

## 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<sub>2</sub> (Sigma-Aldrich, catalog number: 244589)
16. L-arginine monohydrochloride (Sigma-Aldrich, catalog number: A6969)
17. H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, catalog number: 320501)
18. H<sub>3</sub>PO<sub>4</sub> (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<sub>2</sub> 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 thioglycollate 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<sub>2</sub> 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  $\times$  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 ( $1 \times 10^6/\text{cm}^2$ ) in a 12-well plate in 1 ml of RPMI containing 10% heat-inactivated FBS at 37 °C with 5% CO<sub>2</sub> 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  $\mu$ l 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  $\mu$ l 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  $\times$  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  $\mu$ g/ml. (e.g. 25, 50, 100, 250, 500, 1,000 and 1,500  $\mu$ g/ml)
7. Transfer 50  $\mu$ l of cell lysates and standards to a 2 ml Eppendorf tube and add 50  $\mu$ l of 10 mM MnCl<sub>2</sub> 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  $\mu$ l 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  $\mu$ l of stopping solution ( $\text{H}_2\text{SO}_4/\text{H}_3\text{PO}_4/\text{H}_2\text{O} = 1/3/7$ , v/v/v).
11. Next, add 50  $\mu$ l 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  $\mu$ l/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 ( $\mu\text{g}/\text{ml}$ )/Total Protein ( $\mu\text{g}/\text{ml}$ )].

## 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  $\mu\text{g}/\text{ml}$
2. Heat-inactivated FBS  
Inactivate FBS at 55 °C for 30 min  
Stored in aliquots at -20 °C
3. Stopping solution (for 11 ml)  
 $\text{H}_2\text{SO}_4$  1 ml  
 $\text{H}_3\text{PO}_4$  3 ml  
 $\text{H}_2\text{O}$  7 ml

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

## References

1. Jiménez-García, L., Herráñz, S., Luque, A. and Hortelano, S. (2015). [Critical role of p38 MAPK in IL-4-induced alternative activation of peritoneal macrophages](#). *Eur J Immunol* 45(1): 273-286.