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Isolation of Exosome-enriched Extracellular Vesicles Carrying Granulocyte-macrophage Colony-stimulating Factor from Embryonic Stem Cells

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Alisha DSouza, Ph.D.
Senior Review Editor
JoVE,

Dear Dr. DSouza,

We are re-submitting the manuscript “Isolation of Exosome-enriched Extracellular Vesicles Carrying Granulocyte-macrophage Colony-stimulating Factor from Embryonic Stem Cells”. The comments of the reviewers, as well as your suggestions, were very helpful in improving the manuscript. We have made many changes in the manuscript to address these points, which I believe have markedly strengthened the manuscript. The detailed responses to each of comments are described in a separate rebuttal letter.

If there are any questions about the manuscript, please let me know. Your kind help is deeply appreciated.

Sincerely yours,

Chi Li

TITLE:

Isolation of Exosome-Enriched Extracellular Vesicles Carrying Granulocyte-Macrophage Colony-Stimulating Factor from Embryonic Stem Cells

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KEYWORDS:

exosome, extracellular vesicle, embryonic stem cell, GM-CSF, immune-stimulatory, cancer

SUMMARY:

This study describes a method to isolate exosome-enriched extracellular vesicles carrying immune-stimulatory granulocyte macrophage colony-stimulating factors from embryonic stem cells.

ABSTRACT:

Embryonic stem cells (ESCs) are pluripotent stem cells capable of self-renewal and differentiation into all types of embryonic cells. Like many other cell types, ESCs release small membrane vesicles, such as exosomes, to the extracellular environment. Exosomes serve as essential mediators of intercellular communication and play a basic role in many (patho)physiological processes. A granulocyte-macrophage colony-stimulating factor (GM-CSF) functions as a cytokine to modulate the immune response. The presence of GM-CSF in exosomes has the potential to boost their immune-regulatory function. Here, a GM-CSF was stably overexpressed in the murine ESC cell line ES-D3. A protocol was developed to isolate high-quality exosome-enriched

extracellular vesicles (EVs) from ES-D3 cells overexpressing GM-CSF. Isolated exosome-enriched EVs were characterized by a variety of experimental approaches. Importantly, significant amounts of GM-CSF were found to be present in exosome-enriched EVs. Overall, GM-CSF-bearing exosome-enriched EVs from ESCs might function as cell-free vesicles to exert their immune-regulatory activities.

INTRODUCTION:

ESCs are derived from the blastocyst stage of a preimplantation embryo[1]. As pluripotent stem cells, ESCs have the capability to self-renew and differentiate into any type of embryonic cell. Due to their remarkable developmental potential and long-term proliferative capacity, ESCs are extremely valuable for biomedical research[1]. Current research efforts have largely focused on the therapeutic potential of ESCs for a variety of major pathological disorders, including diabetes, heart disease, and neurodegenerative diseases[2-4].

Mammalian cells, including ESCs, are known to release vesicles with variable sizes to the extracellular environment, and these EVs possess many physiological and pathological functions due to their role in intercellular communication[5]. Among different subtypes of EVs, exosomes are small membrane vesicles released from various cell types into the extracellular space upon fusion of intermediate endocytic compartments, multivesicular bodies (MVBs), with the plasma membrane[6]. Exosomes have been reported to mediate intercellular communication and are critically involved in many (patho)physiological processes[7,8]. Exosomes inherit some biological functions from their own parental cells, because exosomes contain biological materials acquired from the cytosol, including proteins and nucleic acids. Thus, the associated antigens or factors stimulating the immune response specific for a given disease are encapsulated in the exosomes from particular types of cells[9]. This paved the way for clinical trials exploring tumor-derived exosomes as an anti-cancer vaccine[10].

A GM-CSF is a cytokine secreted by different types of immune cells[11]. Emerging evidence demonstrates that GM-CSF activates and regulates the immune system and plays an essential role in the antigen-presenting process[12]. For instance, a clinical report suggests that GM-CSF stimulates the immune response to tumors as a vaccine adjuvant[13]. Several GM-CSF-based cancer immunotherapy strategies to exploit the potent immune-stimulatory activity of GM-CSF have been investigated in clinical trials[14]. Among these, a cancer vaccine composed of irradiated GM-CSF-secreting tumor cells has shown some promise in advanced melanoma patients by inducing cellular and humoral antitumor responses and subsequent necrosis in metastasized tumors[15].

Because the exosomes derived from ESCs possess similar biological activities as the original ESCs, maybe GM-CSF-carrying exosomes from ESCs could function as cell-free vesicles to regulate the immune response. In this paper, a detailed method to produce high-quality exosome-enriched EVs from ESCs expressing GM-CSF is described. These exosome-enriched EVs have the potential to serve as immune-regulatory vesicles to modulate the immune response.

PROTOCOL:

1. Culturing ES-D3 cells

1.1. To generate exosome-free fetal bovine serum (FBS), load FBS into an ultracentrifuge and centrifuge at 100,000 x *g* for 16 h at 4 °C. Following centrifugation, collect serum supernatant as exosome-free FBS for culturing the murine ESC cell line ES-D3 and acquiring exosome-enriched EVs.

1.2. Before plating the ES-D3 cells, coat 15 cm tissue culture dishes using gelatin (0.1%) at room temperature for 30 min.

1.3. Following a previously described protocol[16], culture the ES-D3 cells without feeder layer cells in the gelatin-coated 15 cm tissue culture dishes. The ES-D3 cell culture medium is composed of DMEM, exosome-free FBS (15%), nonessential amino acids (0.1 mM), L-glutamine (2 mM), β-mercaptoethanol (0.1 mM), penicillin (50 units/mL), streptomycin (50 µg/mL), and leukemia inhibitory factor (LIF; 100 units/mL). Culture ES-D3 cells at 37 °C in a 5% CO_2 humidified incubator.

1.4. Once the ES-D3 cells reach around 90% confluency in 15 cm tissue culture dishes, remove the medium by aspiration. Wash the cells using trypsin (5 mL; 0.05%). Add trypsin (5 mL) to the dishes and incubate at 37 °C for 5 min. Collect the cells from the dishes.

1.5. Add fresh culture medium (5 mL) to the collected cells to inactivate the trypsin. Centrifuge the cells at 390 x *g* for 5 min. Resuspend the cells in fresh medium and determine the cell number using a hemocytometer.

1.6. For passaging cells, plate the ES-D3 cells (5 x 10^6) in a gelatin-coated (0.1%) 15 cm tissue culture dish with fresh medium (15 mL) and culture for 3 days before subculturing the cells.

1.7. To collect the cell culture supernatant for isolation of exosome-enriched EVs, plate the ES-D3 cells (1 x 10^7) in a gelatin-coated (0.1%) 15 cm tissue culture dish with fresh medium (15 mL) for 3 days prior to collecting cell culture supernatant.

2. Generation of GM-CSF expression plasmid

NOTE: Generate the transfection plasmid pEF1α-mGM-CSF-IRES-hrGFP to overexpress GM-CSF in ES-D3 cells. In this plasmid, expression of murine GM-CSF cDNA along with the marker protein humanized *Renilla reniformis* GFP (hrGFP) is driven by the human polypeptide chain elongation factor 1α (EF1α) promoter[17,18].

2.1. Generate the vector backbone.

2.1.1. Digest the plasmid pEF1α-FD3ER-IRES-hrGFP (20 µg of DNA) using the restriction enzyme EcoRI (100 units) at 37 °C for 2 h to generate two DNA fragments: the vector backbone (6.0 kb) and FD3ER insert (2.5 kb).

2.1.2. Transfer 50% of digested plasmid DNA (10 μg) into one 1.5 mL microcentrifuge tube and treat with alkaline phosphatase (20 units) at 37 °C for 1 h. Resolve both untreated and dephosphorylated DNA using agarose gel electrophoresis (2%).

2.1.3. Purify the vector backbone DNA fragments (6.0 kb) using a DNA gel extraction kit . While the untreated vector backbone will serve as the empty vector (pEF1α-IRES-hrGFP), the dephosphorylated vector backbone will be used to generate GM-CSF-expressing plasmid (pEF1α-mGM-CSF-IRES-hrGFP).

2.2. Generate the GM-CSF cDNA insert.

2.2.1. Digest the plasmid pMSCV-mGM-CSF-IRES-EGFP[19] (20 μg) using the restriction enzyme EcoRI (100 units) at 37 °C for 2 h to produce two DNA fragments: the vector backbone (6.5 kb) and the murine GM-CSF cDNA insert (474 bp).

2.2.2. Resolve the digested DNA through agarose gel electrophoresis (2%). Purify the murine GM-CSF cDNA fragment (474 bp) using a DNA gel extraction kit.

2.3. Generate the expression plasmids.

2.3.1. Set up 2 ligation reactions (10 μL total volume) using a DNA ligation kit.

2.3.1.1. To generate the empty vector pEF1α-IRES-hrGFP, ligate the untreated vector backbone from step 2.1.3 (6.0 kb; 500 ng).

2.3.1.2. To generate pEF1α-mGM-CSF-IRES-hrGFP, ligate the following DNA fragments: (1) the dephosphorylated vector backbone from step 2.1.3 (6.0 kb; 500 ng) and (2) the mGM-CSF cDNA fragment generated in step 2.2.2 (474 bp; 200 ng).

2.3.2. Ligate at 25 °C for 5 min. Transform ligated DNA into DH5α competent *E. coli* cells. Plate the transformed *E. coli* cells on LB-agar plates containing carbenicillin (50 μg/mL).

2.3.3. Purify plasmids from single *E. coli* colonies using a DNA isolation kit. Validate the identities of the plasmids by DNA sequencing.

3. Generation of ES-D3 cells overexpressing GM-CSF

NOTE: Transfect the ES-D3 cells with the plasmid pEF1α-mGM-CSF-IRES-hrGFP to overexpress GM-CS. Cotransfect the plasmid pBabe-Neo into ES-D3 cells to facilitate selection of stably transfected cells[18,20].

3.1. Transfect the plasmids into the ES-D3 cells.

3.1.1. Plate the ES-D3 cells (1.4 x 10^6) in a gelatin-coated (0.1%) 10 cm tissue culture dish with

culture medium (10 mL) for transfection. Culture plated ES-D3 cells at 37 °C for 24 h.

3.1.2. Prepare two plasmid mixtures in 1.5 mL microcentrifuge tubes: (1) pEF1α-IRES-hrGFP (28 μg, vector control) with pBabe-Neo (4 μg) and (2) pEF1α-mGM-CSF-IRES-hrGFP (28 μg, expressing GM-CSF) with pBabe-Neo (4 μg). Carry out transfection using a transfection kit following the manufacturer's protocol.

3.1.3. Add transfection medium (1 mL) and transfection reagent (64 μL) to each tube containing the plasmid mixtures and incubate at room temperature for 5 min.

3.1.4. Add the transfection mixtures to respective 10 cm dishes of ES-D3 cells. Incubate at 37 °C for 5 h.

3.1.5. Replace the medium in 10 cm dishes with fresh culture medium (10 mL). Incubate at 37 °C for 24 h.

3.2. Generate bulk populations of stably transfected ES-D3 cells.

3.2.1. Remove the medium from transfected ES-D3 cells. Wash the cells with trypsin (2 mL; 0.05%). Add trypsin (2 mL) and incubate at 37 °C for 5 min. Transfer the cells to a 15 mL centrifuge tube and add 2 mL of fresh culture medium to neutralize trypsin. Centrifuge at 390 x *g* for 5 min.

3.2.2. Resuspend the transfected cells in fresh culture medium (10 mL). Evaluate the fluorescence intensity of GFP in transfected ES-D3 cells using fluorescence-activated cell sorting (FACS), following manufacturer's protocol.

3.2.3. Transfer the transfected cells into two 10 cm dishes containing fresh culture medium (10 mL). Add neomycin (0.5 mg/mL) to eliminate untransfected cells.

3.2.4. Continue culturing the transfected cells in culture medium containing neomycin (0.5 mg/mL). When the transfected ES-D3 reach 90% confluency, transfer cells to 15 cm tissue culture dishes again. Repeat the procedure for 2 weeks.

3.3. Generate clones of stably transfected ES-D3 cells.

3.3.1. Once bulk populations of stably transfected ES-D3 cells are generated, collect the cells as before. Determine the cell numbers using a hemocytometer. Centrifuge the cells at 390 x *g* for 5 min. Resuspend the cells (1 x 10^7 cells/mL) in fresh culture medium.

3.3.2. Filter the cells through a sterile 40 μm cell strainer. Purify GFP-positive ES-D3 cells using FACS, following manufacturer's protocol.

3.3.3. Plate a single sorted ES-D3 cell into one well of a gelatin-coated (0.1%) 96-well tissue

culture plate containing parental ES-D3 cells (1 x 10^3) in neomycin-free medium (200 µL). Co-culturing transfected ES-D3 cells with their untransfected parental counterparts ensures that stably transfected single ES-D3 cells survive and proliferate as a single clone.

3.3.4. Culture the cells for 48 h, and then add neomycin (0.5 mg/mL) to 96-well plates to eliminate untransfected parental ES-D3 cells.

3.3.5. Continue culturing the GFP-positive ES-D3 cells in 96-well tissue culture plates with medium containing neomycin (0.5 mg/mL) for 1 week. Transfer clonal ES-D3 cell lines to gelatin-coated (0.1%) 6 cm tissue culture dishes with culture medium (5 mL) containing neomycin (0.5 mg/mL) for 1 week.

3.3.6. Determine the intensity of GFP fluorescence in each of transfected ES-D3 cell clones using FACS, following manufacturer's protocol. Select the ES-D3 clones expressing either GM-CSF or the empty vector with high levels of green fluorescence.

3.3.7. Determine the amounts of GM-CSF secreted by ES-D3 cells using a murine GM-CSF ELISA kit, following manufacturer's protocol.

4. Isolation of exosome-enriched extracellular vesicles

4.1. Culture the ES-D3 cells (1 x 10^7) in 15 cm tissue culture dishes for 72 h at 37 °C. Collect the cell culture supernatant. Store collected supernatant at 4 °C up to 1 week to maintain exosomal integrity.

4.2. Centrifuge the cell culture supernatant at 5,000 x *g* for 60 min at 4 °C using a centrifuge to sediment large cell fragments.

4.3. Collect the supernatant and centrifuge at 100,000 x *g* for 90 min at 4 °C using an ultracentrifuge.

4.4. Remove the supernatant. Gently rinse each pellet twice with phosphate-buffered saline (PBS; 1 mL) to remove residual culture supernatant.

4.5. Resuspend each pellet in PBS. Quantify exosome-enriched EVs by their protein content[5].

4.5.1. Measure the protein concentration of exosome-enriched EVs with a bicinchoninic acid (BCA) assay. The expected yield of exosome-enriched EVs from ES-D3 cells is approximately 4 µg protein/mL of cell culture supernatant. Resuspend the exosome-enriched EVs in PBS (protein concentration: ~6 µg/µL). Store the exosome-enriched EVs at -80 °C.

5. Characterization of exosome-enriched extracellular vesicles by transmission electron microscopy

NOTE: Investigate the composition and the structure of the exosome-enriched EVs isolated from ESCs using transmission electron microscopy (TEM)[5].

5.1. Fix the exosome-enriched EVs (3–5 µg/µL) with a final concentration of 2% EM grade paraformaldehyde at room temperature for 2 h.

5.2. Load fixed samples (10 µL) onto copper grids with carbon support film. Incubate the samples with copper grids for 1 min, and then drain the grids with filter paper.

5.3. Stain the grids with a staining solution following manufacturer's protocol.

5.4. Transfer the grids to a piece of filter paper using tweezers. Allow the grids to dry overnight at room temperature.

5.5. Acquire electron microscopy images using a transmission electron microscope (50,000x magnification), following manufacturer's protocol.

6. Evaluation of exosome-enriched extracellular vesicles by western blot analysis

6.1. Prepare whole cell extracts.

6.1.1. Remove the medium from ES-D3 cells cultured in 15 cm dishes. Wash the cells with trypsin (5 mL; 0.05%). Add trypsin (5 mL) to the cells. Incubate at 37 °C for 5 min. Collect the cells and add fresh culture medium (5 mL) to neutralize trypsin. Centrifuge the cells at 390 x *g* for 5 min. Resuspend the cells in PBS.

6.1.2. Determine cell numbers using a hemocytometer. Centrifuge the cells again at 390 x *g* for 5 min. Resuspend the cells in SDS-PAGE loading buffer containing 0.5% SDS (5,000 cells/µL).

6.1.3. Sonicate the samples for 10 s using a sonicator with 10% amplitude (wattage: 500 W; ultrasonic frequency: 20 kHz). Heat the samples at 100 °C for 5 min.

6.2. Prepare the lysates of exosome-enriched EVs.

6.2.1. Resuspend the exosome-enriched EVs in SDS-PAGE loading buffer containing 0.5% SDS at a concentration of 1.2 µg/µL.

6.2.2. Sonicate the samples for 10 s using a sonicator with 10% amplitude (wattage: 500 W; ultrasonic frequency: 20 kHz). Heat the samples at 100 °C for 5 min.

6.3. Detect proteins by Western blot.

6.3.1. Load whole cell extracts (10 µL; 5,000 cells/µL) and exosome-enriched EV lysates (10 µL; 1.2 µg/µL) into each well of a Bis-Tris PAGE gel (4–20%). Transfer proteins onto polyvinylidene

fluoride (PVDF) membranes.

6.3.2. Incubate membranes with appropriate primary and secondary antibodies. Dilute antibodies (at the concentrations indicated below) in blotting buffer containing PBS, Tween-20 (0.2%), and nonfat dry milk (10% w/v).

6.3.2.1. Use the following primary antibodies: anti-Annexin V (200 ng/mL), anti-CD81 (50 ng/mL), anti-Flotillin-1 (200 ng/mL), anti-cytochrome c (100 ng/mL), anti-protein disulfide isomerase (200 ng/mL), anti-GAPDH (33 ng/mL), and anti-Oxphos COX IV-subunit IV (600 ng/mL).

6.3.2.2. Use the following secondary antibodies: peroxidase-conjugated goat anti-rabbit IgG (20 ng/mL) and peroxidase-conjugated goat anti-mouse IgG (20 ng/mL).

6.3.3. Detect proteins using an enhanced chemiluminescence detection kit.

7. Determining GM-CSF concentrations in exosome-enriched extracellular vesicles by ELISA

NOTE: Evaluate the amounts of GM-CSF in exosome-enriched EVs by ELISA using a kit for murine GM-CSF, following manufacturer's protocol with some modifications.

7.1. Coat the ELISA plate with capture antibody. Treat exosome-enriched EVs (0.6 µg) in PBS alone or PBS + 0.05% Tween-20 (100 µL) at room temperature for 30 min. Add treated samples to the coated ELISA plate and incubate at room temperature for 1 h. Wash the plate with PBS alone or PBS + 0.05% Tween-20.

7.2. Add detection antibody to the samples. Incubate at room temperature for 1 h. Wash the plate with PBS alone or PBS + 0.05% Tween-20. Add Avidin-HRP to the samples. Incubate at room temperature for 30 min. Wash the plate with PBS alone or PBS + 0.05% Tween-20.

7.3. Determine the concentrations of GM-CSF in exosome-enriched EVs by measuring the absorbance at 450 nm on a microplate reader.

REPRESENTATIVE RESULTS:

GM-CSF is overexpressed in murine ESCs.

To stably overexpress GM-CSF in ES-D3 cells, murine GM-CSF cDNA was cloned into a transfection vector to generate the expression vector pEF1α-mGM-CSF-IRES-hrGFP (**Figure 1A**). GM-CSF was overexpressed in ES-D3 cells by transfection, and about 20% of transiently transfected ES-D3 cells were GFP-positive. Cell clones stably overexpressing GM-CSF or the empty vector control were acquired by FACS. As shown in **Figure 1B**, the GFP fluorescence intensity of a GM-CSF-expressing ES-D3 cell line or an ES-D3 cell line expressing the empty vector was much higher than that of their parental counterparts. An ELISA assay was carried out to evaluate the GM-CSF concentrations in the cell culture supernatant of different cell lines (**Figure 2**). ES-D3 cells expressing GM-CSF produced markedly higher levels of GM-CSF in the cell culture supernatant than their empty vector control. Furthermore, the amount of GM-CSF generated by GM-CSF-

expressing ES-D3 cells was similar to that of STO fibroblasts expressing GM-CSF, as reported previously[19].

Exosomes are enriched in extracellular vesicles derived from murine ESCs.

Vector control and GM-CSF-expressing ES-D3 cell cultures were expanded, and cell culture supernatant was collected. EVs were isolated after several steps of centrifugation. Single EVs were first evaluated by TEM (**Figure 3**). As shown in the TEM images, isolated EVs contained vesicles of different sizes, which is commonly observed in exosomal preparations[5]. Importantly, the diameters of the individual vesicles were 30–100 nm, consistent with earlier reports describing exosomes[21]. Furthermore, the presence of exosomes in EVs was examined by Western blotting (**Figure 4**). The expression of exosomal markers, including CD81, annexin V, and Flotillin-1, was markedly enhanced in EVs isolated from ES-D3 cells compared with corresponding whole cell extracts (WCE). Importantly, the presence of other subcellular compartment markers in ES-D3-derived EVs was not detected, including (1) the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI), (2) the mitochondrial markers cytochrome c and COX IV-subunit IV, and (3) the cytosolic marker GAPDH. Overall, these data demonstrate that exosomes were highly enriched in EVs derived from ES-D3 cells.

GM-CSF is localized inside exosome-enriched extracellular vesicles isolated from ESCs.

To determine whether exosome-enriched EVs contain GM-CSF molecules, ELISA assay was conducted to evaluate the levels of GM-CSF in exosome-enriched EVs acquired from ES-D3 cells with or without GM-CSF expression (**Figure 5**). To further investigate GM-CSF protein localization within exosome-enriched EVs, the GM-CSF levels were quantitated in exosome-enriched EVs under different washing conditions by ELISA. For this purpose, the detergent Tween-20 (0.05%) was first employed to permeabilize the exosomal membranes, and ELISA assays were carried out in the buffers with or without 0.05% Tween-20. Because Tween-20 is known to reduce protein-protein interactions, the background GM-CSF levels detected in the control EVs were significantly reduced by Tween-20 in the washing buffer. In contrast, GM-CSF levels in the EVs of GM-CSF-expressing cells were significantly increased by Tween-20. These results demonstrate that Tween-20-induced exosomal membrane permeabilization makes GM-CSF molecules inside the vesicles accessible for antibody recognition, providing evidence that the majority of exosomal GM-CSF molecules are localized inside the lumen of isolated vesicles.

FIGURE LEGENDS:

Figure 1. Exogenous GM-CSF is stably overexpressed in ES-D3 cells.

(**A**) The schematic diagram of the plasmid for overexpressing murine GM-CSF in ES-D3 cells, in which an EF1α promoter drives GM-CSF expression and hrGFP serves as an expression marker. (**B**) Fluorescence intensity of GFP in GM-CSF-expressing ES-D3 cells or their empty vector control counterparts was determined by FACS.

Figure 2. ES-D3 cells overexpressing GM-CSF produce high levels of GM-CSF.

GM-CSF concentrations in the medium of the indicated cells were measured by ELISA. The data are presented as mean ± standard deviations (mean ± SD) of three independent ELISA

measurements: **p < 0.001, NS = not significant, ANOVA with Tukey's multiple comparison test.

Figure 3. ES-D3-derived extracellular vesicles are examined by transmission electron microscopy.
Extracellular vesicles were prepared from ES-D3 cells transfected with the plasmid expressing GM-CSF or its empty vector counterpart. Arrows indicate individual vesicles. Scale bar = 100 nm.

Figure 4. Exosomal markers are highly concentrated in extracellular vesicles isolated from ES-D3 cells.
The amounts of markers for exosomes, endoplasmic reticulum (ER), mitochondria, and cytosol in the indicated whole cell extracts (WCE) and EVs were evaluated by Western blotting. PDI = protein disulfide isomerase. Molecular weights markers (kD) are on the left.

Figure 5. Evaluation of GM-CSF levels in exosome-enriched extracellular vesicles.
The levels of GM-CSF in the indicated exosome-enriched EVs were determined under different ELISA conditions. Exosome-enriched EVs were pretreated with or without 0.05% Tween-20. ELISA was carried out using washing buffer containing either PBS only or PBS + 0.05% Tween-20. The data are presented as the mean ± SD of three independent ELISA assays. *p < 0.05, **p < 0.005, ANOVA with Tukey's multiple comparison test.

DISCUSSION:
This study shows a highly efficient method of producing exosome-enriched EVs carrying the immune-stimulatory protein GM-CSF, which can be employed to study the immune-modulatory effects of exosome-enriched EVs. Several studies suggest that exosomes exhibit immune-regulatory and anti-tumor functions[22]. Thus, exosomes from ESCs expressing GM-CSF might also possess biological activities that regulate the immune response. In this protocol, exogenous murine GM-CSF was stably overexpressed in murine ES-D3 cells by transfection (**Figure 1**). Importantly, significant amounts of GM-CSF were detected in exosome-enriched EVs isolated from ES-D3 cells overexpressing GM-CSF (**Figure 5**).

This data suggest that almost all GM-CSF resides within exosome-enriched EVs. As a cytokine, the majority of GM-CSF protein is secreted extracellularly[11]. Like other exosomal cargo material (e.g., mRNAs, miRNAs, and proteins), GM-CSF molecules in the cytosol are encapsulated in the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs)[6,23]. Upon fusion of MVBs with the plasma membrane, GM-CSF-carrying ILVs are released into the extracellular space.

An important step in this protocol is to effectively overexpress GM-CSF in murine ES-D3 cells (**Figure 2**). Earlier efforts to achieve this goal by retroviral infection largely failed, likely because of the suppression of retroviral gene expression at the transcriptional levels in ESCs[24]. Among several viral and cellular promoters examined, the human EF1α promoter showed the most robust activity in ES-D3 cells. Importantly, transgene expression under the control of the EF1α promoter remained stable following long-term cell culture of transfected ES-D3 cells. To express exogenous GM-CSF in another cell type, further studies are needed to evaluate the efficiency of various promoters. The application of this method to isolate GM-CSF-bearing exosome-enriched

EVs can be expanded to other stem cell types as well as tumor cells engineered to express various cytokines. Like GM-CSF, most cytokines are secreted extracellularly[25]. Therefore, a possible limitation of this approach is that the amount of a given cytokine accumulated in exosome-enriched EVs is too low to exhibit its biological activity. For a particular cytokine, new studies need to be carried out to optimize its protein level and biological activity in exosome-enriched EVs.

The most critical hurdle in this study is generating ES-D3 clones overexpressing GM-CSF. To maintain the pluripotential state and promote cell proliferation in vitro, murine ESCs are generally cultured in the presence of feeder cells[26]. To acquire exosomes exclusively from ESCs, a feeder-cell-free protocol to culture ESCs[27] was employed. In this study, ES-D3 cells were cultured in gelatin-coated dishes using medium supplemented with LIF. The plate efficiency and proliferation of single clones of ES-D3 cells under this culture condition were extremely low, making it very challenging to generate ES-D3 clones from single cells. The addition of the conditional medium obtained from parental ES-D3 cells failed to rescue the proliferation deficiency of plated single ES-D3 cells. To overcome this limitation, single ES-D3 cells overexpressing GM-CSF were plated along with parental ES-D3 cells. This plating approach improved the viability of GM-CSF-expressing single ES-D3 clones, facilitating clonal proliferation and expansion. Once transfected, single ES-D3 clones apparently attached to tissue culture plates. They proliferated regardless of the presence of other ES-D3 cells, as parental ES-D3 cells were eliminated 48 h after being plated, allowing only transfected single ES-D3 cells to grow.

Overall, a protocol to successfully generate exosome-enriched EVs carrying GM-CSF from ESCs with the potential to stimulate the immune response in different disease conditions was developed. Furthermore, our recently published study demonstrates that GM-CSF-bearing exosome-enriched EVs from ESCs can serve as a cell-free prophylactic vaccine against cancer[28].

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DISCLOSURES:

Kavitha Yaddanapudi, Chi Li, and John W. Eaton submitted a US patent application “Compositions comprising engineered embryonic stem cell-derived exosomes and methods of use thereof.”

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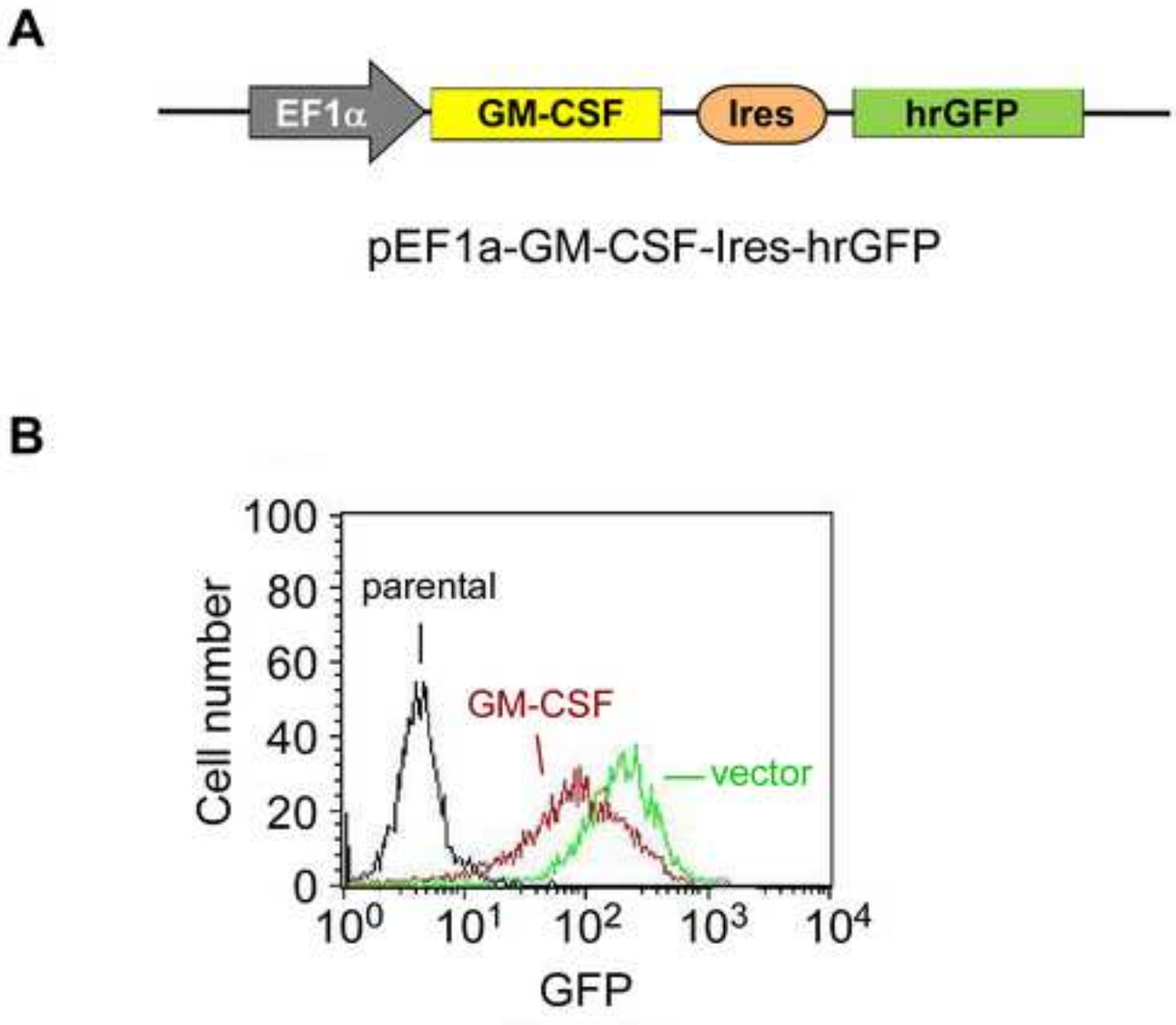
Meng et al., Figure 1



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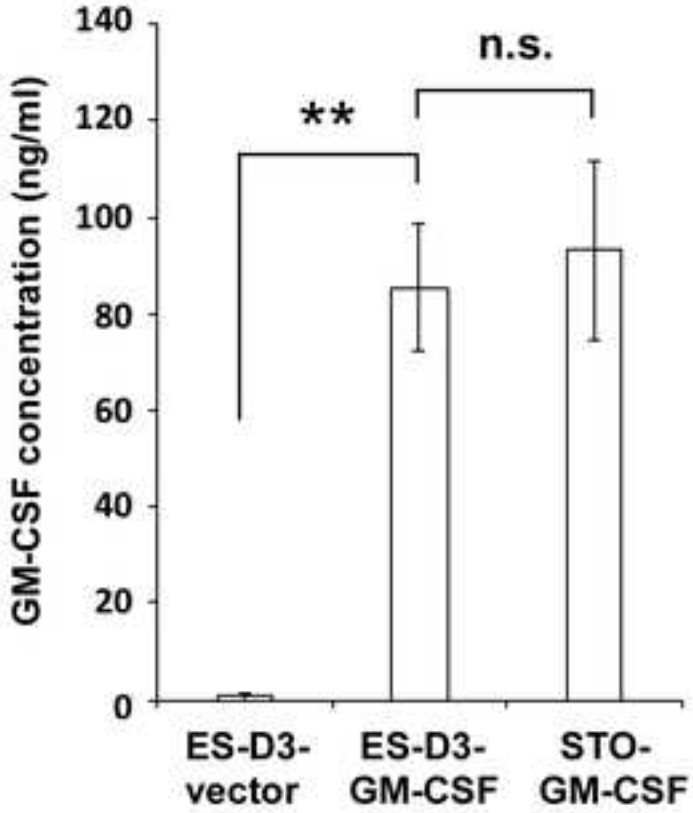
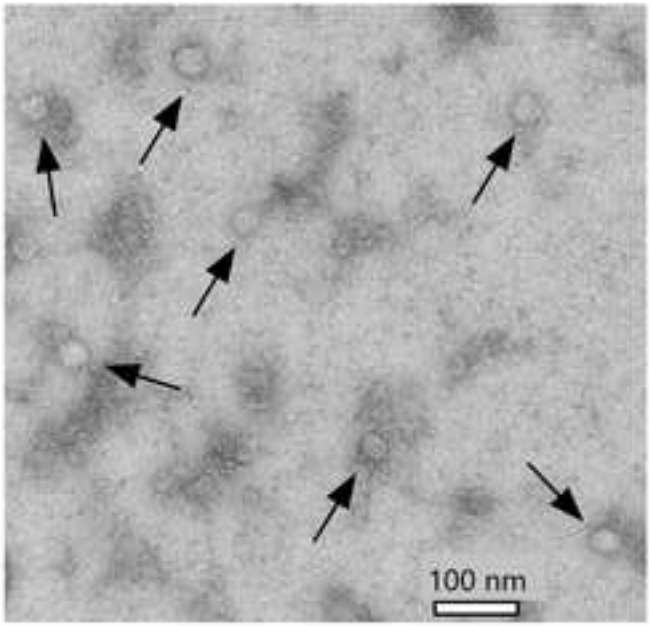
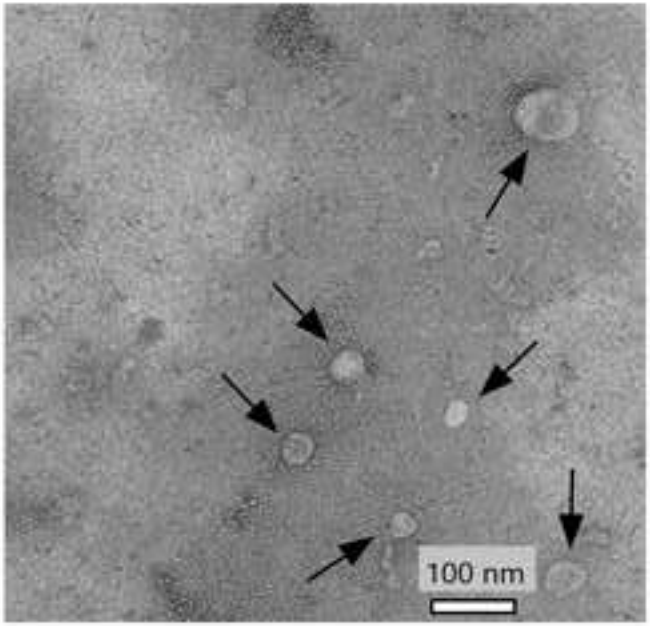


Meng et al., Figure 3



Exosomes (vector)



Exosomes (GM-CSF)

Figure 4

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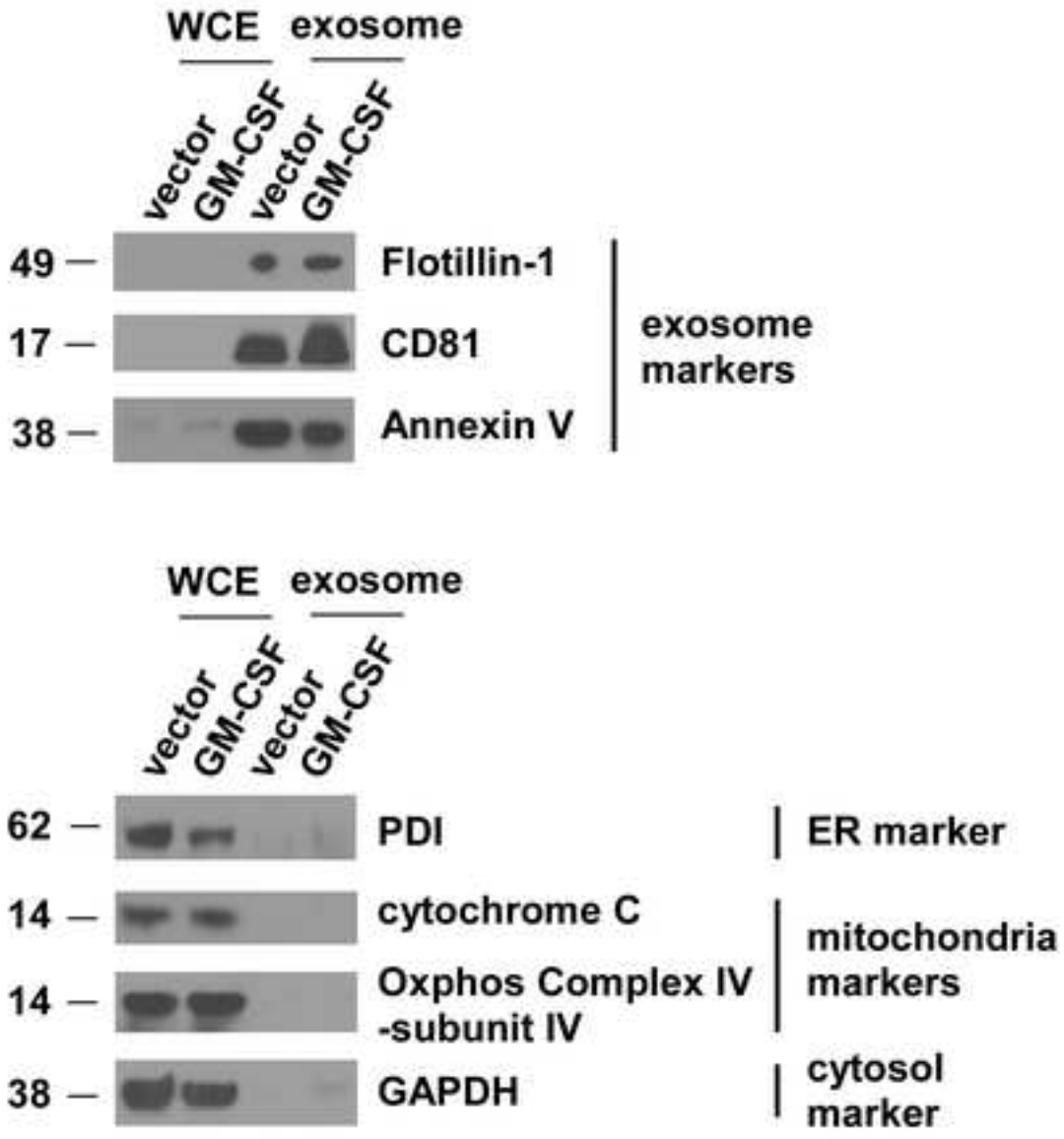

Figure 5

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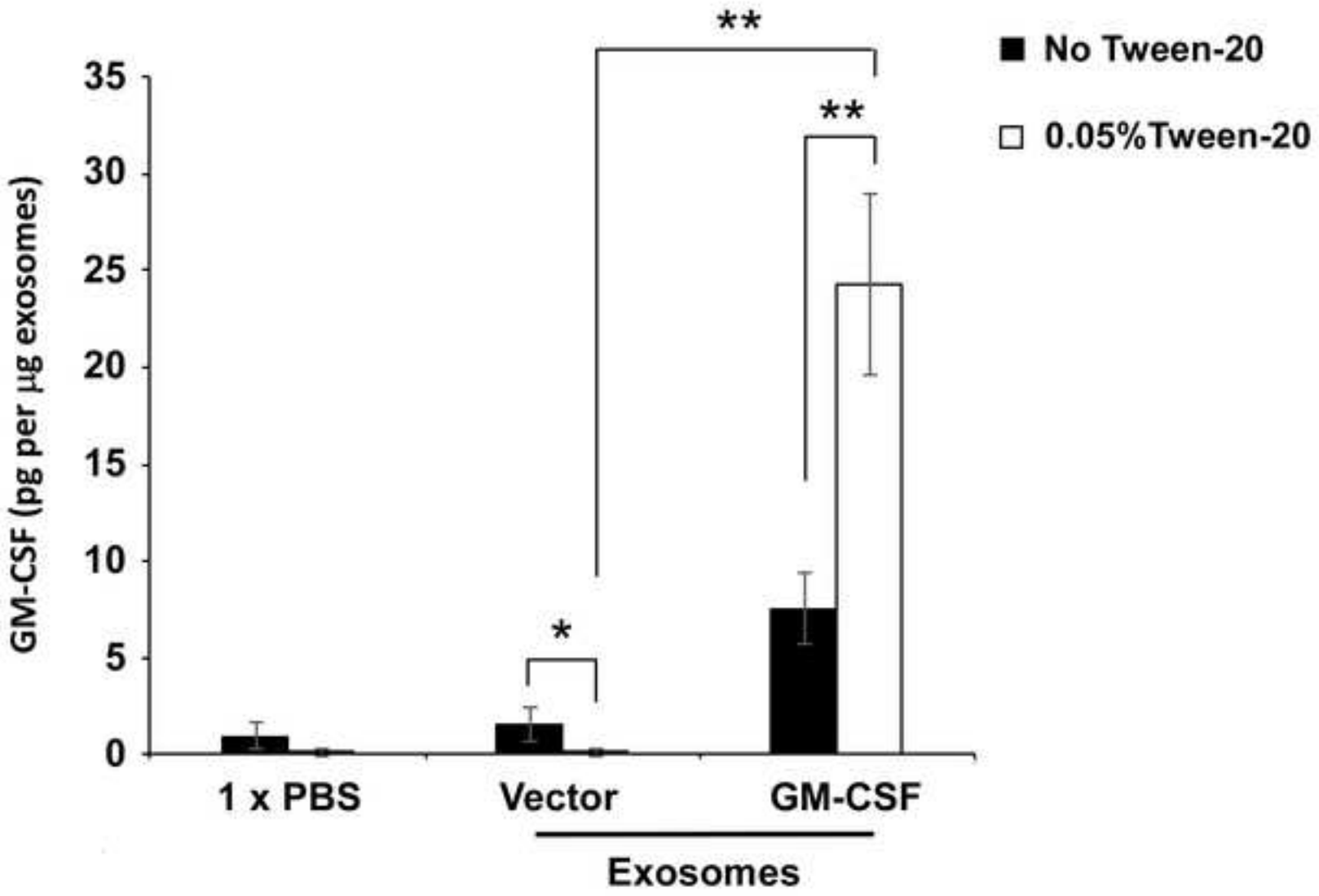

Names of Materials/Reagents/Equipment	Company	Catalog Number	Comments
Alkaline phosphate, Calf Intestinal	New England Biolabs	M0290S	Dephosphorylating DNA plasmid
anti-Annexin V mAb	Santa Cruz Biotechnology	clone H-3, sc-74438	Western blot, RRID:AB_1118989
anti-CD81 mAb	Santa Cruz Biotechnology	clone B-11, sc-166029	Western blot, RRID:AB_2275892
anti-cytochrome c mAb	Santa Cruz Biotechnology	clone A-8, sc-13156	Western blot, RRID:AB_627385
anti-Flotillin-1 mAb	Santa Cruz Biotechnology	clone C-2; sc-74566	Western blot, RRID:AB_2106563
anti-GAPDH pAb	Rockland	600–401-A33S	Western blot, RRID:AB_11182910
anti-mouse IgG, goat, peroxidase-conjugated	Thermo Fisher	31430	Western blot, RRID:AB_228307
anti-Oxphos COX IV-subunit IV mAb	Thermo Fisher	clone 20E8C12 A21348	Western blot, RRID:AB_221509
anti-protein disulfide isomerase (PDI) pAb	Enzo	ADI-SPA-890	Western blot, RRID:AB_10616242
anti-rabbit IgG, goat, peroxidase-conjugated	Thermo Fisher	31460	Western blot, RRID:AB_228341
BCA (bicinchoninic acid) assay	Thermo Fisher	23223	Determining protein concentrations
Bis-Tris PAGE Gel, ExpressPlus, 4-20%	Genscript	M42015	Western blot
Carbenicillin, Disodium Salt	Thermo Fisher	10177012	Selecting *E. coli* colonies
Centrifuge, Avanti J-26 XPI	Beckman Coulter		Low speed centrifugation
Centrifuge rotor, JA-10	Beckman Coulter	09U1597	Low speed centrifugation
Centrifuge bottle, Nalgene PPCO	Thermo Fisher	3120-0500PK	Low speed centrifugation

Cu grids with carbon support film	Electron Microscopy Sciences	FF200-Cu	Acquiring electron microscopy images
EcoRI	New England Biolabs	R0101	Digesting DNA plasmid
Enhanced chemiluminescence detection system	Thermo Fisher	32106	Western blot
FACScalibur flow cytometer	Becton Dickinson		Examining GFP levels of ES-D3 cells
Fetal bovine serum	ATCC	SCRR-30-2020	Medium for ES-D3 cells
Fisherbrand Sterile Cell Strainers; Mesh Size: 40µm	Thermo Fisher	22-363-547	Filtering ES-D3 cells for FACS sorting
Gelatin (0.1%)	Thermo Fisher	ES006B	Culturing ES-D3 cells
GM-CSF ELISA kit	Thermo Fisher	88733422	Determining GM-CSF concentrations
KnockOut Dulbecco's Modified Eagle's Medium	Thermo Fisher	10-829-018	Medium for ES-D3 cells
Leukemia Inhibitory Factor	Thermo Fisher	ESG1106	Medium for ES-D3 cells
L-glutamine	VWR	VWRL0131-0100	Medium for ES-D3 cells
Lipofectamine 2000 transfection reagent	Thermo Fisher	11668019	Transfecting ES-D3 cells
Microplate reader, PowerWave XS	BioTek		Determining GM-CSF concentrations
MoFlo XDP high-speed cell sorter	Beckman Coulter		Isolating single ES-D3 cell clones
NEB 5-alpha Competent E. coli	New England Biolabs	C2988J	Generating GM-CSF expression plasmid
Neomycin	Thermo Fisher	10-131-035	Selecting ES-D3 clones
Non-essential amino acids	Thermo Fisher	SH3023801	Medium for ES-D3 cells

Non-fat dry milk	Thermo Fisher	NC9022655	Western blot
Opti-MEM I Reduced Serum Medium	Thermo Fisher	31985062	Transfecting ES-D3 cells
Paraformaldehyde	Electron Microscopy Sciences	15710	Acquiring electron microscopy images
Penicillin/streptomycin	VWR	sc45000-652	Medium for ES-D3 cells
Plasmid pEF1a-FD3ER-IRES-hrGFP	Addgene	37270	Generating GM-CSF expression plasmid
PVDF membranes	Millipore EMD	IPVH00010	Western blot
QIAprep Spin Miniprep Kit (250)	QIAGEN	27106	Generating GM-CSF expression plasmid
QIAquick Gel Extraction Kit (50)	QIAGEN	28704	Generating GM-CSF expression plasmid
Quick Ligation Kit	New England Biolabs	M2200S	Generating GM-CSF expression plasmid
Transmission electron microscope	Hitachi	HT7700	Acquiring electron microscopy images
Trypsin	VWR	45000-660	Culturing ES-D3 cells
Ultracentrifuge, OptimaTM L-100 XP	Beckman Coulter		High speed centrifugation
Ultracentrifuge rotor, 45Ti	Beckman Coulter	09U4454	High speed centrifugation
Ultracentrifuge polycarbonate bottle	Beckman Coulter	355622	High speed centrifugation
UranyLess staining solution	Electron Microscopy Sciences	22409	Acquiring electron microscopy images

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Author(s): Shuhan Meng, Aaron G. White, Allison Tu, John W. Eaton Cili Li, Kavitha Yaddanapudi

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3. "*Please split long steps (e.g., 2.2.2, 2.3) up*."

Responses:

Following the reviewer's suggestion, we have re-written the "PROTOCOL" section to split long steps in the revised manuscript.

4. "*Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video*."

4.1. "*1.3, 5.1.1: Describe trypsinization. Mention trypsin volume added, incubation duration and temperature, and how trypsin is neutralized*."

Responses:

The information about trypsinization has been provided in the revised manuscript (lines 139-143 in page 3; line 306 in page 6 – line 309 in page 7).

4.2. "*1.4, 1.5, 2.1: mention gelatin%*"

Responses:

We have added the information in the revised manuscript (line 145 in page 3; line 149 in page 3; line 202 in page 4).

4.3. "*2: cite a reference for plasmid use. Cite a reference that describes co-transfection procedure*."

Responses:

For the details of plasmid construction, we have added a new sub-section "Generation of GM-CSF expression plasmid" in the "PROTOCOL" section. A reference (#20) describing co-transfection experiment has also been added to the revised manuscript (line 199 in page 4).

4.4. "*2.3,2.4: Describe the flow cytometry steps in greater detail*."

Responses:

We have added the detailed information about flow cytometer experiments in the revised manuscript (lines 238-243 in page 5).

4.5. "*5.1.2: mention SDS concentration*."
Responses:
The information has been incorporated into the revised manuscript (line 312 in page 7).

4.6. "*5.1.3: mention sonication amplitude (in Watts) and frequency (Hz)*."
Responses:
We have added the information in the revised manuscript (lines 315 and 322 in page 7).

4.7. "*6.2: What are the antibody concentrations*?"
Responses:
The information about the antibody concentrations has been added to the revised manuscript (lines 332-339 in page 7).

5. "*please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps*."
Responses:
We have highlighted the text to be visualized in the revised manuscript.

6. "*JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused*."
Responses:
We have revised the "Discussion" section to fit the requirements of *JoVE*.

7. "References: Please spell out journal names"
Responses:
Journal names have been added to the revised manuscript.

8. "*locate and replace all commercial sounding language in your manuscript* ."
Responses:
Commercial sounding language has been replaced with generic names in the revised manuscript.

9. "*1) Please revise the table of the essential supplies, reagents, and equipment. ……..*
2) List all items in alphabetical order sort by name."
Responses:
"Table of Materials" has been revised as required in the revised manuscript.

COMMENTS FROM PEER-REVIEWERS:
REVIEWER #1:
Major Concerns:

1. "*Last lines of the abstract: ''Overall, GM-CSF-bearing exosomes from ESCs might serve as an alternative prophylactic vaccine against cancer''. This is a big claim, since authors have not tested these exosomes, it is not realistic to make this claim. Authors have only detected GM-SCS in exosomes, detection itself cannot guarantee the application as alternative vaccine unless its potential is tested*."

Responses:

Following the reviewer's suggestion, we have revised the manuscript accordingly. In the revised manuscript, we state that "GM-CSF-bearing exosome-enriched EVs from ESCs might function as cell-free vesicles to exert their immune regulatory activities." at the end of the "Abstract" section. Importantly, we recently published a report showing that GM-CSF-bearing exosome-enriched EVs from ESCs can serve as a cell-free prophylactic vaccine against cancer, which is described in the "Discussion" section of the revised manuscript (lines 481-483 in page 10).

2. "*Page 4. Isolation of exosomes: authors have not used filters below size range of exosomes, which means, it is highly likely that other population of extracellular vesicles (EVs), e.g. microvesicles (up to 1000nm) can also be presented in EV pellets. If true, then why authors prefer to use exosomes instead of EVs. In such experimental settings, MISEV 2018 guidelines suggest using general term EVs. I suggest authors to consult this position paper and cite it in appropriate (PMID: 30637094)*."

Responses:

As the reviewer suggested, we have defined our exosomal preparations as exosome-enriched extracellular vesicles (EVs) in the revised manuscript. The publication mentioned by the reviewer (reference #5) is also cited multiple times in the revised manuscript (line 98 in page 2, lines 278 and 287 in page 6, line 379 in page 8).

3. "*Again, as mentioned above, authors cannot claim their isolates represent only exosomes. Better to apply general term EVs. Follow the guidelines by MISEV 2018*."

Responses:

As mentioned above, we have made appropriate changes in the revised manuscript.

Minor Concerns:

4. "*GM-CSF in title: avoid abbreviations which are uncommon for readers. Write full name.*"

Responses:

Following the reviewer's suggestion, we have added full name of GM-CSF in the title.

5. "*Culturing ES-D3 cells. Avoid uncommon abbreviations in headings. Write full name*".

Responses:

As required by the reviewer, we have revised the manuscript accordingly.

6. "*Page 3, section 1.3. cell reach confluence, collect cell…. Write the % of confluence*,"

Responses:

We have provided the information about the confluency of ES-D3 cells in the revised manuscript (line 139 in page 3).

7. "*Page 3, line 147: Provide the source of plasmid. Whether constructed in lab or purchased from? In case it was constructed in lab, write the protocol*."
Responses:
A new sub-section (Generation of GM-CSF expression plasmid) has been added to the "PROTOCOL" section to describe the construction of GM-CSF expression plasmid.

8. "*Page 3, line 150. Transfection was carried for ''how many hours''*?
Responses:
We have provided more detailed information about transfection in the revised manuscript (lines 215-216 in page 4).

9. "*section 2.3: mention if the media was changed during 2 weeks' time period, or indicate if cells behaved normally inspite of deprivation of nutrients over two weeks period.*"
Responses:
Following the reviewer's suggestion, we have provided more detailed information about cell culture in the revised manuscript (lines 253-256 in page 5).

10. "*Page 5.3:……. How much was ug of exosome lysate in 10 ul from each sample?*"
Responses:
The information has been added to the revised manuscript (line 326 in page 7).

11. "*Figure 1: what was the transfection efficiency*."
Responses:
The information about the transfection has been added to the "REPRESENTATIVE RESULTS" section of the "revised manuscript (line 365 in page 8).

REVIEWER #2:
Minor Concerns:
"*The authors state that GM-CSF-bearing exosomes derived "might serve as an alternative prophylactic vaccine against cancer." To show that exosomal GM-CSF fits the above criterion, the authrs need to demonstrate its biologic activity in vitro, perhaps using a reported cell line that is GM-CSF-dependent or by delivery of GM-CSF-carrying exosomes to monocytes and show the capability of these recipient cells to differentiate into DCs*."
Responses:
As mentioned in the responses to #2 major concern from the Reviewer #1, we have revised the statement about the potential of GM-CSF-bearing exosome-enriched extracellular vesicles (EVs) in the manuscript. Now we claim that these exosome-enriched EVs have the potential to serve as immune regulatory vesicles to modulate immune responses.