Chapter 6

Evaluation of Classical, Alternative, and Regulatory Functions of Bone Marrow-Derived Macrophages

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Abstract

The role of macrophage subsets in allergic diseases in vivo is under current investigation. These cells perform sentinel functions in the lung, the skin, and the gastrointestinal mucosa. Their interface with environmental cues influences the initiation, progression, development, and resolution of allergic diseases. Researchers often culture bone marrow-derived macrophages to study macrophage biology. The in vitro maturation of bone marrow precursor cells into mature macrophages is a powerful technique used to study macrophage biology. The polarization or differential activation of macrophages into functionally distinct subsets can provide insight into allergic disease pathologies. Classically activated, alternatively activated, and regulatory macrophages have different effector functions that can affect allergic responses. Understanding macrophage biology during allergen exposure, host sensitization, and disease progression/resolution may lead to improved therapeutic interventions. The purpose of this chapter is to outline protocols used for the culture and polarization of classically activated, alternatively activated, and regulatory macrophages. In addition, the techniques to measure macrophage-specific effector molecules by ELISA, RT-PCR, and immunoblotting are reviewed.

Key words M1 macrophage, M2 macrophage, Regulatory macrophage, Inflammation, Cytokine, Allergy and asthma

1 Introduction

Macrophages may play different roles in allergic responses depending on their location, predisposing genetic factors, and environmental factors. Macrophages are a predominant immune cell of tissues exposed to the environment such as the lung, intestine, and skin [1] and are likely to be one of the first effector cells to come into contact with allergens. Therefore, the response of the macrophage will have profound primary effects on the microenvironment and secondary effects on downstream cell types. After subsequent allergen exposure following an initial sensitization phase, the macrophage can secrete inflammatory cytokines such as interleukin-1β, interferon-γ, tumor necrosis factor-α, and

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interleukin-6. Elaboration of these cytokines causes an influx of additional inflammatory cells and can alter smooth muscle and epithelial cell function. Alternatively, macrophages can secrete anti-inflammatory mediators such as interleukin-10 and prostaglandin E_2 that can dampen allergic responses [2]. Experimental models of asthma and allergy, typically mouse models, have revealed a role for cytokines associated with T helper 2 (Th2) inflammation: interleukin-4, -5, and -13 [3, 4]. Although ex vivo analysis of patient samples has corroborated some conclusions from animal models, they have also illustrated that there is a more complex and variable disease process than was previously understood [5].

The heterogeneity of macrophage functions has led to the classification of three phenotypically distinct populations, the classically activated macrophage (CAM) or M1 macrophage, the alternatively activated macrophage (AAM) or M2 macrophage, and the regulatory macrophage. These designations are analogous with the T helper subsets Th1, Th2, and Treg, respectively. The M1 macrophage is characterized by secretion of proinflammatory cytokines (IL-1β, IL-12, and TNF-α) and increased amounts of reactive oxygen and nitrogen species [6]. These cells become polarized in the presence of IFN-γ and TNF-α or lipopolysaccharide (LPS) and are maintained by Th1 T lymphocytes. In contrast, M2 macrophages are characterized by increased expression of l-arginase, YM1, and RELMα/FIZZ1, which facilitate wound healing and angiogenesis. These cells are induced in the presence of interleukin-4 or interleukin-13. Regulatory macrophages are less well characterized. These cells are anti-inflammatory and secrete immunosuppressive cytokines such as interleukin-10 and transforming growth factor-β. These cells can be generated in vitro by incubation with immune complexes and Toll-like receptor agonists [8]. Their existence in vitro may be reflected in myeloid-derived suppressor cells and tumor-associated macrophages in vivo.

Recent evidence has provided support for the in vivo differentiation of these macrophage subsets [9, 10]. Nonetheless, the dynamic nature of macrophage plasticity suggests that these phenotypes may not be stable in vivo. Transcriptome analysis of in vitro-polarized macrophages and of ex vivo-purified macrophage populations has revealed striking differences in transcriptional programs of these cells [11]. During allergic diseases, aberrant macrophage activation and/or polarization is sometimes seen. These cells play a key role in vivo in the development and resolution of allergic diseases. Macrophages populate the interface of the host with the environment, specifically, the lung, the skin, and the gastrointestinal mucosa. These cells are one of the first cells to come in contact with environmental allergens and provide biomolecules to modulate an allergic response.

The role of macrophage subsets in allergic diseases in vivo is currently under investigation. Much of the data from experimental

models fails to present a clear picture. Depending on the model system, each subset has been shown to either promote or inhibit allergic responses. Ex vivo analysis of macrophages from atopic patients has revealed conflicting results [11–14]. The complexity of macrophage responses in allergic disease reflects the relative contributions and balance of M1, M2, and regulatory macrophages. As this area of research will ultimately improve treatment of allergic diseases, the culture of bone marrow-derived macrophages is an important laboratory technique. This is especially true when combined with ex vivo analyses of tissue-specific or tissue-resident macrophages, which will lead to a more complete understanding of disease pathologies.

2 Materials

2.1 Harvest

1. Personal protective equipment, including but not limited to laboratory coat, gloves, and goggles.
2. Age- and sex-matched mice: We typically use 8–12-week-old male mice.
3. Laminar flow hood.
4. Surgical instruments: Forceps and scissors.
5. 20–27 gauge needles.
6. 4. 3 or 5 cc syringes.
7. 100 μm cell strainer.
8. Hemocytometer.
9. Light microscope.

2.2 Culturing

1. Tissue culture incubator.
2. Tabletop tissue culture centrifuge equipped to spin 15 and 50 ml conical tubes.
3. Sterile and pyrogen-free PBS (without Ca^{2+} and Mg^{2+}).
4. Hank's Balanced Salt Solution.
5. Trypsin:EDTA.
6. Base media: DMEM, 10 % heat-inactivated fetal bovine serum, 1 % l-glutamine, 1 % sodium pyruvate, 1 % nonessential amino acids, 1 % penicillin/streptomycin.
7. Macrophage media: 20 % L929 cell (from American Type Culture Collection; CCL-1) conditioned media as a source of Macrophage-Colony-Stimulating Factor (M-CSF), plus base media. Alternatively M-CSF can be purchased commercially and used to supplement base media at 1×10^4 U/ml. L929 cells are a reliable source of inexpensive M-CSF. L929 cells should be cultured in base media until they are 90 % confluent.

Harvest the media by centrifugation and filter through a 0.45 μm filter to sterilize. Store filtered media at −80 °C until use.

8. 1 % Penicillin/streptomycin.
9. Fetal bovine serum (FBS), certified and low endotoxin tested.
10. Ethylenediaminetetraacetic acid (EDTA).
11. 100 × 20 mm tissue culture-treated plates.
12. 150 × 25 mm tissue culture-treated plates.
13. Multi-well tissue culture-treated plates: 6-, 12-, and 24-well plates.
14. Ultralow-bind non-treated tissue culture plates or petri dishes.
15. Pipettes, pipettors, pipette aids.
16. Tubes: 1.5 ml; 15 ml BD Falcon™ conical tubes; 50 ml BD Falcon™ conical tubes.

2.3 Functional Assays

1. Cell scraper.
2. Diff-Quick staining reagents.
3. Microscope slides.
4. Flow cytometer (optional).
5. Anti-F4/80-FITC antibody.
6. Anti-CD11b (Mac-1)-PE antibody.
7. Flow cytometry wash buffer: 1× PBS plus 2 % FBS.
8. Isotype control antibodies.
9. Recombinant mouse interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-13, or Macrophage-Colony Stimulating Factor (M-CSF) (Peprotech).
10. Ultrapure LPS.
11. Superscript III (Invitrogen).
12. Oligo $dT_{16–18}$ primer.
13. Phusion™ DNA polymerase (New England Biolabs).
14. ELISA plate reader.
15. ELISA kits capable of quantifying IL-1β, IL-12, and TNF-α.
16. Cell lysis buffer: 1× PBS plus 1 % Triton-X100 and protease inhibitors.
17. BCA assay kit (Thermoscientific) or Bradford protein assay (Biorad).
18. Standard materials for immunoblotting.
19. Endofit ovalbumin (Invivogen).
20. Dialyzed Rabbit anti-ovalbumin (Fitzgerald Industries International).

3 Methods

3.1 Isolation

1. Mice from specific pathogen-free housing should be used (*see* **Note 1**). Euthanize mice according to current Institutional Animal Care and Use Committee (IACUC) guidelines. Animals should be sex matched for minor histocompatibility antigens. We use 8–12-week-old donor mice for all of our experiments.
2. Prepare one mouse (*see* **Note 2**) at a time on a dissection tray and spray down the carcass with 70 % ethanol to sterilize the field.
3. Pin the carcass down with dissecting pins or large-gauge needles with the ventral side facing up.
4. Apply forceps to the skin anterior to the urethral opening. With scissors, cut skin along the ventral midline from the groin to the chin, carefully avoiding the underlying musculature.
5. Next, with scissors, make an incision from the start of the first incision caudally to the ankle on both sides of the animal. Carefully peel the skin off the appendages to the ankle joint.
6. Remove tissue from the legs with scissors and dissect the leg away from the body.
7. Denude the remaining soft tissue from the pelvic and femoral bones and separate proximal to the knee joint and the pelvic girdle (*see* **Note 3**).
8. Immerse the dissected femurs in 70 % ethanol for 1 min (*see* **Note 4**).
9. Wash twice in DPBS with penicillin (500–1,000 U/ml) and streptomycin (500–1,000 μg/ml).
10. While supporting the femur with forceps, use a 25 gauge (*see* **Note 5**) needle fitted to either a 3 or a 5 cc syringe filled with 2 ml of DPBS (*see* **Note 6**). Carefully insert the needle into the bone marrow cavity and gently expel the bone marrow from the bone with a jet of liquid directed into a 15 ml screw top tube with 5 ml of prewarmed 1× DPBS. Repeat and articulate the needle along the bone shaft to ensure that a majority of the bone marrow has been evacuated from the cavity.
11. Centrifuge cells for 10 min at $500 \times g$ at 10 °C. Discard the supernatant.
12. Count bone marrow cells in a hemocytometer and adjust the cells to a density of 5×10^6/ml in macrophage media.

3.2 Culturing

1. Add between 2 and 5×10^5 cells to a sterile tissue culture (100×15 cm) or petri dish (*see* **Note 7**).
2. Incubate for 6–7 days (*see* **Note 8**) in a 5 % CO_2-humidified tissue culture incubator. Check cells daily (*see* **Note 9**) and

wash cells one time every 2–3 days with DPBS. Resuspend the washed cells with macrophage media and replate on the same dish.

3. On day 6 or 7, discard the media in the tissue culture dish and wash the adherent cells with DPBS. Add 5–7 ml of 0.05 % trypsin–EDTA solution and incubate for 15–20 min at 37 °C (*see* **Note 10**).
4. Dislodge cells with gentle washing with a pipette aid.
5. Centrifuge the cells to wash and resuspend the pellet in base media.
6. Two femurs from a single mouse (12 weeks of age) should yield $2–6 \times 10^7$ macrophages.

3.3 Phenotyping

1. Resuspend $1–5 \times 10^5$ cells in 100 μl of 1× DPBS supplemented with 2 % FBS and 2 mm EDTA in a 1.5 ml tube.
2. Add fluorescently labeled anti-F4/80 and Mac-1 antibodies (*see* **Note 11**) and incubate on ice in the dark for 30 min.
3. Wash twice with 1× DPBS supplemented with 2 % FCS.
4. Resuspend cells in 500 μl of wash buffer.
5. Analyze the cells by flow cytometry. Macrophages should be positive for both F4/80 and CD11b. Cell purity ranges from 90 to 99 %, as indicated by double-positive staining.

3.4 Polarization to Classically Activated Macrophages (M1) (See Note 12)

1. Culture macrophages for 6–8 days.
2. Add $0.5–1.0 \times 10^6$ cells in 1 ml of media to each well in a 6-well tissue culture plate. Add 10–200 U/ml of recombinant mouse IFN-γ (*see* **Note 13**) for 6–18 h depending on the functional endpoint.
3. The following day add 1–100 ng of ultrapure LPS to stimulate cells. Stimulation times will vary depending on the endpoint assay.
4. For gene induction studies measuring transcription of proinflammatory cytokines, 2–6 h of stimulation with LPS works well. Briefly, cells are washed in 1X DPBS and cells are removed via physical scraping. Total RNA can be isolated using standard techniques. 1 μg of total RNA is reverse transcribed using Superscript III with oligo $dT_{16–18}$ primer following the manufacturer's suggested protocol. We use 2 μl of cDNA reaction for amplification with Phusion™ DNA polymerase (*see* **Note 14**) to amplify the following genes (with primers listed): *Il-12p40*, *Tnfa*, *iNos*, and *Gapdh* (*see* Table 1).
5. For cytokine elaboration: Harvest cell-free tissue culture supernatants (*see* **Note 15**) 6–18 h after stimulation depending on the cytokine assayed. Perform cytokine ELISA (*see* **Note 16**) on serially diluted supernatants.

Table 1
RT-PCR primers

Gene	Forward primer	Reverse primer
Tnfa	5′-CAGCCTCTTCTCATTCCTGCTTGTC-3′	5′-TCTGGAAGACTCCTCCCAGGGTATAT-3′
iNos	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′
Il12p40	5′-ATGGCCATGTGGGAGCTGGAGAAAG-3′	5′-GTGGAGCAGCAGATGTGAGTGGCT-3′
Arg1	5′-CAGAAGAATGGAAGAGTCAG-3′	5′-CAGATATGCAGGGAGTCACC-3′
Fizz1	5′-GGTCCCAGTGCATATGGATGAGACCATAGA-3′	5′-CACCTCTTCACTGCAGGGACAGTTGGCAGA-3′
Il-10	5′-CCAGTTTTACCTGGTAGAAGTGATG-3′	5′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′
SK-1	5′-ACAGCAGTGTGCAGTTGATGA-3′	5′-GGCAGTCATGTCCGGTGATG-3′
Gapdh	5′-GCACTTGGCAAAATGGAGAT-3′	5′-CCAGCATCACCCCATTAGAT-3′

6. Alternatively, harvest the cell pellet for western blot analysis. Wash cells twice with 1× DPBS. Add 1 ml of 1× DPBS, scrape cells from their respective wells, and place in a 1.5 ml microcentrifuge tube. Centrifuge at 4 °C for 1 min at maximum speed (>10,000 × *g*). Aspirate the supernatant. Lyse cells in 100 μl of lysis buffer with protease inhibitors for 30 min on ice. Spin lysed cells for 15 min at maximum speed (>10,000 × *g*) at 4 °C. Transfer lysate to a new tube and quantitate protein concentration by BCA assay or Bradford protein assay. Load 20–100 μg of total cell lysate on an SDS-PAGE gel (*see* **Note 17**). Gels can be transferred to nitrocellulose or PVDF membranes for immunoblotting. We used standard immunoblotting techniques for the detection of cytokines, cell surface receptors, and intracellular signaling molecules.
7. Functional assays such as phagocytosis, reactive oxygen or nitrogen species generation, and migration are generally performed 24–72 h after polarization.

3.5 Polarization to Alternatively Activated Macrophages (M2) (See Note 12)

1. Culture macrophages for 6–8 days.
2. Add 0.5–1.0 × 10^6 bone marrow-derived macrophages in 1 ml of media to each well in a 6-well tissue culture plate. Add 10–20 U/ml of recombinant mouse IL-4 or IL-13 for 18 h (*see* **Note 13**).
3. Stimulate cells with 1–100 ng ultrapure LPS and incubate (*see* **Note 18**).
4. For gene induction studies measuring transcription of proinflammatory cytokines, 2–6 h of stimulation with LPS works well. Briefly, cells are washed in 1× DPBS and cells are removed via physical scraping. Total RNA can be isolated using standard techniques.
5. 1 μg of total RNA is reverse transcribed using Superscript III with oligo $dT_{16–18}$ primer following the manufacturer's suggested protocol. We use 2 μl of cDNA reaction for amplification with Phusion™ DNA polymerase (*see* **Note 14**) to amplify the following genes: *Arg1*, *Fizz1*, and *Gapdh* (*see* Table 1).
6. It has been reported in the literature that activity and soluble collagen production can be measured in AAM lysates [15].

3.6 Polarization to Regulatory Macrophages (See Note 12)

Regulatory macrophages might represent a heterogenous population of macrophages that arise from different stimulation/polarization protocols. Indeed, there have been regimens that produce "regulatory" macrophages that include immune complexes, glucocorticoids, IL-10, and others [16, 17]. Here, we focus on regulatory macrophages generated in the presence of immune complexes.

1. Add 0.5–1.0 × 10^6 bone marrow-derived macrophages in 1 ml of media to each well in a 6-well tissue culture plate.

2. Prepare ovalbumin immune complexes by adding 20 μl of 1 mg/ml of endofit ovalbumin (*see* **Note 19**) to 500 μl of DMEM. Add 75 μl of 4 mg/ml rabbit anti-ovalbumin IgG dropwise. Nutate for 30–60 min at room temperature to allow complexes to form.
3. Stimulate macrophages with 1–50 ng of ultrapure LPS and with 100 μl endotoxin-free ovalbumin:IgG complexes as prepared above. Control stimulations (including unstimulated, LPS only, OVA only, and OVA-specific IgG only) should be done in parallel.
4. Incubate the macrophages for 18–24 h in a 37 °C incubator.
5. For gene induction studies measuring transcription of proinflammatory cytokines, 2–6 h of stimulation with LPS works well. Briefly cells are washed in 1× DPBS and removed via physical scraping. Total RNA can be isolated using standard techniques.
6. 1 μg of total RNA is reverse transcribed using Superscript III with oligo $dT_{16–18}$ primer following the manufacturer's suggested protocol.
7. We use 2 μl of cDNA reaction for amplification with Phusion™ DNA polymerase (*see* **Note 14**) to amplify the following genes: *Il10*, *Il12p40 SK-1*, and *Gapdh* (*see* Table 1).
8. Collect cell-free supernatants for ELISA measurement of IL-12 p40, IL-10, and either TNF-α or IL-6 (*see* **Note 16**).

4 Notes

1. We use mice housed exclusively in specific pathogen-free (SPF) containment. Mice with underlying inflammatory conditions or infections may affect macrophage function.
2. It is imperative that all solutions remain sterile and pyrogen-free. Bone marrow-derived macrophages are exceptionally sensitive to bacterial components. If possible, all manipulations should be carried out in a laminar flow hood using aseptic techniques. The generation of bone marrow-derived macrophages from novel, transgenic, or gene ablation mice may require individual optimization.
3. Tissue-specific macrophages can be harvested in parallel. Tissues commonly used for macrophage isolation include, but are not limited to, spleen, liver, lung, and intestine. Other immunologically relevant tissues such as spleen, lymph nodes, and thymus can also be harvested at this time to assay different cellular components, making full use of the experimental animal.

4. Tibia bones can be used as an additional source of bone marrow precursor cells.
5. Smaller or larger gauge needles can be used.
6. Different isotonic solutions such as HBSS or DMEM can be used in place of DPBS.
7. We have used both treated and non-treated tissue culture plasticware to cultivate bone marrow-derived macrophages. Using treated plasticware avoids possible confusion while growing different cell types. As a result of using treated tissue culture plasticware, bone marrow-derived macrophages adhere tightly to these dishes and may require physical dissociation with a cell scraper or prolonged treatment with trypsin:EDTA solution.
8. Slight variability in bone marrow-derived macrophage growth and maturation may be due to variability of growth factors (M-CSF) in L929 conditioned media.
9. Daily inspection of cells allows for visual confirmation of cell growth, adherence, and rapid assessment of contamination.
10. Bone marrow macrophages adhere tightly to tissue culture-treated plasticware and may require additional incubation time with 0.05 % trypsin:EDTA, increased concentration (0.25 % vs. 0.05 %) of trypsin:EDTA solution, or mechanical detachment with a cell scraper.
11. We have used many different fluorophores and antibody sources. The fluorophores must not overlap in emission spectra and must be compatible with the flow cytometer laser(s) and filters.
12. After 6–8 days in culture, macrophages can be polarized into one of the three main populations: classically activated, alternatively activated, or regulatory macrophages.
13. Commercial sources of recombinant growth factors such as interleukins are typically expressed in *E. coli*. These preparations have varying amounts of microbial contaminants (i.e., LPS) that may alter macrophage function. Source and lot variation should be evaluated.
14. Other DNA polymerases can be used (for example, Takara LA Taq). We have had success with Phusion™ using different source material and amounts, primer sets, and amplifying conditions.
15. Different effector molecules have different kinetic secretion profiles. Initial time point experiments will better define the appropriate stimulation periods.
16. A series of four twofold serial dilutions of supernatants will allow for experimental values to fall within the linear range of the assay.

17. The level of sensitivity of analyte will depend on the reagents used. Optimization with different detection antibodies and lysate concentrations might be necessary.
18. We have seen lot, source, and experimenter variability with LPS preparations. Single-use aliquots should be stored at −80 °C and quality assured before experimentation.
19. Commercial preparations of ovalbumin contain varying amounts of LPS. We have used endofit ovalbumin; other sources of ovalbumin should be tested for LPS before experimentation.

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