Chapter 14

Measuring Phagocytosis in Bone Marrow-Derived Macrophages and Peritoneal Macrophages with Aging

Ryan Lu, Nirmal K. Sampathkumar, and Bérénice A. Benayoun

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.

Key words Phagocytosis, Macrophages, Aging, Peritoneal macrophages, Bone marrow-derived macrophages, Zymosan, Innate immunity

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,

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Sean P. Curran (ed.), *Aging: Methods and Protocols*, Methods in Molecular Biology, vol. 2144,
https://doi.org/10.1007/978-1-0716-0592-9_14,

aging is associated with increased macrophage infiltration into tissues [6]. Phagocytosis is a phenomenon where a cell engulfs a foreign particle (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 [5, 9]. Resident macrophagepopulations exist across tissues (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 proinflammatory state vs. prorepair or wound-healing environment [5]. Macrophages activated with proinflammatory 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 extracellular 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 (1) isolate bone marrow-derived macrophages (BMDMs) in cohorts of aging mice (based on the protocol described in [12]) and peritonealmacrophages [13, 14], and (2) 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 500 μL centrifuge tube, cut off the cap and make few holes at the bottom of the tube using a 20 G 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 (1× D-PBS, 2 mM EDTA) (Miltenyi Biotec) and 0.5% BSA (Miltenyi Biotec).
3. Red blood cell lysis buffer: dilute 10× RBC lysis buffer (Miltenyi Biotec) to 1× using ddH_2O.
4. MACS filters, 30 μm (Miltenyi Biotec) and 70 μm (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 0.22 μm membrane.
2. MACS rinsing buffer: MACS resuspension buffer (1× D-PBS, 2 mM EDTA) (Miltenyi) and 0.5% BSA (Miltenyi Biotec).
3. (Recommended) Red blood cell lysis buffer: dilute 10× RBC lysis buffer (Miltenyi) to 1× using ddH_2O (*see***step 10** of Subheading 3.2).
4. Plastics and other supplies: 10 mL sterile syringes, 20 G needles, sterile 15 mL 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 100× Solution (Genesee Scientific).
4. L929 cells (ATCC CCL-1).
5. Recombinant mouse M-CSF (Miltenyi Biotec).
6. D-PBS (VWR).
7. Cell cultureplates and other reagents: tissue-culture treated T75 flasks, sterile 15 and 50 mL conical tubes, and serological pipettes.

8. Macrophageculture medium: DMEM/F12, 10% FBS, 10% L929 conditioned medium, 1% Penicillin/Streptomycin, 1 ng/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 12 mm (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. Macrophageculture medium (*see* Subheading 2.3).
5. 10% paraformaldehyde (Makron Fine Chemicals). Dilute to 4% with 1× D-PBS.
6. Microscope charged glass slides (Springside Scientific) and ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific).

2.5 Animals

All animals are treated and housed in accordance to the Guide for Care and Use of Laboratory Animals. All experimental procedures are approved by the University of Southern California's Institutional Animal Care and Use Committee (IACUC), and are in accordance with institutional and national guidelines. All mice are 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 are 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 (*see* **Note 1**). No live animal is 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 (Figs. 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.

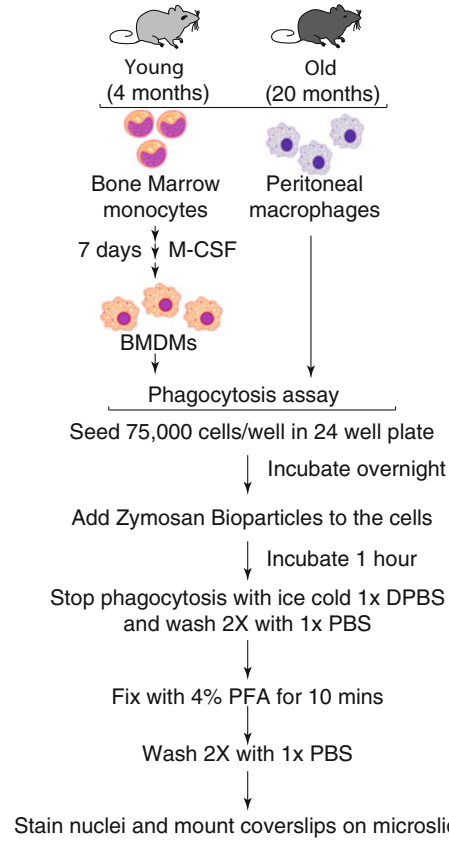

Fig. 1 Overview of phagocytosis assay steps using BMDMs and peritoneal macrophages. Flowchart depicting the steps involved in performing phagocytosis assay using bone marrow-derived macrophages and peritoneal macrophages. *BMDM* bone marrow-derived macrophages. *M-CSF*: macrophage colony-stimulating factor. *PFA* paraformaldehyde solution

3.1 Production of Conditioned Media Containing M-CSF

1. L929 cells are seeded at 50% confluency in a 600 mL cell culture flask (VWR) with 25 mL of DMEM/F12 medium supplemented with FBS.
2. Conditioned media (CM) is collected 2 days after cell seeding.
3. CM is filtered through a 0.45 μm filter to remove cells, and collected into 50 mL conical tubes.
4. CM is stored at −20 °C until use.

3.2 BMDM Isolation, Differentiation, and Culture

1. Euthanize mouse by CO_2 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**).

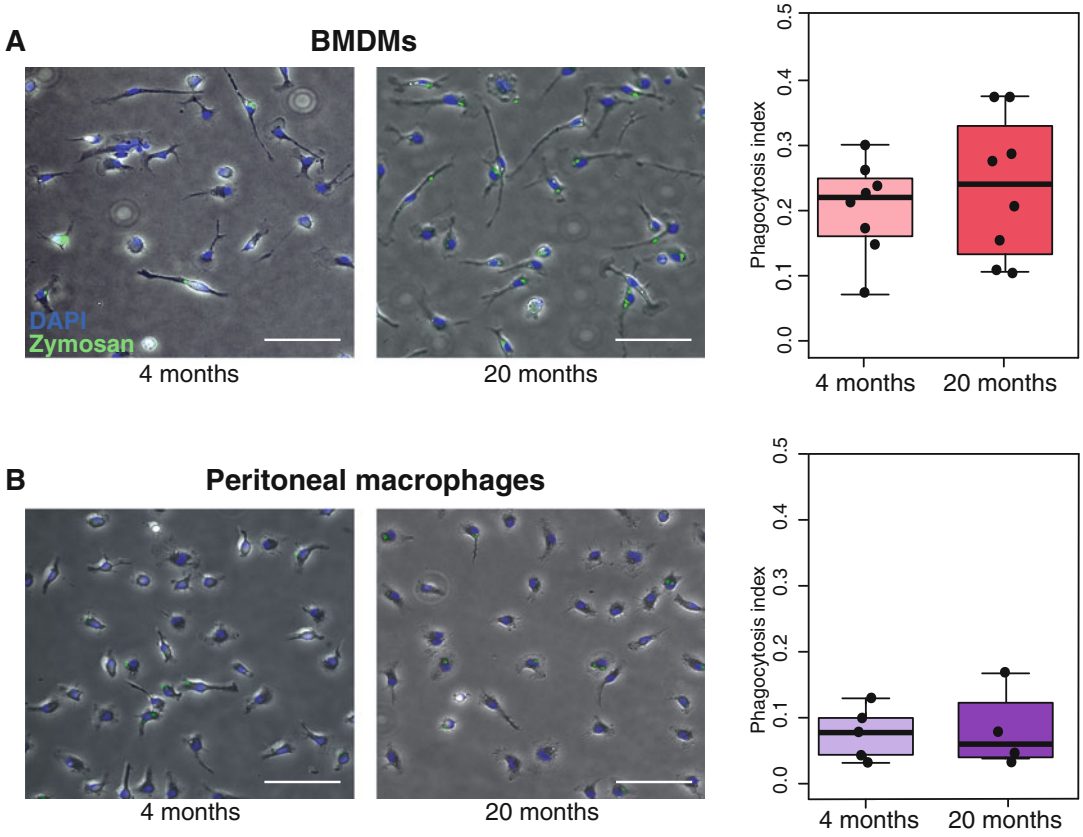

Fig. 2 Microscopy images of BMDM and peritoneal macrophages with Zymosan bioparticles phagocytosed. Representative microscopic images of BMDMs (**a**) or peritoneal macrophages (**b**) from 4- and 20-month-old mice, together with quantification of phagocytosis index. The phagocytosis index is defined as the ratio of cells with visible cargo after 1 h. Scale bar—75 μm

4. Place the cleaned bones in ice-cold D-PBS supplemented with 1% penicillin/streptomycin.
5. Repeat **steps 1–4** 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 four cleaned hind limb bones in the 'Column tube for bone marrow cells isolation'.
8. Centrifuge the bones in their column tubes at 10,000 × *g* for 30 s in a tabletop centrifuge (*see* **Note 3**). All the bone marrow cells will be collected in the 1.5 mL microcentrifuge tubes, and bones will appear white (emptied of the marrow).
9. Resuspend the collected cells in 1 mL of MACS rinsing buffer and filter any chunks of muscle/nondisassociated cells using 70 μm cell strainers.

10. Add 10 mL of 1× RBC lysis buffer (Miltenyi Biotec), mix gently by pipetting up and down. Incubate at room temperature for 2 min. Do not vortex, which can cause undesired immune cell activation.
11. Centrifuge cells at 300 × *g* for 10 min, 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 300 × *g* for 10 min and proceed to **step 13**.
13. Resuspend the cell pellet in 10 mL of macrophageculture 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% CO_2.
15. Do not disturb the flask, until replacement of the differentiation macrophage medium with fresh medium on day 3.
16. On days 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 15 min. 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 Macrophage Isolation and Culture

1. Euthanize mouse by CO_2 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 10 mL of ice-cold Peritoneal Wash medium into the peritoneum using a 10 mL syringe with a 20 G needle. Take care to not puncture any internal organs.
4. Gently shake the mice for 30 s to dislodge any loose cells present in the peritoneal cavity.
5. Collect the peritoneal lavage using a 10 mL syringe with a 20 G 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 15 mL conical tube. Place tubes on ice until processing.
7. Repeat **steps 1–6** for all remaining animals.
8. Centrifuge peritoneal lavage at 300 × *g* for 10 min. Carefully aspirate the supernatant while avoiding the cell pellet.
9. Resuspend cells in 1 mL of MACS resuspension buffer.
10. If a red pellet is visible, perform the RBC lysis step (as described in Subheading 3.2).
11. Isolate macrophages following the manufacturer's instruction for MACS peritoneal macrophages isolation kit.
12. Collect the eluted cells, centrifuge cells at 300 × *g* for 10 min. Resuspended cells in 1–2 mL macrophageculture medium.
13. Check cell yield and viability using a hemocytometer or the COUNTESS automated cell counter. We routinely get a yield of 7×10^5–1.2×10^6 cells/mL for both 4- and 20-month-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 macrophageculture medium for 36–48 h prior to performing phagocytosis assay to allow for the cells to recover (*see* **Note 4** for peritonealmacrophages). We recommend preparing at least duplicate wells for each independent macrophageculture to account for well-to-well variability.
2. Dilute fluorescently labeled Zymosan BioParticles® to a concentration of 1 μg/mL in the complete macrophageculture medium.
3. Remove the medium from the 24-well plate and replace it with 250 μL of the bioparticle mixture in each well.
4. Incubate in the humidified cell culture incubator for 1 h at 37 °C and 5% CO_2.
5. After incubation, quench the phagocytosis process by adding 500 μL 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 min 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 h, 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 1000 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 DAPIstaining 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 Alexa Fluor 488-positive cells to total number of cells to determine the phagocytosis index.

4 Notes

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.
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.
4. Never centrifuge the bones more than 30 s 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.

Acknowledgments

This work was supported by NIA R00AG049934, an innovator grant from the Rose Hills foundation and a generous gift from the Hanson-Thorell Family to B.A.B.

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