Video Article

Isolation and Characterization of Neutrophil-derived Microparticles for Functional Studies

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Abstract

Polymorphonuclear neutrophil-derived microparticles (PMN)-MPs) are lipid bilayer, spherical microvesicles with sizes ranging from 50–1,000 nm in diameter. MPs are a newly evolving, important part of cell-to-cell communication and signaling machinery. Because of their size and the nature of their release, until recently MP existence was overlooked. However, with improved technology and analytical methods their function in health and disease is now emerging. The protocols presented here are aimed at isolating and characterizing PMN-MPs by flow cytometry and immunoblotting. Moreover, several implementation examples are given. These protocols for MP isolation are fast, low-cost, and do not require the use of expensive kits. Furthermore, they allow for the labeling of MPs following isolation, as well as pre-labeling of source cells prior to MP release, using a membrane-specific fluorescent dye for visualization and analysis by flow cytometry. These methods, however, have several limitations including purity of PMNs and MPs and the need for sophisticated analytical instrumentation. A high-end flow cytometer is needed to reliably analyze MPs and minimize false positive reads due to noise or auto-fluorescence. The described protocols can be used to isolate and define MP biogenesis, and characterize their markers and variation in composition under different stimulating conditions. Size heterogeneity can be exploited to investigate whether the content of membrane particles versus exosomes is different, and whether they fulfill different roles in tissue homeostasis. Finally, following isolation and characterization of MPs, their function in cellular responses and various disease models (including, PMN-associated inflammatory disorders, such as Inflammatory Bowel Diseases or Acute Lung Injury) can be explored.

Video Link

The video component of this article can be found at https://www.jove.com/video/56949/

Introduction

Recently, "microparticles/microvesicles" originating from the cell cytosol or plasma membrane have become of great scientific interest, as emerging data suggest that these structures, ranging from 50-1,000 nm in diameter, can carry biological information and serve as a non-canonical method of cellular communication. Immune cell-derived MPs and particularly those produced by polymorphonuclear neutrophils (PMNs) are of great interest given the important role of PMNs in host defense[1,2], inflammatory responses[3], and wound-healing[4]. Intriguingly, thus far, numerous reports have shown both pro-inflammatory and anti-inflammatory functions of PMN-MPs[5], suggesting a potential context-, disease-, species-, and organ-specific role of MPs.

Described protocols in this communication provide a cost-effective, innovative, and adaptable method to study the function of MPs in health and disease. They are applicable to many model organisms, organs, and stimulation conditions. They allow for the identification of several types of MPs and can be used in the future to address their pro-inflammatory and anti-inflammatory functions. As an example, described here is how to study the function of PMN-MPs in epithelial wound healing *in vitro* and *in vivo*. The presented protocol for isolation of mouse bone marrow-derived PMNs was adapted with some modifications from a previously described method[6].

Furthermore, protocols described in this study allow for the detection and characterization of specific markers that can be found on PMN-MPs by two complementary methods: Western blot and flow cytometry. We find that immunoblotting of MPs using standard protocols[5] is easy and reliable, however, recent advances in sensitivity of flow cytometry instruments, and improved noise-to-signal ratio now allow for further analysis of MPs using this method. The described protocols in this study incorporate recent advances and recommendations from original research articles, including modifications to centrifugation speed and time, the addition of sample filtering and freezing/storage conditions[7,8], and how to reduce the "background noise", improve the detection limit of PMN-MPs, and discriminate between different sizes of MPs.

Protocol

All animal work was approved by the Northwestern IACUC. All experiments were completed in accordance and compliance with all relevant regulatory and institutional guidelines. For human subjects donating blood, an informed consent was presented and signed; in addition, all human subjects in this study were treated in accordance with the institutional and federal guidelines for human welfare.

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NOTE: The protocol steps are listed under the following subsections: (i) Mouse Bone Marrow Cell Isolation; (ii) PMN Isolation from Murine Bone Marrow; (iii) PMN Isolation from Human Blood; (iv) MP Isolation from PMN Supernatants (by Ultracentrifugation); (v) Characterization of MPs by Western Blotting; (vi) Characterization of MPs by Flow Cytometry; (viii) Application of isolated MPs to Study Wound Healing

1. Mouse Bone Marrow Cell Isolation

1. **Dissection of the femur and tibia from the mouse hind legs**
    1. Prepare a euthanasia box (*e.g.*, an empty 1 mL tip box) for inhalation of anesthesia. Add a few drops of 100% isoflurane onto a sterile cloth taped to the inside of a plastic box lid. As per new IACUC guidelines, animals should not come in direct contact with isoflurane, thus place a tip holder between the mouse and the gauze containing the isoflurane.
    NOTE: Isoflurane is a powerful inhalational anesthetic and should be handled with extreme caution inside a fume hood.
    2. Euthanize mice as per IACUC recommendation. Place a mouse inside the euthanasia box described in step 1.1.1 for 1–5 min until it has ceased breathing. Ensure the animal's death by performing a cervical dislocation.
    3. Lift the abdominal skin using stainless steel, 12 cm curved, 0.17 mm X 0.1 mm tweezers and cut the skin half way between the upper and lower extremities. Peel the skin from here to the most distal section of the mouse body including the lower extremities. With 10 cm long, straight dissecting scissors, remove the muscles from the hind legs and dislocate the hip joint leaving the femur head intact.
    4. Use scissors to dissect the muscles from the femur and the tibia and separate the femur from the tibia. Make sure the ends of the bones remain intact as they contain significant amounts of bone marrow.
    5. Remove all remaining flesh by rolling the bones inside a paper towel. Place the clean bones in a Petri dish containing Serum-Free Medium (SFM, *i.e.*, Dulbecco's Modified Eagle's medium (DMEM) without serum or antibiotics).
    6. Rinse the bones in 70% ethanol and then in SFM.
    7. Prepare 50 mL of SFM in a 50 mL conical tube. Load 10 mL of SFM into a 10 mL syringe and attach it to a 25 and 5/8 inch gauge needle.
    8. Trim the end of the bones with scissors until an opening to the bone marrow (reddish color) is visible.
    9. Flush the bone marrow cells into a new 50 mL tube. Use a 10 mL syringe with a 26 and 5/8 inch gauge needle with approximately 3 mL/bone of SFM. When finished flushing, resuspend the cells in the tube by pipetting (use disposable 1 mL plastic tips) to break cell aggregates.

2. PMN Isolation from Murine Bone Marrow

1. **Red cell lysis**
    1. Pellet the collected bone marrow by centrifuging at 350 x *g* for 8 min at 4 °C.
    2. Resuspend the cell pellet in 20 mL of 0.2% NaCl for approximately 20-30 s to lyse the erythrocyte fraction. Do not exceed 30 s as this will result in PMN lysis. Add 20 mL of 1.6% NaCl to restore osmolality.
    NOTE: This step may lead to very little PMN activation.
    3. Wash the cells with 2 mL of PBS and centrifuge as in step 2.1.1 above.Remove the supernatant.
    4. Proceed to isolating PMNs by density gradient centrifugation.

2. **Isolation of neutrophils by density gradient centrifugation**
    1. Warm polysucrose and sodium diatrizoate solutions, with densities of 1.119 g/mL and 1.077 g/mL (see **Table of Materials**), to room temperature (RT) before use.
    2. Prepare polysucrose and sodium diatrizoate gradients fresh, immediately prior to bone marrow cell application by slowly layering 3 mL of the 1.077 g/mL density solution over 3 mL of the 1.119 g/mL density solution in 15 mL conical tubes.
    NOTE: Preparation of the gradient solution too far in advance will result in mixing of the layers and poor neutrophil purity and recovery.
    3. Resuspend the bone marrow cell pellet in 1 mL of ice-cold PBS and overlay the resulting cell suspension on top of the polysucrose and sodium diatrizoate gradient (slowly, to avoid mixing of the layers).
    4. Centrifuge for 30 min at 850 x g at 25 °C at RT, **without brake**. Keep the reagents at RT for effective separation of the neutrophils from other cells present in the bone marrow. Set the centrifuge at slow deceleration to allow for the gradient to be efficiently formed and maintained after the centrifugation step.
    5. Visually locate a mononuclear cell layer at the interface of PBS and 1.077 g/mL density solution (upper layer). Remove this layer and the rest of the density solution without disturbing the PMN band.
    6. Collect an entire PMN layer and some of the underlying polysucrose and sodium diatrizoate 1.119 g/mL solution into 15 mL tubes using a transfer pipette.
    NOTE: The purity of the isolated PMNs will depend on a complete removal of the upper layer.
    7. Top the tubes with filtered PBS (filtered through a 0.1 µm pore size filter) and centrifuge at 300 x g for 5 min, at 4 °C.
    8. Wash the pelleted PMNs with 2 mL of PBS using centrifugation conditions as in step 2.2.7.
    9. Resuspend in 1 mL of filtered PBS and count the isolated PMNs by hemocytometer.
    NOTE: For this isolation method, the resulting PMN purity amounts to ~ 85-90%, as was assessed by flow cytometry. The remaining contaminating fractions consists primarily of mononuclear lymphocytes.

3. **PMN stimulation to induce MP release**
NOTE: PMNs can be stimulated to release MPs using various activating conditions, including N-formyl-l-methionyl-leucyl-l-phenylalanine (fMLF), phorbol-12-myristate-13-acetate (PMA), or Interferon gamma (IFNγ). Importantly, prior to simulation, PMNs should be kept on ice at all times.
    1. Pellet PMNs (300 x g, 5 min, 4 °C) and resuspend in 0.1 µm filtered Hank's Balanced Salt solution (HBSS) solution, containing the stimulating agent of choice. For optimal stimulation, use 100 µL stimulation solution per 10 million PMNs for 20-30 min at 37 °C in 15 mL tubes.

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NOTE: The following concentrations of activators were successfully used in our previous work: fMLF(0.5-1 µM for human and 2-5 µM for mouse PMNs), PMA (200 nM), and IFNγ (100 ng/mL). If the release of pre-labeled MPs is desired, incubate unstimulated PMNs (1-5 x 10^6) with N-(2-aminoethyl) maleimide-FITC (see **Table of Materials**) (1 µM in 100 µL HBSS, 15 min, 37 °C). Proceed with steps 2.3.1-2.3.3.

2. Spin down at 850 x g for 5 min at 4 °C, to remove PMNs and transfer cell-free supernatants to new 1.5 mL microcentrifuge tubes.
3. For MP isolation from cell-free supernatants, continue to step 4.2.

3. PMN Isolation from Human Blood

1. **Recruit healthy volunteers for blood extraction according to Institutional IRB guidelines.**
    1. Collect blood from the forearm of a healthy volunteer into commercially available 5 mL sodium citrate-containing tubes (see **Table of Materials**).
    2. Pipette 5 mL of dextran and sodium diatrizoate solution (density of 1.113 g/mL, see **Table of Materials**) into sterile 15 mL tubes and slowly layer 5 mL of freshly isolated human peripheral blood on top of it.
    3. Centrifuge the tubes for 50 min at 400 x g at RT (25 °C) with low acceleration and no brake.
    4. At the end of the centrifugation, remove the plasma and mononuclear cells (upper layer) using a sterile plastic suction pipette.
    5. Transfer the bottom layer (PMNs) into a new sterile 50 mL conical tube.
    6. Add an equal volume of 0.45% NaCl to PMNs (mix well and gently by rotating the tube).
    7. Centrifuge for 10 min at 400 x g at 25 °C.

2. **Red cell lysis**
    1. Add 10 mL of ice-cold, sterile water to pelleted cells for 45 s followed by 10 mL of ice-cold sterile 1.8% NaCl to restore osmolality.
    NOTE: All following steps for PMN isolation should be performed on ice to avoid PMN activation and premature MP release. The lysis step itself results in minor but not significant PMN activation, however, it is essential for isolating a pure PMN population for functional assays.
    2. Centrifuge the cells for 10 min at 250 x g at 4 °C with soft deceleration.
    3. Repeat steps 3.2.1 and 3.2.2.
    4. Resuspend PMNs in 5 mL of ice-cold HBSS without calcium or magnesium. Count total live cells (use a hemocytometer and viability staining at 1:2 dilution, see **Table of Materials**) before stimulation.
    NOTE: Isolated PMNs should be used for stimulation or other experiments within 2 h of isolation to avoid functional and cellular changes and cell death.

4. MP Isolation from Activated PMN Supernatants

NOTE: A similar protocol is used for the isolation of MPs from murine and human PMNs.

1. Pellet PMNs (300 x g, 5 min, 4 °C) and resuspend in 0.1 µm filtered HBSS solution, containing the stimulating agent of choice (for example 1 µM fMLF, see Note: 2.3.1). For optimal stimulation use 100 µL stimulation solution per 10 million PMNs for 20-30 min at 37 °C in 15 mL tubes.
2. Following stimulation, spin down activated PMNs at 600 x g for 5 min at 4 °C and transfer cell-free supernatants to new 1.5-mL microcentrifuge tubes.
3. Centrifuge the PMN-cleared supernatant at 13,000 x g, 10 min, 4 °C to remove cell debris and transfer the cleared supernatant into a new ultracentrifuge tube.
4. Centrifuge for 1 h at 100,000 x g, at 4 °C. Remove supernatants before MP storage. As needed, store pelleted MPs in paraffin sealed ultracentrifuge tubes at -80 °C until use in experiments.

5. Examination of PMN-MPs by Western Blotting

1. Add 1% SDS buffer (50-200 µL with 100 mM Tris pH: 7.4) to MPs pellet (from step 4.4), transfer to new 1.5 mL microcentrifuge tubes and boil the lysed MPs for 5 min.
NOTE: The number of MPs and the lysis buffer volume must be adjusted and optimized for each protein of interest.
2. **Load equal amounts of PMN-MPs per lane in loading buffer containing 10% β-mercapto-ethanol.**
NOTE: With our method we load MPs that were isolated from the same number of stimulated PMNs.
    1. Separate the lysates by SDS-PAGE on 10% polyacrylamide gel (use 50-100 V for 1-1.5 h) and transfer total proteins onto nitrocellulose membranes as described[9].

3. Block membranes for 1 h with 5% non-fat milk in 0.05% Tween-20 Tris-buffered saline, and incubate it with an appropriate primary (overnight at 4 °C), followed by secondary HRP-conjugated antibodies.
4. Visualize the bands by adding chemoluminescence solution to the membrane and exposure to an X-ray film inside a light-proof cassette (1-24 h).

6. Examination of PMN-MPs by Flow Cytometry

1. **For antibody staining, dilute antibodies appropriately in FACS buffer (PBS with 0.1 mM EDTA, 0.1% sodium azide). Resuspend pelleted PMN-MPs (derived from 1-3 x 10^6 PMNs/condition) in 100 µL antibody solution.**
    1. If staining for Annexin V, resuspend pelleted PMN-MPs in Annexin V buffer containing 5 µL of FITC-conjugated Annexin V. If co-staining with antibodies, add the desired antibodies (for an example see **Table of Materials**) directly to the Annexin V solution. Add

fluorescent lipid dye, N-(2-aminoethyl) maleimide (see **Table of Materials**), at 1 µM concentration in 100 µL of FACS buffer to label and identify MPs.

2. Incubate the MPs in staining solution for at least 20 min at 4 °C.
3. Wash the MPs by ultracentrifugation (1 h at 100,000 x g, at 4 °C); discard the supernatant.
4. Resuspend in FACS buffer and run the samples on a designated flow cytometer equipped with an upgraded electronic unit for increased sensitivity (see **Table of Materials**).

7. Applications for Isolated PMN-MPs to Study PMN Function in Wound Healing

1. ***In vivo* administration of PMN-MPs to colonic wounds**
    1. Anesthetize mice by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Confirm anesthesia by pedal reflex (firm toe pinch) and adjust as necessary.
    2. Place the mouse on its stomach and using 28-cm biopsy forceps and an endoscope equipped with a high-resolution camera (such as a veterinary endoscope for small animal use, see **Table of Materials**), generate 3-5 superficial wounds along the dorsal side of the colon. Upon completion of wounding, return mice to a cage placed on a temperature-controlled (37 °C) mat until recovered.
    3. Anesthetize mice (as in step 7.1.1). Use an endoscope to acquire images of inflicted wounds 24 h (day 1) post-wounding. Let mice recover as in step 7.1.2.
    4. At the same time (24 h post-wounding), administer murine PMN-MPs derived from 2 x 10^6 PMNs in 100 µL of HBSS+ directly into each wound site using a colonoscopy-based microinjection system (use a 29 G needle)[9]. Three days later (day 4 post-wounding) anesthetize mice (as in step 7.1.1) and use an endoscope to reacquire images of healing wounds. Let mice recover as in step 7.1.2.
    5. Using preferred image analysis software (see **Table of Materials**), outline visible wound regions in acquired images, measure the area of the same wound at days 1 and 4 post-wounding, and calculate the percentage of wound closure.

2. **Human epithelial cells *in vitro* wound healing**
    1. Count Caco-2 BBe or T84, human intestinal epithelial cells by hemocytometer and seed 5 x 10^5 cells/well in 24-well culture plates. Incubate the cells in a standard incubation chamber at 37 °C and 5% CO_2. Caco-2 BBe or T84 reach confluency within 48 h[9]. To culture epithelial cells, use DMEM and DMEM-F12 (50:50) with supplements as previously described[9].
    2. Generate mechanical scratch wounds in the monolayer using a pipette tip and low suction as previously described[4,9]. Acquire images of wound areas immediately after scratching using an inverted phase-contrast differential interference microscope (use 5X or 10X objectives), to be used as a time = 0 reference point.
    3. Add MPs (derived from 2-4 X 10^6 PMNs) to scratched-wounded epithelial cell monolayers, and incubate for an additional 24 h (48 h post-wounding, at 37 °C with 5% CO_2). At this time re-acquire images of the scratch-wounds.
    4. Use preferred image analysis software (see **Table of Materials**) to measure wound areas at t = 0 and t = 48 h. Calculate the percentage of wound closure by determining the difference in wound area at the selected time points.

Representative Results

Representative flow cytometric analysis of MPs that were isolated from human and mouse PMNs are shown in **Figure 1**. The size heterogeneity of PMN-MPs can be assessed by comparison to known sized beads as shown in **Figure 1A, B** for human MPs. Note, no significant differences in size heterogeneity were observed between mouse and human MPs. Similarly, using flow cytometry and fluorescence labeling, expression of protein/s of choice by isolated MPs of either mouse or human origin can be determined. For example, expression of Phosphatidyl serine (PhS) can be examined by Annexin V staining. Murine bone marrow PMN-MPs are shown in **Figure 1C, D**. Expression of several markers of choice can also be assessed as shown for Annexin V and CD11b staining of human MPs, **Figure 2A, B**. Another method to detect PMN-MPs by flow cytometry is to stain freshly-isolated PMNs prior to stimulation as shown in **Figure 2C**. For example, PMN staining with the lipid marker N-(2-aminoethyl) maleimide-FITC prior to fMLF stimulation results in the release of green MPs, that can be readily detected by flow. Of note, while staining with N-(2-aminoethyl) maleimide-FITC has been previously used to label and detect MPs that were obtained from peripheral human blood[10] or injected into animals[11], the use of other markers for labeling purposes for *in vitro* or *in vivo* application should be determined by each investigator. In addition to flow cytometry, PMN-MPs can be analyzed by immunoblotting for proteins of interest. As shown in **Figure 3,** MPs derived from human PMNs that were stimulated with several known activators, were examined for the expression of key inflammatory (matrix metallopeptidase 9 (MMP-9) and myeloperoxidase (MPO)) and anti-inflammatory (Annexin A1) molecules. As evident from representative immunoblots, PMN stimulation with IFNγ (50 ng/mL), PMA (200 nM), and fMLF (1 µM) resulted in MPs expressing varying levels of MMP-9. However, only perturbation of the actin cytoskeleton by Latrunculin B (1 µM) prior to stimulation with fMLF (5 µM) led to abundant presence of MPO in PMN-MPs. Similarly, varying levels of Annexin A1 (high to no detection) were detected on MPs following the described activating conditions. These results suggest that PMN-MP composition is stimulus-dependent.

Finally, **Figure 4** shows how isolated PMN-MPs can be used to study wound healing *in vitro* and *in vivo* in colonic injury. PMN-MPs can be added to scratch-wounded epithelial monolayers in cultures, where healing can be monitored by imaging acquisition at pre-determined time points. MPs can further be microinjected directly into colonic wounds, that were generated by biopsy forceps and endoscopic imaging[9], and their effect on healing can be assessed. For both *in vitro* and *in vivo* analysis of wound healing, images of inflicted wounds are acquired immediately post wounding (or otherwise as specified) and continuously through the healing process at pre-determined time points. Using commercially available image analysis software, changes in wound area (size) are measured and used to determine the wound closure rate. Application of PMN-MPs that contain MPO to either cultured epithelial monolayers or *in vivo* to colonic wounds has detrimental effects, leading to delayed healing[9].

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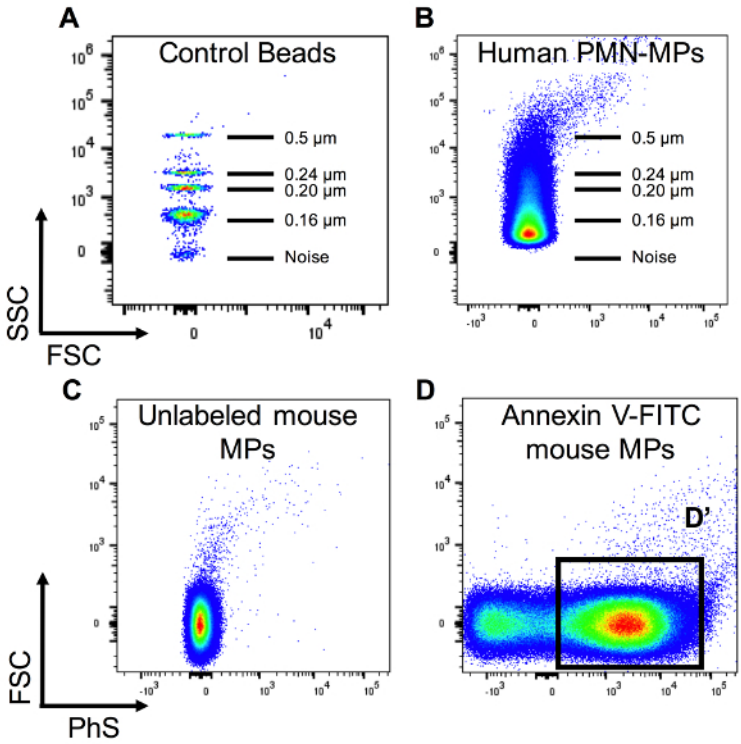

Figure 1. Analysis of PMN-MP size and surface markers by flow cytometry. (**A**) Flow cytometer optimized control beads (see **Table of Materials**) of known sizes and scatter values are shown in the SSC and FSC orthogonal representation. The beads are used to get a relative size comparison with a PMN-MP sample shown in B. (**B**) Human PMN-MPs were isolated and analyzed by flow cytometry using conditions described for the beads. The heterogeneity in MP sizes can be seen. (**C-D**) MPs derived from fMLF-stimulated murine bone marrow PMNs were analyzed by flow cytometry. Representative flow diagrams show unstained (**C**) or Annexin V-FITC stained (**D**) MPs. Rectangular area shows Annexin V-positive MPs (FITC-positive MPs). SSC: Side Scatter; FSC: Forward Scatter; PhS: Phosphatidyl serine. Please click here to view a larger version of this figure.

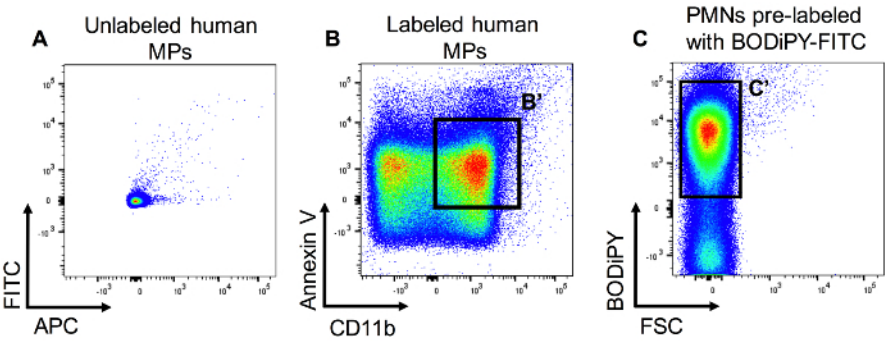

Figure 2. PMN-MP staining and analysis by flow cytometry. (**A**) Unstained and (**B**) Annexin V-FITC- and hCD11b-APC-stained MPs. Square area encloses an Annexin V/CD11b double positive population of PMN-MPs. (**C**) Freshly isolated human PMNs (1 x 10^6) were stained with a fluorescent dye, N-(2-aminoethyl) maleimide-FITC and stimulated with fMLF. MPs were isolated from cell supernatants and analyzed by flow cytometry. Rectangular area encloses a N-(2-aminoethyl) maleimide-FITC positive MP population (M-FITC, Y-axis label). Please click here to view a larger version of this figure.

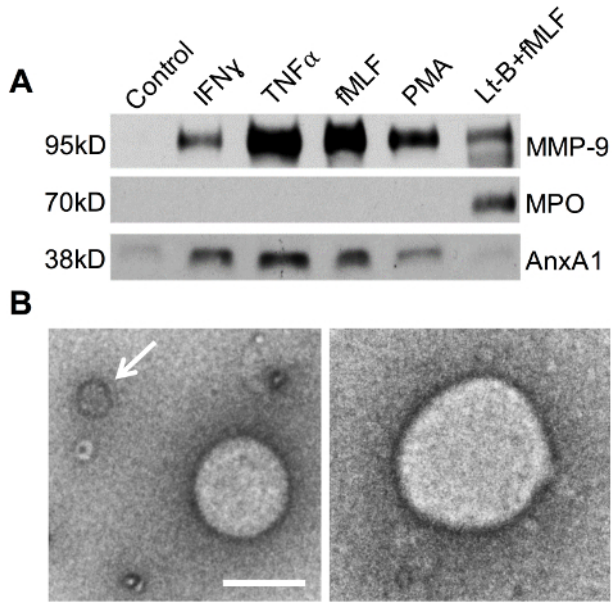

Figure 3. Composition of PMN-MPs is stimulus-dependent. (**A**) Human PMNs were stimulated with either IFNγ, TNFα, fMLF, PMA, or a combination of latranculin B followed by fMLF (LtB- fMLF). MPs were isolated from the resulting cell supernatants by ultracentrifugation and protein lysates were prepared in 1% SDS buffer. Proteins were separated by size electrophoretically in a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed for either an MMP-9, MPO, or Annexin A1 primary antibody followed by the appropriate HRP-conjugated secondary antibodies. (**B**) Representative Electron Microscopy images of human PMN-MPs depict size heterogeneity. An exosome <100 nm in size is indicated by the white arrow. Scale bar = 250 nm (left and right panels). Please click here to view a larger version of this figure.

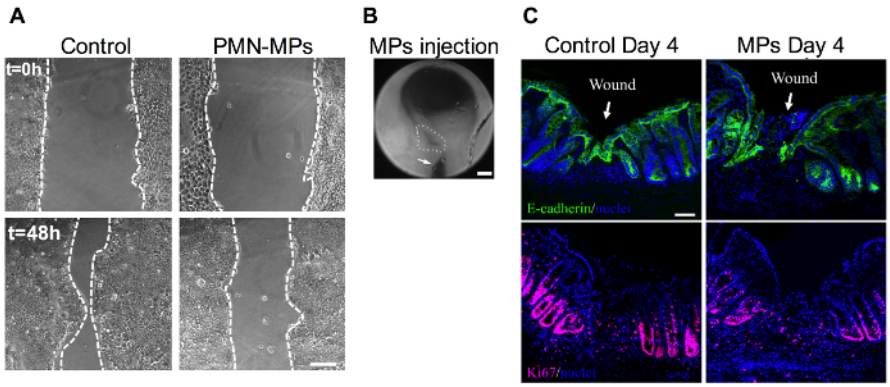

Figure 4: The use of isolated PMN-MPs in studying the role of PMNs in epithelial wound healing. (**A**) Caco-2 BBe human intestinal epithelial cells were plated to confluency, scratch-wounded, and subjected to MPs derived from 3 million PMNs, which were added immediately post-wounding. Representative images show wound closure (48 h post-wounding) in control (left panels) and PMN-MP-treated epithelial cells (right panels). Scale bar = 100 µm. (**B**) To examine the effect of PMN-MPs on colonic wound healing *in vivo*, isolated PMN-MPs were injected directly into the wound area using an endoscopy-based microinjection system (at 24 h post-wounding, the colonic wound is outlined by a dashed line and the injection needle site is shown by a white arrow). Wound closure was assessed 3 days later (4 days post-wounding) by endoscopic imaging. Scale bar = 300 µm. (**C**) 4 days post-wounding mice were euthanized and colonic mucosal wounds were extracted with scissors, embedded in optimum cutting temperature (O.C.T) compound and frozen with liquid nitrogen. Eight micrometer sections of the wounds were stained for E-cadherin (green) and the nuclear stain DAPI (blue) to assess the level of re-epithelialization (upper panels), or for DAPI (blue) and Ki67 (red) to visualize the total and proliferating epithelial cells at the wound edge (lower panels). Scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

Protocols for the isolation and characterization of PMN-derived MPs are described in this communication. Several key critical points must be taken into account for the success of the procedure. First, PMNs must be isolated fresh and used in experiments within 2 h of isolation to prevent spontaneous activation and degranulation. All handling of PMNs during isolation and up to the point of stimulation must be performed on ice to prevent activation and premature MP release[12]. Second, ultracentrifugation for 1 h or more maximizes the pelleting of MPs. Third, for analysis by flow cytometry, the instrument must be properly calibrated with a mix of instrument-specified fluorescent beads (the beads type is adjusted to a particular instrument) to correctly define MP sizes. For example, while one manufacturer of flow cytometers recommends using FSC beads as a

size-related parameter, others have been optimized for SSC beads. In addition, the flow cytometer to be used for MP analysis must be equipped with upgraded electronics to best resolve MPs from the background noise.

To correctly identify MPs in addition to size parameters, the use of fluorescence staining as described in the procedures above is highly recommended. Furthermore, all solutions during the preparation and isolation should be filtered through a 0.1 µm filter system to minimize inclusion of dust particles or other precipitates that will increase the background noise during acquisition by flow cytometry. Importantly, storage conditions of MPs should be considered. Little evidence[8], and our own unpublished observations, suggest that MP freezing leads to membrane rupture and MP breakage. Finally, varying PMN activation conditions and timing may change and improve MP yield.

There are some limitations to this method. Isolation of mouse PMNs from bone marrow is typically not pure (~85–90%) and is contaminated by other immune cells (primarily mononuclear lymphocytes) as was determined by flow cytometric analysis. Thus, the resulting isolates may include small amounts of MPs released by other immune cells. Alternatives are available and include isolation of PMNs by commercially available magnetic beads, however, this would be a longer and a significantly costlier procedure. Finally, without fluorescence labeling, in addition to size parameters, definitive differentiation of MPs from dust or other soluble or airborne particles of similar sizes that can contaminate the sample is challenging and error-prone.

In the future, sorting of PMN-MPs labeled with specific markers will help elucidate MP composition under specific stimulatory conditions or models of disease, and hopefully allow for the use of MPs as diagnostic markers and potential therapeutic targets to treat inflammatory diseases.

Disclosures

The authors have no conflict of interest of any kind related to this communication

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