Video Article

In Vitro Phagocytosis of Myelin Debris by Bone Marrow-Derived Macrophages

Alyssa J Rolfe[1], Dale B Bosco[1], Erynn N Broussard[1], Yi Ren[1]

[1]Department of Biomedical Sciences, College of Medicine, Florida State University

Correspondence to: Yi Ren at yi.ren@med.fsu.edu

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Abstract

Bone marrow-derived macrophages (BMDMs) are mature leukocytes that serve a critical physiological role as professional phagocytes capable of clearing a variety of particles. Normally, BMDMs are restricted from the central nervous system (CNS), but following an injury, they can readily infiltrate. Once within the injured CNS tissue, BMDMs are the primary cell type responsible for the clearance of injury-derived cellular debris, including large quantities of lipid rich myelin debris. The neuropathological ramifications of BMDM infiltration and myelin debris phagocytosis within the CNS are complex and not well understood. The protocols described here, allow for the direct *in vitro* study of BMDMs in the context of CNS injury. We cover murine BMDM isolation and culture, myelin debris preparation, and assays to assess BMDM myelin debris phagocytosis. These techniques produce robust quantifiable results without the need for significant specialized equipment or materials, yet can be easily customized to meet the needs of researchers.

Video Link

The video component of this article can be found at https://www.jove.com/video/56322/

Introduction

Bone marrow-derived macrophages (BMDMs) are an important link between the innate and adaptive immune systems. As antigen presenting cells (APCs), they can communicate with lymphocytes via both antigen presentation and cytokine release[1,2,3]. However, as professional phagocytes, their primary function is to clear pathogens, aged cells, and cellular debris[1,4]. Following a spinal cord injury (SCI), substantial quantities of myelin debris is generated from dying oligodendrocytes, the cell type responsible for CNS axon myelination[5]. We and others have shown that clearance of myelin debris is primarily the responsibility of infiltrating BMDMs[5,6,7]. However, within spinal cord injury sites engulfment of myelin debris has been suggested to shift these normally anti-inflammatory cells towards a pro-inflammatory state[5,8,9]. As key mediators of neuro-inflammation in the injured spinal cord, BMDMs are important clinical targets.

To help investigate the influence of BMDMs in the injured spinal cord, we have developed an *in vitro* model to directly study how BMDMs respond to myelin debris. To improve biological relevance, both primary murine BMDMs and freshly isolated myelin debris are used in these investigations. As such, the methods presented here also detail the isolation and culture of primary murine BMDMs, and a modified sucrose gradient technique used to isolate murine CNS derived myelin debris[10,11,12]. Myelin debris can be readily labeled with a fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), to track its internalization by BMDMs. CFSE is well suited for this application because it is non-cytotoxic, and its narrow fluorescent spectrum permits multiplexing with other fluorescent probes[13,14]. Following phagocytosis, myelin debris lipids are transported through the lysosomes and packaged as neutral lipids into intracellular lipid droplets[5]. To quantify this intracellular lipid accumulation, we present an Oil Red O (ORO) staining method optimized for quantitative image analysis. This simple staining method produces robust reproducible results and quantification[15]. These methods facilitate the study of myelin debris phagocytosis and lipid retention with limited specialized equipment.

Protocol

The methods described here and in Section 2 have been approved by the Florida State University Institutional Animal Care and Use Committee (IACUC) and follows the guidelines set forth in the *Guide for Care and Use of Laboratory Animals, 8th edition*. All animals used in this in this protocol are house in a dedicated laboratory animal facility until use. No *in vivo* experimentation was performed prior to sacrifice. Animal numbers were based on experimental need using average cell and myelin collections as a guide in order to minimize usage.

NOTE: This protocol describes the generation of bone marrow-derived macrophages (BMDMs) (Section 1), the preparation of fluorescently labeled brain-derived myelin debris (Section 2), the general procedure for analyzing myelin debris phagocytosis (Section 3), and the general procedure for analysis of myelin debris lipid accumulation (Section 4). Reagent preparation, cell harvesting and manipulation, myelin collection and labeling, and assay performance should be completed in a laminar airflow biosafety cabinet.

1. Generation of Primary Bone Marrow-Derived Macrophages

1. **Preparation of complete macrophage culture medium (CMCM)**
   NOTE: Macrophage generation from bone marrow requires the use of macrophage colony-stimulating factor (M-CSF). This preparation utilizes L929 murine fibroblast conditioned media as the source of M-CSF.
   1. Generate L929 murine fibroblast conditioned media by culturing cells in 145 mm dishes with 50 mL of high glucose (4500 mg/L L-glucose) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% new born calf serum (NCS) and Penicillin/Streptomycin for 7 days.
   2. Collect media from cultures into 50 mL conical centrifuge tubes and centrifuge for 30 minutes at 3500 x g, 4 °C.
   3. Filter supernatants through a 0.2 µm syringe filter into a new 50 mL conical centrifuge tube. Conditioned media can be stored at -80 °C for up to 6 months.
   4. To high glucose DMEM, add 5% vol/vol NCS, 15% vol/vol L929 murine fibroblast conditioned DMEM, and 1% Penicillin/Streptomycin. Media can be stored at 4 °C for 2-3 months. Warm to 37 °C before use.

2. **Collection of primary bone marrow cells and macrophage induction**
   NOTE: Using this protocol one mouse will generate approximately 20 million bone marrow-derived macrophages (BMDMs).
   1. Euthanize the desired number of 8-10 week old mice using standard CO_2 asphyxiation guidelines followed by cervical dislocation.
   2. Sanitize the animal using 70% (vol/vol) ethanol:H_2O, saturating the fur.
   3. Cut a small opening in the abdomen and carefully pull back the skin to reveal the underlying tissue. Take care to not puncture the peritoneal cavity.
   4. Remove both hind legs starting at the hip and ending at the ankle. Place the collected tissue into a 100 mm Petri dish containing 5-10 mL sterile phosphate buffered saline (PBS) supplemented with 1% Penicillin/Streptomycin.
      NOTE: When processing several animals make sure to keep dishes on ice to limit tissue degradation.
   5. Remove muscle and connective tissue from the bone using a scalpel. Only the femur and tibia are used for cell collection, the fibula can be discarded.
   6. Expose the marrow cavity of the collected bones by trimming a small section from each end with a scalpel.
   7. Using a 25g needle, flush the marrow cavities with CMCM into a 50 mL conical centrifuge tube. The bones will appear white once they have been sufficiently flushed.
   8. Once collection is completed, agitate aspirates with an 18g needle for 30-90 s to generated a single cell suspension.
      NOTE: An optional red blood cell (RBC) lysis step may be performed at this point. It has recently been suggested that intracellular iron content may influence the effects of myelin debris upon macrophage polarization[16]. For information regarding the inclusion of a RBC lysis step refer to Trouplin *et al.*[17].
   9. Filter the suspension through a 70 µm sterile cell strainer into a new 50 mL conical centrifuge tube.
   10. Seed collected cells evenly into 145 mm cell culture dishes containing 15-20 mL CMCM. Use approximately three culture dishes per mouse sacrificed. Incubate cultures at 37 °C, 5% CO_2.
   11. After 72 hours wash plates once with sterile PBS to remove non-adherent cells, then add 15-20mL fresh CMCM. Incubate cultures for an additional 4 days at 37 °C, 5% CO_2. After 7 days of total culture, the hematopoietic bone marrow cells initially isolated will be mature BMDMs (**Figure 1**).

2. Generation of Fluorescently Labeled Brain-Derived Myelin Debris

NOTE: All reagents can be stored at 4 °C for up to 1 month.

1. **Preparation of myelin debris collection reagents**
   1. Prepare Tris·Cl buffer solution.
      1. To 800mL distilled deionized H_2O (ddiH_2O) add 20 mL of 1 M Tris·Cl, pH 7.45 (20 mM final concentration) and 20 mL of 100 mM Na_2EDTA (2 mM final concentration).
      2. Adjust pH to 7.45. Adjust volume to 1000 mL with ddiH_2O. Filter solution through a 0.2 µm filtration unit.
   2. Prepare 1 M sucrose solution.
      1. To 100 mL of Tris·Cl buffer solution, add 68.46 g of sucrose. Adjust volume to 200 mL with Tris·Cl buffer solution. Filter solution through a 0.2 µm filtration unit.
   3. Prepare 200 mL of 0.32 M sucrose solution by diluting the 1 M solution with the Tris·Cl buffer solution 0.83:0.16 (vol/vol).
   4. Prepare 150 mL of 0.83 M sucrose solution by diluting the 1 M solution with the Tris·Cl buffer solution 0.83:0.16 (vol/vol).

2. **Collection of brain-derived crude myelin debris**
   NOTE: The collection method described here is for the isolation of crude myelin debris.
   1. Euthanize 10-12 mice 8-10 weeks of age using standard CO_2 asphyxiation guidelines followed by cervical dislocation.
   2. Dissect the brains and place them in a 100 mm dish containing 10 mL of 0.32 M sucrose solution. Keep the dish on ice.
   3. Using sterile surgical scissors cut the brains into pieces approximately 5 mm^3 in size.
   4. Transfer the tissue to a 50 mL conical centrifuge tube and add approximately 30 mL of 0.32 M sucrose solution.
   5. Homogenize with a sterile hand-held rotary homogenizer, until a smooth solution is achieved.
   6. Dilute the homogenized brains to a final volume of 90 mL with 0.32 M sucrose solution.
   7. To six 38.5-mL thin-walled polypropylene ultracentrifuge tubes, add 20 mL of 0.83 M sucrose solution.
   8. Gently add the homogenized brain solution to the top of the 0.83 M sucrose solution, taking care not to mix the two layers.
   9. Balance each tube with the 0.32 M sucrose solution.

10. Centrifuge at 100,000 x g for 45 min at 4°C using an appropriate pre-cooled ultracentrifuge rotor. Set rotor acceleration and deceleration to their minimum values to reduce myelin debris loss.
11. Collect the myelin debris from the interface of the two sucrose densities.
    NOTE: Debris should appear as a white band towards the center of the tube.
12. Combine the crude myelin debris into a 50 mL conical centrifuge tube and adjust the volume to approximately 35 mL using Tris·Cl buffer solution.
13. Homogenize the crude myelin debris with a sterile hand-held rotary homogenizer for 30-60 s.
14. Evenly divide the suspension between 6 clean ultracentrifuge tubes and balance with an appropriate volume of Tris·Cl buffer solution.
15. Centrifuge at 100,000 x g for 45 min at 4°C using an appropriate pre-cooled ultracentrifuge rotor. Set rotor acceleration and deceleration to their maximum values.
16. As solid white pellets will now be visible, discard the supernatant and re-suspend the pellets in 10-15 mL of Tris·Cl buffer solution.
17. Evenly divide the suspension between 2 clean ultracentrifuge tubes and balance with an appropriate volume of Tris·Cl buffer solution.
18. Centrifuge again at 100,000 x g for 45 min at 4 °C using an appropriate pre-cooled ultracentrifuge rotor. Set rotor acceleration and deceleration to their maximum values.
19. Discard the supernatant and resuspend the pellets in 5-6 mL of sterile PBS and divide the suspension between an appropriate number of pre-weighed 1.5 mL micro-centrifuge tubes.
20. Centrifuge at 22,000 x g for 10 min at 4 °C.
21. Discard the supernatant and determine the weight of the myelin debris pellets.
22. Re-suspend the pellets in PBS to a final concentration of 100 mg/mL.
    NOTE: Ten to twelve brains is enough to produce 10-15 mL of 100 mg/mL myelin debris. Myelin debris can be store at -80 °C for 6 months.

3. **Myelin debris fluorescent labeling**
    1. Prepare the 50 µM carboxyfluorescein succinimidyl ester (CFSE) solution immediately before use.
        1. Using sterile PBS, dilute a 5 mM stock solution of CFSE prepared with 100% dimethyl sulfoxide (DMSO) to a final working concentration of 50 µM.
        2. Filter solution through a 0.2 µm syringe filter.
    2. Thaw the desired amount of 100 mg/mL myelin debris and re-suspend with a sterile 29g needle.
       NOTE: Freezing the myelin debris will cause it to drop out of solution requiring resuspension before use.
    3. Transfer myelin debris to a pre-weighed 1.5 mL micro-centrifuge tube.
    4. Centrifuge at 14,800 x g for 10 min at 4°C. Discard the supernatant.
    5. Resuspend the myelin debris in 200 µL of CFSE solution per 100 µL myelin debris pelleted.
    6. Incubate for 30 min at room temperature (RT) protected from light.
    7. Centrifuge at 14,800 x g for 10 min at 4 °C. Discard the supernatant.
    8. Resuspend the pellet in 600-800 µL of wash buffer (0.2 µm filter sterilized 100 mM glycine in PBS).
    9. Centrifuge at 14,800 x g for 10 min at 4 °C. Discard the supernatant.
    10. Repeat steps 2.3.8-2.3.9 twice more.
    11. After the final wash, determine the weight of the myelin debris pellet and re-suspend to 100 mg/mL with sterile PBS.
        Note: Labeled myelin debris can be store at -80 °C for up to 6 months.

3. Myelin Debris Phagocytosis Assay

NOTE: The following is the basic method for observing phagocytosis of fluorescently labeled myelin debris. Addition of other treatments and experimental conditions will need to be optimized by the investigator.

1. **Preparation of treatment plates**
    1. For each 145 mm plate, collect mature BMDMs into a 15 mL conical centrifuge tube using 5-6 mL of 10 mM ethylenediaminetetraacetic acid (EDTA) in Dulbecco's phosphate-buffered saline (dPBS) (pH 7.4).
       NOTE: Do not use trypsin as it may alter the activation state of the cells[18].
    2. Add an equal volume of pre-warmed CMCM to each tube of collected cells.
    3. Centrifuge at 180 x g for 8 min at 20 °C. Discard the supernatant.
    4. Re-suspend cells in CMCM and count.
    5. To each well of a 24-well cell culture plate, add $1x10^5$ cells in 1 mL of CMCM.
    6. Incubate at 37 °C, 5% CO_2 for 24 h.

2. **Myelin debris treatment and analysis**
    1. Add 10 µL of 100 mg/mL CFSE labeled myelin debris to each well (1 mg/mL final concentration).
    2. Incubate for 1-3 hours at 37 °C, 5% CO_2.
    3. Wash plate 3 times with sterile PBS to remove non-engulfed myelin debris.
    4. Add 400 µL of 4% paraformaldehyde to each well. Incubate for 30 min at RT.
    5. Wash plate 2 times with sterile PBS.
    6. Add 400 µL of Hoechst 33258 solution to each well. Incubate for 5 min at RT protected from light.
    7. Wash plates 2 times with sterile PBS.
    8. Add 200 µL of Fluoro-gel with Tris buffer to each well to reduce photobleaching.
    9. Image cells using an inverted epi-fluorescent capable microscope.
       NOTE: The excitation and emission wavelengths of CFSE are 494 nm and 521 nm, respectively.
    10. Divide the number of CFSE positive cells by the number of nuclei to determine relative myelin debris uptake.

11. Prior to imaging, maintain plates for up to 7 days at 4 °C protected from light.

4. Quantification of Intracellular Lipids Via Oil Red-O Staining

NOTE: The following is the basic method for observing intracellular myelin-debris-derived lipids. The use of CFSE labeled myelin debris is not recommended for fluorescent quantification of ORO staining due to spectral overlap. Addition of other treatments and experimental conditions will need to be optimized by the investigator.

1. **Preparation of staining solutions**
    1. Prepare 0.5% Oil Red-O (ORO) staining solution.
        1. Slowly add 100 mL of 100% propylene glycol (1,2-Propanediol) to 0.5 g of ORO (1-([4-(Xylylazo)xylyl]azo)-2-naphthol) while stirring.
        2. Heat solution at 95 °C for 15 min or until all large particles are dissolved.
        NOTE: Do not heat over 100 °C.
        3. Filter solution through a piece of filter paper while still warm into a new container.
        4. Allow solution to cool overnight at RT. Solution can be stored for up to 1 year at RT.
    2. Prepare 85% propylene glycol solution.
        1. Add 85 mL of 100% propylene glycol to 15 mL of ddiH_2O. Stir until mixed. Solution can be stored for up to 1 year at RT.
2. **Preparation of treatment plates**
    1. Prepare a 24-well plate following the method outlined in Section 3.
    2. Incubate at 37 °C, 5% CO_2 for 24 h.
3. **Myelin debris treatment**
    1. Add 10 µL of 100 mg/mL myelin debris to each well (1 mg/mL final concentration).
    2. Incubate for 1-3 h at 37 °C, 5% CO_2.
    3. Wash plate 3 times with sterile PBS to remove non-digested myelin debris.
    Note: At this point, plates can either undergo immediate fixation or fresh CMCM can be added, and cells returned to incubation for additional time points.
    4. Add 400 µL of 4% paraformaldehyde to each well. Incubate for 30 min at RT.
    5. Wash plate 2 times with sterile PBS then procced to staining.
4. **ORO staining and analysis**
    1. Wash fixed plate 3 times with ddiH_2O.
    2. Add 400 µL of 100% propylene glycol to each well and incubate for 5 min at RT.
    NOTE: This will reduce carryover of ddiH_2O.
    3. Aspirate the propylene glycol and add 400 µL of ORO solution to each well.
    4. Incubate for 8 min at 60 °C.
    5. Aspirate the ORO solution. Then, add 400 µL of 85% propylene glycol to each well and incubate for 5 minumintes at RT.
    6. Wash plate 3 times with ddiH_2O.
    7. Add 400 µL of Hoechst 33258 solution to each well. Incubate for 5 min at RT, protected from light.
    8. Wash plates 2 times with sterile PBS.
    9. Add 200 µL of Fluoro-gel with Tris buffer to each well.
    10. Image cells using an inverted epi-fluorescent capable microscope. ORO can be imaged using a standard Texas Red or DsRed filter set.
    11. Quantify relative lipid retention by determining the ORO positive area in each image field and dividing it by the number of nuclei.
    12. Maintain plates for 7 days at 4 °C protected from light.

Representative Results

Treatment of BMDMs with CFSE labeled myelin debris should yield clear internalization (**Figure 2**). While a 3-hour interaction time is sufficient for BMDMs to phagocytose enough added myelin debris for robust downstream detection, intracellular accumulation can be observed with as little as 1 hour of interaction. However, some myelin debris may still be present on the cell surface after washing. This may be due to insufficient washing, or particles not being fully internalized during the early stages of phagocytosis.

While BMDMs can rapidly internalize myelin debris, lysosomal processing is required before ORO stainable lipid droplets form. To demonstrate, BMDMs were incubated with myelin debris for 90 minutes, washed, and cultured for additional amounts of time prior to fixation and staining (**Figure 3**). **Figure 4** shows there is a delay between treatment initiation and neutral lipid appearance. It should also be noted that lipid retention is not stable during culture. BMDMs will begin to metabolize accumulated lipids soon after droplet formation. As such, we recommend waiting no longer than 24 hours between washing away non-engulfed myelin debris and fixation.

Quantification of ORO stained area demonstrates the rate of myelin lipid metabolism in BMDMs (**Figure 5**). Following a 90-minute interaction period, cells were returned to incubation in fresh CMCM. During the early chase period in fresh medium, the BMDMs metabolize the phagocytosed myelin debris lipid component into neutral ORO stainable lipids. A steady increase in ORO positive area is typical in the hours following the interaction. After 24 hours however, the BMDMs will have effluxed or metabolized a significant portion of the intracellular lipids.

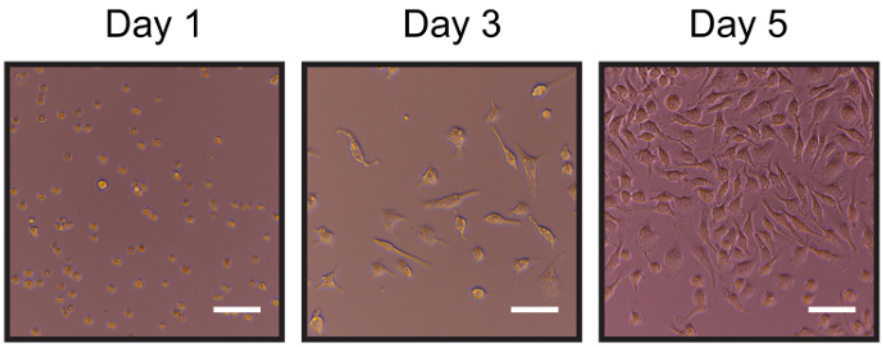

Figure 1: Representative BMDM Cultures. Few adherent cells will be initially observed. On day 3, any non-adherent cells are removed. By Day 7, mature bone marrow-derived macrophages are observed. Scale = 50 µm. Please click here to view a larger version of this figure.

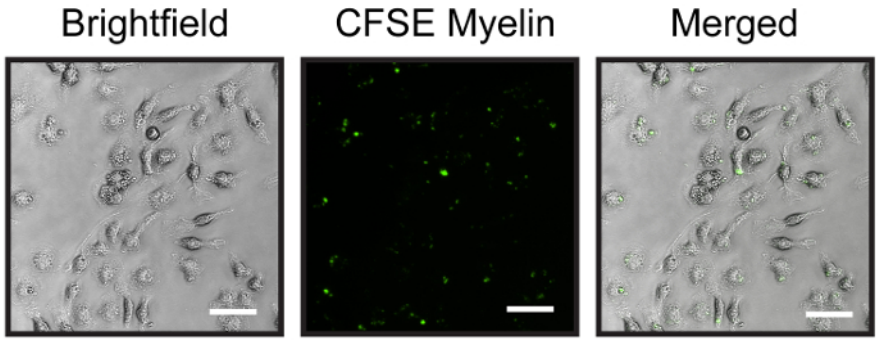

Figure 2: Representative Images of BMDM Myelin Debris Phagocytosis Assay. Cells were treated with 1mg/mL CFSE labeled myelin debris for 1 hour prior to washing and fixation with 4% PFA. Internalized myelin debris can be visualized using standard GFP filter sets on an epi-fluorescent capable microscope. Scale = 50 µm. Please click here to view a larger version of this figure.

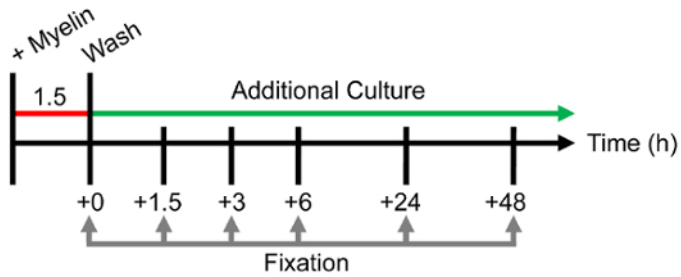

Figure 3: Diagram of Oil Red O (ORO) Experimental Design. BMDM are treated with 1mg/mL myelin debris (1:100 dilution) for 90 minutes, followed by washing and culture in fresh medium prior to fixation. Please click here to view a larger version of this figure.

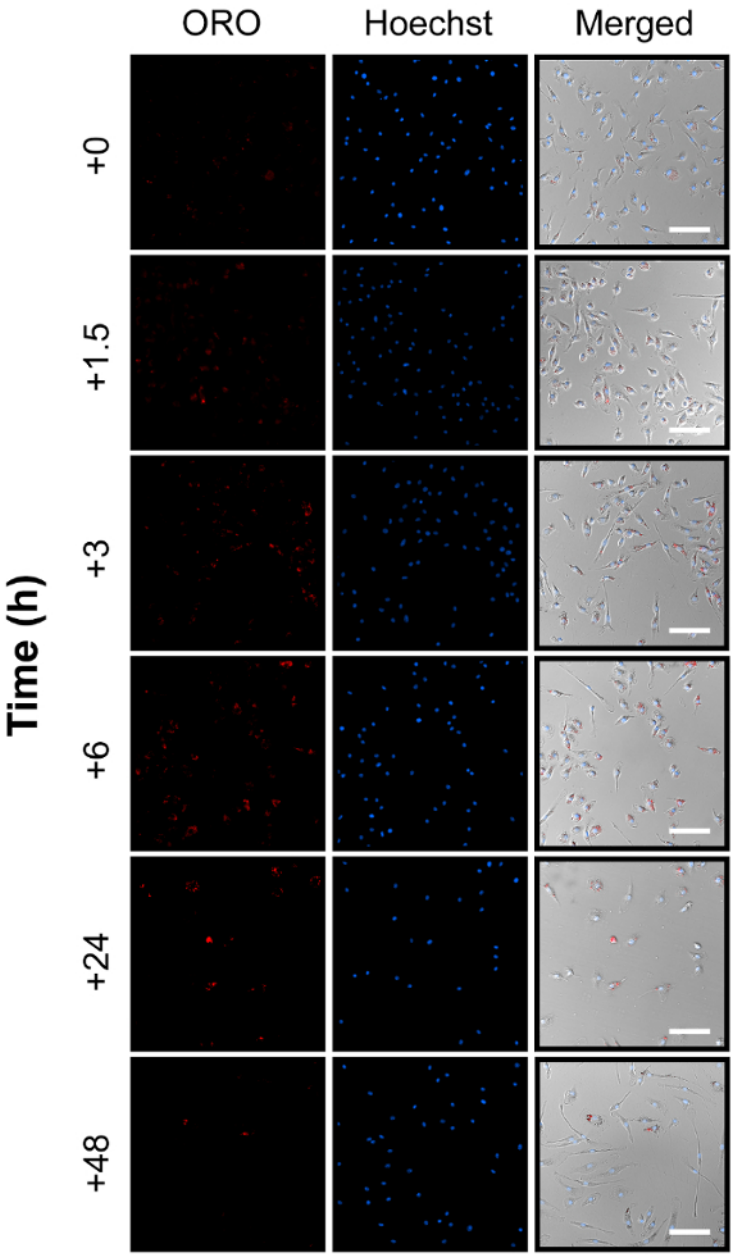

Figure 4: ORO Staining of BMDM Following Myelin Debris Phagocytosis. Following fixation with 4% PFA at the indicated time-points, BMDMs were stained with ORO and Hoechst 33258. Representative images for each time-point are shown. Scale = 100 µm. Please click here to view a larger version of this figure.

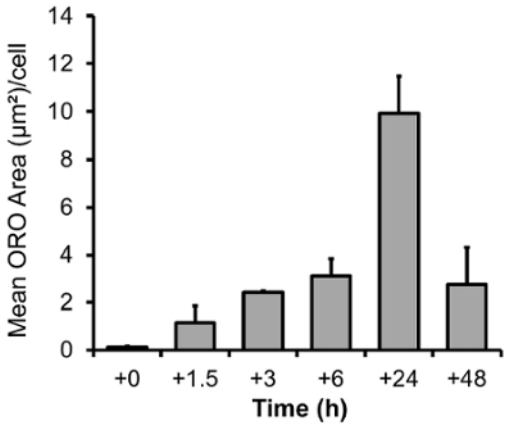

Figure 5: Quantitative Image Analysis of ORO Staining. For quantification of ORO staining, 3 wells were imaged at 20X with 5 images per well. All image acquisition settings were identical. Error bars = SEM. (n=3). Please click here to view a larger version of this figure.

Discussion

The procedures described here utilize both freshly isolated crude CNS myelin debris and primary bone marrow-derived macrophages. To reduce animal expenditures, we recommend that both brains and bone marrow cells be harvested from each mouse at the time of sacrifice. Two researchers working together can prepare both materials simultaneously. Alternatively, brains can be stored at -80 °C in PBS supplemented with antibiotics prior to myelin debris isolation. It has been our experience that brains can be maintained in these conditions for up to 1 month with no appreciable loss of myelin debris generation potential.

CFSE is used to label myelin debris because of its ease of use and florescent intensity. The dye remains quenched until cellular esterases cleave the acetate group on the molecule[14]. The freed succinimidyl ester group can then react with primary amides, resulting in covalent attachment to cellular proteins[14]. While CFSE is excitable using standard green fluorescent channels, there are several derivatives commercially available with different spectral properties. Thus, it is possible to design a similar phagocytosis assay using multiple fluorescent dyes. It should also be noted that because the dye readily crosses plasma membranes, it can be used to label apoptotic cells in a similar manner as myelin debris[19]. Moreover, while our method relies upon standard image analysis, with user optimization, quantification can be performed using flow cytometry or a high throughput plate imaging system.

Oil Red O (also known as Solvent Red 27, or Sudan Red 5B) is a fat-soluble dye that has been used extensively to stain neutral lipids in histological samples. Its deep red pigmentation allows for visualization of intracellular lipids via both bright field and fluorescent microscopy[5,20]. Using fluorescent microscopy allows for single channel imaging and robust quantification of ORO stained lipid droplets. While the staining method is simple and highly reproducible, care must be taken during sample preparation. It is critical that alcohol or acid-based fixatives not be used, as they can dissolve intracellular lipids, leading to a significant reduction in staining intensity. Additionally, as with any quantitative image analysis, the importance of standardized image capture settings and sufficient unbiased sampling cannot be understated. The use of staining controls is recommended to account for any sample auto-fluorescence. Such staining controls should be treated identically, but left unstained with ORO to measure the level of background auto-fluorescence. Even so, our image analysis method requires less optimization than other lipid quantification techniques, such as the ORO extraction method[21], which requires standard curve preparation, is more prone to experimental variability, and necessitates destruction of the sample. The use of image based analysis has several advantages over traditional ORO extraction methods in that it non-destructive and is compatible with immunostaining[21,22] .

Finally, while the procedures described are relatively straight-forward, it is critical that proper aseptic technique be used, since macrophages express multiple receptors that recognize pathogen associated molecular patterns (PAMPS) such as the endotoxin lipopolysaccharide (LPS)[23]. Interaction with bacterial contaminates will result in macrophage activation and can dramatic change their function[24,25]. In regards to the collected myelin debris, testing for contamination is recommended. Endotoxin detection methods such as the limulus amebocyte lysate (LAL) assay have high sensitivity, and have been used previously for this application[5,19].

Macrophages play an important role in both health and disease, yet the role of BMDMs in the context of CNS injury is currently an important topic of investigation. Using the methods described here, researchers can begin to better understand the mechanism of myelin debris phagocytosis by BMDMs.

Disclosures

The authors have no disclosures.

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