Macrophage Phagocytosis Assay of *Staphylococcus aureus* by Flow Cytometry

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[Abstract] This protocol describes a straightforward technique to evaluate the phagocytotic capacity of murine macrophages for *Staphylococcus aureus* (*S. aureus*). By staining *S. aureus* with Hexidium Iodide and staining murine bone marrow-derived macrophages (BMDMs) with FITC, the macrophage bacterial up-taking ability can be rapidly analyzed by flow cytometry. *S. aureus* is a Gram-positive bacteria causing severe human and animal infections. Host immune cells such as macrophages serve to eliminate *S. aureus* by phagocytosing the pathogen and save the host from life-threatening diseases. Study of host macrophage ability to phagocytose *S. aureus* is important for understanding the host-pathogen interaction and can help to elucidate the pathogenesis of *S. aureus* infection. This protocol may also be applied for macrophage phagocytotic assay of other gram-positive bacteria.

Materials and Reagents

1. Cell line-L929 (ATCC, catalog number: CCL-1)
2. *Staphylococcus aureus* strain-Sanger 476 (Wellcome Trust Sanger Institute, catalog number: MSSA476)
3. Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Gibco®, catalog number: 11965-092)
4. L-Glutamine (Life Technologies, Gibco®, catalog number: 25030-081)
5. Penicillin-Streptomycin (Life Technologies, Gibco®, catalog number: 15140-148)
6. Fetal bovine serum (Hyclone, catalog number: SH30070.03)
7. DPBS (Life Technologies, Gibco®, catalog number: 14190)
8. EDTA (Sigma-Aldrich, catalog number: E6758)
9. Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 25200-056)
10. ACK lysis buffer (Life Technologies, Gibco®, catalog number: A10492-01)
11. Tissue-culture water (Hyclone, catalog number: SH3052902)
12. Tryptic Soy Broth powder (TSB) (BD, catalog number: 211768)
13. Hexidium Iodide (Life Technologies, Invitrogen™, catalog number: H7593)
14. FITC-anti-mouse-F4/80 (BioLegend, catalog number: 123107)
15. DMEM complete media with 10% FBS (D10) (see Recipes)

16. Supernatant of L-cell cultural media (L-sup) (see Recipes)
17. Bone marrow macrophage medium (BMM medium) (see Recipes)
18. 500 mM-EDTA (see Recipes)
19. 5 mM-EDTA (see Recipes)
20. TSB (see Recipes)
21. Hexidium Iodide stock (see Recipes)

Equipment

1. 26G needle (Sigma-Aldrich, catalog number: Z192392-100EA)
2. Syringe (Terumo Medical Corporation, catalog number: SS-30L)
3. Cell culture dish (Corning, catalog number: 430293)
4. 6-Well Plate (Falcon, catalog number: 351146)
5. 75 cm flask (Corning, catalog number: 430641)
6. Vacuum filtration system (Corning, catalog number: 430758)
7. 50 ml Corning tube (Corning, catalog number: 430290)
8. 5 ml flow cytometry tube (BD, catalog number: 352054)
9. Bacterial incubator shaker
10. CO_2 incubator
11. Sorvall RT7 centrifuge
12. Flow cytometer (FACSCanto)

Software

1. FlowJo

Procedure

A. Differentiation of bone-marrow derived macrophage (BMDM)
   1. Euthanize three mice by CO_2. Sterilize the abdomen and hind legs of the mice with 70% ethanol.
   2. Make an incision in the midline of the abdomen, and expose the hind limbs by clipping outward. Remove all muscle from the bones and cut bones at both ends.
   3. Flush out bone marrow progenitor cells from bones using DPBS by syringe with a 26G needle. Flush 3-5 times for each bone by 2 ml DPBS. Spin down cells in Sorval RT7 centrifuge (4 °C, 1,500 rpm, 5 min).
   4. After aspirating out the supernatant using vacuum suction, treat cells by 5 ml ACK lysis buffer to lyse the erythrocytes (1 min, room temperature).

5. Next, add 45 ml of DPBS to the lysed cells to balance the osmotic pressure.
6. Spin cells down again, count cells by hemocytometer, and adjust them to a concentration of 5 x 10^5 cells/ml in BMM medium. Seed 10 ml cells in each 10-cm tissue culture dish. Expect to yield 1 x 10^8 of bone marrow cells from three mice.
7. Culture the cells for 3 days in CO_2 incubator at 37 °C. Then change for fresh BMM medium and culture for another 4 days (10 ml/dish).
8. The macrophages are now ready for functional assay.
9. Place the dishes in a sterile tissue culture hood, and wash the dishes twice with 5 ml cold DPBS.
10. Add 2 ml 5 mM-EDTA (room temperature) to each dish and wait around 5 min to detach the cells. Add 5 ml D10 to each dish and harvest all the cells. Expect to yield ~5 x 10^6 of BMDMs per dish.
11. Using this preparation, more than 95% of the differentiated cells should be macrophages based on F4/80 and CD11b expression. Analyze the expression of these markers by routine flow cytometry techniques. Choose different dyes for each marker (*e.g.* FITC for F4/80 and APC for CD11b).

B. Culture of *S. aureus* to exponential period

1. One day before the experiment, inoculate *S. aureus* into 3 ml TSB in a bio-safety cabinet and culture the bacteria overnight in a bacterial incubator shaker (37 °C, 225 rpm). *S. aureus* is a bio-safety level 2 pathogen which can cause severe human infections.
2. On the next morning, dilute 100 µl overnight culture into 10 ml new TSB. The approximate OD_{600} is around 0.1. Shake the bacteria approximately 2 hours until OD_{600} reach around 0.8 (37 °C, 225 rpm). OD_{600} of 1.0 is approximately 1.5 x 10^9 CFU/ml.
3. Spin down the bacteria (4 °C, 3,000 rpm, 10 min) and wash twice with 50 ml cold DPBS.
4. Adjust bacterial concentration to 5 x 10^8/ml in DPBS. Place the bacteria on ice before assay.

C. Stain *S. aureus* with Hexidium Iodide

1. Aliquot 1 ml *S. aureus* (5 x 10^8/ml) into a 50 ml Corning tube.
2. Add 20 µl Hexidium Iodide stock and mix it well.
3. Incubate the bacteria for 15 min in darkness (room temperature).
4. Wash twice with 50 ml cold DPBS (4 °C, 3,000 rpm, 10 min).
5. Re-suspend the bacteria in BMM medium at the concentration of 2 x 10^7/ml. The bacteria are ready for assay. Use them right away.

D. Macrophage phagocytosis assay

One day before the assay, seed 2 x 10^6 BMDMs into single wells in a 6-well plate. Apply the multiplicity of infection (MOI) 10 for *S. aureus* infection.

1. Briefly, on the day of assay remove the old medium and replace it with new BMM medium containing *S. aureus* (2 x 10^7/ml) to each well.
2. Quickly spin the 6-well plate (room temperature, 500 rpm, 5 min). Incubate the cells in a CO_2 incubator for 30 min to allow bacterial uptake by BMDMs.
3. Then add 200 µl cold Trypsin-EDTA to the cells and incubate for 10 min at room temperature without agitation to remove residual bacteria at macrophage surface.

   Note: Trypsin-EDTA solution treatment is to remove the excess bacteria. Prolonged treatment may also affect BMDM adherence, so the treating time shouldn't exceed 10 min. Trypsin-EDTA treatment will not interfere with F4/80 analysis.
4. Wash the plate three times with DPBS, using 2ml DPBS for each wash.
5. Add 0.5 ml 5mM-EDTA (room temperature) to the well to detach the cells in about 5 min and then 2 ml D10. Harvest the BMDMs by spinning (4 °C, 1,500 rpm, 5 min). Wash cells in 50 ml DPBS and re-suspend cells in 10 ml D10. Count the cells by hemocytometer.

E. Stain macrophages with FITC-anti-mouse-F4/80

1. Add 1 x 10^6 BMDMs from procedure D to 5 ml flow cytometry tube and spin down in Sorval RT7 centrifuge (4 °C, 1,500 rpm, 5 min). Aspirate the supernatant. Re-suspend cells in 100 µl DPBS. Three additional control tubes are required for flow cytometry analysis, including one blank tube without fluorescence dye, one FITC-anti-mouse-F4/80 single staining tube and one Hexidium Iodide single staining tube.
2. Add 1 µl FITC-anti-mouse-F4/80 to the tube following manufacturer's instruction (final concentration: 5 µg/ml). And incubate 20 min in darkness without agitation (room temperature).
3. Then add 500 µl DPBS. The cells are ready for flow cytometry assay.

F. Flow cytometry

Routine flow cytometry techniques are applied for phagocytosis analysis following manufacturer's instruction. In a FACSCanto machine, the fluorescence produced from Hexidium Iodide staining falls into FL2 channel (Excitation 488/Emmision 575) and fluorescence produced from FITC staining falls into FL1 channel (Excitation 488/Emmision 519). Results can be analyzed by FlowJo. The performer should be familiar with FlowJo software to analyze 4-color flow cytometry data. Briefly, open the flow cytometry data in FlowJo software. First, plot FSC and SSC in X-axis and Y-axis. Then gate single live cells. Finally plot FITC and PE in X-axis and Y-axis of the single live cells.

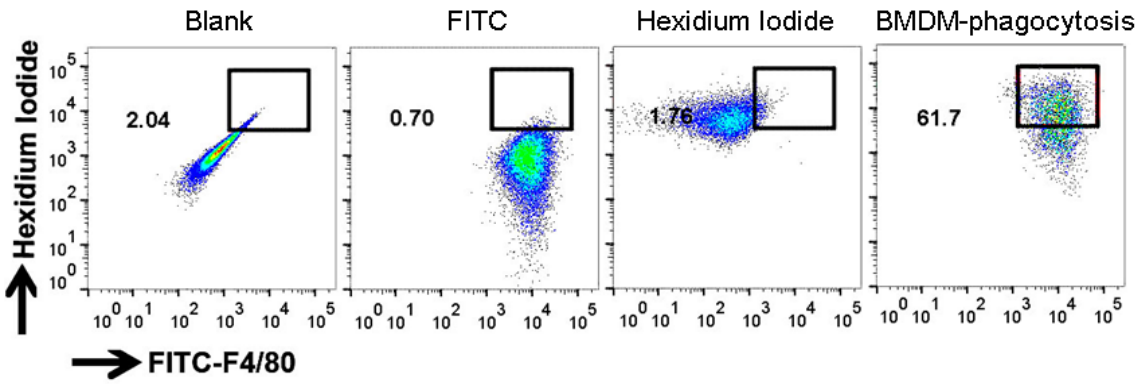

Figure 1. Practical example of a bone marrow-derived macrophage (BMDM) phagocytosis experiment. Blank: BMDMs without both FITC-anti-F4/80 staining and bacterial uptake. FITC: BMDMs with FITC-anti-F4/80 staining but without bacterial uptake. Hexidium Iodide: BMDMs with bacterial uptake but without FITC-anti-F4/80 staining. BMDM-phagocytosis: BMDMs with both FITC-anti-F4/80 staining and bacterial uptake.

Recipes

1. DMEM complete media with 10% FBS (D10)
   440 ml DMEM
   50 ml FBS
   5 ml L-Glutamine (Stocking concentration: 200 mM)
   5 ml penicillin-streptomycin (stocking concentration: 10,000 U/ml)
   Stored at 4 °C
2. Supernatant of L-cell cultural media (L-sup)
   Seed 5 x 10^5 L-929 cells in 75 cm flask in 55 ml D10
   Culture the cells for more than one week
   Culturing medium was harvested and filtered through vacuum filtration system and frozen down and stored at -20 °C, and use them within 6 months
3. BMM medium
   350 ml D10
   150 ml L-sup
   Stored at 4 °C
4. 500 mM-EDTA
   146 g EDTA dissolved in tissue-culture water, adjust pH to 8.0
5. 5 mM-EDTA
   5 ml 500 mM-EDTA
   495 ml DPBS

Stored at 4 °C

6. TSB
   Dissolve 30 g Tryptic Soy Broth powder into deionized water, and autoclave at 121 °C for 30 min
   Stored at room temperature
7. Hexidium Iodide stock (5 mg/ml)
   Dissolve 5mg Hexidium Iodide in 1 ml DMSO in darkness
   Aliquot and stored at -20 °C

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