Thioglycollate-elicited Peritoneal Macrophages Preparation and Arginase Activity Measurement in IL-4 Stimulated Macrophages

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[Abstract] Macrophages are an essential cell population of innate immunity that plays important roles in inflammatory processes. Two main different phenotypes have been described with opposing activities: The classically activated macrophages (M1) and the alternatively activated macrophages (M2). Alternative activation of mouse macrophages can be induced by type 2 cytokines such as IL-4 and it is characterized by the regulation of the L-arginine metabolism. M2 macrophages convert arginine to ornithine and urea through the action of Arginase-1. Here we described a method for the isolation of peritoneal macrophages from thioglycollate-elicited mice and alternative activation by stimulation with IL-4. Intraperitoneal injection of thioglycollate elicits large numbers of macrophages into peritoneal cavity.

Materials and Reagents

1. Mice C57BL/6 male (age 8-12 weeks)
2. Difco Fluid Thioglycollate medium (BD, catalog number: 225650)
3. 70% and 100% ethanol
4. PBS (Lonza, catalog number: BE 17-515Q)
5. RPMI 1640 (Lonza, catalog number: BE 12-115F)
6. FBS (endotoxin<10 EU/ml) (Hyclone, catalog number: SV30160.03)
7. Penicillin-Streptomycin (Lonza, catalog number: BE 17-602E)
8. Murine IL-4 (Peprotech, catalog number: 214-14)
9. Tris (Panreac Applichem, catalog number: 131940.1211)
10. NaCl (Merck KGaA, catalog number: 1.06404.1000)
11. EDTA (Sigma-Aldrich, catalog number: ED255)
12. Triton X-100 (Sigma-Aldrich, catalog number: 8787)
13. Protease inhibitor mixture (Sigma-Aldrich, catalog number: P8340)
14. Urea (Sigma-Aldrich, catalog number: 4883)
15. $MnCl_2$ (Sigma-Aldrich, catalog number: 244589)
16. L-arginine monohydrochloride (Sigma-Aldrich, catalog number: A6969)
17. H_2SO_4 (Sigma-Aldrich, catalog number: 320501)
18. H_3PO_4 (Sigma-Aldrich, catalog number: 345245)

19. Isonitrosopropiophenone (Sigma-Aldrich, catalog number: I3502)
20. 12-well plates
21. 1.5 ml Eppendorf tubes
22. 50 ml sterile Falcon tubes
23. Cell strainer (Becton Dickinson, catalog number: 352340)
24. 10 ml syringe
25. 21G Needles
26. 25G needles
27. Lysis buffer (see Recipes)
28. Heat-inactivated FBS (see Recipes)
29. Stopping solution (see Recipes)

Equipment

1. Bench-top refrigerated centrifuge
2. Scissors
3. Incubator (37 °C, 5% CO_2 and 95 % humidity)
4. Tissue culture hood (biosafety cabinet)
5. Optical microscopy
6. Neubauer cell counting chamber
7. Espectrophotometer plate reader
8. Thermoblock (Eppendorf Thermomixer Compact) or water bath

Procedure

A. Injection of thyoglicollate into the peritoneum
   1. Preparation of 3% thioglycollate medium.
      a. Suspend 30 grams of thioglycollate medium in 1,000 ml of pyrogen-free water.
      b. Aliquot to 100 ml sterile bottles.
      c. Autoclave (15 psi/121 °C/15 min).
      d. After cooling, store in a dark place at room temperature for 2 months before using. *Note: Thioglycollate solution needs to age for several weeks until it turns to brown in color. This process is important to increase the extravasation of peritoneal cells.*
   2. Inject 2.5 ml of 3% thioglycollate medium i.p. per mouse using a 10 ml syringe with a 25 G needle. Wait for 4 days and harvest peritoneal cells (see step B).

B. Isolation and culture of thioglycollate elicited peritoneal macrophages
   1. Sacrifice mouse with CO_2 or isofluorane (the use of cervical dislocation is not indicated in this protocol in order to avoid potential internal bleeding).
   2. Clean abdomen with 70% ethanol.

3. Remove skin to expose the peritoneal wall. Practice a small incision in the skin and pull firmly.
4. Inject 10 ml of RPMI 1640 into the peritoneal cavity with a 25 G needle.
5. Massage abdomen for approximately 30 sec.
6. Recover peritoneal fluid as much as possible using a 10 ml syringe with a 21 G needle. (Usually approximately 8-10 ml fluid can be recovered from one mouse.)
7. Remove needle from syringe and put fluid into a 50 ml conical centrifuge tube on ice.
8. Centrifuge peritoneal cells 5 min at 300 *x g* (1,500 rpm, 4 °C). Discard the supernatant and collect the pellet.
9. Resuspend cell pellet in 10 ml of RPMI 1640 medium and count cells. (Usually approximately 30-50 million cells can be recovered from one mouse.)
10. Culture peritoneal cells (1 x 10^6/cm^2) in a 12-well plate in 1 ml of RPMI containing 10% heat-inactivated FBS at 37 °C with 5% CO_2 for 3 h.
11. Remove non-adherent cells by extensive washing with RPMI containing 10% heat-inactivated FBS at 37 °C.
12. Proceed to overnight cell starvation in the presence of 1 ml of RPMI 2% FBS per well.

C. M2 activation of peritoneal macrophages
1. After cell starvation, maintain the cells in RPMI 2% FBS and stimulate macrophages by adding IL-4 (20 ng/ml) to the 12-well plate and incubate at 37 °C for 24 h.

D. Arginase activity measurement
1. For cell dissociation, wash cells once with PBS and add an appropriate volume of lysis buffer at RT (see Recipes).
   Note: Usually 200 µl in each 12-well plate.
2. Remove cells with a cell scraper, collect them into a 1.5 ml Eppendorf and pipette up and down 5 times with a 200 µl tip for complete suspension.
3. Lyse the cells for 15 min at 4 °C.
4. Then spin down cell lysates at full speed (10 min, 14,000 rpm, 16,800 *x g*) at 4 °C and isolate the supernatant. Store at 4 °C before use.
5. Meanwhile, prepare a stock of 8 M urea in 50 mM Tris-HCl (pH 7.5).
6. Dilute stock of urea in 50 mM Tris-HCl (pH 7.5) to yield a standard range from 25 to 1,500 µg/ml. (*e.g.* 25, 50 100, 250, 500, 1,000 and 1,500 µg/ml)
7. Transfer 50 µl of cell lysates and standards to a 2 ml Eppendorf tube and add 50 µl of 10 mM $MnCl_2$ diluted in 50 mM Tris-HCl (pH 7.5).
8. Incubate tubes in a thermoblock or water bath for 10 min at 55 °C to trigger arginase-1 activity.
9. Then add 50 µl of 0.5 M L-arginine to the tubes and incubate in a thermoblock or water bath at 37 °C for 60 min. This step induces arginine hydrolysis.

10. Stop the reaction by adding 400 µl of stopping solution ($H_2SO_4/H_3PO_4/H_2O$ = 1/3/7, v/v/v).
11. Next, add 50 µl of 9% isonitrosopropiophenone in 100% ethanol to each sample and standard, and incubate the tubes in a thermoblock at 100 °C for 60 min.
12. Place the tubes in the dark at RT for 30 min.
13. Transfer 100 µl/well of samples and standards in triplicate to a 96-well plate and read optical density at 540 nm with a 690 nm correction.
14. Calculate sample concentrations from the standard curve and converted to Arginase Units using the following formula: [Urea Produced (µg/ml)/Total Protein (µg/ml)].

Recipes

1. Lysis buffer
   20 mM Tris (pH 7.5)
   150 mM NaCl
   2 mM EDTA
   0.1% Triton X-100
   Protease inhibitor mixture 1 µg/ml
2. Heat-inactivated FBS
   Inactivate FBS at 55 °C for 30 min
   Stored in aliquots at -20 °C
3. Stopping solution (for 11 ml)
   H_2SO_4 1 ml
   H_3PO_4 3 ml
   H_2O 7 ml

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Note: The study was performed under an ISCIII approved protocol.

References

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