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Measuring phagocytosis in bone marrow-derived macrophages and peritoneal macrophages with aging.

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Abstract

The majority of age-related diseases share common inflammatory mechanisms, a phenomenon which has been described as “inflamm-aging”, and genetic variants in immune and inflammatory genes are significantly associated with exceptional human longevity and/or age-related diseases. Consistently, aging is associated with increased macrophage infiltration into tissues. Macrophages are a key component of the innate immune system and the inflammatory response, which accomplish key tasks such as phagocytosis, antigen presentation and cytokine production. Phagocytosis is the process by which specialized cells that can clear harmful foreign particles, pathogens, and dead or dying cells. Upon phagocytosis, foreign particles are internalized in vesicles, forming phagosomes. Phagosomes go on to fuse with lysosomes, and the ingested particles are neutralized by lysosomal enzymes. Macrophages have two main origins: tissue-resident macrophages differentiate from specific embryonic progenitors, whereas monocyte-derived macrophages differentiate from bone-marrow progenitors. Because of their key role in inflammation and damage repair, macrophages are a key cell type in age-related inflammatory diseases. Here, we describe an efficient method to quantify the phagocytotic ability of two types of primary macrophages in aging mice: bone marrow derived macrophages (BMDMs) and tissue-resident peritoneal macrophages.

Keywords

aging; macrophages; phagocytosis; Zymosan; innate immunity

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¹-It is best to avoid getting blood into the peritoneal cavity when performing cervical dislocation, however blood in the peritoneal cavity will not affect the final cell viability. Performing a red blood cell lysis step is then helpful to limit column clogging.

1. Introduction

The majority of age-related diseases share common inflammatory mechanisms [1,2], a phenomenon which has been described as “inflamm-aging” [1,2], and genetic variants in immune and inflammatory genes are significantly associated with exceptional human longevity and/or age-related diseases [3,4]. Macrophages are a key component of the innate immune system and the inflammatory response, which accomplish key tasks such as phagocytosis, antigen presentation and cytokine production [5]. Consistently, aging is associated with increased macrophage infiltration into tissues [6]. Phagocytosis is a phenomenon where a cell engulfs a foreign particle (*e.g.* bacteria, fungi, *etc.*) or dead cells, subsequently forming a phagosome, which later fuses with lysosome to process the cargo, then known as the phagolysosome. Macrophages are one of the key components of innate immunity and play a major role in cytokine production, antigen presentation, and clearing of pathogens/dead cells due to infection/injury through phagocytosis [7,8].

Macrophages have two main origins: tissue-resident macrophages differentiate from specific embryonic progenitors, whereas monocyte-derived macrophages differentiate from bone-marrow progenitors [9,5]. Resident macrophage populations exist across tissues (*e.g.* microglia in the brain, Kupffer cells in the liver, osteoclasts in the bone matrix, *etc.*) [9]. Because of their key role in inflammation and damage repair, macrophages are a key cell type in age-related inflammatory diseases [10]. Depending on the context, activated macrophages can promote a more pro-inflammatory state *vs.* pro-repair or wound-healing environment [5]. Macrophages activated with pro-inflammatory signals (*e.g.* Lipopolysaccharide, Interferon gamma), are known as “classically activated” or “M1” macrophages [5]. In contrast, macrophages activated with tissue-remodeling signals (*e.g.* IL-4) are classified as “alternatively activated” or “M2” macrophage subsets [5]. Interestingly, M1 and M2 macrophages are associated to distinct metabolic and secretory outputs [5]. For instance, classically activated M1 macrophages secrete inflammatory cytokines (*e.g.* IL-6) and promote extra-cellular matrix hydrolysis through secretion of metalloproteinases, whereas alternatively activated M2 macrophages secrete anti-inflammatory cytokines, and promote extra-cellular matrix deposition [5]. Macrophages are diverse in their morphology, number and response to LPS activation with aging depending on their origin. For instance, macrophages from lungs show elevated response to LPS with age while the macrophages from spleen showed the opposite [11]. Therefore, it is critical to follow a robust method to determine the phagocytotic potential of macrophages throughout life, to evaluate the potential to mount an efficient immune response. Here, we describe an effective method to (i) isolate bone marrow derived macrophages (BMDMs) in cohorts of aging mice (based on the protocol described in [12]) and peritoneal macrophages [13,14], and (ii) to perform phagocytosis assays to evaluate the phagocytic potential of these cells throughout life.

2. Materials

2.1 Isolation of bone marrow derived macrophages (BMDMs)

1. “Column tube” for isolation of bone marrow cells: take a 500µL centrifuge tube, cut off the cap and make few holes at the bottom of the tube using a 20G needle.

Place this tube in a sterile 1.5 mL microcentrifuge tube. (based on the protocol described in [12])

2. MACS rinsing buffer: MACS resuspension buffer (1x D-PBS, 2mM EDTA) (Miltenyi Biotec) and 0.5% BSA (Miltenyi Biotec)
3. Red blood cell lysis buffer: dilute 10xRBC lysis buffer (Miltenyi Biotec) to 1x using ddH₂O.
4. MACS filters, 30µm (Miltenyi Biotec) and 70µm (Miltenyi Biotec)
5. (Optional) Monocyte isolation kit (Miltenyi Biotec)
6. (Optional) quadroMACS magnet (Miltenyi Biotec)
7. (Optional) LS columns (Miltenyi Biotec)
8. COUNTESS automated cell counter (Thermo Scientific) and slides (or hemocytometer), and Trypan blue solution.

2.2 Isolation of peritoneal macrophages

1. Peritoneal Wash Buffer: 3% BSA (Akron), in D-PBS (Mg/Ca Free), filter sterile on 0.22µm membrane.
2. MACS rinsing buffer: MACS resuspension buffer (1x D-PBS, 2mM EDTA) (Miltenyi) and 0.5% BSA (Miltenyi Biotec)
3. (Recommended) Red blood cell lysis buffer: dilute 10xRBC lysis buffer (Miltenyi) to 1x using ddH₂O (see step 3.2.10).
4. Plastics and other supplies: 10mL sterile syringes, 20G needles, sterile 15mL conicals and serological pipettes.
5. Macrophages isolation kit (Peritoneum) – Miltenyi Biotec
6. OctoMACS magnet (Miltenyi Biotec)
7. MS columns (Miltenyi Biotec)

2.3 Macrophage culture medium

1. DMEM/F12 medium with stabilized glutamine (VWR)
2. Fetal bovine serum [FBS] (Sigma)
3. Penicillin/Streptomycin 100X Solution (Genesee Scientific)
4. L929 cells (ATCC CCL-1)
5. Recombinant mouse M-CSF (Miltenyi Biotec)
6. D-PBS (VWR)
7. Cell culture plates and other reagents: tissue-culture treated T75 flasks, sterile 15mL and 50mL conical tubes, and serological pipettes.

8. Macrophage culture medium: DMEM/F12, 10 % FBS, 10% L929 conditioned medium, 1% Penicillin/Streptomycin, 1ng/mL recombinant M-CSF.
9. 0.25% trypsin (Corning)

2.4 Phagocytosis assay

1. Plastics and other supplies: 24-well tissue-culture plates (VWR), glass coverslips diameter 12mm (Carolina), forceps to handle coverslips.
2. Ice-cold D-PBS: to quench the phagocytosis reaction and wash cells.
3. Fluorescently labeled Zymosan BioParticles® with Alexa-488 (Thermo Fisher scientific).
4. Macrophage culture medium (see 2.3).
5. 10% paraformaldehyde (Makron Fine Chemicals). Dilute to 4% with 1x D-PBS.
6. Microscope charged glass slides (Springside Scientific) and ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific).

2.5 Animals

All animals were treated and housed in accordance to the Guide for Care and Use of Laboratory Animals. All experimental procedures were approved by the University of Southern California's Institutional Animal Care and Use Committee (IACUC), and were in accordance with institutional and national guidelines. All mice were maintained under specific pathogen-free (SPF) conditions in the AAALAC-accredited Ray R Irani Hall Animal Facility at USC. C57BL/6N mice at different ages were obtained from the National Institute on Aging (NIA) colony at Charles Rivers and acclimated at the USC animal facility for at least 2 weeks before any processing. All mice are euthanized between 9 and 11 am to limit circadian effects. No live animal was censored.

We have successfully performed the described procedures in mice of both sexes and across a range of ages (3-29 months), with reasonable viability and visible Zymosan uptake. For the purpose of this method's description, we are focusing on data from male mice aged 4 and 20 months (Figure 1 and 2).

3. Methods

All the steps involved in isolation of bone marrow cells and peritoneal macrophages are done on ice (unless indicated otherwise) and all centrifugation steps should be performed in centrifuges pre-cooled to 4°C.

3.1 Production of conditioned media containing M-CSF.

- a. L929 cells are seeded at 50% confluency in a 600mL cell culture flask (VWR) with 25mL of DMEM/F12 medium supplemented with FBS.
- b. Conditioned media (CM) is collected two days after cell seeding.

- c. CM is filtered through a 0.45µm filter to remove cells, and collected into 50mL conical tubes.
- d. CM is stored at -20°C until use.

3.2 BMDM isolation, differentiation and culture

1. Euthanize mouse by CO₂ asphyxiation followed by cervical dislocation as a secondary means of euthanasia.
2. Carefully isolate the hind limbs without disturbing/breaking the bones from the euthanized mouse.
3. De-skin the isolated hind limbs and remove all the muscle (See note 2).
4. Place the cleaned bones in ice-cold D-PBS supplemented with 1% penicillin/streptomycin.
5. Repeat steps 3.2.1- 3.2.4 for all remaining animals (See note 2).
6. Cut at the one end of the bones (knee joint) to expose the bone marrow cells to be collected by centrifugation. Cutting one side is sufficient.
7. Place all 4 cleaned hind limb bones in the 'Column tube for bone marrow cells isolation'
8. Centrifuge the bones in their column tubes at 10,000g for 30 seconds in a tabletop centrifuge (See note 3). All the bone marrow cells will be collected in the 1.5mL microcentrifuge tubes, and bones will appear white (emptied of the marrow).
9. Resuspend the collected cells in 1mL of MACS rinsing buffer. and filter any chunks of muscle/non-disassociated cells using 70µm cell strainers.
10. Add 10mL of 1xRBC lysis buffer (Miltenyi Biotec), mix gently by pipetting up and down. Incubate at room temperature for **2 minutes**. Do not vortex, which can cause undesired immune cell activation.
11. Centrifuge cells at 300g for 10 minutes, and carefully remove the supernatant. Wash the pellet twice with MACS rinsing buffer
12. (Optional) For a purer cell fraction, use the Miltenyi Biotec MACS Monocyte separation kit, according to the manufacturer's instructions. Collect the eluted cells, centrifuge cells at 300g for 10 minutes and proceed to step 13.
13. Resuspend the cell pellet in 10mL of macrophage culture medium and plate it in a T75 tissue culture flask. Check cell yield using a hemocytometer or COUNTESS automated cell counter (Thermo Scientific).
14. Incubate cells in a humidified cell culture incubator at 37°C and 5% CO₂.

².Bone marrow isolation and peritoneal lavage are always done at a cold temperature to minimize cell death.

³.Bones can be kept in D-PBS on ice for few hours without overall impact on cell viability and yield.

15. Do not disturb the flask, until replacement of the differentiation macrophage medium with fresh medium on day 3.
16. On day 7-8, all live cells are considered to be differentiated BMDMs.
17. Wash the cells twice with D-PBS, and detach the cells using 0.25 % trypsin at 37°C for 15min. Flush the flask with a serological pipette several times to help with cell detachment, as macrophages are highly adherent to cell culture plastics.
18. Estimate the cell concentration. Check cell yield and viability using a hemocytometer or COUNTESS automated cell counter.
19. (Optional) Perform flow cytometry analysis using CD11b and/or F4/80 antibodies to estimate the purity of the cells.

3.3 Peritoneal macrophages isolation and culture

1. Euthanize mouse by CO₂ asphyxiation followed by cervical dislocation as a secondary means of euthanasia.
2. Retract the abdominal skin manually to expose the peritoneal wall taking care to avoid puncturing it.
3. Carefully inject 10mL of ice-cold Peritoneal Wash medium into the peritoneum using a 10mL syringe with a 20G needle. Take care to not puncture any internal organs.
4. Gently shake the mice for 30 seconds to dislodge any loose cells present in the peritoneal cavity.
5. Collect the peritoneal lavage using a 10mL syringe with a 20G needle. This suspension contains peritoneal cells including macrophages, B-cells and T-cells.
6. Remove the needle from the syringe, and slowly dispense peritoneal lavage into a 15mL conical tube. Place tubes on ice until processing.
7. Repeat steps 3.3.1- 3.3.6 for all remaining animals.
8. Centrifuge peritoneal lavage at 300g for 10 minutes. Carefully aspirate the supernatant while avoiding the cell pellet.
9. Resuspend cells in 1mL of MACS resuspension buffer.
10. If a red pellet is visible, perform the RBC lysis step (as described in 3.2).
11. Isolate macrophages following the manufacturer's instruction for MACS peritoneal macrophages isolation kit.
12. Collect the eluted cells, centrifuge cells at 300g for 10 minutes. Resuspended cells in 1-2 mL macrophage culture medium.
13. Check cell yield and viability using a hemocytometer or the COUNTESS automated cell counter. We routinely get yield of 7×10^5 to 1.2×10^6 cells/mL for both 4 months and 20 months old animals.
14. Proceed to plating cells for different assays.

3.4 Phagocytosis assay

1. Place one sterile coverslip per well of a 24-well tissue culture plate. Plate 75,000 cells per well in macrophage culture medium for 36-48 hours prior to performing phagocytosis assay to allow for the cells to recover (See note 4 for peritoneal macrophages). We recommend preparing at least duplicate wells for each independent macrophage culture to account for well-to-well variability.
2. Dilute fluorescently-labeled Zymosan BioParticles® to a concentration of 1µg/mL in the complete macrophage culture medium.
3. Remove the medium from the 24-well plate and replace it with 250µL of the bioparticle mixture in each well.
4. Incubate in the humidified cell culture incubator for **1 hour** at 37°C and 5% CO₂.
5. After incubation, quench the phagocytosis process by adding 500µL of ice-cold D-PBS.
6. Wash coverslip twice with ice-cold D-PBS to remove free bioparticles and decreased background.
7. Fix the cells using a 4% paraformaldehyde solution for 10 minutes at Room Temperature.
8. Wash coverslip twice with D-PBS.
9. Mount each coverslip onto a glass slide using a drop of ProLong Diamond Antifade Mounting medium. Allow the microscope slides dry in dark at room temperature for 24 hours, and use nail polish to seal coverslips for long term storage at 4°C.
10. Use an epifluorescence microscope to image the cells. Acquire images for at least 1,000 cells per animal across replicate coverslips.
11. Using the ImageJ software (<https://imagej.nih.gov/ij/>), count the number of green positive cells (Zymosan-internalized cells), and total number of cells in any given image (based on DAPI-staining of nuclei). To note, because a cell may contain more than one phagosome, green positive cells need to be counted by hand in the software rather than with the count particle function.
12. Calculate the ratio of AlexaFluor-488 positive cells to total number of cells to determine the phagocytosis index.

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4. Never centrifuge the bones more than 30 seconds to get the bone marrow cells and immediately resuspend cells with MACS cell resuspension buffer to avoid clotting of red blood cells from the bone marrow.

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Fig. 1. Overview of phagocytosis assay steps using BMDMs and peritoneal macrophages. Flowchart depicting the steps involved in performing phagocytosis assay using Bone marrow Derived Macrophages and peritoneal macrophages. BMDM: Bone marrow Derived Macrophages. M-CSF: Macrophage Colony Stimulating Factor. PFA: paraformaldehyde solution.

Fig. 2. Microscopy images of BMDM and peritoneal macrophages with Zymosan bioparticles phagocytosed.

Representative microscopic images of BMDMs (A) or peritoneal macrophages (B) from 4 and 20 months old mice, together with quantification of phagocytosis index. The phagocytosis index is defined as the ratio of cells with visible cargo after 1 hour. Scale bar – 75 μ m.