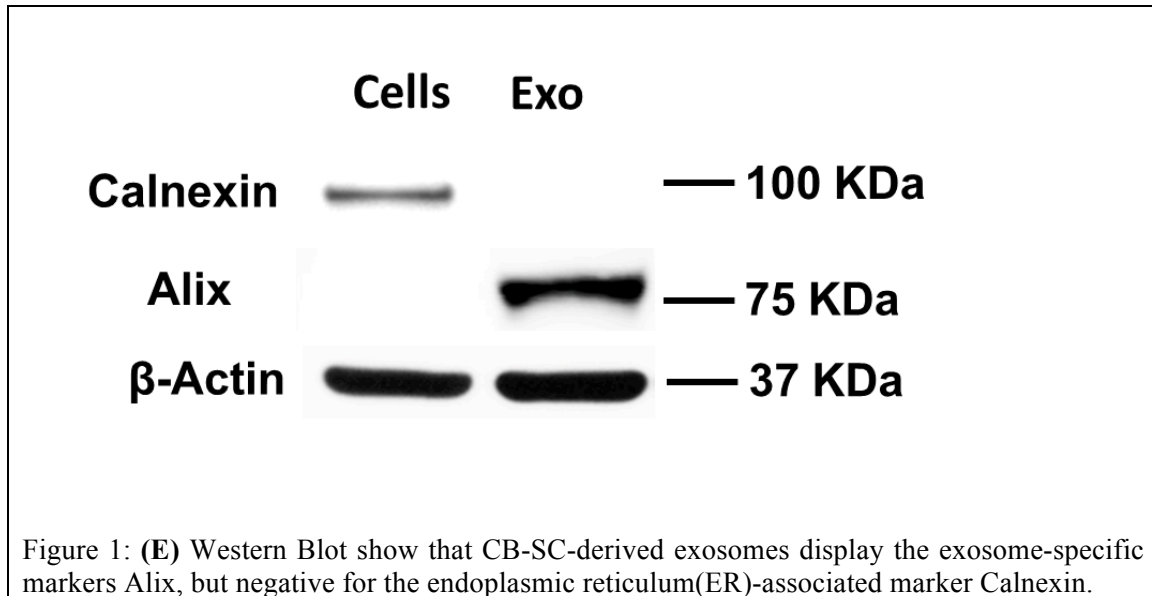


1000 ml Culture medium, store at 4 °C

**Response to Editorial Comments:**

1. Please review Figure 1E. The boxes around parts of the figure look like image artifacts. If possible, can this Figure 1E be recreated?

**Answer:** Thank you for your suggestion. This has been revised as following.



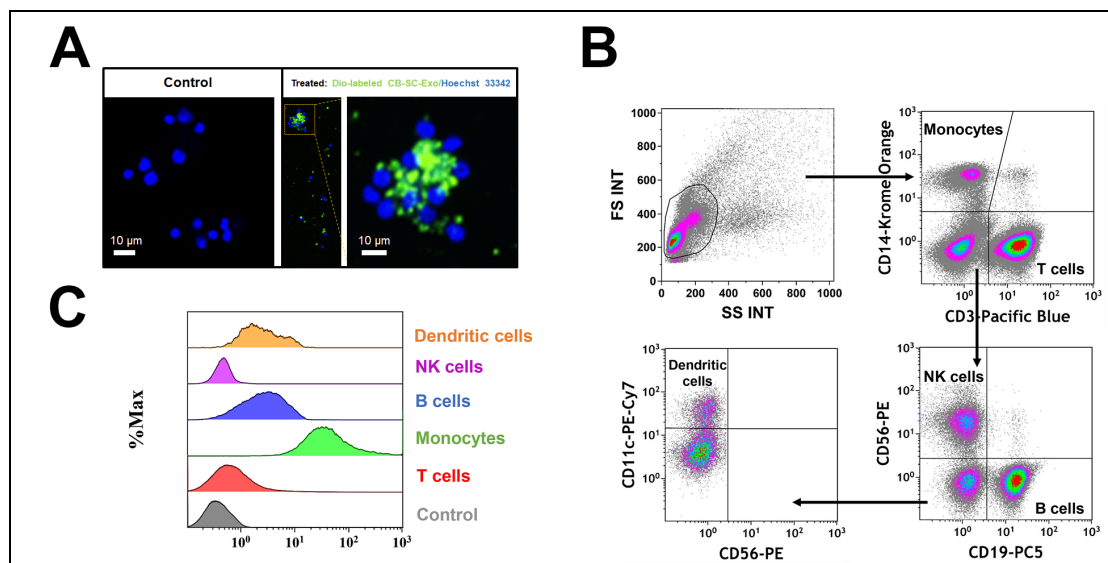
## Responses to Reviewer 4:

### 1. I didn't receive the video about the process of this new method.

**Answer:** Many thanks for your comment. The video will be scheduled to produce after this manuscript is accepted for publication.

### 2. In section of Result, there are no images demonstrating that exosomes released from CB-SC tagged with Dio into PBMC.

**Answer:** Many thanks. We performed this experiment according to your comments. PBMC were treated with 3, 3'-Dioctadecyloxycarbocyanine perchlorate (Dio)-labeled CB-SC-derived exosomes for 4 hours at 37°C in 5% CO<sub>2</sub> in the non-tissue culture-treated 24-well plate. The microscopy observation demonstrated the direct interaction of Dio-labeled exosomes with PBMC (**Figure 2A**). To better define which cell population interacted with the Dio-labeled exosomes, different cell compartments were gated with cell-specific markers such as CD3 for T cells, CD11c for myeloid dendritic cells (DC), CD14 for monocytes, CD19 for B cells, and CD56 for NK cells (**Figure 2B**). After an incubation for 4 hr, flow cytometry demonstrated that different blood cell compartments displayed at different median fluorescence intensity (MFI) of Dio-positive exosomes (**Figure 2C**). Notably, monocytes exhibited higher median fluorescence intensity of Dio-positive exosomes than those of other immune cells (**Figure 2C**), highlighting the monocytes were primarily targeted by the CB-SC-derived exosomes.



**Figure 2: Interaction of CB-SC-derived exosomes with different population of PBMC.** (A) The interaction of Dio-labeled CB-SC-derived exosomes (green) with PBMC (blue, nuclear staining with Hoechst 33342) was photographed with Nikon Eclipse Ti2 microscope with NIS-Elements software version 5.11.02, with a high magnification showing the distribution of Dio-labeled exosomes (green) in the PBMC cells after the co-incubation for 4 hours 5% CO<sub>2</sub> in the tissue culture-treated 24-well plate. *n* = 2. (B) Gating strategy for flow cytometry analysis with cell-specific surface markers for different subpopulation in PBMC, including CD3 for T cells, CD14 for monocytes, CD19 for B cells, CD56 for NK cells and CD11c for DCs. (C) Display different median fluorescence intensity (MFI) of Dio-labeled exosome among different PBMC subpopulations (e.g., T cells, Monocytes, B cells, NK cells, DCs).

3. **There are still many grammatical mistakes in this draft manuscript. It would greatly benefit from English language editing.**

**Answer:** Thanks for your comments. The manuscript has been revised by English editing.

1 **TITLE:**

2 **Differentiation of Monocytes into Phenotypically Distinct Macrophages After Treatment with**  
 3 **Human Cord Blood Stem Cell (CB-SC)-Derived Exosomes**

4

5 **AUTHORS & AFFILIATIONS:**

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7

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20

21 **KEYWORDS:**

22 CB-SC, Stem Cell Educator (SCE) therapy, exosomes, monocyte, type 2 macrophage,  
 23 differentiation, immune modulation

24

25 **SUMMARY:**

26 Application of exosomes is an emerging tool for drug development and regenerative medicine.

27 We establish an exosome isolation protocol with high purity to isolate exosomes from novel  
 28 identified stem cells called CB-SC for mechanistic studies. We also coculture CB-SC-derived  
 29 exosomes with human monocytes, leading to their differentiation into phenotypically distinct  
 30 macrophages.

31

32 **ABSTRACT:**

33 Stem Cell Educator (SCE) therapy is a novel clinical approach for the treatment of type 1 diabetes  
 34 and other autoimmune diseases. SCE therapy circulates the isolated patient's blood mononuclear  
 35 cells (e.g., lymphocytes and monocytes) through an apheresis machine, co-cultures the patient's  
 36 blood mononuclear cells with adherent cord blood-derived stem cells (CB-SC) in the SCE device  
 37 and then returns these "educated" immune cells to the patient's blood. Exosomes are nano-sized  
 38 extracellular vesicles between 30–150 nm existing in all biofluid and cell culture media. To further  
 39 explore molecular mechanisms underlying SCE therapy and determine the actions of exosomes  
 40 released from CB-SC, we investigate which cells phagocytize these exosomes during the  
 41 treatment with CB-SC. By co-culturing Dio-labeled CB-SC-derived exosomes with human  
 42 peripheral blood mononuclear cells (PBMC), we found that CB-SC-derived exosomes were  
 43 predominantly taken up by human CD14-positive monocytes, leading to the differentiation of  
 44 monocytes into type 2 macrophages (M2), with spindle-like morphology and expression of M2-

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50 associated surface molecular markers. Here, we present a protocol for the isolation and  
51 characterization of CB-SC-derived exosomes and the protocol for the co-culture of CB-SC-derived  
52 exosomes with human monocytes and the monitoring of M2 differentiation.

53

#### 54 **INTRODUCTION:**

55 Cord blood stem cells (CB-SC) are a unique type of stem cell identified from human cord blood and  
56 are distinguished from other known types of stem cells such as mesenchymal stem cells (MSC)  
57 and hematopoietic stem cells (HSC)<sup>1</sup>. Based on their unique properties of immune modulation  
58 and their ability to tightly adhere to the surface of petri dishes, we developed a new technology  
59 designated as Stem Cell Educator (SCE) therapy in clinical trials<sup>2,3</sup>. During SCE therapy, a patient's  
60 peripheral blood mononuclear cells (PBMC) are collected and circulated through a cell separator  
61 and co-cultured with adherent CB-SC in vitro. These "educated" cells (CB-SC-treated PBMC) are  
62 then returned to the patient's circulation in a closed-loop system. Clinical trials have already  
63 demonstrated the clinical safety and efficacy of SCE therapy for the treatment of autoimmune  
64 diseases including type 1 diabetes (T1D)<sup>2,4</sup> and alopecia areata (AA)<sup>5</sup>.

65

66 Exosomes are a family of nanoparticles with diameters ranging 30–150 nm and exist in all biofluid  
67 and cell culture media<sup>6</sup>. Exosomes are enriched with many bioactive molecules including lipids,  
68 mRNAs, proteins and microRNAs (miRNA), and play an important role in cell to cell  
69 communications. Of late, exosomes have become more attractive for researchers and  
70 pharmaceutical companies due to their therapeutic potentials in clinics<sup>7-9</sup>. Recently, our  
71 mechanistic studies demonstrated that CB-SC-released exosomes contribute to the immune  
72 modulation of SCE therapy<sup>10</sup>.

73

74 Here, we describe the protocol to explore the mechanism of SCE therapy through targeting  
75 monocytes by CB-SC-released exosomes. First, CB-SC-released exosomes were isolated from CB-  
76 SC-derived conditioned media using ultracentrifugation methods and validated by flow  
77 cytometry, western blot (WB) and dynamic light scattering (DLS). Second, CB-SC-derived  
78 exosomes were labeled with a green fluorescent lipophilic dye: Dio. Third, they were co-cultured  
79 with PBMC to examine the positive percentages of Dio-labeled CB-SC-derived exosomes at the  
80 different subpopulations of PBMC by flow cytometry. This protocol provides a guidance to study  
81 the action of exosomes underlying the immune modulation of stem cells.

82

#### 83 **PROTOCOL:**

84 The protocol follows the guidelines of institutional human research ethics committee at Center  
85 for Discovery and Innovation, Hackensack Meridian Health. Human buffy coat blood units were  
86 purchased from the New York Blood Center (New York, NY). Human umbilical cord blood units  
87 were collected from healthy donors and purchased from Cryo-Cell International blood bank  
88 (Oldsmar, FL). Both New York Blood Center and Cryo-Cell have received all accreditations for  
89 blood collections and distributions, with IRB approval and signed Consent Forms from donors.

90

#### 91 **1. Cell culture and preparation of CB-SC-derived conditioned medium**

92

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94 1.1. Transfer 25 mL cord blood (See **Table of Materials**) over 20 mL of density gradient medium  
95 ( $\gamma = 1.077$ ) into a 50 mL conical tube.  
96  
97  
98 1.2. Centrifuge at 1,690 x *g* for 20 min at 20 °C in a swinging-bucket rotor without brake.  
99  
100 1.3. Carefully transfer the mononuclear cell layer (buffy coat) to a new 50 mL conical tube. Fill  
101 the conical tube with phosphate buffered saline (PBS) to 40 mL. Mix and centrifuge to pellet cells  
102 at 751 x *g* for 10 min at 20 °C.  
103  
104 1.4. Discard the supernatant and add 15 mL of ACK lysis buffer (See **Table of Materials**) to the  
105 cell pellet. Re-suspend cells through pipetting. Then incubate for 10 min at room temperature.  
106  
107 NOTE: This step removes the red blood cells.  
108  
109 1.5. Fill the conical tube with 25 mL of PBS. Centrifuge at 751 x *g* for 5 min and discard  
110 supernatant to obtain pelleted mononuclear cells.  
111  
112 1.6. Wash 2x with 40 mL of PBS to remove the remaining lysis buffer.  
113  
114 1.7. Centrifuge at 751 x *g* for 5 min to pellet the cells.  
115  
116 1.8. Discard supernatant and re-suspend cord blood mononuclear cells with 10 mL of chemical-  
117 defined serum-free medium (See **Table of Materials**) per tube.  
118  
119 1.9. Combine cord blood mononuclear cells to one tube.  
120  
121 1.10. Take 20  $\mu$ L cell suspension and mix with 20  $\mu$ L of 0.4% trypan blue solution (See **Table of**  
122 **Materials**) in 1.5 mL tube.  
123  
124 1.11. Load into the chamber slide and quantify cell number and cell viability with an automated  
125 cell counter.  
126  
127 NOTE: Cell suspension is diluted at 1:10 if cell concentration is above  $1 \times 10^7$  cells/mL.  
128  
129 1.12. Seed mononuclear cells in 150 mm x 15 mm Petri dishes at  $1 \times 10^6$  cells/mL, 25 mL/dish in  
130 chemical-defined serum-free cell culture medium.  
131  
132 1.13. Incubate at 37 °C under 8% CO<sub>2</sub> conditions for 10–14 days until CB-SC reach more than 80%  
133 confluence.  
134  
135 1.14. Discard the supernatant and wash with 15 mL PBS per Petri dish, then remove the PBS.  
136  
137 NOTE: CB-SC attached to Petri dishes tightly.

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1.15. Repeat the step 1.14 two times.

1.16. Add 25 mL of chemical-defined serum free medium per Petri dish.

1.17. Incubate at 37 °C under 8% CO<sub>2</sub> conditions for 3–4 days.

1.18. Collect the CB-SC-derived conditioned medium into 50 mL conical tubes.

## 2. Characterization of CB-SC

2.1. Detach CB-SC by pipetting 10 mL of PBS-based cell dissociation buffer up and down with a 5 mL pipette tip (see **Table of Materials**).

2.2. Centrifuge at 1,690 x *g* for 5 min to pellet cells and resuspend in 200 µL of PBS.

2.3. Fix and permeabilize cells for intracellular staining via staining preparation kit (see **Table of Materials**).

2.4. Add 5 µL of Fc blocker (see **Table of Materials**) per sample and incubate for 15 min at room temperature.

2.5. Add fluorescence-conjugated mouse anti-human monoclonal antibodies including CD34, CD45, SOX2, OCT3/4, CD270, and Galectin 9 at 25 µg/mL (see **Table of Materials**) to 100 µL volume of cells. Incubate for 30 min at room temperature with light protection.

2.6. After staining, wash cells with 1 mL of PBS and centrifuge at 751 x *g* for 10 min to pellet cells.

2.7. Re-suspend cells with 200 µL of PBS and transfer into a 5 mL tube.

2.8. Perform flow cytometry to validate the expression of CB-SC-associated above specific markers.

## 3. Isolation of CB-SC-derived exosomes

3.1. Centrifuge the conditioned medium collected from step 1.18. at 300 x *g* at 4 °C for 10 min. Transfer the supernatant to a new 50 mL conical tube.

3.2. Centrifuge the supernatant collected from step 3.1 at 2,000 x *g* at 4 °C for 20 min. Transfer the supernatant to a new 50 mL conical tube.

3.3. Centrifuge the supernatant collected from step 3.2 for 30 min at 10,000 x *g* at 4 °C. Transfer the supernatant to a new 50 mL conical tube.



182 NOTE: The fixed angle rotor is used so that the cell pellets are precipitated to the side of the tube.  
183 Mark the side of the cap and draw a circle on the side of the tube where the pellet is expected.

184 3.4. Filter supernatants collected from step 3.3 with a 0.22  $\mu\text{m}$  filter (see **Table of Materials**).

186 3.5. Transfer 15 mL of media to each 10 kDa centrifugal filter unit (see **Table of Materials**).

188 3.6. Centrifuge at 4,000  $\times g$  for 30 min to isolate the concentrated exosome media.

190 3.7. Transfer concentrated exosomes to ultracentrifuge tube. Then, pellet exosomes at 100,000  
191  $\times g$  for 80 min at 4  $^{\circ}\text{C}$ .

193 3.8. Discard the supernatant and re-suspend the pellet exosomes in 10 mL of PBS.

194 3.9. Centrifuge at 100,000  $\times g$  for 80 min at 4  $^{\circ}\text{C}$  to collect exosomes pellet.

196 3.10. Re-suspend exosomes pellet in 200  $\mu\text{L}$  of PBS by pipetting up and down.

#### 199 4. Characterization of CB-SC-derived exosomes

201 4.1. Quantifying total protein concentration of exosome preparation by bicinchoninic acid  
202 assay (BCA) kit

204 4.1.1. Pipette 10  $\mu\text{L}$  of each albumin standard and isolated exosome sample prepared in 3.10.  
205 into a 96-well plate in duplicate.

207 4.1.2. Add 200  $\mu\text{L}$  of the working reagent from the BCA kit to each well. Mix contents of the plate  
208 thoroughly on the plate shaker for 10 s.

209 NOTE: Working reagent (WR): 50-part reagent A with 1-part reagent B.

211 4.1.3. Cover the plates with foil and incubate them at 37  $^{\circ}\text{C}$  for 30 min.

213 4.1.4. Cool plates to room temperature (RT).

215 4.1.5. Measure sample absorbance at 562 nm via a plate reader.

217 4.2. Preparation and staining of exosomes for flow cytometry

219 4.2.1. Capture exosomes by adding 20  $\mu\text{L}$  of anti-human CD63 magnetic beads (4.5  $\mu\text{m}$  size) (see  
220 **Table of Materials**) into 25  $\mu\text{g}$  of CB-SC-derived exosomes prepared in step 3.10. in total 100  $\mu\text{L}$   
221 volume of PBS.

223 4.2.2. Incubate the tube overnight (18–22 h) at 4  $^{\circ}\text{C}$  on the shaker at 800 rpm.

226  
227 4.2.3. Centrifuge the tube at 300 x *g* for 30s to collect the sample at the bottom of the tube.  
228  
229 4.2.4. Add 300  $\mu$ L of isolation buffer (0.1% bovine serum albumin (BSA) in PBS) and mix gently by  
230 pipetting.  
231  
232 NOTE: This step washes the bead-bound exosomes.  
233  
234 4.2.5. Place the tube on a magnet stand for 1 min (see **Table of Materials**) and discard the  
235 supernatant.  
236  
237 4.2.6. Repeat steps 4.2.4–4.2.5.  
238  
239 4.2.7. Re-suspend the bead-bound exosomes with 400  $\mu$ L of isolation buffer.  
240  
241 4.2.8. Aliquot 100  $\mu$ L of bead-bound exosomes to each tube.  
242  
243 4.2.9. Add fluorescence-conjugated antibodies (CD9-FITC, CD81-PE and CD63-FITC at 25  $\mu$ g/mL  
244 respectively) to each flow tube with CD63 bead-captured exosomes.  
245  
246 NOTE: Isotype-matched IgGs serve as negative control.  
247  
248 4.2.10. Incubate for 45 min at room temperature with light protection on shaker at 800 rpm.  
249  
250 4.2.11. Repeat steps 4.2.4–4.2.5.  
251  
252 4.2.13. Re-suspend the bead-bound exosomes in 200  $\mu$ L of isolation buffer and transfer to 5 mL  
253 flow tubes.  
254  
255 4.2.15. Place the tubes in the sample carousel of the flow cytometer.  
256  
257 4.2.16. Open the protocol for the exosome testing.  
258  
259 4.2.17. Run the sample automatically by flow cytometer.  
260  
261 **4.3. Exosome detection by western blot**  
262  
263 NOTE: Western blot is a well-established method and we will not go into detail for the method  
264 itself.  
265  
266 4.3.1. Lyse the pellets of CB-SC-derived exosomes from step 3.9. with 100  $\mu$ L of RIPA buffer,  
267 pipette 20x, then place on ice for 5 min.  
268

269 4.3.2. Quantify the protein concentration of exosome lysate by BCA kit and load 25 µg of protein  
270 per well.  
271

272 4.3.3. Separate protein by gel electrophoresis for 40 min at 150 V.  
273

274 4.3.4. Transfer the protein to polyvinylidene fluoride (PVDF) membrane by semi-dry transferring  
275 method<sup>11</sup>.  
276

277 4.3.5. Block the membrane with 5% non-fat milk for 30 min.  
278

279 4.3.6. Incubate with 2 µg/mL anti-human ALIX (see **Table of Materials**) and 1 µg/mL anti-human  
280 CALNEXIN antibodies (see **Table of Materials**).  
281

282 4.3.7. Detect the protein by chemiluminescence with a digital imaging system.  
283

284 **4.4. Exosome validation by dynamic light scattering (DLS)**  
285

286 4.4.1. Dilute 10 µg of CB-SC-derived exosome samples in 1 mL of PBS.  
287

288 4.4.2. Transfer the 1 mL of diluted sample into disposable semi-micro cuvette (see **Table of**  
289 **Materials**).  
290

291 4.4.3. Place the cuvette in the DLS instrument. Set the refractive index (RI) as 1.39 for all the  
292 sample monitor.  
293

294 4.4.4. Run samples at 25 °C and acquire three measurements per fraction to get an average size  
295 distribution.  
296

297 **4.5. Exosome validation by transmission electron microscopy (TEM)**  
298

299 4.5.1. Coat formvar on 300 mesh copper grids <sup>12</sup>(see **Table of Materials**).  
300

301 4.5.2. Strengthen the formvar with the additional layer of evaporated carbon on copper grids<sup>12</sup>.  
302

303 NOTE: Such coating approach is excellent for specimen support.  
304

305 4.5.3. Load 10 µL of exosome samples onto grids and leave to air dry.  
306

307 4.5.4. Negatively stain samples with uranyl acetate for 5 min.  
308

309 4.5.5. Wash three times with DI water and leave to air dry.  
310

311 4.5.6. Observe and photograph the samples under TEM. Set the accelerating voltage at 200 kV  
312 and spot size at 2.

313  
314 **5. Measure Dio-labeled CB-SC-derived exosomes up taken by different subpopulation of PBMC**  
315  
316 **5.1. Label CB-SC-derived exosomes with green fluorescent lipophilic dye Dio**  
317  
318 5.1.1. Transfer 100 µg of CB-SC-derived exosomes (prepared in step 3.10) into a 15 mL centrifuge  
319 tube.  
320  
321 5.1.2. Dilute sample with PBS to 5 mL.  
322  
323 5.1.3. Add green fluorescent lipophilic dye Dio (see **Table of Materials**) until working  
324 concentration reaches 5 µM.  
325  
326 5.1.4. Incubate for 15 min at room temperature protected from light.  
327  
328 5.1.5. Transfer the sample into an ultracentrifuge tube.  
329  
330 5.1.6. Centrifuge at 100,000 x g for 80 min to pellet Dio-labeled CB-SC-derived exosomes.  
331  
332 5.1.7. Re-suspend the labeled exosomes in 200 µL of PBS.  
333  
334 **5.2. Preparation of human PBMC**  
335  
336 5.2.1. Transfer 25 mL of human buffy coat (See **Table of Materials**) over 20 mL of density gradient  
337 medium ( $\gamma = 1.077$ ) into a 50 mL conical tube.  
338  
339 5.2.2. Repeat step 1.2. to 1.11.  
340  
341 5.2.3. Transfer  $1 \times 10^6$  PBMC into non-tissue-treated hydrophobic 24-well plate (1 mL/well).  
342  
343 NOTE: Non-tissue-treated plate was utilized to avoid adhering of monocytes.  
344  
345 **5.3. Co-culture Dio-labeled exosomes with PBMC**  
346  
347 5.3.1. Transfer 40 µL Dio-labeled CB-SC-derived exosomes prepared in 5.1.7 to each PBMC-  
348 containing well in a 24-well plate using a 200 µL pipette. Add the same volume of PBS to control  
349 wells.  
350  
351 5.3.2. Mix by pipetting 10x. Incubate for 4 h.  
352  
353 5.3.3. Collect 200 µL exosome-treated PBMC and label with Hoechst 33342 for 10 min at room  
354 temperature.  
355 ▲

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357 5.3.4. Centrifuge at 300 x g for 10 min at room temperature. Discard the supernatant and re-  
358 suspend the cell pellet in 100 µL of PBS.

359 5.3.5. Mount cells onto microscope slides.

360 5.3.6. Observe and photograph the interaction of Dio-labeled CB-SC-derived exosomes with  
361 Hoechst 33342-labeled PBMC by using microscope.

362 5.3.7. Transfer remaining cells from step 5.3.3 into 1.5 mL tube.

363 5.3.8. Centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant and re-suspend the cell  
364 pellet in 200 µL of PBS.

365 5.3.9. Add 5 µL of Fc blocker (see Table of Materials) per sample. Incubate for 15 min at room  
366 temperature.

367 NOTE: Fc blocker inhibits non-specific binding when staining with antibodies.

368 5.3.10. Add antibodies (CD3, CD4, CD8, CD11c, CD14, CD19, CD56 at 25 µg/mL) (see Table of  
369 Materials) to stain PBMC.

370 NOTE: Isotype-matched IgGs serve as negative control.

371 5.3.11. Incubate for 30 min at room temperature with light protection.

372 5.3.12. Add 1 mL of PBS and centrifuge at 300 x g for 10 min at 4 °C to pellet the cells.

373 5.3.13. Re-suspend the cells with 200 µL PBS. Add 5 µL of propidium iodide.

374 5.3.14. Use flow cytometry evaluate the level of Dio-labeled exosome uptake in different  
375 subpopulation of PBMC.

## 376 **6. Examine the action of CB-SC-derived exosomes on monocytes**

### 377 **6.1. Isolation human CD14-positive monocytes**

378 6.1.1. Transfer 3 x 10<sup>7</sup> human PBMC into a 15 mL tube.

379 6.1.2. Centrifuge at 300 x g for 10 min at 4 °C.

380 6.1.3. Place the separation column (see Table of Materials) in the magnet separator (see Table  
381 of Materials)

382 6.1.4. Wash separation columns three times with 2 mL cold running buffer (see Table of  
383 Materials).

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406  
407 6.1.5. Re-suspend the cells in 300  $\mu$ L of cold PBS. Add 60  $\mu$ L of CD14 microbeads. Mix well and  
408 incubate on ice for 15 min.  
409  
410 6.1.6. Add 6 mL of cold PBS. Centrifuge at 300 x *g* for 10 min at 4 °C.  
411  
412 6.1.7. Re-suspend the pelleted cells in 500  $\mu$ L of cold running buffer.  
413  
414 6.1.8. Transfer cells into the separation column (prepared in step 6.14) and let them pass  
415 through.  
416  
417 6.1.9. Wash the separation column three times with 2 mL of running buffer per wash. Lift the  
418 column from magnet separator and place it in a 15 mL centrifuge tube.  
419  
420 NOTE: The 15 mL tube should be placed on ice due to the adherence of CD14-positive monocytes  
421 to the tube at room temperature.  
422  
423 6.1.10. Transfer 2 mL of cold running buffer to the top of column then isolate the CD14-positive  
424 cells into the 15 mL tube.  
425  
426 6.1.11. Centrifuge at 300 x *g* for 10 min at 4 °C to pellet the CD14-positive cells.  
427  
428 6.1.12. Re-suspend the cells with 2 mL of cold chemical-defined serum free medium (see **Table**  
429 **of Materials**).  
430  
431 6.1.13. Transfer 50  $\mu$ L of cells into a 1.5 mL tube.  
432  
433 6.1.14. Stain with 10  $\mu$ L of Krome Orange-conjugated anti-human CD14 mAb (see **Table of**  
434 **Materials**) for 20 min.  
435  
436 NOTE: Isotype-matched IgGs serve as negative controls.  
437  
438 6.1.15. Add 1 mL PBS to the cells. Centrifuge at 300 x *g* for 10 min to pellet cells.  
439  
440 6.1.16. Re-suspend cells in 200  $\mu$ L of PBS and transfer to 5 mL tube. Determine the purity of CD14-  
441 positive monocytes by flow cytometry.  
442  
443 **6.2. Treatment of monocytes with CB-SC-derived exosomes**  
444  
445 6.2.1. Seed 1 x 10<sup>6</sup> purified monocytes with chemical-defined serum free culture medium (see  
446 **Table of Materials**) in tissue culture-treated 6-well plate (2 mL/well).  
447  
448 6.2.2. Incubate for 2 h at 37 °C under 5% CO<sub>2</sub>.  
449

450 6.2.3. Discard the supernatant with 1 mL pipette. Add 2 mL of 37 °C pre-warmed chemical-  
451 defined serum free culture medium (see **Table of Materials**) gently.

452

453 NOTE: Monocytes were adhered to the plate within 2 h. Floating cells were identified as dead or  
454 other cell contaminations.

455

456 6.2.4. Add 80 µg CB-SC-derived exosomes isolated from step 3.10 to monocyte cultures in a 6-  
457 well plate with total volume of 2 mL.

458

459 NOTE: The same volume of PBS was added to control wells.

460

461 6.2.5. Incubate at 37 °C under 5% CO<sub>2</sub> for 3–4 days.

462

463 6.2.6. Photograph the cell morphology using an inverted microscope at 200× magnification (see  
464 **Table of Materials**).

465

466 6.2.7. Detach cells by pipetting up and down in 1 mL of a PBS based cell dissociation buffer with  
467 1 mL pipette tip.

468

469 6.2.8. Harvest the remaining attached cells via a cell scraper.

470

471 NOTE: Since primary monocytes or differentiated macrophages attach tightly, some cells remain  
472 adhered to the bottom after the treatment with dissociation buffer. Therefore, these cells are  
473 harvested with a cell scraper.

474

475 6.2.9. Collect cells at 1,690 x g for 5 min. Re-suspend cells in 200 µL of PBS.

476

477 6.2.10. Add 5 µL of Fc blocker (25 µg/mL) to block non-specific binding.

478

479 6.2.11. Add antibodies (CD14, CD80, CD86, CD163, CD206, and CD209 at 25 µg/mL, see **Table of**  
480 **Materials**) to cells. Incubate for 30 min at room temperature.

481

482 NOTE: Isotype-matched IgGs serve as negative control

483

484 6.2.12. Add 1 mL of PBS to cells and centrifuge at 300 x g for 10 min. Discard the supernatant and  
485 re-suspend with 200 µL of PBS.

486

487 6.2.13. Add 5 µL of propidium iodide per sample (200 µL) and transfer cells to new 5 mL flow  
488 tube.

489

490 6.2.14. Perform the flow cytometry and evaluate the levels of CD14, CD80, CD86, CD163, CD206,  
491 and CD209 expressions.

492

493 **REPRESENTATIVE RESULTS:**

494 Initially, the phenotype and purity of CB-SC were examined by flow cytometry with CB-SC-  
495 associated markers such as leukocyte common antigen CD45, ES cell-specific transcription factors  
496 OCT3/4 and SOX2. CB-SC display high levels of CD45, OCT3/4, SOX2, CD270, and galectin 9  
497 expression, but no expression of CD34 expression (Figure 1A). Flow cytometry analysis confirmed  
498 the expression of exosome-specific markers including CD9, CD81 and CD63 were on CB-SC-  
499 derived exosomes (Figure 1B). Morphology and size distribution of exosomes were characterized  
500 by TEM and DLS (Figure 1C, D), with the size of  $85.95 \pm 22.57$  nm. Western blot further proved  
501 the expression of the exosome-associated marker Alix, without expression of the ER-associated  
502 marker Calnexin (Figure 1E).

503  
504 PBMC were treated with Dio-labeled CB-SC-Exo. The microscopy observation demonstrated the  
505 direct interaction of Dio-labeled CB-SC-Exo with PBMC (Figure 2A). To better define which cell  
506 population interacted with the Dio-labeled CB-SC-Exo, different cell compartments were gated  
507 with cell-specific markers such as CD3 for T cells, CD11c for myeloid dendritic cells (DC), CD14 for  
508 monocytes, CD19 for B cells, and CD56 for NK cells (Figure 2B). After an incubation for 4 hr, flow  
509 cytometry demonstrated that different blood cell compartments displayed at different median  
510 fluorescence intensity (MFI) of Dio-positive exosomes (Figure 2C). Notably, monocytes exhibited  
511 higher median fluorescence intensity of Dio-positive CB-SC-Exo than those of other immune cells  
512 (Figure 2C), highlighting the monocytes were primarily targeted by the CB-SC-derived exosomes.

513  
514 To explore the direct effects of CB-SC-derived exosomes on monocytes, the purified CD14<sup>+</sup>  
515 monocytes were co-cultured with CB-SC-derived exosomes for three days. The exosome-treated  
516 monocyte successfully differentiated into spindle-like morphologies (Figure 3A). Next,  
517 phenotypes of the CB-SC-Exo treated or untreated monocytes were tested, revealing the  
518 expressions of M2-associated markers including CD163, CD206, CD209 were markedly increased  
519 among the exosome-treated group (Figure 3B, red histogram). Comparing with the conventional  
520 M2 macrophages generated by M-CSF + IL-4, CB-SC-Exo-treated monocytes expressed the similar  
521 levels of M2-associated markers such as CD163, CD206, CD209, with no significant differences  
522 (Figure 3C). Therefore, these data indicate that monocytes differentiate into macrophages with  
523 M2 phenotype after the treatment with CB-SC-derived exosomes.

#### 524 525 **FIGURE LEGENDS:**

526  
527 **Figure 1: Characterization of CB-SC-derived exosomes.** (A) Phenotypic characterization of CB-SC,  
528 highly expression of CD45, OCT3/4, SOX2, CD270 and Galectin, no expression of CD34. (B)  
529 Expressions of exosome-associated markers (CD63, CD9, CD81) on CB-SC-derived exosomes.  
530 Isotype-matched IgGs served as control for flow cytometry (gray histogram). (C) Transmission  
531 Electron Microscopy (TEM) image of the CB-SC-derived exosomes. (D) Size distribution of CB-SC-  
532 derived exosomes using Dynamic Light Scattering (DLS). (E) Western blots show that CB-SC-  
533 derived exosomes display the exosome-specific marker Alix, but negative for endoplasmic  
534 reticulum (ER)-associated marker Calnexin.

535  
536 **Figure 2: Interaction of CB-SC-derived exosomes with different population of PBMC.** (A)  
537 The interaction of Dio-labeled CB-SC-Exo (green) with PBMC (blue, nuclear staining with

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544 Hoechst 33342) was photographed with Nikon Eclipse Ti2 microscope with NIS-Elements  
545 software version 5.11.02, with a high magnification showing the distribution of Dio-labeled  
546 exosomes (green) in the PBMC cells after the co-incubation for 4 hours 5% CO<sub>2</sub> in the non-tissue  
547 culture-treated 24-well plate, *n* = 2. (B) Gating strategy for flow cytometry analysis with cell-  
548 specific surface markers for different subpopulation in PBMC, including CD3 for T cells, CD14  
549 for monocytes, CD19 for B cells, CD56 for NK cells and CD11c for DCs. (C) Display different  
550 median fluorescence intensity (MFI) of Dio-labeled exosome among different PBMC  
551 subpopulations (e.g., T cells, Monocytes, B cells, NK cells, DCs).

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552  
553 **Figure 3; Effects of CB-SC-derived exosomes on monocytes.** (A) Morphological change of  
554 monocytes into the spindle-like cells after treatment with CB-SC-derived exosomes. (B) Up-  
555 regulated the level of M2-associate markers' expression after the treatment with CB-SC-derived  
556 exosomes, such as CD163, CD206 and CD209 (red line). Untreated monocytes (green line) served  
557 as control. Isotype-matched IgG served as negative control (gray line). (C) Phenotypic comparison  
558 between conventional M2 macrophages and the CB-SC-Exo-induced M2 macrophages. To  
559 generate the conventional M2 macrophages, the purified CD14<sup>+</sup> monocytes were treated with  
560 50 ng/mL macrophage colony-stimulating factor (M-CSF) at 37 °C, 5% CO<sub>2</sub> conditions for 7 days,  
561 and followed by the overnight treatment with 10 ng/mL IL-4. M2-associated markers including  
562 CD14, CD80, CD86, CD163, CD206, and CD209 were evaluated by flow cytometry. Isotype-  
563 matched immunoglobulin G (IgG) serve as control. The data are presented as mean + SD; N = 3.

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#### 565 **DISCUSSION:**

566 Application of exosomes is emerging field for clinical diagnosis, drug developments and  
567 regenerative medicine. Here, we present a detailed protocol regarding the preparation of CB-SC-  
568 derived exosomes and the functional study of exosomes on the differentiation of human  
569 monocytes. Current protocol demonstrated that functional CB-SC-derived exosomes are isolated  
570 by sequential centrifugation and ultracentrifugation with high purity and exhibiting the immune  
571 modulation on monocytes.

572  
573 Comparing with other conventional protocols, ultrafiltration is an established approach for the  
574 isolation and purification of exosomes from different cells or media, based upon the molecular  
575 weight and exclusion sizes that are different from other extracellular vesicles (EVs). While  
576 ultrafiltration isolation is more time-saving than the ultracentrifugation-based separation, it may  
577 cause structural damage to vesicles at large sizes. Exosomes can also be collected by polyethylene  
578 glycol (PEG)-mediated precipitation at low cost, though this method risks the exosome purity due  
579 to the protein contamination<sup>13,14</sup>. Therefore, the current protocol was cost-effective to produce  
580 exosomes at high purity. Based on the immune modulations of CB-SC-derived exosomes<sup>10</sup>,  
581 characterization of CB-SC-derived exosomes may offer a valuable biomarker to evaluate the  
582 potency of Stem Cell Educator with CB-SC before clinical applications.

583  
584 Macrophages are professional antigen-presenting cells against viral and bacterial infections, with  
585 varied biological functions and heterogeneities. Based on their differences in surface markers  
586 and immune function, macrophages are categorized with two sub-populations: type 1

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589 macrophages (M1, conventional macrophages causing inflammation) and type 2 macrophages  
590 (M2, displaying anti-inflammation)<sup>15</sup>. This study established that purified human monocytes were  
591 differentiated into type 2 macrophages after the treatment with CB-SC-derived exosomes,  
592 displaying an anti-inflammation phenotype<sup>10</sup>. CB-SC-derived exosome-treated monocytes  
593 exhibited the elongated morphology and expressed the M2-associated surface markers (e.g.,  
594 CD163, CD206 and CD209), with the similar phenotype as the conventional M2 macrophages  
595 generated by using cytokines M-CSF + IL-4. Such phenotypic changes of monocytes highlight the  
596 new mechanism underlying the immune modulation of CB-SC for the treatment of type 1  
597 diabetes and other autoimmune diseases. During the SCE therapy, patient's immune cells were  
598 co-cultured with CB-SC around 8–9 h. The SCE-treated monocytes carried the CB-SC-derived  
599 exosomes back into the body, which contributed to the M2 differentiation and the expansion of  
600 the induction of immune tolerance, leading to the improvement of clinical outcomes after the  
601 treatment with SCE therapy.

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#### 606 **DISCLOSURES:**

608 Dr. Zhao is a founder of Tianhe Stem Cell Biotechnology Inc. Dr. Zhao is an inventor of Stem Cell  
609 Educator technology. All other authors have no financial interests that may be relevant to the  
610 submitted work.

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Dear Dr. Nguyen,

Please find our revised manuscript (JoVE61562R3), titled “Differentiation of monocytes into phenotypically distinct macrophages after the treatment with human cord blood stem cell (CB-SC)-derived exosomes”. We appreciate editorial and reviewers’ comments that are very helpful for us to improve the quality of this manuscript. We have revised the manuscript according to editorial and peer reviewer’s comments, with tracking changes. Please feel free to let me know if you have additional comments. Many thanks for this invitation again and give us this opportunity to publish our work in JoVE.

We appreciate your kind consideration.

Yours sincerely,

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