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ISOLATION OF MOUSE NEUTROPHILS

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Abstract

Neutrophils represent the first line of defense against bacterial and fungal pathogens. Indeed, patients with inherited and acquired qualitative and quantitative neutrophil defects are at high risk for developing bacterial and fungal infections and suffering adverse outcomes from these infections. Therefore, research aiming at defining the molecular factors that modulate neutrophil effector function under homeostatic conditions and during infection is essential for devising strategies to augment neutrophil function and improve the outcome of infected individuals. This unit describes a reproducible density gradient centrifugation-based protocol that can be applied in any laboratory to harvest large numbers of highly enriched and highly viable neutrophils from the bone marrow of mice both at the steady state and following infection with *Candida albicans* as described in UNIT 19.6. In another protocol, we also present a method that combines gentle enzymatic tissue digestion with a positive immunomagnetic selection technique or Fluorescence-activated cell sorting (FACS) to harvest highly pure and highly viable preparations of neutrophils directly from mouse tissues such as the kidney, the liver or the spleen. Finally, methods for isolating neutrophils from mouse peritoneal fluid and peripheral blood are included. Mouse neutrophils isolated by these protocols can be used for examining several aspects of cellular function *ex vivo* including pathogen binding, phagocytosis and killing, neutrophil chemotaxis, oxidative burst, degranulation and cytokine production, and for performing neutrophil adoptive transfer experiments.

INTRODUCTION

Neutrophils comprise the main cellular component of the innate immune system and the first line of defense against pathogens. Immune recognition of the invading pathogen triggers a local innate immune response that results in the prompt expansion of neutrophils in the bone marrow and their recruitment to the site of infection. Once mobilized at the infected tissue, neutrophils become activated and mediate a variety of effector functions including binding, uptake and killing of pathogens by oxidative and/or non-oxidative mechanisms, degranulation of proteases in the extracellular space and production of pro-inflammatory and anti-inflammatory mediators that shape and modulate the outcome of the innate and adaptive antimicrobial immune response (Amulic et al., 2012). On the other hand, some of these

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neutrophil functions, when in excess or dysregulated, may promote tissue injury and immunopathology instead of appropriate antimicrobial activity (Kruger et al., 2015; Narasaraaju et al., 2011). Therefore, continued research is needed to elucidate the molecular mechanisms by which neutrophils induce protective or detrimental immune responses in the context of various infectious and non-infectious inflammatory conditions.

This unit describes methods for isolating and enriching neutrophils from mouse bone marrow, peripheral blood, peritoneal fluid or various mouse tissues. Basic Protocol 1 describes a density gradient centrifugation-based method for harvesting large numbers of neutrophils from the bone marrow of mice both at steady state and under inflammatory conditions. The latter is achieved by infecting mice with *Candida albicans* (see UNIT 19.6) although other pathogens can be used to suite experimental conditions. Basic Protocol 2 describes a method for harvesting of neutrophils from mouse kidney, liver or spleen. Neutrophils are isolated using immunomagnetic selection that relies on Ly6G, a mouse neutrophil-specific cell surface marker. Tissue neutrophils can also be enriched by FACS using antibodies targeted against surface expression of CD45, Ly6G, and CD11b (Alternate Protocol 1). Basic Protocol 3 describes a protocol for isolating neutrophils from peripheral blood. Finally, Basic Protocol 4 can be used for purification of polymorphonuclear leukocytes (PMN) from peritoneal exudate cells or peripheral blood by Histopaque density gradient centrifugation. An alternative method (see Alternate Protocol 2) for the purification of PMN from peritoneal exudate cells or peripheral blood by Histopaque density gradient centrifugation is also provided. The number and purity of neutrophils obtained from these protocols are sufficient for investigating neutrophil phagocytosis, intracellular and extracellular killing, oxidative burst, chemotaxis, degranulation, survival, cytokine and chemokine production; for performing transcriptional profiling of isolated neutrophils; for adoptive transfer experiments of isolated neutrophils into recipient mice; and for labeling of neutrophils with dyes and tracking their trafficking in various tissues *in vivo* following adoptive transfer into recipient mice.

NOTE: Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1: ISOLATION OF NEUTROPHILS FROM MOUSE BONE MARROW

This protocol describes a technique to isolate neutrophils from the bone marrow of naive or infected mice using Histopaque separation media by a density gradient centrifugation method.

Materials needed

C57BL/6 mice (control and *Candida albicans*-infected; see unit 19.6; optional)

70% (v/v) ethanol solution

1 × RPMI 1640 with l-glutamine and 25 mM HEPES (Mediatech, cat. no. 10-041-CV)

Heat-inactivated fetal bovine serum (FBS; Gemini Bioproducts, cat. no. 100-106)

Penicillin/streptomycin (10,000 U penicillin/10,000 µg/ml streptomycin) (Mediatech, cat. no. 30-002-CI)

Ice

Phosphate-buffered saline (PBS) without calcium and magnesium (Mediatech, cat. no. 21-040-CV)

EDTA (Mediatech, cat. no. 46034-CI)

0.2% and 1.6% sodium chloride (NaCl) solutions in distilled water (J.T. Baker, cat. no. 3624-01)

Sterile Histopaque 1119 (Sigma-Aldrich, cat. no. 11191)

Sterile Histopaque 1077 (Sigma-Aldrich, cat. no. 10771)

LIVE/DEAD fixable blue dead cell stain kit (Invitrogen, cat. no. L-23105)

Anti-mouse CD45 APC (clone 30-F11; BD Biosciences, cat. no. 559864)

Anti-mouse Ly6G PE (clone 1A8; BD Biosciences, cat. no. 551481)

Anti-mouse CD11b APC-eFluor® 780 (clone M1/70; eBioscience, cat. no. 47-0112-82)

Tweezers, scalpels, and scissors, kept in 70% ethanol solution

100 × 15-mm petri dishes (BD Falcon, cat. no. 351029)

25-G × 5/8-in. needles (BD Biosciences, cat. no. 305122)

12-ml syringes (Kendall, cat. no. 512878)

50-ml conical centrifuge tubes (BD Falcon, cat. no. 352098)

100-µm cell strainers (BD Biosciences, cat. no. 352360)

Combitips Plus Biopur (Eppendorf, cat. no. 2249608-5)

Refrigerated and room temperature centrifuges

15-ml conical centrifuge tubes (Corning, cat. no. 430053)

Tissue culture hood

25-ml serological pipets (BD Falcon, cat. no. 356525)

10-ml serological pipets (BD Falcon, cat. no. 356551)

3-ml Pasteur pipets (BD Falcon, cat. no. 357575)

Additional reagents and equipment for euthanasia of mice (*unit 1.8*), infection of mice with *Candida albicans* (*unit 19.6*), and counting viable cells by trypan blue exclusion (appendix 3B)

Harvesting of bone marrow cells from mouse femurs and tibias

1. Euthanize mice using an approved IACUC procedure and spray the animal surface with 70% ethanol.
2. Make an incision in the mid-abdomen on the ventral side and remove the skin in order to expose the abdomen and lower extremities.
3. Remove the muscles from both legs using scissors and carefully dislocate the acetabulum from the hip joint.

NOTE: Exercise caution to avoid breaking the femur head.

4. After separating the bones, place them in a sterile petri dish containing ice-cold RPMI 1640 1X supplemented with 10% FBS and 1% penicillin/streptomycin. Keep petri dish on ice.
5. Further remove the remaining muscles from the femurs and tibias using a scalpel and separate the femur from the tibia at the knee joint.

NOTE: Exercise caution to avoid breaking the femur and tibia bone ends while separating them.

6. Sterilize each bone by rinsing in a 70% ethanol solution within a petri dish followed by three subsequent washes in ice-cold sterile PBS within corresponding petri dishes in order to rinse off the ethanol from the surface of the bones.
7. Cut off the epiphyses of the bones and keep them aside in an empty sterile petri dish.
8. Using a 25-gauge $\times$ 5/8 in. needle and a 12 cc syringe filled with RPMI supplemented with 10% FBS and 2 mM EDTA, flush the bone marrow cells onto a 50 ml conical tube through a 100 μ m cell strainer.

NOTE: EDTA is used to avoid blood clotting and cell clumping.

NOTE: Blanching of bones typically indicates that the bones have been sufficiently scraped to provide a good yield of bone marrow cells; to that end, approximately 10 ml of RPMI supplemented with 10% FBS and 2 mM EDTA is required to flush each femur and tibia pair.

9. Cut the bone epiphyses in small 0.5–1 mm³ pieces with a scalpel and smash them through the 100 μ m cell strainer using the back end of a 2.5 ml Eppendorf Combitip Plus pipette tip.
10. Collect the bone marrow cells by centrifugation at 427 $\times$ g for 7 minutes at 4°C.
11. Resuspend the cell pellet in 20 ml of 0.2% NaCl for approximately 20 seconds followed by addition of 20 ml of 1.6% NaCl to lyse the red blood cells.

NOTE: Do not incubate the cells in 0.2% NaCl for more than 20 seconds before adding equal volume of 1.6% NaCl to avoid bone marrow cell lysis.

12. Centrifuge for 7 minutes at 427 $\times$ g at 4°C to collect the bone marrow cells.

13. Wash the cells with RPMI 1640 1X supplemented with 10% FBS and 2 mM EDTA and centrifuge again as in step 12.

Purification of neutrophils from bone marrow cells using Histopaque-based density gradient centrifugation

- 14 Count the bone marrow cells and resuspend in 1–3 ml of ice-cold sterile PBS.
NOTE: Resuspend cells in 1 ml if harvesting bone marrow from one uninfected mouse. Resuspend cells in 3 ml if harvesting bone marrow from more than one uninfected mouse or from infected mice. Investigators should perform pilot experiments tailored to the specific conditions used, as the density of bone marrow cells can affect neutrophil purity after centrifugation.
- 15 Add 3 ml of Histopaque 1119 (density, 1.119 g/ml) in a 15-ml conical centrifuge tube.
NOTE: Histopaque 1119 should be brought to room temperature before use.
- 16 Overlay with 3 ml of Histopaque 1077 (density, 1.077 g/ml).
NOTE: Histopaque 1077 should be brought to room temperature before use.
NOTE: Exercise caution to avoid disturbing the interface between Histopaque 1119 and Histopaque 1077.
- 17 Overlay the bone marrow cell suspension on top of the Histopaque 1077 layer.
NOTE: Exercise caution to avoid disturbing the interface between the cell suspension and Histopaque 1077.
- 18 Centrifuge for 30 minutes at $872 \times g$ at room temperature without brake.
- 19 Collect the neutrophils at the interface of the Histopaque 1119 and Histopaque 1077 layers.
- 20 Wash the collected neutrophils twice with RPMI 1640 1X supplemented with 10% FBS and 1% penicillin/streptomycin and centrifuge at 1400 rpm for 7 minutes at 4°C.
- 21 Take a small aliquot before the last wash to determine neutrophil count, viability by trypan blue exclusion and FACS analysis using a fluorescent viability dye (Lionakis et al., 2011), and purity by flow cytometry using staining with APC-conjugated anti-CD45, PE-conjugated anti-Ly6G and APC-Cy7-conjugated anti-CD11b.

BASIC PROTOCOL 2: ISOLATION OF NEUTROPHILS FROM MOUSE TISSUES

Under inflammatory conditions, neutrophils traffic to tissue and acquire a different phenotype relative to the neutrophils produced in the bone marrow, which is modulated by the local inflammatory milieu. This protocol describes a technique to isolate neutrophils from various tissues (kidney, liver, or spleen) of *Candida albicans*-infected mice using

gentle enzymatic digestion of tissue and subsequent positive immunomagnetic selection or FACS sorting that relies on Ly6G, a mouse neutrophil-specific cell surface marker.

Materials needed

C57BL/6 mice

10 × phosphate-buffered saline (PBS; Mediatech, cat. no. 46-013-CM)

1 × RPMI 1640 with l-glutamine and 25 mM HEPES (Mediatech, cat. no. 10-041-CV)

Heat-inactivated fetal bovine serum (FBS; Gemini Bioproducts, cat. no. 100-106)

Penicillin/streptomycin (10,000 U penicillin/10,000 µg/ml streptomycin) (Mediatech, cat. no. 30-002-CI)

Ice

RPMI without serum

Liberase TL (Roche, cat. no. 05401020001)

DNase I (Roche, cat. no. 10104159001)

FACS buffer: 1X Phosphate-buffered saline (PBS) without calcium and magnesium supplemented with 0.5% bovine serum albumin (BSA) and 0.01% sodium azide [10% (w/v) sodium azide (Teknova, cat. no. S0209)]

Percoll (GE Healthcare, cat. no. 17-0891-01)

Magnetic-activated cell sorting (MACS) buffer: PBS containing 2 mM EDTA (Mediatech, cat. no. 46034-CI) and 0.5% bovine serum albumin (BSA)

Anti-Ly6G microbead kit for mouse neutrophils (Miltenyi, cat. no. 130-092-332)

LIVE/DEAD fixable blue dead cell stain kit (Invitrogen, cat. no. L-23105)

Anti-mouse CD45 APC (clone 30-F11; BD Biosciences, cat. no. 559864)

Anti-mouse Ly6G PE (clone 1A8; BD Biosciences, cat. no. 551481)

Anti-mouse CD11b APC-eFluor® 780 (clone M1/70; eBioscience, cat. no. 47-0112-82)

Anti-mouse CD16/CD32 (clone 93; eBioscience, cat. no. 14-0641-86)

Surgical instruments (kept in 70% ethanol solution) including:

Tweezers

Scalpels

Scissors

100 × 15-mm petri dishes (BD Falcon, cat. no. 351029)

50-ml conical centrifuge tubes (BD Falcon, cat. no. 352098)

37°C water bath

40-µm cell strainers (BD Biosciences, cat. no. 352340)

70-µm cell strainers (BD Biosciences, cat. no. 352350)

100-µm cell strainers (BD Biosciences, cat. no. 352360)

Refrigerated and room temperature centrifuges

15-ml conical centrifuge tubes (BD Falcon, cat. no. 352097)

LS+ positive selection columns (Miltenyi, cat. no. 130-042-401)

QuadroMACS Separator (Miltenyi, cat. no. 130-090-976)

MACS MultiStand (Miltenyi cat # 130-042-303)

Tissue culture hood

25-ml serological pipets (BD Falcon, cat. no. 356525)

10-ml serological pipets (BD Falcon, cat. no. 356551)

3-ml Pasteur pipets (BD Falcon, cat. no. 357575)

12-ml syringes (Kendall, cat. no. 512878)

25-G × 5/8-in. needles (BD Biosciences, cat. no. 305122)

NOTE: All prepared solutions can be stored up to 3 months at 4°C. Please follow manufacturer's instructions for storage of antibodies and media purchased.

Single cell suspension from mouse kidney, liver or spleen by gentle enzymatic tissue digestion and Percoll-based density gradient centrifugation

1. Infect mice with *Candida* yeast cells or other pathogen of interest (*see unit 19.6*).
2. Anesthetize mouse after infection using an approved Animal Care and Use Committee procedure and perfuse mice with PBS to remove circulating blood cells from the organs of interest.
3. Excise organ of interest and place it in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin on ice until ready to use.
4. Place the organ of interest in a petri dish and add 5–10 ml of digesting solution (RPMI without serum containing Liberase TL and DNase I).

NOTE: The concentration of Liberase TL in the digesting solution is 0.2125 mg/ml for kidney and liver and 0.125 mg/ml for spleen; the concentration of DNase I in the digesting solution is 0.1 mg/ml for all organs (Lionakis et al., 2012).

NOTE: Liberase is deactivated in the presence of serum.

5. Finely mince the organ of interest into 1 mm³ size pieces using a scalpel and collect them in a 50 ml conical centrifuge tube.

NOTE: 10 ml of digesting solution is required for kidney and spleen digestion, while 20 ml of digesting solution is needed for liver digestion.

6. Incubate the organs at 37°C in a water bath in digesting solution for 20 minutes.

NOTE: Longer digestion times result in decreases in cell viability.

7. At the end of the 20 minute incubation, add an equal volume of RPMI with 10% FBS (i.e., 10 ml for kidney/spleen and 20 ml for liver) and place the 50 ml conical centrifuge tube on ice to stop the digestion.

NOTE: Both addition of serum and placement of tube on ice inhibit the activity of Liberase TL.

8. Pass the digested tissue through a cell strainer.

NOTE: 70 μ m for kidney and liver; 100 μ m for spleen.

9. Centrifuge at $427 \times g$ for 7 minutes to collect the cells.

10. Wash the cells with FACS buffer and collect cells by centrifuging at $427 \times g$ for 7 minutes. The spleen cells are now ready for magnetic or FACS separation. For liver and kidney cells, the following density centrifugation step is required to obtain a single cell suspension.

11. Resuspend the kidney or liver cells in 8 ml of 40% Percoll and overlay them onto 3 ml of 70% Percoll in a 15 ml conical centrifuge tube using a transfer pipette.

NOTE: 40% and 70% Percoll dilutions are made from a “100% Percoll” solution using FACS buffer. The “100% Percoll” solution is generated by adding 5 ml of 10X PBS in 45 ml of FACS buffer.

NOTE: Exercise caution to avoid disturbing the interface between 40% and 70% Percoll solutions.

12. Centrifuge for 30 minutes at $872 \times g$ without brake at room temperature.

13. Harvest cells from the 70%/40% Percoll interface, wash twice with FACS buffer and resuspend in MACS or FACS buffer for proceeding with magnetic separation of neutrophils or FACS sorting, respectively.

Isolation of neutrophils using Ly6G-based positive immunomagnetic selection

1. Count cells and resuspend up to 10^8 cells in 200 μ l of MACS buffer.

NOTE: If $>10^8$ cells are to be used (e.g., from spleen), adjustments are required in the amounts of each reagent added per the manufacturer’s instructions.

2. Add 50 μ l of anti-Ly6G biotin beads per sample.

3. Mix well with a pipette and incubate for 10 min in a refrigerator (2–8°C).

NOTE: Do not incubate on ice.

4. Add 150 μ l of MACS buffer in each sample.

5. Add 100 μ l of anti-biotin microbeads in each sample.

6. Mix well with a pipette and incubate 15 minutes in a refrigerator (2–8°C).

NOTE: Do not incubate on ice.

7. Wash the cells with 10 ml of MACS buffer and centrifuge at 300 g for 10 min.
8. Aspirate the supernatant completely and resuspend in 500 µl of MACS buffer.
9. Place a LS column per sample on the magnet.

NOTE: Similar results with high neutrophil numbers, purity and viability are obtained by using LS columns and a QuadroMACS separator as well as by using autoMACS columns and an autoMACS or an autoMACS Pro separator.

10. Rinse with 3 ml of MACS buffer per column and discard the flow through.
11. Apply the cell suspension to the column and collect the negative flow through.
12. Wash the column with 3 ml of MACS buffer each for three times and collect the negative flow through.
13. Remove the column from magnet and place onto a new 15 ml conical centrifuge tube.
14. Wash the column with 5 ml of MACS buffer and flush the cells with the plunger provided with the columns.
15. Centrifuge at 300 g for 10 min.
16. Resuspend cells in PBS and take an aliquot to determine neutrophil count, viability by the trypan blue exclusion method and FACS analysis using a fluorescent viability dye (Lionakis et al., 2010), and purity by flow cytometry using staining with APC-conjugated anti-CD45, PE-conjugated anti-Ly6G and APC-Cy7–conjugated anti-CD11b.

Alternative Protocol 1: Isolation of neutrophils using FACS

As an alternative to Ly6G-based positive immunomagnetic selection, tissue neutrophils can also be enriched by FACS using antibodies targeted against surface expression of CD45, Ly6G and CD11b.

Additional Materials (also see Basic Protocols [1](#) and [2](#))

Anti-mouse CD45 APC (clone 30-F11; BD Biosciences, cat. no. 559864)

Anti-mouse Ly6G PE (clone 1A8; BD Biosciences, cat. no. 551481)

Anti-mouse CD11b APC-eFluor® 780 (clone M1/70; eBioscience, cat. no. 47-0112-82)

Anti-mouse CD16/CD32 (clone 93; eBioscience, cat. no. 14-0641-86)

Sorting buffer: PBS without calcium and magnesium supplemented with 10% FBS and 0.5 mM EDTA

FACS tubes (BD Biosciences, cat. no. 352053)

Additional reagents and equipment for flow cytometry and FACS (Chapter 5)

1. Count cells and resuspend in FACS buffer (PBS + 0.5% BSA + 0.01% NaN₃) at 4°C at a concentration of 1×10^7 cells/ml in a sterile FACS tube (5 ml polystyrene round-bottom tube).
2. Proceed with Fc-blockade by adding anti-CD16/CD32 and incubate for 15 min at 4°C in the dark.

NOTE: The optimal amount of antibody required is approximately 1 μ l per 1×10^6 cells.
3. Add APC-conjugated anti-CD45, PE-conjugated anti-Ly6G and APC-Cy7–conjugated anti-CD11b and incubate for 30 min at 4°C in the dark.

NOTE: The optimal amount of antibody required is approximately 1 μ l per 1×10^6 cells for each antibody.
4. Wash off excess antibodies by adding 1 ml FACS buffer to each tube and centrifuge for 7 min at $472 \times g$ at 4°C.
5. Repeat wash and centrifugation step as in #4.
6. Resuspend cell pellet at a concentration of 5×10^6 cells/ml in sorting buffer (PBS without calcium and magnesium supplemented with 10% FBS and 0.5mM EDTA).
7. Sort CD45⁺Ly6G⁺CD11b⁺ cells using standard sorting procedures.

BASIC PROTOCOL 3: ISOLATION OF NEUTROPHILS FROM PERITONEAL FLUID

This protocol is a rapid and efficient method for isolation of highly purified murine neutrophils.

Materials Needed

Casein solution (see [recipe](#))

Donor mice (2- to 4-month-old male mice of any conventional strain)

Harvest solution: 0.02% EDTA in $1 \times$ PBS (see [recipe](#) for $10 \times$), filter-sterilized through 0.2- μ m filter (room temperature)

70% ethanol

$1 \times$ PBS (see [recipe](#) for $10 \times$)

Percoll gradient solution (see [recipe](#)), room temperature

Medium to be used with neutrophils in subsequent experimental procedures, room temperature

Medium to be used with neutrophils in subsequent experimental procedures, containing 50% (v/v) FBS, room temperature

Diff-Quik staining solutions (Baxter)

10-ml syringes and 21-G needles

Forceps

Small, straight surgical scissors

15- or 50-ml conical polypropylene centrifuge tubes

Benchtop centrifuge

10-ml ultracentrifuge tubes (Beckman or equivalent)

Sorvall RC70 ultracentrifuge with T-1270 fixed-angle rotor (or equivalent)

Cytospin cytocentrifuge including chambers and filter cards (Shandon/Lipshaw)

Microscope slides appropriate for use with Cytospin centrifuge

Additional reagents and equipment for intraperitoneal injection ([unit 1.6](#)), euthanasia of mice ([unit 1.8](#)), harvesting peritoneal exudate cells ([unit 14.1](#)), and counting viable cells by trypan blue exclusion (appendix 3B)

Elicit and collect peritoneal cells

1. Fill a 10-ml syringe with sterile casein solution and inject 1.0 ml into the peritoneal cavity of each mouse ([unit 1.6](#)) using a 21-G needle. Allow inflammatory response to develop overnight and repeat injection of 1.0 ml casein solution the next morning.
2. Three hours after the second injection, euthanize animals by carbon dioxide asphyxiation or other approved method ([unit 1.8](#)). *To avoid introducing blood into the peritoneum, the cervical dislocation method should be avoided. It should be noted that this procedure primarily induces a neutrophil infiltrate; to obtain a macrophage-enriched population refer to [unit 14.1](#).*
3. Clean abdomen of each mouse with 70% ethanol. Make a ventral midline incision with scissors, and then retract abdominal skin with forceps to expose the intact peritoneal wall (see [unit 14.1](#)).
4. Place 3 to 5 ml sterile harvest solution in a 10-ml syringe with a 21-G needle. Insert needle through the peritoneal wall of the first mouse with the beveled edge of the needle facing up and inject the entire volume.
5. Gently massage the abdomen of the donor animal. Using the same syringe and needle as in step 4, with the beveled tip of needle facing down, slowly withdraw all the peritoneal fluid and transfer to a 15- or 50-ml conical polypropylene centrifuge tube.
6. Inject another 3 to 5 ml of harvest solution as in step 4 and repeat the procedure in step 5 to remove the remaining cells from the peritoneum and transfer them to the tube. Repeat steps 4 and 5 for each mouse used and pool the peritoneal fluid.
7. Centrifuge the pooled peritoneal fluids 10 min at $200 \times g$, room temperature, in a benchtop centrifuge.

8. Remove the supernatant and wash the peritoneal exudate cells three times, each time by resuspending the cells in 10 ml of $1 \times$ PBS, centrifuging for 10 min at $200 \times g$, room temperature, and then removing the supernatant. Resuspend the washed pellet in 1 ml sterile room temperature $1 \times$ PBS, and then count the number of viable cells by trypan blue exclusion (appendix 3B).

Isolate neutrophils using continuous density gradient

1. Mix $3\text{--}5 \times 10^7$ peritoneal exudate cells (suspended in 1 ml of $1 \times$ PBS; see step 8) with 9 ml Percoll gradient solution at room temperature in a 10-ml Beckman ultracentrifuge tube.
2. Ultracentrifuge the mixture 20 min at $60,650 \times g$ (25,700 rpm in a Sorvall T-1270 fixed-angle rotor), 4°C . Discard the thin, faint upper layer (which contains macrophages and lymphocytes). Collect the neutrophils (which will be found in the second opaque layer; see Fig. 3.20.1).
3. Wash the cells once by adding 10 ml of $1 \times$ PBS and then centrifuging 5 min at $200 \times g$, room temperature, and removing the supernatant. Resuspend pellet in appropriate medium for subsequent experimental procedures at room temperature.

Determine cell purity and viability

1. Take a 20- μl aliquot of cell suspension and add 180 μl of medium containing 50% FBS.
2. Centrifuge the samples onto slides for 5 min at 1000 rpm in a Cytospin cytocentrifuge. Stain with Diff-Quik staining solution. Examine the cells with conventional light microscopy at $400 \times$ to evaluate neutrophil purity. *Cells showing fine granules and multilobed nuclei are counted as neutrophils.*
3. Count the number of viable cells by trypan blue dye exclusion (appendix 3B).

BASIC PROTOCOL 4: ISOLATION OF NEUTROPHILS FROM PERIPHERAL BLOOD

This protocol is a rapid method for isolation of nonelicited neutrophils from mouse peripheral blood (Devi et al., [1995](#)).

Materials Needed

- Naive mice
- Methoxyflurane (Metofane)
- Heparin
- $1 \times$ phosphate-buffered saline (PBS; see [recipe](#) for $10 \times$)
- 1-ml syringe with 20- to 22-G needle
- $12 \times 75\text{-mm}$ glass tube

Benchtop centrifuge

Additional reagents and equipment for methoxyflurane anesthesia of mice ([unit 1.4](#)), cardiac puncture of mice ([unit 1.7](#)), euthanasia of mice ([unit 1.8](#)), counting cells using a hemacytometer (appendix 3A), and isolation of neutrophils by density gradient centrifugation (as for peritoneal fluid; see [Basic Protocol 3](#), steps 9 to 14)

1. Anesthetize naive mice by inhalation of methoxyflurane ([unit 1.4](#)).
2. Draw 5 U heparin into a 1-ml syringe with a 20- to 22-G needle, and then use the syringe to bleed mice by cardiac puncture ([unit 1.7](#)). Euthanize the mice ([unit 1.8](#)). Transfer blood to a 12 × 75-mm glass tube.
3. Centrifuge the blood 10 min at $200 \times g$, room temperature, in a benchtop centrifuge. Collect the whitish buffy coat layer, which appears at the plasma interface.
4. Count the cells in buffy coat layer using a hemacytometer (appendix 3A). Resuspend $3\text{--}5 \times 10^7$ buffy coat cells in 1 ml of $1 \times \text{PBS}$.
5. Isolate neutrophils by density gradient centrifugation (see [Basic Protocol 3](#), steps 9 to 14).

Alternative Protocol 2: Isolation of neutrophils from peritoneal fluid or peripheral blood

An alternative method for isolation of neutrophils using a discontinuous density gradient composed of two solutions of a radiopaque medium of differential density (Histopaque-1077 and -1119) is commercially available from Sigma. Following the manufacturer's procedure, neutrophils can be enriched from mouse peritoneal exudate or buffy coat cells from peripheral blood.

Additional Materials (also see [Basic Protocols 3 and 4](#))—Histopaque-1119 and -1077 (polysucrose/sodium diatrizoate, densities 1.1119 and 1.077, respectively; store at 4°C; Sigma).

15-ml polypropylene centrifuge tubes

1. Prepare peritoneal exudate cells (see [Basic Protocol 3](#), steps 1 to 8) or peripheral blood buffy coat cells (see [Basic Protocol 4](#), steps 1 to 4).
2. Add 3 ml Histopaque 1119 to a 15-ml tube. NOTE: *Histopaque 1119 should be brought to room temperature before use.*
3. Gently pipet 3 ml Histopaque 1077 on top of the Histopaque 1119 layer. NOTE: *Histopaque 1077 should be brought to room temperature before use.*
IMPORTANT NOTE: *Exercise caution to avoid disturbing the interface between Histopaque 1119 and Histopaque 1077.*
4. Carefully layer 1 ml of cell suspension (from [Basic Protocol 3](#), step 8 or [Basic Protocol 4](#), step 4) over the upper gradient, adding the cell suspension slowly along the side of tube so as not to break the gradient surface. IMPORTANT NOTE:

Exercise caution to avoid disturbing the interface between the cell suspension and Histopaque 1077.

5. Centrifuge 30 min at $700 \times g$, room temperature, without using a brake.
6. Using a pipet, collect neutrophils from the interface of the Histopaque 1077 and 1119 layers and transfer to 15-ml tubes.
7. Wash the cells three times, each time by adding 10 ml of $1 \times$ PBS, centrifuging 5 min at $200 \times g$, 4°C , and then removing the supernatant.
8. Determine cell purity and number of viable cells (see [Basic Protocol 3](#), steps 12 to 14).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions.

Casein solution

Slowly add 9 g casein (from bovine milk, sodium salt; Sigma) with stirring to 100 ml hot $1 \times$ PBS, pH 7.2 (see [recipe](#) for $10 \times$) containing 0.9 mM CaCl_2 and 0.5 mM MgCl_2 .

Bring solution to a boil, then autoclave 1 hr at 125°C . Store casein solution 1 to 2 weeks at 4°C , but warm to room temperature before use. Discard solution if pink color fades.

Patience is required to get casein into solution. First heat PBS to 80° to 90°C , then, while stirring, add small (0.5 to 1 g) aliquots casein. After each aliquot has dissolved, add the next aliquot until all 9 g are in solution.

Percoll gradient solution

Add 10 ml of sterile $10 \times$ PBS, pH 7.2 (see [recipe](#)) to 90 ml sterile Percoll (Pharmacia Biotech). Store up to 3 months at 4°C ; discard if turbidity develops.

Phosphate-buffered saline (PBS), pH 7.2, $10 \times$

0.23 g NaH_2PO_4 (anhydrous)

1.15 g Na_2HPO_4 (anhydrous)

9 g NaCl

Add H_2O to 90 ml

Adjust to pH 7.2 using 1 M NaOH or 1 M HCl

Add H_2O to 100 ml

Store up to 3 months at room temperature

Dilute 1 part $10 \times$ PBS with 9 parts H_2O for $1 \times$ working solution. For PBS with calcium and magnesium, add 0.9 mM CaCl_2 and 0.5 mM MgCl_2 to $1 \times$ working solution. Sterilize by filtration.

COMMENTARY

Background Information

In contrast to human neutrophils, which can be easily harvested in large numbers from peripheral blood (Clark and Nauseef, 2001), isolation of mouse neutrophils from blood is hindered by the small volume of mouse blood that does not allow for the isolation of sufficient numbers of neutrophils for performing downstream functional immunological studies and/or adoptive transfer experiments. Therefore, mouse neutrophil isolation has traditionally relied on eliciting granulocytes from the peritoneal cavity of mice following intraperitoneal injection of thioglycollate or casein, because the peritoneal cavity does not contain significant numbers of neutrophils at steady state (Watt et al., 1979). The yield of peritoneal neutrophils under these “inflammatory” conditions is indeed amenable to downstream functional immunological studies and adoptive transfer experiments (Luo and Dorf, 2001). However, thioglycollate-elicited and casein-elicited peritoneal neutrophils exhibit an activated phenotype, which is driven by a non-physiological stimulus, and their purity in the inflammatory peritoneal lavage varies between 50–90%, which requires a downstream gradient centrifugation step using Percoll or Histopaque-based cell separation media in order to achieve acceptable neutrophil purity (Luo and Dorf, 2001).

In contrast to blood and the peritoneal cavity, the mouse bone marrow is a convenient reservoir for isolating large numbers of neutrophils both at steady state under homeostatic conditions and during activation under a variety of infectious and non-infectious inflammatory conditions. It is the anatomical location of mice from where large numbers of neutrophils can be harvested at the steady state for immunological assays and adoptive transfer experiments. Bone marrow neutrophils have been shown to be functionally similar to blood neutrophils in mice and have been reported to survive for a longer period of time in culture (Boxio et al., 2004), thus making them a very useful resource for research studies of neutrophil biology and physiology.

The method we present in Basic Protocol 1 utilizes Histopaque cell separation media, which consist of sodium diatrizoate and Ficoll. This method is easier to layer compared to the density gradient centrifugation method that utilizes discontinuous Percoll gradients consisting of 55%/65%/75% Percoll in PBS, which often results in intermixing of the Percoll interfaces due to the small density differences between the 55%, 65% and 75% Percoll densities. Bone marrow neutrophils obtained using this method from uninfected and infected mice can be used for experiments to assess binding, uptake and killing of pathogens by neutrophils intracellularly and extracellularly, to examine neutrophil chemotaxis (using Boyden chambers), survival, degranulation and oxidative burst, to perform transcriptional analyses of harvested neutrophils and measure their secretory potential of cytokines and chemokines, to perform adoptive transfer of cells into recipient mice, and to label the neutrophils with dyes before adoptive transfer, which provides an opportunity to track neutrophil trafficking in various tissues *in vivo* (Lionakis et al., 2012).

Importantly, under inflammatory conditions it becomes critical to investigate the effector function of neutrophils from the site of infection as their activity may differ from that of bone marrow neutrophils, driven by factors present in the local immunological

microenvironment of the infected tissue (Whitney et al., 2014). Therefore, the method we present in Basic Protocol 2 utilizes a Ly6G-based strategy to achieve positive immunomagnetic selection or FACS-based sorting of neutrophils from mouse kidney, liver or spleen; this method can be adapted to also allow neutrophil isolation from other mouse tissues. Ly6G is a neutrophil-specific cell surface marker in mice (Daley et al., 2008) and, therefore, these Ly6G-based positive selection strategies can result in high yield, high purity and high viability of neutrophils from mouse tissues under a variety of infectious and non-infectious inflammatory conditions that mimic human disease models in mice.

Besides bone marrow and tissue neutrophils, harvesting neutrophils from the mouse peritoneal cavity and peripheral blood are also useful depending on the experimental design and site of infection or inflammation. Therefore, the methods we present in Basic Protocols 3 and 4 and in [Alternate Protocol 2](#) utilize Percoll- or Histopaque-based density gradient centrifugation to harvest highly pure and viable neutrophils from these compartments.

Critical Parameters and Troubleshooting

Care should be taken to maintain sterile techniques throughout all steps of the described protocols because LPS can activate neutrophils, which once activated they can secrete cytokines and undergo apoptosis. While performing Histopaque-based density gradient centrifugation, caution should be taken to ensure that Histopaque 1119 and Histopaque 1077 have reached room temperature before overlaying, in order to maximize neutrophil purity. In addition, the layering of the Histopaque 1119 and Histopaque 1077 gradients should be performed immediately before overlaying the bone marrow cell suspension and not in advance, as after a few minutes on the bench the two layers intermix. Caution should be also taken when layering Histopaque 1077 onto Histopaque 1119 to avoid intermixing in the Histopaque 1119 and Histopaque 1077 interface, as such intermixing of the two densities will significantly decrease the purity of neutrophils. Furthermore, centrifugation at room temperature, not at 4°C, is essential to maximize purity of neutrophils.

Moreover, overlaying more than 100 million bone marrow cells may result in reduction of neutrophil purity from >90% to ~80%. Increasing the volume of PBS for bone marrow cell suspension from 1 ml to 3 ml may ameliorate the decrease in neutrophil purity when large numbers of bone marrow cells are layered over Histopaque 1077. However, it is advised that investigators should perform pilot studies tailored to the specific conditions of their experiments in order to maximize neutrophil purity after Histopaque-based centrifugation.

During magnetic separation, care should be taken to follow the manufacturer's protocol and to use the appropriate column/magnet separator pair. Incubation with the MACS reagents should be performed in the refrigerator, not on ice, per the manufacturer's recommendation to avoid non-specific binding on the reagents to cells other than neutrophils, which can adversely affect cell purity. Also, it is important to ensure that the column is emptied in between washing steps with MACS buffer and does not contain any residual liquid before proceeding to the next washing steps, per the manufacturer's guidelines. Conversely, it is important to avoid excessive drying of the column in between washing steps with MACS buffer.

Anticipated Results

The typical yield of bone marrow cells from an 8–12 week-old C57BL/6 mouse is between 60 and 80 million. The typical yield of neutrophils from the bone marrow (i.e., 2 femurs and 2 tibias) of an uninfected 8–12 week-old C57BL/6 mouse following Histopaque-based gradient centrifugation is ~6–12 million cells. Neutrophils isolated from the bone marrow of an 8–12 week-old uninfected C57BL/6 mouse using this technique are typically >90% pure and >95% viable, as determined by FACS analysis. Infection with *Candida* results in a significant expansion of bone marrow neutrophils, and up to 30–40 million neutrophils with high purity (80–95%) and high viability (>95%) can be recovered per *Candida*-infected mouse bone marrow, depending on the infecting inoculum and the timing of bone marrow harvesting post-infection (Lionakis et al., 2012).

Immunomagnetic or FACS-based separation of neutrophils from both kidneys of an 8–12 week old C57Bl/6 mouse infected with *Candida* yields between to 1–5 million cells, depending on the infecting inoculum and the timing of bone marrow harvesting post-infection (Lionakis et al., 2010). Magnetically-enriched tissue neutrophils are >90% pure and >95% viable, as determined by FACS analysis (Lionakis et al., 2012). FACS-enriched tissue neutrophils are >98% pure and >95% viable.

Neutrophil yield from the blood or the peritoneal cavity varies considerably among individuals. In general, two casein injections will yield $2\text{--}7 \times 10^6$ purified neutrophils per mouse. The purity of neutrophils after Percoll gradient separation is >95%. Contaminating cells primarily include other granulocytes, lymphocytes, and macrophages. $0.5\text{--}1.0 \times 10^6$ blood neutrophils per mouse can be expected after Percoll centrifugation.

Viability of neutrophils (by trypan blue dye exclusion) should be >95%.

Time Considerations

Bone marrow cells can be harvested from the femurs and tibias of each mouse in 15–20 minutes and can then be used for isolation of neutrophils. The entire protocol of obtaining enriched neutrophils using Histopaque-based density gradient centrifugation can be completed within 2 hours.

Single cell suspensions from mouse organs can be obtained within 3–4 hours depending on the number of mice used and the organ of interest; for example, harvesting of cells from spleen does not require a Percoll-based centrifugation step following tissue digestion and is faster. After a single cell suspension has been obtained, an additional 60–90 minutes are required to obtain enriched neutrophils via positive immunomagnetic selection; the exact time varies depending on the number of organs and the number of LS columns used.

Once tissue cells are in a single cell suspension, sorting of neutrophils using FACS can be completed within 1–2 hours depending on the number of samples and the percent of neutrophils within a given cell suspension.

Harvest of peritoneal exudate cells from a single mouse can be completed in 10 min. It usually takes 30 to 45 min to harvest peritoneal cells from a group of ten mice. Isolation of

neutrophils from peritoneal exudate cells, gradient preparation, cell separation, and cell counting should take <2 hr.

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Figure 3.20.1.

Separation of casein-induced peritoneal neutrophils following purification by Percoll density gradient centrifugation. The faint upper band contains primarily lymphocytes and macrophages (L,M), the broad middle band includes neutrophils and other granulocytes such as eosinophils (polymorphonuclear leukocytes; PMN), and the bottom band includes erythrocytes (E).

Figure 3.20.2. Purity (left panel) and viability (right panel) of neutrophils isolated from the bone marrow of an uninfected C57BL/6 mouse using the Histopaque-based gradient centrifugation method.

Figure 3.20.3. Purity (left panel) and viability (right panel) of neutrophils isolated from the kidneys of a *Candida albicans*-infected C57BL/6 mouse using the Ly6G-based positive immunomagnetic selection method.