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Measuring Opsonic Phagocytosis via Fcγ Receptors and Complement Receptors on Macrophages

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Abstract

Phagocytosis is a cellular process that plays crucial roles in the removal of dead or dying cells, tissue remodeling, and host defense against invading pathogens. Most eukaryotic cells are decorated with glycoproteins containing terminal sialic acids, whose negative charges tend to repel cells, making so-called “non-specific” phagocytosis a relatively inefficient process. Professional phagocytes are so designated because they express two major classes of receptors on their surfaces that are primarily involved in phagocytosis. Paradoxically, these receptors do not recognize microbes directly, but rather endogenous proteins that become tethered to microbes and target them for destruction. These are the Fcγ receptors that bind to the Fc portion of IgG and the complement receptors (CRs), which bind primarily to cleavage products of the third component of complement, C3. This unit describes assays that are used to measure these two types of macrophage phagocytosis.

INTRODUCTION

Immunoglobulin G antibodies recognize their cognate antigens via their antigen binding domains, leaving their Fc tails available to bind to FcγR on phagocytic cells. FcγRs fall into two general classes; those that mediate effector functions and those that transport immunoglobulin across epithelial barriers. This chapter will focus on the former receptors. There are four types of Fcγ receptors that activate effector functions, and a single receptor that inhibits these functions. FcγRs that fall within the activation class include FcγRI (CD64), FcγRIIA (CD32a), FcγRIII (CD16) and FcγRIV (Nimmerjahn and Ravetch 2006). There is no mouse counterpart of FcγRIIA and no known human counterpart to FcγRIV. The activating FcγRs recruit an ITAM-containing γ chain to transduce activating signals through the protein kinase Syk. FcγIIB (CD32b) is an inhibitory receptor that does not transduce activating signals. This receptor contains an ITIM domain in its cytoplasmic tail that recruits phosphatases to inhibit FcγR activation. Macrophages utilize the activating receptors to recognize antigens and pathogens opsonized with IgG. IgG-mediated uptake generally results in enhanced killing of internalized pathogens and more efficient antigen presentation (Swanson and Hoppe 2004). The expression of all of the FcγR on macrophages appears to be independently regulated and receptor expression can change dramatically with macrophage activation. Thus, the expression levels of individual FcγRs can sometimes be used as an indirect indicator of cellular activation. FcγR are expressed primarily on monocytes/macrophages, neutrophils, and dendritic cells. Dendritic cell maturation results in a dramatic down-regulation of FcγR expression. FcγR-mediated adhesion results in the rapid and efficient particle internalization, even in resting (non-stimulated) macrophages. Thus, most of the particles targeted to FcγR will be routed to phagolysosomes. In addition to IgG some acute phase proteins in the pentraxin family have been shown to bind to FcγR and promote phagocytosis.

Complement activation can result in the covalent modification of the target surface, depositing opsonic fragments of complement that can bind to CRs on phagocytic cells. Two

of the three pathways by which complement can be activated (alternative and lectin) are considered “innate” and can be initiated in non-immune individuals. The third classical pathway of complement activation requires antibody. During complement activation the third component of complement, C3, is cleaved to C3b which can bind to cell surfaces. C3 is a metastable thioester that binds covalently to exposed hydroxyl and amino groups. C3 is the most abundant of the complement proteins and the C3 convertases represent one of the principal amplification steps in the pathway. Bound C3b is rapidly cleaved to an inactivated form that is designated iC3b. Professional phagocytes have receptors for C3b (CR1) and iC3b (CR3, Mac-1, CD18/CD11b). The CR1 on phagocytes can also bind to C4b. A recently described complement receptor that has immunoglobulin-like folds (CRIg) has been reported to bind to bound C3b and iC3b (Helmy et al. 2006; He et al. 2008). This receptor has been identified on Kupffer cells. There are several other complement activation fragments that bind to specific receptors on leukocytes, but their role in mediating phagocytosis is relatively minor compared to the receptors mentioned above, and therefore they will not be addressed in this chapter.

Unlike Fc γ R-mediated phagocytosis, complement-mediated binding generally does not lead to efficient particle internalization by resting macrophages (Aderem and Underhill, 1999). Complement coated erythrocytes (E-IgMC) generally remain attached to the surface but not taken up. Treatment of macrophages with PMA or LPS, or priming with IFN- γ can enhance complement-mediated phagocytosis. The CRIg on liver Kupffer cells, in contrast, mediates the phagocytosis of complement-opsonized particles in non-immune hosts.

This unit describes assays to quantify the binding and phagocytosis of particles opsonized with either IgG or complement. In these assays, sheep red blood cells (SRBC) are used as the indicator particle because they are easy to visualize and count and because the hemoglobin released from lysed RBC can provide a convenient colorimetric way to quantitate SRBC binding and phagocytosis. The basic protocol describes light microscopic and colorimetric methods to measure macrophage binding and phagocytosis mediated either by Fc γ R or CR. SRBC opsonized with IgG (IgG-SRBC) are the targets for Fc γ R-mediated activity, whereas SRBC opsonized by IgM and complement (E-IgMC) are used for CR specificity.

NOTE: All reagents and equipment coming into contact with live cells must be sterile. For these assays, it is important to use fresh SRBC that should not exhibit measurable binding to macrophages when not opsonized. Old SRBC become “sticky” and will bind to macrophages in the absence of opsonization. Furthermore, the loss of sialic acid on aged RBC can lead to the activation of the alternative complement pathway. Thus, for all these assays, it is important to incorporate negative controls that should not bind to specific receptors in order to establish receptor-specificity of the binding event.

Basic Protocol: Measurement of Fc γ R- or Complement Receptor-Mediated Phagocytosis

In the basic protocol, adherent macrophages are incubated with IgG-opsonized SRBC and phagocytosis is measured. Following the incubation period, monolayers are typically washed to remove unbound erythrocytes, and then the amount of cell associated RBC are quantitated. This can be done by visually counting the number of cell-associated SRBC on giemsa-stained monolayers, or by spectrophotometrically quantitating the amount of SRBC-derived hemoglobin associated with the monolayer.

Materials

For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see appendix 2A; for suppliers, see appendix 5.

Primary macrophage cultures (see Unit 14.1) or macrophage cell lines (see Units 7.6)

Macrophage activation (see Unit 14.2 for activating agents)

ACK lysis solution (see recipe below)

0.1% (w/v) SDS solution

2,7-diaminofluorene (DAF) stock solution: 100 mg of DAF (Product number: D17106. Sigma-Aldrich, www.sigmaaldrich.com) are dissolved into 10 mL of glacial acetic acid with vigorous vortexing at room temperature. The solution can be kept at 4°C for a month in the dark.

DAF working solution: Add 1 mL of DAF stock solution and 0.1 mL of 30% of hydrogen peroxide (Catalog number: H323-500. Fisher Scientific. www.fishersci.com) into 10 ml of 0.2 M Tris-HCl (pH 7.4) containing 6 M urea.

Phosphate-buffered saline (PBS) (see recipe), ice cold

12-channel pipettor (to deliver 50- and 100- μ l aliquots) and disposable reservoirs (PGC Scientific).

96-well flat-bottom tissue culture plates (e.g., Falcon or Costar)

96-well microtiter plates (e.g., Falcon or Costar)

15- and 50-ml conical centrifuge tube

Centrifuge (Eppendorf Centrifuge 5810R or equivalent)

37°C water bath

Additional reagents and equipment for counting cells with a hemacytometer (appendix 3A)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Prepare macrophages

- 1 Bone marrow-derived macrophages are prepared (see Unit 14.1) and resuspended in culture medium to a concentration of $\sim 1 \times 10^6$ cells/ml. Approximately 100 μ l of macrophage cell suspension are seeded onto each well ($\sim 1 \times 10^5$ cells/well) of a 96-well flat-bottom tissue culture plate. Three replicates per treatment should be prepared. Cells are allowed to adhere for an hour, washed to remove non-adherent cells and then incubated overnight at 37°C in 5% CO₂. For peritoneal exudate cells, 100 μ l of a suspension of approximately 2×10^6 cells/ml are added to the wells. Wells are washed after 1 hour and incubated overnight. For macrophage-like cell lines, an amount of cells that yields a monolayer that is 50 ~ 75% confluent after overnight incubation should be used. This will vary depending on the cell line used, but a reasonable starting concentration is approximately 5×10^5 cells/ml $\times$ 100 μ l.
- 2 Macrophages can be activated with various reagents (see Unit 14.2) that can affect phagocytosis. Several cytokines are known to influence Fc γ R expression, including interferon- γ (IFN- γ) and M-CSF. Cells treated with medium alone

should be used as controls. Unopsonized SRBC should be used as negative controls, and they should be included in all experiments. Whenever working with macrophages, it is important to avoid any contaminating LPS that is frequently found on conventional laboratory glassware.

- 3 Prepare IgG-opsonized SRBC for FcγR-mediated phagocytosis (E-IgG) or SRBC opsonized with IgM and complement (E-IgM-C) for complement receptor-mediated binding (*See Supporting Protocol for SRBC preparation*).

Incubate opsonized SRBC with macrophages

- 4 Aspirate medium from the 96-well plate containing the macrophages (from step 2).

One should be careful to aspirate medium from macrophages with minimal vacuum to prevent removal of cells from the plate. Tilt the plate at an angle of ~30–45° and lower the aspirator into the medium until it almost touches the bottom. Microscopic examination of the plate after washing should be performed to verify that there was no loss of cells during washing. Cells may be lost from a small spot if the aspirator inadvertently touches the plate. For 96 well plates, a 200μl pipette tip or a tuberculin syringe with a 25 g needle attached to a vacuum hose are convenient ways to aspirate.

- 5 Add 100μl opsonized SRBC suspension (SRBC: at $\sim 1 \times 10^7$ cells/ml. see **Supporting Protocol**) to each well containing macrophages and incubate for a period of time.

The ratio of SRBC to macrophages should be empirically determined depending on the assay. A ratio of 10:1 is typical and will be used here. This generally results in 2–3 erythrocytes bound per macrophage after one-hour incubation. A preliminary time course can determine the amount of time during which the reaction remains linear. Choose an incubation time within the linear range that allows significant phagocytosis. The linear range may extend from 30 min to as much as 3 hr. E-IgMC bind to CR3 poorly at 4°C. At 37°C, resting cells will bind E-IgMC but will not initiate CR3-mediated phagocytosis. To facilitate CR3-mediated phagocytosis, PMA can be added to each well at a final concentration of 100 ng/ml.

Colorimetric quantitation of the phagocytosis of opsonized SRBC by macrophages

- 6 After incubation, aspirate the medium from the macrophages and wash the monolayers 3 times with 100μl culture medium.
- 7 To determine the number of SRBC that were internalized by the monolayer, aspirate culture medium and add 100μl ACK lysis solution to each well. Gently swirl the plate in a circular motion on the laboratory bench and examine the wells in an inverted microscope for evidence of lysis.

ACK lysis solution will disrupt SRBC that have not been phagocytosed and those that are bound to the exterior of the cells. The ACK lysis solution should be allowed to remain in the wells just long enough for the SRBC to lyse: the time required for lysis generally ranges from 15–30 seconds and should not be left on more than 1 min as this will begin to lyse the macrophages.

- 8 Aspirate the ACK lysis solution and rinse each well with 100μl culture medium.
- 9 To determine total number of monolayer-associated SRBC (bound and internalized) delete the ACK lysis step (step 7 above).

- 10 Add 100 μ l of 0.1% SDS to all wells and incubate 5 to 10 min at room temperature to allow macrophages to be lysed.
- 11 Transfer 50 μ l of macrophage lysate of each well to a 96-well microtiter plate. Add 50 μ l of DAF working solution to each well that contains 50 μ l of macrophage lysate. Mix well and incubate for 10 min at room temperature.
Negative controls should include macrophages that have incubated with unopsonized SRBC.
- 12 To set up a standard curve of OD as a function of SRBC, harvest and resuspend SRBC in sterile PBS at 1×10^8 cells/mL and dilute this SRBC solution 10-fold with 0.1% SDS to 1×10^7 cells/mL. Prepare a 2-fold serial dilution in 0.1% SDS, including 0.1% SDS blank. Transfer 50 μ l of each dilution in a 96-well microtiter plate. Add 50 μ l of DAF working solution to each well. Mix well and incubate for 10 min at room temperature.
- 13 Measure the absorbance at 620 nm in a 96-well microtiter plate reader. The absorbance is stable for 10 to 15 minutes.

In the presence of hydrogen peroxide, hemoglobin catalyzes the formation of fluorene blue from DAF, which has a broad spectrum range between 500 and 690 nm. The linear relationship between the optical density at 620 nm and the concentration of hemoglobin has been demonstrated.

Visual quantitation of the phagocytosis of opsonized SRBC by macrophages

- 14 Wash the monolayers three times with warm medium after their incubation with opsonized SRBC.
- 15 Fix monolayers by adding 2.5% fresh cold glutaraldehyde in PBS for 30 minutes in the cold.
- 16 Wash monolayers with PBS and add 100 μ l Giemsa for 10 min.
- 17 Wash monolayer 3 $\times$ with PBS and leave PBS in the wells.
- 18 Visualize monolayer on inverted microscope

Data analysis

- 19 The colorimetric results can reveal the total number of erythrocytes associated with the monolayer (bound and internalized), and the number of SRBC internalized by the monolayer (after ACK lysis). For IgG-SRBC, these numbers should be fairly similar, provided the input number of SRBC is not too high. For complement-mediated SRBC, there should be much more SRBC outside the cell than inside. If it is not then one should consider the possibility of LPS contamination. The number of SRBC is determined from the standard curve. Controls consisting of unopsonized SRBC should be used and subtracted from all values. If this control is high, then fresh new SRBC should be ordered. Calculate average replicate values from individual experiments and use an appropriate statistical test for analysis of data. The manual quantitation using light microscopy can determine the number of SRBC phagocytosed *per* 100 phagocytes and the percentage of macrophages with one or more SRBC associated with them. Ideal variability among replicates should be below 10%. Differences between different preparations of SRBC contribute the largest source of variability between individual experiments.

Alternate Protocol: Measurement of Receptor-mediated Binding Activity following the addition of phagocytosis inhibitors

This alternative protocol is similar to the basic protocol for phagocytosis assay (above) except that inhibitors to prevent the phagocytosis of the target cells by macrophages are added. Thus, opsonized SRBC cells bind to macrophages but cannot be ingested. The phagocytosis assay employing ACK lysis step (previous step 7) measures the number of erythrocytes that have been ingested, whereas the assay omitting ACK lysis (step 9) measures total bound and ingested RBC. The assay described here measures total SRBC bound to the receptor but not ingested. The assay is performed exactly as described above, except that 30 min before the addition of erythrocytes, macrophages are treated with either cytochalasin or latrunculin to prevent phagocytosis. After the incubation period, monolayers are washed four times to remove unbound erythrocytes. Care should be taken to not wash the monolayer too vigorously causing the bound RBC to lyse or release.

Additional Materials (also see Basic Protocol and Supporting Protocol)

Cytochalasin D: 30 μ M (Cat# 250255, Calbiochem, www.emdbiosciences.com)

Latrunculin-A: 10 μ M (Cat# T119, BIOMOL-Enzo Life Sciences, www.biomol.com)

24-well flat-bottom tissue culture plates (e.g., Costar or Falcon)

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Prepare macrophages

- 1 Macrophages are prepared (see Unit 14.1) and resuspended in the culture medium to a concentration of 1×10^6 cells/mL. Add 100 μ l of suspension to each well of a 24-well tissue culture plate (1×10^5 cells/well). Prepare at least duplicate wells for each treatment and control. Culture cells in a humidified 37°C, 5% CO₂ incubator overnight.
- 2 Activate macrophages (see Unit 14.2) to modify receptor expression if desired.
- 3 Prepare IgG-SRBC (see **Supporting Protocol**).
- 4 Add 0.1 ml of 10 μ M Latrunculin-A to each 9.9 ml opsonized SRBC (SRBC: $\sim 1\text{--}2 \times 10^7$ cells/ml), or cytochalasin D at a concentration of 30 μ M.
Latrunculin-A, at a final concentration of 100 nM, completely prevents the bound SRBC from becoming ingested via inhibition of actin polymerization and microfilament-mediated processes (Oliveira, et al. 1996).

Incubate opsonized SRBC with macrophages

- 5 Aspirate medium from macrophages. Add 100 μ l of opsonized SRBC suspension to each well ($\sim 1\text{--}2 \times 10^6$ cells/well). Incubate plate in a humidified 37°C, 5% CO₂ incubator for 30 min to 1 hour.

Measure opsonized SRBC bound to macrophages

- 6 Aspirate SRBC suspension from macrophages carefully. With a 1-ml pipettor, gently wash each well with 100 μ l culture medium. Repeat wash procedure three more times.

Opsonized SRBC cells that do not bind the macrophage are washed off without being lysed. This step needs a more thorough washing than the basic phagocytosis assay, but extra cares should be taken to avoid aspirating too vigorously. Direct 100 µl of medium against the side of the well so as not to dislodge the cells from the plate.

- 7 Add 100 µl of 0.1% SDS to each well and gently shake the plate for 10 min at room temperature to completely disrupt the macrophages.
- 8 Transfer 50 µl of macrophage lysate of each well to a 96-well microtiter plate. Add 50 µl of DAF working solution to each well that contains 50 µl of macrophage lysate. Mix well and incubate for 10 min at room temperature.
- 9 Set up a standard curve of OD as a function of SRBC (see **Basic Protocol**, step 12).
- 10 Measure the absorbance at 620 nm in a 96-well microtiter plate reader. The absorbance is stable for 10 to 15 minutes.

Analyze data

- 11 The results can be defined as the number of SRBC bound to the monolayer, provided that the number of bound SRBC is determined from the standard curve. Calculate average replicate values from individual experiments and use an appropriate statistical test for analysis of data (see **Basic Protocol**).

Supporting Protocol: Preparation of IgG-SRBC and E-IgM-C

In this supporting protocol, SRBC are opsonized with either anti-SRBC IgG (IgG-SRBC) for the measurement of FcγR-mediated phagocytosis, or SRBC opsonized with IgM and complement (E-IgMC) to measure binding mediated by complement receptors. Complement is “fixed” via the classical pathway using IgM antibody to SRBC. It is important that the antibody used to opsonize SRBC be free of IgG, because this will mediate binding to the Fcγ receptors. There should be little to no binding of IgM-opsonized SRBC to macrophages unless complement is added.

Additional Materials (also see Basic Protocol)

For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see appendix 2A; for suppliers, see appendix 5.

Sheep red blood cells: SRBC (Cat#: 55876, Washed, preserved sheep red blood cells, MP Biomedicals, Inc. www.mpbio.com).

Rabbit Polyclonal IgG against SRBC: Cat#: 55806, MP Biomedicals, Inc. www.mpbio.com) or a monoclonal IgG2a against SRBC (ATCC® number: TIB-111™, hybridoma clone S.S-1, www.atcc.org) or a monoclonal IgG2b against SRBC (ATCC® number: TIB-109™, hybridoma clone N-S.8.1, www.atcc.org).

Rabbit IgM antibody to SRBC is obtained from Cedarlane Laboratories (#CL9000-M) (www.cedarlanelabs.com). Alternatively a hybridoma secreting IgM monoclonal antibody to SRBC is obtained from the American Type Culture Collection (ATCC number TIB-112, hybridoma clone S.S-3 (www.atcc.org)).

Human complement component C5-deficient serum: Catalog number A501, Quidel® Corporation, www.quidel.com; or mouse C5-deficient serum can be obtained from C5-deficient mice such as AKR/J mouse (Cat#: 000648, Jackson Laboratories, www.jaxmice.jax.org).

GVB⁺⁻ buffer (see **Reagents and Solutions**)

GVBNi⁺ buffer (see **Reagents and Solutions**), ice cold

50-ml conical plastic centrifuge tubes (e.g., Falcon)

Centrifuge (Eppendorf Centrifuge 5810R or equivalent)

Prepare and Count SRBC

1. Wash 5 ml of SRBC with 40 ml ice-cold PBS by centrifuging at $500 \times g$ at 4°C for 5 min. Aspirate PBS and repeat wash with an additional 40 mL PBS.
2. Wash SRBC once with 40 mL ice-cold GVB⁺⁻ solution.
3. Resuspend SRBC in 20 mL GVB⁺⁻ solution and transfer 100 μ l of the SRBC suspension to count. Count SRBC in a hemocytometer and adjust to a concentration of 2×10^8 /ml.

Alternatively, SRBC can be quantitated by measuring hemoglobin content. Resuspend 100 μ l SRBC in 2.9 mL of distilled H₂O (1/30 dilution). Read the absorbance of the water-lysed sample at OD 540 nm versus that of water and calculate the SRBC concentration: the value of the lysed SRBC suspended at 1×10^9 cells/ml should be ~0.350 OD 540 nm.

Preparation of IgG-SRBC

1. Place 2×10^8 SRBC in a 50 ml conical tube and centrifuge at $500 \times g$ at 4°C for 10 min at 4°C. Carefully aspirate the supernatant and resuspend the SRBC pellet to 1 ml in PBS. Add anti-SRBC IgG to the pellet and vortex. This preparation should be enough to assay two 96-well plates of macrophages.

The optimal (subagglutinating) IgG concentration is empirically determined by adding different amounts of IgG to a constant number (2×10^8) SRBC in parallel microfuge tubes. If the agglutination titer is provided with a commercially supplied IgG, it is best to start at the agglutination titer and work downward using two-fold dilutions. The highest titer that yields minimal agglutination should be used. For convenience, the optimal titer is recorded for future use and the antibody is aliquotted in small volumes for subsequent experiments. The titer need only be determined once, provided that fresh SRBC are routinely used. The use of SRBC without opsonization should always be included as a negative control.

2. Gently mix SRBC with anti-SRBC IgG mixture and incubate 30 min at room temperature with gentle rotation to resuspend the cells.
3. Add PBS to 25 ml final volume and centrifuge 10 min at $500 \times g$, room temperature.
4. Aspirate supernatant and wash cells one additional time with PBS. SRBC will not form a tight pellet. Be careful not to aspirate SRBC.
5. Resuspend the final SRBC-IgG at a concentration of 1×10^7 SRBC /ml in PBS and add desired amount to monolayer.

Opsonized SRBC should be used within hours of completing this step.

Prepare E-IgM-C (optional)

1. If measuring complement receptor-mediated phagocytosis in Basic Protocol 1, prepare I-IgM-C by placing 2×10^8 SRBC in a 50-ml conical plastic centrifuge

tube and centrifuge at $500 \times g$ for 10 min at 4°C. Carefully aspirate the supernatant and resuspend in 1 ml GVB.

2. Add anti-SRBC IgM to the SRBC and vortex. Incubate at 37°C for 30 min with gentle agitation.

The optimal concentration of IgM should be predetermined as described above for IgG.

3. Wash E-IgM in 25 ml PBS and resuspend pellet in 1 ml GVB⁺⁺. Add 50 µl C5D serum (5% v/v final) and incubate at 37°C for 30 min.
4. Resuspend the E-IgM-C to a concentration of 1×10^7 SRBC /ml and add the desired amount to the monolayer.

Reagents and Solutions

For common stock solutions, see appendix 2A; for suppliers, see appendix 5.

ACK lysis solution: Dissolve 1.66 g of ammonium chloride (31 mM final), 0.2 g of potassium bicarbonate (2 mM final), and 40 µl of 0.5 M EDTA (20 µl final). Adjust the volume to 1 liter with distilled H₂O. Store for 1 year at room temperature

Calcium chloride stock solution, 200× Dissolve 0.44 g of CaCl₂×2H₂O in 100 ml of distilled H₂O. Store 1 to 2 years at room temperature.

GVB⁺⁺ buffer (gelatin/veronal buffer with Mg²⁺): In a 1-liter graduated cylinder or volumetric flask, mix: 200 ml of 5× veronal-buffered saline, pH 7.2 (see recipe), 5 ml of 200× magnesium chloride stock (see recipe), and 2 g of gelatin powder. Heat on stirrer hot plate to dissolve gelatin. Add distilled H₂O to 1 liter. Check pH is 7.2 to 7.4 at 22°C. Store 12 hr at 4°C. If necessary, the pH may be adjusted with a small amount of 1 M HCl or 1 M NaOH. If stock solutions are prepared properly, little or no adjustment should be required. Large adjustments to pH should be avoided as they may change the critical magnesium cation concentration. This may be prepared in larger quantities if it is filter sterilized to prevent bacterial contamination.

MgCl₂ (magnesium chloride) stock, 200× Dissolve 2.03 g of MgCl₂×6H₂O in 100 ml distilled H₂O. Store 1 year at room temperature.

Phosphate-buffered saline (PBS): Dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, and 0.24g of KH₂PO₄ in 800 ml of distilled H₂O. Adjust pH to 7.4. Adjust volume to 1L with additional distilled H₂O. Sterilize by autoclaving.

Saline solution: 0.9% (w/v) NaCl

Veronal-buffered saline (VBS), pH 7.2 to 7.4, 5× Dissolve 4.6 g barbituric acid in 800 ml boiling distilled H₂O. In a separate container, dissolve 2.0 g sodium barbital and 83.8 g sodium chloride in 1 liter of distilled H₂O. Mix the two solutions and add distilled H₂O to 2 liters. Store for 1 month at 4°C. A 1/5 dilution of the stock solution with water (3.5 mM veronal buffer) should have a pH of 7.2 to 7.4. 1 ml (100 mg/ml) kanamycin stock solution can be added but is not essential. For use in making of BDVA and BDVEA buffers (see recipes), prepare this stock solution without NaCl.

Background Information

The macrophage FcγRs mediate phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). Signals mediated by FcγR receptors can also modulate macrophage cytokine production (Mosser and Edwards, 2008). FcγR expression on macrophages can reflect the state of activation (Politis and Vogel, 1990), and on dendritic cells FcγR can promote DC

maturation and facilitate antigen cross-presentation. The use of SRBC opsonized with rabbit polyclonal IgG (described above) measures the total activity of all Fc γ R subtypes. It is difficult to measure the activity of individual FcRs using assays such as this because of the high avidity of IgGs within an immune complex. Similarly, the complement-dependent binding observed in the assays described above cannot be assigned to a single complement receptor. Complement fragments C3b and C4b will bind to the CR1, whereas iC3b will bind to the CR3. However the rapid cleavage of surface-bound C3b to iC3b means that the majority of complement-dependent binding to macrophages will occur via the CR3.

Because of their uniform size and morphology opsonized SRBC have been traditionally counted via light microscopy. This technique allows an analysis of both the percentage of macrophages in the monolayer with associated SRBCs, as well as the average number of SRBC associated with each macrophage. This type of analysis enables one to discriminate between one monolayer in which 50% of the macrophages have bound 1 SRBC and another monolayer in which 25% have bound 2 SRBC. These two monolayers would yield essentially the same results by the colorimetric assay described above. Another convenient way to visualize SRBC attachment to macrophages is to utilize fluorescently labeled SRBC. There are now several amine-reactive fluorescent dyes that are rapidly taken up by cells and metabolized to remain cell-associated (Greenberg and Grinstein 2002). Macrophages are typically counterstained with propidium iodide and the monolayers visualized by fluorescence light microscopy.

The colorimetric assay to measure SRBC-associated Hb takes advantage of the fact that hemoglobin possesses a pseudoperoxidase activity. In the presence of peroxide, it acts as an enzyme to catalyze the conversion of 2,7-diaminofluorene into fluorene blue (Worthington, et al. 1987). Fluorene blue has a broad absorption spectrum from 500 nm to 690 nm and its absorption between 610 nm and 630 nm correlates in a linear fashion with the hemoglobin concentration. Such properties are applied to measure phagocytosis activity and ADCC of macrophages (Montaño and Morrison. 1999). An alternative way to quantitate SRBC attachment is to measure the amount of radioactive chromium (⁵¹Cr) from preloaded SRBC (Vogel, et al. 1983). This assay is more sensitive, but it raises concerns because of the hazards associated with radioactivity.

The major difference between the binding assay and phagocytosis assay are that in the binding assay, the phagocytosis inhibitor is included to prevent receptor-mediated phagocytosis; and hypotonic washing step by ACK solution in the phagocytosis assay is eliminated in the binding assay so that the extracellular membrane receptor-bound SRBC can be measured. Latrunculin-A, as a phagocytosis inhibitor, is 10- to 100-fold stronger than cytochalasin compounds (de Oliveira and Mantovani 1988; Oliveira, et al. 1996) ATP depleting agents such as iodoacetic acid (IAA) and 2,4-dinitrophenylhydrazine (2,4-DNAP) can also be used to inhibit phagocytosis (Politis and Vogel, 1990). The protocols described herein are not only for mouse macrophages but also for any adherent cells that express the receptors. With minor modification of the wash step, the protocol is applicable for non-adherent cells of which the cells are centrifuged and the supernatants are aspirated. Overall, the phagocytic activity and binding assays described herein are much easier, faster and cheaper and provide a high-throughput method to accurately measure opsonic phagocytosis and binding-mediated by Fc γ R or complement receptors.

Critical Parameters and Troubleshooting

The optimal concentration of antibody for opsonizing SRBC needs to be determined prior to the experiment. Too much antibody can cause agglutination whereas too little will result in inefficient opsonization. High variability (>10%) among replicate wells usually indicates

unequal numbers of macrophages in the wells, which can be minimized by precise and consistent pipetting of macrophage suspensions into the wells of culture plate. Microscopic examination of cell viability of macrophages should be performed to monitor any loss of cells caused by treatment-related changes to cell adherence. Another variable that must be considered is the age of the SRBC used in the assay. In general, older SRBC become more sticky and adhere to macrophages even without opsonization. Thus, controls that include unopsonized SRBC should be performed with every assay.

Anticipated Results

IgG-SRBCs should bind avidly to macrophages and be efficiently internalized. The engulfed SRBC will remain intact for some time and retain hemoglobin. These cells can be visualized under bright-field microscopy. The efficiency of complement-mediated phagocytosis is much lower than that of Fc γ R-mediated phagocytosis. Many cytokines have been shown to modulate receptor expression and activity (Politis, et al. 1990). The type I interferons are able to increase Fc γ R expression in mouse macrophages, whereas IFN- γ may actually decrease some Fc γ R expression. This may not be the case with human macrophages (Pearse et al., 1992).

Time Considerations

The generation and culture of macrophages is the most time-consuming part of the procedure that must be set up before the assays are performed. Murine bone marrow derived macrophages take seven days to develop, whereas peritoneal macrophages can be used the same day (details see Unit 14.1). The assays *per se* can easily be performed within a single working day. The time needed for stimulation with cytokines can vary, but generally an overnight cytokine priming step is used.

Literature Cited

- Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 1999; 17:593–623. [PubMed: 10358769]
- de Oliveira CA, Mantovani B. Latrunculin A is a potent inhibitor of phagocytosis by macrophages. *Life Sci.* 1988; 43:1825–1830. [PubMed: 3200109]
- Greenberg S, Grinstein S. Phagocytosis and innate immunity. *Curr. Opin. Immunol.* 2002; 14:136–145. [PubMed: 11790544]
- He JQ, Wiesmann C, van Lookeren Campagne M. A role of macrophage complement receptor CR1g in immune clearance and inflammation. *Mol Immunol.* 2008; 45:4041–4047. [PubMed: 18752851]
- Helmy KY, Katschke KJ Jr, Gorgani NN, Kljavin NM, Elliott JM, Diehl L, Scales SJ, Ghilardi N, van Lookeren Campagne M. CR1g: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell.* 2006; 124:915–927. [PubMed: 16530040]
- Montaño RF, Morrison SL. A colorimetric-enzymatic microassay for the quantitation of antibody-dependent complement activation. *J Immunol Methods.* 1999; 222:73–82. [PubMed: 10022374]
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–969. [PubMed: 19029990]
- Nimmerjahn F, Ravetch JV. Fc γ receptors: old friends and new family members. *Immunity.* 2006; 24:19–28. [PubMed: 16413920]
- Oliveira CA, Kashman Y, Mantovani B. Effects of latrunculin A on immunological phagocytosis and macrophage spreading-associated changes in the F-actin/ G-actin content of the cells. *Chem Biol Interact.* 1996; 100:141–153. [PubMed: 8646787]
- Pearse RN, Feinman R, Ravetch J. Characterization of the promoter of the human gene encoding the high-affinity IgG receptor: Transcriptional induction by γ -interferon is mediated through common DNA response elements. *Proc. Natl. Acad. Sci. U.S.A.* 1992; 88:11305–11309. [PubMed: 1837149]

- Politis AD, Vogel SN. Pharmacologic evidence for the requirement of protein kinase C in IFN-induced macrophage Fc receptor and Ia antigen expression. *J. Immunol.* 1990; 145:3788–3795. [PubMed: 1700995]
- Swanson JA, Hoppe AD. The coordination of signaling during Fc receptor-mediated phagocytosis. *J. Leukocyte Biology.* 2004; 76:1093–1103.
- van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cell Microbiol.* 2007; 9:2095–2102. [PubMed: 17590164]
- Vogel SN, Finbloom DS, English KE, Rosenstreich DL, Langreth SG. Interferon-induced enhancement of macrophage Fc receptor expression: -interferon treatment of C3H/HeJ macrophages results in increased numbers and density of Fc receptors. *J. Immunol.* 1983; 130:1210–1214. [PubMed: 6218203]
- Wiesmann C, Katschke KJ, Yin J, Helmy KY, Steffek M, Fairbrother WJ, McCallum SA, Embuscado L, DeForge L, Hass PE, van Lookeren Campagne M. Structure of C3b in complex with CR1g gives insights into regulation of complement activation. *Nature.* 2006; 444:217–220. [PubMed: 17051150]
- Worthington RE, Bossie-Codreanu J, Van Zant G. Quantitation of erythroid differentiation in vitro using a sensitive colorimetric assay for hemoglobin. *Exp Hematol.* 1987; 15:85–92. [PubMed: 3490994]