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Differentiation and Characterization of Myeloid Cells

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Abstract

Recent molecular studies of myeloid differentiation have utilized several in vitro models of myelopoiesis, generated from either ex vivo differentiated bone marrow progenitors or induced immortalized myeloid cell lines. Ex vivo differentiation begins with an enriched population of bone marrow-derived hematopoietic stem cells generated by lineage depletion and/or positive selection for CD34⁺ antigen (human) or Sca-1+ (mouse) cells, which are then expanded and subsequently induced in vitro in a process that recapitulates normal myeloid development. Myeloid cell lines include two human leukemic cell lines, NB-4 and HL-60, which have been demonstrated to undergo retinoic acid-induced myeloid development, however, both cell lines exhibit defects in the upregulation of late-expressed neutrophil-specific genes. Multiple murine factor-dependent cell models of myelopoiesis are also available that express the full range of neutrophil maturation markers, including: 32Dcl3 cells, which undergo G-CSF-induced myeloid maturation, EML/EPRO cells, which develop into mature neutrophils in response to cytokines and retinoic acid, and ER-Hoxb8 cells, which undergo myeloid maturation upon removal of estradiol in the maintenance medium. In this unit, the induction of myeloid maturation in each of these model systems is described, including their differentiation to either neutrophils or macrophages, if applicable. Commonly used techniques to test for myeloid characteristics of developing cells are also described, including flow cytometry and real time RT-PCR. Together, these assays provide a solid foundation for in vitro investigations of myeloid development with either human or mouse models.

Keywords

stem cell progenitors; promyelocytes; myeloid induction; neutrophil characteristics; macrophages; cell-surface markers; granule protein gene expression

This unit describes the induction of bone marrow-derived hematopoietic stem cell progenitors (e.g., CD34⁺ or lineage-depleted cells) and myeloid progenitor cell lines towards terminal myeloid differentiation, and assays that will determine the myeloid characteristics of the resulting populations.

Myeloid differentiation of stem cell progenitors

Purified progenitors [typically isolated from bone marrow or peripheral blood as a purified CD34⁺ population (human) or a lineage-depleted population (mouse)] or progenitor cell

lines can be induced to undergo myeloid differentiation in suspension culture using a select set of cytokines and interleukins. For CD34⁺ human progenitors, the differentiation process occurs in two steps as described in Basic Protocol 1. First, progenitors are expanded in medium supplemented with stem cell factor (SCF) and interleukin-3 (IL-3), which gives rise to an expanded population of early promyelocytes. Second, the promyelocytes are induced to become fully mature neutrophils by granulocyte-colony stimulating factor (G-CSF), which is added to the medium containing both SCF and IL-3. For lineage-depleted mouse progenitors, the differentiation process is similar to that used for human cells, except that an additional step of induction in G-CSF alone is used as described in Basic Protocol 2. In addition, a population of macrophages can also be generated from the mouse progenitors by inducing the SCF/IL-3-induced population in both granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF), and then just M-CSF. Maturation of each population is assessed by examining Wright-Giemsa-stained cytospin smears for morphologic changes, and by examining changes in myeloid-specific cell surface protein and/or gene-expression profiles as described in Support Protocols 2 and 3. An Alternate protocol for the expansion of colony forming units in methylcellulose is also provided, however, assessment of myeloid characteristics is qualitative and is typically performed using neutrophil function assays such as NBT staining (UNIT 7.23).

Differentiation of progenitor cell lines

A variety of human and mouse myeloid cell lines have been established and well characterized for their ability to undergo neutrophil-specific myeloid differentiation (Fig. 22F.5.1). Basic Protocol 3 describes the differentiation of two human leukemic cell lines, HL-60 and NB-4. These cell lines undergo morphologic maturation and acquire many characteristics of mature neutrophils, however, they fail to express secondary granule proteins. An alternate protocol is also included that describes the induction of HL-60 (or NB4) cells toward mature macrophages with phorbol myristate acetate or 1 α ,25-dihydroxyvitamin D³ (Vit D₃) (see Fig. 22F.5.2). Differentiation of a murine myeloblastic cell line, 32Dcl3 cells, is detailed in Basic Protocol 4. This cell line demonstrates morphologic maturation that is accompanied by upregulated expression of multiple secondary granule protein genes. Basic Protocol 5 describes the differentiation of a unique multipotent murine progenitor cell line, EML (erythroid, myeloid, and lymphoid) cells, and the subsequent induction of a derived early promyelocytic cell line, EPRO, towards fully mature neutrophils. Upon differentiation, these cells also undergo gene expression changes observed in maturing myeloid progenitors in vivo. Finally, recently developed models of either neutrophil or macrophage differentiation are described in Basic Protocol 6, both based on the overexpression of an estrogen receptor (ER)-Hoxb8 fusion protein that blocks the differentiation of bone marrow-derived progenitors. The SCF ER-Hoxb8 cell line is induced toward mature neutrophils, whereas the GM-CSF ER-Hoxb8 cell line is induced toward macrophages; both inductions only require removal of β -estradiol from their maintenance medium.

Analysis of myeloid characteristics

Upon induction of differentiation, myeloid cells exhibit specific changes in their gene expression profiles that result in the expression of specific cell surface markers. In addition, during differentiation of neutrophils, granule protein gene expression is also modulated: early precursors express only primary granule protein genes, whereas more mature precursors show a loss of primary granule protein gene expression concomitant with the activation of secondary granule protein gene expression. Support Protocol 2 details the analysis of undifferentiated versus differentiated cells for cell surface expression of myeloid markers, specifically the neutrophil-specific marker Gr-1 and the integrin binding protein CD11b/CD18 (Mac-1) for neutrophils, and the macrophage marker F4/80 and Mac-1 for macrophages. Finally, details to analyze the expression of secondary versus primary granule protein genes by either Northern or real time RT-PCR analyses are described in Support Protocol 3.

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

Basic Protocol 1

Neutrophil Differentiation of Human CD34⁺ Progenitors

In this protocol, primary hematopoietic stem cell progenitors are induced to differentiate towards early promyelocytes by ex vivo culture in suspension medium supplemented with SCF and interleukin-3. After 3 days, the population consisting primarily of promyelocytes is transferred to a medium supplemented with SCF, IL-3, and G-CSF, which induces terminal differentiation into mature neutrophils. During the culturing procedure, it is important to transfer cells to fresh medium every 2 to 3 days of culture, and to maintain the cells at a density between 2×10^5 cells/ml and 8×10^5 cells/ml.

Materials—Purified hematopoietic stem cell progenitors (for human cells, CD34⁺ progenitors isolated from bone marrow or peripheral blood; *UNIT 7.4*)

IMDM supplemented with 10% heat-denatured FBS (*APPENDIX 2A*), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

Recombinant cytokines: SCF (Amgen), IL-3 (ICN Biomedicals), and G-CSF (Neupogen, Amgen)

6-well tissue culture plates

Tabletop centrifuge

Additional reagents and equipment for counting cells (*APPENDIX 3A*), trypan blue exclusion test for cell viability (*APPENDIX 3B*), Wright-Giemsa staining (*APPENDIX 3C*)

1. Determine cell concentration of the purified CD34⁺ progenitors (*UNIT 7.4*) using an automated counter (e.g., Coulter counter or a hemacytometer (*APPENDIX 3A*), using 0.1% trypan blue exclusion for cell viability (*APPENDIX 3B*).

Although the immunomagnetic separation steps outlined in unit 7.4 focus on the isolation of T cell subpopulations, a similar procedure can be applied to isolate CD34⁺ using appropriate monoclonal antibodies (e.g., anti-CD34 MoAb, Becton Dickinson).

2. Pellet progenitors in 15 mL conical tubes by centrifuging 5 min at $250 \times g$, room temperature. Aspirate and resuspend the pellet at a concentration of 1×10^5 cells/ml in IMDM supplemented with 10% heat-inactivated FBS, 100 ng/ml IL-3, and 100 ng/ml SCF. Incubate cells in 5 ml of medium in each well of a 6-well tissue culture plate. Incubate for 2 days.
3. Following 2 days of culture, renew the medium by collecting cells, centrifuging 5 min at $250 \times g$, room temperature, aspirating the supernatant, and resuspending the pellet in the growth medium described in step 1 at a final cell density of $2\text{--}5 \times 10^5$ cells/ml. Incubate 24 hr.
4. On the third day of culture, pellet cells (comprised primarily of promyelocytes) by centrifuging as in step 3 and resuspending cell pellet in fresh IMDM plus antibiotics supplemented with 10% FBS plus 100 ng/ml IL-3, 100 ng/ml SCF, and 10 ng/ml of G-CSF.
5. Monitor the maturation of the promyelocytes towards mature neutrophils by Wright-Giemsa staining of cytospin smears (APPENDIX 3c). Renew medium every 2 to 3 days throughout the differentiation process and maintain cell densities between 2×10^5 cells/ml and 8×10^5 cells/ml.

Typically, mature neutrophils will emerge by 10 days of culture, with maximum numbers observed by 12 days. Mature neutrophils should be viable up to 14 days of total culture, any further incubation will be accompanied by spontaneous apoptosis.

Analyses of morphologic maturation requires the use of a Cytospin cytocentrifuge, including chambers, filter cards (Shandon/Lipshaw), and microscope slides appropriate for use with a Cytospin cytocentrifuge.

Basic Protocol 2

Neutrophil Differentiation of Lineage-Depleted Mouse Progenitors

This protocol begins with the extraction of bone marrow from mice femurs and tibiae, and then a magnetic separation system is used to deplete lineage positive cells. The resulting population enriched for hematopoietic stem cells is then expanded in medium supplemented with SCF plus IL-3 to yield high numbers of early myeloid progenitors plus promyelocytes. The addition of G-CSF to the mixture then further increases the numbers of promyelocytes, followed by a final induction with just G-CSF. During the initial induction in SCF/IL-3, the cells will rapidly proliferate so dilutions must be performed to maintain the cells at a density between 2×10^5 cells/ml and 1×10^6 cells/ml.

Materials—Dissection supplies for cleaning bones from hind legs (sterile scalpels and forceps)

IMDM supplemented with 2% heat-denatured FBS or 10% heat-denatured HS (APPENDIX 2A), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

1× PBS (APPENDIX 2A)

Recombinant cytokines: SCF, IL-3 and G-CSF (PeproTech, resuspended from lyophilized material with 1× PBS plus 0.1% FBS to a final concentration of 10 µg/ml)

Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set (BD Biosciences, Cat. # 558451)

Mouse BD Fc Block purified anti-mouse CD16/CD32 (BD Biosciences, Cat. # 553141)

23 gauge needles and syringes (3 or 5 ml)

60mm and 6-well tissue culture plates

Tabletop centrifuge

Additional reagents and equipment for euthanasia (UNIT 1.8), counting cells using a hemacytometer (APPENDIX 3A), counting cells (APPENDIX 3A), trypan blue exclusion test for cell viability (APPENDIX 3B), Wright-Giemsa staining (APPENDIX 3C)

1. Euthanize mice. Using aseptic technique, peel skin from the top of each hind leg, down over the foot. Remove the femur and tibia bones and store in IMDM + 2% FBS medium on ice until all the mice have been prepared. Remove the muscles from the bones (usually on a sterile gauze pad) by scraping bone with a sterile gauze and place the cleaned bones in a new tissue culture dish containing ice-cold RPMI 1640.

Male mice are preferred because they have bigger bones and therefore yield greater numbers of bone marrow cells.

Intravenous injection of a barbituric acid derivative is the preferred method of euthanasia.

2. Cut off the ends of each bone with scissors.
3. Attach a 10-ml syringe to a 25-G needle and fill with ice-cold IMDM + 2% FBS.
4. Insert needle into the bone marrow cavity of the femur or tibia. Flush the bone cavity with ~2 to 5 ml IMDM + 2% FBS or until the bone cavity appears white. Allow the flowthrough to collect into a sterile 50-ml conical centrifuge tube.
5. Strain cells through a 70 µm nylon cell strainer (BD Falcon) into a new conical tube. Pellet progenitors by centrifuging in 15 ml conical tubes for 10 min at 250 × g, room temperature. Aspirate medium and resuspend the pellet in 10 ml of 1× PBS and perform a cell count. Pellet cells and then resuspend the pellet in 1× PBS + 3% FBS and 0.1% Sodium azide (cell staining buffer) at a concentration of 20×10^6 cells/ml.

6. Add anti-mouse CD16/CD32 monoclonal antibody (e.g. Mouse Fc Block, BD Pharmingen, Cat. No. 553141) using 0.25 μ g per 10^6 cells (e.g. 10 μ L of stock reagent per 20×10^6 cells total in 1 ml of cell staining buffer). Allow the mixture to incubate for 15 min. on ice.
7. Add biotinylated lineage depletion cocktail at 100 μ L per 20×10^6 cells total. Mix well and incubate an additional 15 min. on ice.
8. Add 9.9 ml of 1 $\times$ BD Imag Buffer (prepared from 10 $\times$ stock solution) and centrifuge cells for 7 min. at $300 \times g$.
9. Resuspend cell pellet in 100 μ L of BD Imag Streptavidin Particles (pre-vortex particles to prepare a mixed suspension) and incubate at 6-12°C for 30 min.
10. Add 900 μ L of BD IMag buffer and transfer cells to a sterile _snap-cap_ 12 $\times$ 75 mm round bottom polypropylene tube (BD Falcon 352058 or equivalent tubes), place the tube in the IMagnet and let incubate for 10 min. at room temperature.
11. Using a sterile 1 ml pipette, carefully remove medium and cells without disrupting streptavidin particles that adhere to the side of the tube, and transfer lineage-depleted cells to a fresh round bottom tube.
12. Remove the first tube with streptavidin particles from the IMagnet and resuspend the remaining particles in 1 ml of BD IMag buffer, return the tube to the IMagnet and repeat the incubation at room temperature from step 9.
13. Carefully remove the second round of lineage-depletion and add these cells to the first round of lineage depletion performed in step 8. The lineage depleted cells will be in a final volume of ~ 2 ml.
14. Place the final lineage depleted cells back into the IMagnet, let the cells incubate for 8 min. to remove any residual streptavidin particles, and transfer lineage negative cells to a fresh 15 ml conical tube. Centrifuge cells at $250 \times g$ for 5 min.
15. Resuspend the pellet in IMDM + 20% Horse Serum, perform cell count, and transfer cells to wells of a 6-well plate, using 5 ml total volume per well. Typical starting concentrations are $3-5 \times 10^5$ cells per ml in a 6-well cell culture plate.

Typical yields from one mouse are 1×10^6 total cells, which should be dispersed in 2 wells of a 6-well plate for the initial incubation.
16. Add 50 ng/ml each of SCF and IL-3, and let cells incubate for 3 days, checking cells each day for expansion and maintaining final concentrations between $2 - 8 \times 10^5$ cells/ml.

Typical yields after 3 days of culture from the initial 10^6 cells are $\sim 3 \times 10^6$ total cells, divided equally into 4 wells of a 6-well plate.
17. After 3 days of culture, cells are expanded into medium with 50 ng/ml each of SCF plus IL-3, plus 50 ng/ml of G-CSF. Incubate cells an additional 2 days, maintaining concentrations between $2 - 8 \times 10^5$ cells/ml. This will induce the expanded population of myeloid progenitors toward promyelocytes.

Typical yields after 2 days of culture in all three cytokines are $\sim 1.2 \times 10^7$ cells total from the initial 10^6 lineage depleted cells.

18. After 2 days, collect all cells by centrifugation at $250 \times g$ for 5 min, and wash cells twice in $1 \times$ PBS. After the second wash, resuspend the pelleted cells in IMDM + 20% HS supplemented with 50 ng/ml of G-CSF only. Let cells incubate an additional two days, for a total of 7 days from day of harvest.

The final induction in G-CSF will yield $\sim 1.5 \times 10^7$ cells per mouse.

Alternate Protocol

Monocyte / Macrophage Differentiation of Lineage-Depleted Mouse Progenitors

This protocol begins with the same steps used to generate lineage-depleted mouse progenitors from bone marrow and the initial expansion of the progenitors in SCF plus IL-3. However, instead of continuing to incubate the cells in this cocktail plus G-CSF, the cells are washed in $1 \times$ PBS and then transferred into medium with GM-CSF plus M-CSF to induce monocyte differentiation. After 2 days of culture, the cells are then washed in $1 \times$ PBS and transferred to medium with M-CSF only. At the completion of the induction process, a population highly enriched with mature macrophages is generated. As previously indicated, dilutions must be performed to maintain the cells at a density between 2×10^5 cells/ml and 1×10^6 cells/ml.

1. After inducing the lineage-depleted bone marrow cells in SCF plus IL-3 for 3 days (up to step 13 in Protocol 2, which yields $\sim 3 \times 10^6$ cells per mouse), the cells are collected and centrifuged at $250 \times g$ for 5 min., and then washed twice in $1 \times$ PBS.
2. After the second wash, resuspend the cells in IMDM + 20% HS at a concentration of 3×10^5 cells/ml in 5 ml per well of a 6-well TC plate. Add 50 ng each of GM-CSF and M-CSF and incubate cells for 2 days.

Typical yields from the initial population of lineage depleted cells is $\sim 1 \times 10^7$ cells total.

3. Collect cells in 15 ml conical tubes, centrifuge at $250 \times g$ for 5 min at room temperature, and wash cells twice in $1 \times$ PBS.
4. After the second wash, resuspend the cell pellet in IMDM + 20% HS supplemented with 50 ng/ml of M-CSF only. Further incubate the cells for 3 additional days.

The final yield per mouse is $\sim 1.5 \times 10^7$ cells total.

Alternate Protocol

CFU-GM Colony Formation

Colonies of developing myeloid cells can also be produced by growing progenitors in IMDM containing a combination of growth factors and 1% methylcellulose. The type of growth factors employed will depend on the desired result; e.g., Douer et al. (2000) reported a high production number of CFU-GM from $CD34^+$ cells by a combination of GM-CSF (50 ng/ml) plus 3×10^{-7} M all-trans retinoic acid (ATRA), or by G-CSF (75 ng/ml) alone, whereas Tocci et al. (1996) reported a combination of Epo, IL-3, GM-CSF, KL (SCF) and 1

$\times 10^{-7}$ M ATRA produced primarily CFU-G. Methylcellulose can be purchased as a prepared mixture to which cytokines can be added. The methylcellulose medium is mixed by drawing medium with a 10-ml sterile syringe without a needle, and carefully ejecting the medium to minimize the introduction of air bubbles. Cells are then added to this mixture and again mixed by carefully drawing and ejecting the medium with a syringe. Medium/cell mixture is transferred to 35-mm tissue culture dishes or wells of 6-well tissue culture plates using a 3-ml syringe with a 16-G sterile needle. Cells are allowed to develop for 10 to 14 days, and analysis for differentiation is performed by picking cells with a micropipet, washing the cells in PBS, and analyzing cells for oxidative burst by NBT reduction staining (UNIT 7.23).

NOTE: Use of a blunt-ended 16-G needle during addition of cells/methylcellulose mixture to plates is more convenient due to safety concerns, but a sharp-tipped needle also will work with added caution during handling.

Additional Materials (also see Basic Protocol 1)

IMDM supplemented with heat-denatured 2% FBS (APPENDIX 2A), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 μ g/ml streptomycin sulfate

Methylcellulose medium (without cytokines available for human or murine cells; Stem Cell Technologies)

Cytokines (depending on the conditions required; SCF, Amgen; IL-3, MP Biomedicals; G-CSF, Neupogen, Amgen; EPO, Epoitin, Amgen; and GM-CSF, MP Biomedicals)

3- and 10-ml sterile syringes

6-well tissue culture plates or 35-mm tissue culture dishes

16-G needles

Additional reagents and equipment for NBT staining assay (UNIT 7.23)

1. Add IMDM to the concentrated methylcellulose medium to yield a final concentration of 1.1% methylcellulose, 15% FBS, 1% BSA, 2 mM L-glutamine, 5 U/ml penicillin G, and 5 μ g/ml streptomycin sulfate. Add the desired growth factors to this mixture. Gently mix the solution with a 10-ml sterile syringe without a needle avoiding the introduction of bubbles. Let this mixture stand 5 to 10 min at 37°C to let bubbles migrate to the top of the medium.
2. Dilute purified CD34⁺ cells to a final concentration of 2×10^5 cells/ml with IMDM plus 2% FBS.
3. Add 0.3 ml cells to 3 ml IMDM medium with cytokines (from step 1), and mix gently with a 3-ml syringe. Allow cell mixture to sit for 5 to 10 min at room temperature to allow any bubbles to rise to the top of the tube.
4. Pass 1.1 ml of methylcellulose/cell mixture into either 35-mm tissue culture plates or wells of a 6-well tissue culture plate, using a 3-ml syringe with a 16-G needle.

Disperse the medium by tipping the plates back and forth. Incubate for 10 to 14 days.

If using 35-mm tissue culture plates, place each plate into a larger petri dish containing an additional 35-mm plate filled with sterile water. For 6-well tissue culture plates, add 5-ml sterile water to all empty wells.

5. Count colonies of developing granulocytes after 10 to 14 days, and qualitatively analyze cells by assays of neutrophil function (e.g., NBT staining assay, *UNIT 7.23*).

Basic Protocol 3

Neutrophil Differentiation of Human Leukemic Cell Lines

Currently, there are two human leukemic cell lines commonly used to examine myeloid differentiation: HL-60 cells, which are at the myeloblast (AML-M2) stage of development, and NB-4 cells, which are at the promyelocyte (AML-M3) stage of development. Both types are maintained in the same growth medium.

Materials—HL-60 or NB-4 cells (HL-60 available from ATCC; NB4 cells can be obtained by contacting Dr. Michel Lanotte, mlanotte@chu-stlouis.fr)

RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

DMSO

10 mM stock ATRA solution (see recipe)

Additional reagents and equipment for Wright-Giemsa staining (*APPENDIX 3C*)

1. Grow cultures of HL-60 or NB-4 cells to $5\text{--}8 \times 10^5$ cells/ml in RPMI-1640 culture medium supplemented with 10% FBS (complete medium).
2. Dilute cells to 3×10^5 cells/ml in RPMI-1640 culture medium supplemented with 10% FBS, and incubate overnight to obtain a population of exponentially growing cells.
3. Dilute exponentially growing cells to a density of 3×10^5 cells/ml in RPMI-1640 culture medium supplemented with 10% FBS supplemented with the following: for HL-60 cells, induce with a final concentration of 1.25% DMSO and/or 1 µM/liter ATRA; for NB-4 cells, induce with a final concentration of 5 µM ATRA (5 µl of stock 10 mM ATRA per 10 ml of medium).

HL-60 cells have been reported to undergo neutrophil maturation in either 1.25% DMSO or 1 µM ATRA, but a combination of both factors has been suggested to provide the most efficient differentiation (see Le Cabec et al., 1997).

4. Renew the medium and dilute cells to a density of $3\text{--}5 \times 10^5$ cells/ml after 2 days of induction.

5. After 3 to 4 days of induction, inspect the morphology of differentiated cells by preparing Wright-Giemsa stained cytospin smears (*APPENDIX 3c*).

Most cells should appear as band cells, although some metamyelocytes and segmented cells can be observed.

Analyses of morphologic maturation requires the use of a Cytospin cytocentrifuge, including chambers, filter cards (Shandon/Lipshaw), and microscope slides appropriate for use with a Cytospin cytocentrifuge.

Alternate Protocol

Monocyte / Macrophage Differentiation of Human Leukemic Cell Lines

Although originally described as promyelocytic cells, HL-60 cells were later characterized as myeloblasts and therefore capable of differentiation toward either neutrophils or macrophages. Additional studies confirmed that the monocyte/macrophage differentiating agents phorbol 12-myristate 13-acetate (PMA) and $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), either alone or in combination, can efficiently induce monocyte/macrophage differentiation of HL-60 cells (for recent examples, see Olins et al, 2008, and Xu et al, 2010). By comparison, promyelocytic NB4 cells carry the classic t(15;17) translocation but also retain some differentiation plasticity and can be induced toward macrophages, however the combined effects of PMA and $1,25(OH)_2D_3$ are best used to achieve maximal differentiation toward this lineage (see Song and Norman, 1998, as an example).

1. Expand and passage cells in RPMI-1640 culture medium supplemented with 10% FBS as described in Protocol 3.
2. Induce differentiation toward monocytes as follows: for HL-60 cells, supplement medium with either 16 nM PMA or $1\alpha,25(OH)_2D_3$ at 10-100 nM; for induction of NB4 cells, supplement medium with a combination of 10-100 nM each of PMA and $1\alpha,25(OH)_2D_3$

Reports on the use of PMA to induce monocyte/macrophage differentiation in either HL-60 or NB4 cell lines indicate concentrations that range from 1.6 nM to 100 nM, but the majority indicate that 16 nM is sufficient to cause rapid differentiation with 3 days of induction. NB4 cells tend to require both PMA and $1\alpha,25(OH)_2D_3$, typically 16 nM and 100 nM, respectively.

3. Monitor differentiation over 24 to 48 hours, maintaining cell density at $3 - 5 \times 10^5$ cells/ml. Cells will become attached to the culture plate surface as cell adhesion proteins become upregulated.
4. Maturation to fully mature macrophages is complete by day 5 (see Fig. 22.5.2); harvesting of cells can be accomplished by direct lysis after removing medium (and collecting any detached cells in medium by centrifugation at $250 \times g$ for 5 min.) or by using a cell scraper in $1 \times$ PBS (for protein extraction, addition of proteinase inhibitor cocktails is recommended).

Basic Protocol 4

Differentiation of 32Dcl3 Cells to Myeloid Cells

Myeloblastic 32Dcl3 cells are an IL-3-dependent cell line that can be induced with G-CSF to undergo complete neutrophil differentiation. Myeloid differentiation is induced by washing the cells in PBS to remove the IL-3 and subsequent growth in G-CSF.

During the initial stages of G-CSF induction (in the absence of IL3), a significant proportion of 32Dcl3 cells will undergo apoptosis. Therefore, expect a significant decrease in cell number during the first 1 to 2 days of induction. By the second or third day of induction, however, the remaining G-CSF responsive cells will begin to proliferate, and will then undergo differentiation after 6 to 8 days of induction. Dead cells can be removed by layering cells over calf serum and collecting live cells by slow centrifugation (10 min at $200 \times g$), however, this step is optional and does not improve the differentiation process.

Materials—Myeloblastic 32Dcl3 cells

IMDM supplemented with 10% FBS, 10% WEHI-conditioned medium (see Support Protocol 1), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

PBS (APPENDIX 3A)

G-CSF (Neupogen, Amgen)

Additional reagents and equipment for Wright-Giemsa staining (APPENDIX 3C)

1. Grow myeloblastic 32Dcl3 cells to a density of $5\text{--}8 \times 10^5$ cells/ml. Add fresh IMDM supplemented with 10% FBS and 10% WEHI-conditioned medium to dilute the cells to $2\text{--}3 \times 10^5$ cells/ml and grow overnight.

Typically, a 1:1 dilution 1 day prior to induction suffices.

2. Centrifuge an appropriate amount of exponentially growing cells to give a final concentration of $6\text{--}8 \times 10^5$ cells/ml in 15 ml conical tubes for 6 min at $250 \times g$, room temperature. Wash cells two times by resuspending in 10 ml of $1 \times$ PBS and centrifuging 5 to 6 min at $250 \times g$, room temperature.

3. Resuspend pelleted cells after washes in IMDM medium supplemented with 10% FBS and recombinant human G-CSF to a final concentration of 100 ng/ml.

The final concentration of resuspended cells should be 5×10^5 cells/ml.

4. Renew G-CSF-containing medium every 2 to 3 days during the induction by collecting cells by centrifuging 6 min at $250 \times g$, room temperature, washing cells in 10 ml of PBS, and resuspending cells in fresh G-CSF-containing medium at a final concentration of $5\text{--}8 \times 10^5$ cells/ml.

During the first 24 to 48 hr of induction, 50% of the cells will undergo apoptosis, whereas the remaining cells will begin to expand after 3 to 4 days of culture.

5. After 6 to 7 days, assess the maturation of cells by Wright-Giemsa stained cytospin smears (*APPENDIX 3C*).

Cells with segmented nuclei should be apparent by 6 days of induction.

Analyses of morphologic maturation requires the use of a Cytospin cytocentrifuge, including chambers, filter cards (Shandon/Lipshaw), and microscope slides appropriate for use with a Cytospin cytocentrifuge.

Basic Protocol 5

Myeloid Differentiation of Multipotent EML Cells

EML cells can be obtained directly from ATCC (EML cell line no. CRL-11691) and are maintained in 6-well tissue culture plates in IMDM supplemented with 20% heat-inactivated horse serum (HS) plus 15% BHK/MKL-conditioned medium (as a source of SCF, see Support Protocol 1).

Myeloid differentiation of EML cells is performed in a two-step process. First, cells are passed to 25-cm² tissue culture flasks and induced with IL-3 plus ATRA in the presence of SCF for 3 days. After washing the induced cells with PBS, early promyelocytes (EPRO) cells are then selected in IMDM plus 20% HS supplemented with either 10 ng/ml GM-CSF or 10% BHK/HM-5-conditioned medium (as a source of GM-CSF, see Support Protocol 1) in either 25- or 75-cm² tissue culture flasks. Finally, EPRO cells are induced with ATRA in the presence of GM-CSF to mature neutrophils. Alternatively, MPRO cells (murine promyelocytes; available from ATCC clone 2.1 no. CRL-11422) can also be used as a source of promyelocytes. MPRO cells are similar to EPRO cells in that they are derived from bone marrow cells transduced with a dominant negative retinoic acid receptor alpha, but are isolated by direct culturing in GM-CSF (Tsai and Collins, 1993).

EML cells are particularly sensitive to cell density, requiring maintenance of $1-5 \times 10^5$ cells/ml by passage of 0.5 to 1.0 ml of cells into 4.5 ml of maintenance medium in 6-well tissue culture plates every 2 to 3 days. Do not let EML cells overgrow (e.g., $>8 \times 10^5$ cells/ml), and when passing cells, dilute to no less than 0.5×10^5 cells/ml.

EPRO cells grown in IMDM plus 20% horse serum and GM-CSF can undergo spontaneous differentiation (see Lawson et al., 1998b). To minimize this effect, cells can be grown in Aim V medium (GIBCO) supplemented with 1% to 5% FBS plus 10% BHK-HM5-conditioned medium. However, EPRO cells grown in this medium become more sensitive to density, and therefore require more frequent passage. This medium is also used to culture MPRO cells. However, EPRO and MPRO cells grown in this medium must be cultured in 6-well tissue culture plates, and maintained at a density of $2-8 \times 10^5$ cells/ml by the passage of cells every 2 to 3 days of culture.

Materials—EML cells

IMDM supplemented with 20% heat-denatured horse serum, 15% BHK/MKL-conditioned medium (see Support Protocol 1), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

IMDM supplemented with 20% heat-denatured horse serum, 10% BHK/HM5-conditioned medium (see Support Protocol 1) or 10 ng/ml mouse GM-CSF (Peprotech), 2 mM L-glutamine, 5 U/ml penicillin G, and 5 µg/ml streptomycin sulfate

WEHI-conditioned medium (see Support Protocol 1) or mouse IL-3 (Peprotech, prepared with 1× PBS plus 0.1% FBS at 50 µg/ml for stock solution)

10 mM ATRA stock solution (see recipe)

PBS (APPENDIX 2A) without Ca²⁺ and Mg²⁺

6-well tissue culture plates

25- and 75-cm² tissue culture flasks

Microscope slides

Cytospin centrifuge

Additional reagents and equipment for Wright-Giemsa staining (APPENDIX 3C)

Induce EML cells to early promyelocytes (EPRO)

1. Expand EML cells to 5×10^5 cells/ml in 6-well tissue culture plates and dilute 1:1 in IMDM supplemented with 20% heat-denatured horse serum and 15% BHK/MKL-conditioned medium 16 to 24 hr prior to induction.
2. Induce exponentially growing EML cells to promyelocytes by diluting cells to $2\text{--}3 \times 10^5$ cells/ml in growth medium (with SCF) in a 25-cm² tissue culture flask and adding a final concentration of 10 µM ATRA plus 5% (v/v) WEHI-conditioned medium (as a source of IL-3) or 25 ng/ml mouse IL-3.
3. After 2 days, renew growth medium supplemented with 10 µM ATRA plus 5% (v/v) WEHI-conditioned medium (or 25 ng/ml IL-3) by collecting cells in 15 ml conical tubes and centrifuging 6 min at $250 \times g$, room temperature, and then resuspending cells at a density of 5×10^5 cells/ml.

At this stage, the cells can be expanded into a 75-cm² tissue culture flask to maximize the number of cells that will emerge during GM-CSF selection.

4. After 3 days of induction, wash cells two times in PBS, and resuspend cells at 1×10^6 cells/ml in base IMDM medium supplemented with 20% heat-denatured HS and 10% BHK/HM-5-conditioned medium or 10 ng/ml mouse GM-CSF (EPRO growth medium). Incubate 5 to 7 days.

After 1 to 2 days, ~50% to 70% of the cells will undergo apoptosis, but the GM-CSF-responsive EPRO cells will emerge after 5 to 7 days of culture. During the selection process, renew the medium every 3 days of selection. EPRO cells are then maintained in EPRO growth medium by diluting cells at $6\text{--}8 \times 10^5$ cells/ml to $0.5\text{--}1.0 \times 10^5$ cells/ml every 2 to 3 days of culture.

Induce EPRO cells to neutrophils

5. Expand cells in EPRO growth medium to $5\text{--}10 \times 10^5$ cells/ml the day prior to induction.
6. Induce exponentially growing cells by adding 10 μM ATRA solution (final concentration) to EPRO growth medium for 3 days. Centrifuge 1×10^4 cells onto microscope slides for 5 min at 500 rpm with a Cytospin cytocentrifuge and stain cells with Wright-Giemsa (APPENDIX 3c).

Cells should appear as a band and segmented neutrophils after 3 to 4 days of induction. MPRO cells also undergo rapid differentiation into mature neutrophils when induced with 10 μM ATRA, however, the induction is complete by 72 hr.

Basic Protocol 6

Myeloid Differentiation of ER-Hoxb8 Progenitors

ER-Hoxb8 cells are maintained in medium that contains β -estradiol, which causes continuous transcriptional function of the ER-Hoxb8 fusion protein and thereby maintains the cells in a proliferating progenitor state. The original lines derived from bone marrow were selected in either SCF or GM-CSF, which yielded progenitors committed to either neutrophil or monocyte/macrophage pathways, respectively. Maintenance is therefore performed in either SCF plus β -estradiol for SCF ER-Hoxb cells, or GM-CSF plus β -estradiol for GM-CSF ER-Hoxb8 cells. Upon removal of β -estradiol, each cell line undergoes rapid differentiation that is typically complete within 5 days of induction.

Both cell lines tend to be sensitive to the type of FBS used during culture, and therefore it is recommended to use “certified” or “defined” FBS for routine maintenance. Cytokines can be obtained either by purchasing purified versions of SCF or GM-CSF (e.g. from Peprotech), or conditioned medium can be used; for example, CHO cells that constitutively express SCF can be obtained, as can B16 cells that constitutively express GM-CSF.

Materials—SCF ER-Hoxb8 and GM-CSF ER-Hoxb8 cell lines (obtained directly from M. Kamps, UCSD School of Medicine, La Jolla, San Diego, CA)

Opti-MEM medium (Invitrogen) supplemented with 10% heat-inactivated certified FBS, 2 mM L-glutamine, 5 U/ml penicillin G, and 5 $\mu\text{g}/\text{ml}$ streptomycin sulfate

RPMI-1640 medium supplemented with 10% heat-inactivated certified FBS, 2 mM L-glutamine, 5 U/ml penicillin G, and 5 $\mu\text{g}/\text{ml}$ streptomycin sulfate

10 mM stock solution β -estradiol (Sigma, E-2758, dissolve in 100% ethanol with shaking for 30 min. at room temperature, stored at -20°C)

50 $\mu\text{g}/\text{mL}$ Stem Cell Factor (SCF) (Peprotech, stock solution prepared with $1\times$ PBS + 0.1% FBS)

50 $\mu\text{g}/\text{ml}$ Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (Peprotech, stock solution prepared with $1\times$ PBS + 0.1% FBS)

Induce SCF ER-Hoxb8 cells toward neutrophils

1. Thaw cells in Opti-MEM plus 10% FBS supplemented with 10 ng/ml SCF and 1 μ M β -estradiol. Once established, expand SCF ER-Hoxb8 cells to $6-8 \times 10^5$ cells/ml in 6-well tissue culture plates and dilute 1:1 in maintenance medium.
2. Collect cells in 15 mL conical tubes and centrifuge at $250 \times g$ for 5 min. Resuspend in 5 ml of 1 $\times$ PBS, centrifuge and repeat to wash cells.
3. Resuspend washed cell pellet in Opti-MEM plus 10% FBS supplemented with 10 ng/ml SCF ONLY (no β -estradiol) and culture in 5 ml per well in 6-well culture plates.
4. Maintain cells at a concentration of $2-6 \times 10^5$ cells/ml, diluting cultures as required. Monitor morphologic maturation using Wright-Giemsa-stained cytopins; lobulated nuclei should be identified by 4-5 days of induction.

Induce GM-CSF ER-Hoxb8 cells toward monocytes/macrophages

1. Thaw cells in Opti-MEM plus 10% FBS supplemented with 20 ng/ml GM-CSF and 1 μ M β -estradiol. Once established, expand GM-CSF ER-Hoxb8 cells to $6-8 \times 10^5$ cells/ml in 6-well tissue culture plates and dilute 1:1 in maintenance medium.
2. Collect cells in 15 ml conical tubes and centrifuge at $250 \times g$ for 5 min. Resuspend in 5 ml of 1 $\times$ PBS, centrifuge and repeat to wash cells.
3. Resuspend washed cell pellet in Opti-MEM plus 10% FBS supplemented with 10 ng/ml SCF ONLY (no β -estradiol) and culture in 5 ml per well in 6-well culture plates.
4. Maintain cells at a concentration of $2-6 \times 10^5$ cells/ml, diluting cultures as required. Monitor morphologic maturation using Wright-Giemsa-stained cytopins; macrophage morphologic features should be identified by 4-5 days of induction (see Figure 22.F.2).

Support Protocol 1

Preparation of WEHI-3B Conditioned Medium

WEHI-3B cells can be obtained from the American Tissue Culture Collection ([APPENDIX 5](#)) and are grown in RPMI supplemented with 10% heat-denatured FBS. BHK/MKL and BHK/HM-5 cell lines can be obtained from ATCC and are grown in DMEM supplemented with 10% heat-inactivated FBS. All medium also contains 2 mM L-glutamine, 5 U/ml penicillin G, and 5 μ g/ml streptomycin sulfate.

Materials—Cells of interest (e.g., WEHI-3B, BHK/MKL, BHK/HM-5) and corresponding growth medium supplemented with 2 mM L-glutamine, 5 U/ml penicillin G, and 5 μ g/ml streptomycin sulfate

PBS ([APPENDIX 2A](#))

1 $\times$ Trypsin-EDTA

15 ml conical centrifuge tubes

75- and 150-cm² tissue culture flasks

0.45- μ m filters

Passage adherent producer cells

1. Grow cells to a density of 70% to 80% confluency. Aspirate growth medium and wash cells with 10 ml PBS by slowly adding PBS to one side of the flask, gently washing medium over the cells, and aspirating off the wash from the lower edge of the flask.
2. Add 2 to 3 ml of 1 $\times$ Trypsin-EDTA to the flask and incubate 5 min.
3. Collect cells by adding 10 ml growth medium and repeated pipetting of cells down the bottom surface of the flask. Transfer cells to a 15-ml conical centrifuge tube and centrifuge 5 min at 250 $\times$ g, room temperature.
4. Decant the supernatant, resuspend cells in 10 ml growth medium and add 1 to 5 ml of cells to 30 ml of fresh growth medium in a 75-cm² flask.

Make conditioned medium

5. Expand cells in a 75-cm² flask to a density of 80% to 90% confluence.
6. Collect cells as in step 3 and pass all cells to a 150-cm² flask with a final volume of 100 ml of growth medium. Allow cells to expand overnight to 80% to 90% confluence.
7. Collect cells and pass to four 150-cm² tissue culture flasks, each with a total volume of 150 ml of growth medium.
8. Allow cells to grow for 4 days.

During the expansion stage, the pH indicator in the conditioned medium may become yellowish in color.

9. Collect conditioned medium and filter through a 0.45- μ m filter.

To decrease clogging of the filter in the filter flasks by cell debris, a fresh pre-filter disc can be placed into the top filter unit between each addition of conditioned medium.

Alternatively, medium can be centrifuged in 50-ml conical tubes 10 min at 250 $\times$ g, room temperature, to pellet debris.

10. Dispense filtered conditioned medium into 50-ml aliquots to sterile 50-ml conical tubes and store medium at -20°C . Prior to use, thaw medium for several minutes at 37°C .

Conditioned medium may be stored up to 1 year at -20°C . Once thawed, the conditioned medium is stable up to 3 months at 4°C . Do not refreeze.

Support Protocol 2

Analysis of Cell Surface Myeloid Markers

Expression of several cell surface markers of myeloid differentiation can be examined to characterize the extent of myeloid differentiation during the induction process of either bone marrow progenitors or cell line models. First, a loss of CD34 expression has been documented during myeloid induction of CD34⁺ cells isolated from bone marrow, and during the differentiation of progenitor EML cells towards early promyelocytes (e.g., EPRO cells) (Berliner et al., 1995; Lawson et al., 1998b). For definitive myeloid characteristics, progenitors induced toward neutrophils can show increased cell surface expression of the neutrophil-specific markers Ly-6G and Ly-6C (Gr-1) and the heterodimeric integrin binding protein CD11b/CD18 (Mac1); progenitors induced toward macrophages exhibit increased expression of Mac-1 and F4/80 (expression of CD14 also works well, but it is expressed by both granulocytes and monocytes/macrophages, see Choi et al, 2012). Typical results from differentiated murine bone marrow progenitors and model cell lines are depicted in Fig. 22.5.3; similar results can be generated in NB4 and HL-60 cells (see Lee et al, 2002, as an example). This protocol describes the analysis of cell surface marker expression, and can be performed with either flow cytometry (FCM) or whole cell imaging system (e.g. Cellometer Vision CBA Analysis System, Nexcelom Biosciences, or equivalent) analyses.

Materials—Cell samples: undifferentiated versus differentiated cells, 1×10^6 cells/sample

Cell labeling buffer (see recipe), ice cold

PBS (APPENDIX 2A), ice cold Fc receptor blocking antibody (anti-CD16/CD32; for mouse cells bearing Fc γ II and Fc γ III receptors; e.g., Fc block, Pharmingen)

Fluorochrome-labeled antibody to Gr-1, Mac-1 or F4/80 (Pharmingen or Santa Cruz)

12 $\times$ 75-mm flow cytometry tubes (e.g., Falcon)

Microcentrifuge

Additional reagents and equipment for trypan blue exclusion (APPENDIX 3B)

1. Induce cells as described in appropriate protocols above, maintaining cells at $3\text{--}5 \times 10^5$ cells/ml. After induction, count the number of viable cells for each induced cell line to be analyzed by trypan blue exclusion (APPENDIX 3B).
2. Pellet a total of 2×10^6 differentiated versus undifferentiated cells (per antibody) by collecting cells in 15 ml conical tubes and centrifuging 6 min at $250 \times g$, room temperature, resuspend pellet in 1000 μ l ice-cold PBS and then transfer 500 μ l of resuspended cells into two 1.5-ml microcentrifuge tubes (one for test antibody and a second for the isotype control). Centrifuge cells 5 min at $1000 \times g$, 4°C, and resuspend cells in each tube with 100 μ l of ice-cold cell labeling buffer.

All washes during this step and subsequent steps can be performed in either ice cold PBS or cell labeling buffer, but the final resuspension of cells prior to addition of antibodies and cytometry analyses should be performed with cell labeling buffer.

3. Add 0.5 μg / 100 μl cells of rat anti-mouse CD16/CD32 Fc receptor blocking antibody (e.g., Fc block) to block non-antigen-specific antibody binding. Incubate 15 min on ice.

Fc block reduces binding of the Fc portions of anti-mouse antibodies to FcR-bearing cells, including granulocytes. This step is therefore recommended for analyzing mouse cells with an anti-mouse monoclonal antibody.

4. Add 0.4 – 1.0 μg of a fluorochrome (e.g., FITC- or PE-) conjugated monoclonal antibody (e.g., anti-Gr-1 or anti-Mac-1) or primary antibodies that are not supplied as conjugated derivatives (e.g. rabbit anti-F4/80) to one tube of cell solution containing Fc block. Incubate 45 min on ice and protected from light.

A sample incubated with 0.5 μg of an isotype-matched immunoglobulin standard serves as a negative control.

5. For samples that require a fluorochrome-conjugated secondary antibody, pellet cells by centrifuging at $1000 \times g$ for 5 min at 4°C and resuspend cells in 500 μl of ice-cold PBS. Repeat centrifugation and resuspend pellet in 100 μl of cell labeling buffer. Add 0.4 μg of fluorochrome-conjugated secondary antibody (e.g. FITC goat anti-rabbit antibody) and incubate on ice for 45 min protected from light.
6. Wash cells two times by resuspending the cells in 400 μl of ice-cold cell labeling buffer, centrifuge 5 min at $1000 \times g$, 4°C , resuspend cells in 200 to 300 μl of cell labeling buffer, and transfer to pre-labeled $12 \times 75\text{-mm}$ flow cytometry tubes. Samples can be analyzed immediately but can be stored for up to 2 to 3 hr on ice until analyzed by flow cytometry (*UNITS* 5.3 & 5.4).

Table-top cell analyses systems, such as that available from Nexcelom (Cellometer), can yield high quality results also, but smaller volumes are required for such analyses; when resuspending cells during the final wash, use only 100 μl of the cell labeling buffer.

Support Protocol 3

Analysis of Neutrophil-Specific Granule Protein Gene Expression

A well-accepted alternate approach to examining the extent of myeloid differentiation that does not require flow cytometry is to examine levels of RNA expression of select neutrophil-specific genes, such as the secondary granule protein genes lactoferrin and neutrophil gelatinase. C/EBP ϵ , a transcriptional regulator critical to the expression of neutrophil-specific genes, is also upregulated during myeloid maturation and therefore levels of C/EBP ϵ expression can also be assessed to determine the extent of maturation (see Fig. 22F.5.2). Expression assays of these genes can be accomplished by northern blot analysis (*UNITS* 10.11 & 10.12) as described by Lawson et al. (1998b), by qualitative RT-PCR (*UNIT* 10.20) as described by Berliner et al. (1995), or by real time RT-PCR as described below.

RNA isolation is performed using standard procedures as outlined below using TRI reagent (Molecular Research Center, also available as TRIzol from Life Technologies). For RT-PCR of human granule protein genes, induced cells grown in liquid culture can be directly lysed in TRI reagent, and then total RNA isolated. Approximately 20 to 200 μg of total RNA can be isolated from 1×10^7 cells, 5 μg of which is used to generate cDNAs (e.g., by using SuperScript III Reverse Transcriptase, Invitrogen). For real time PCR analysis, 1 μl of the RT reaction is used. Alternatively, colonies may be isolated from methylcellulose by adding 1 to 2 ml of IMDM with 2% fetal bovine serum to each 35-mm dish containing methylcellulose culture, mixing the contents, and transferring the mixture to 15-ml centrifuge tubes. After washing the plates several times to harvest all cells, the cells are centrifuged 7 to 10 min at $250 \times g$. Single colonies may also be harvested using sterile 200- μl pipet tips on a pipettor, with the volume set to 50 μl , and drawing up the colonies in a 25- to 30- μl volume while viewing the colonies under an inverted microscope (magnification $80\times$). However, to yield sufficient numbers of cells for RNA isolation, colonies may need to be pooled. Example primers for RT-PCR of human granule protein genes and C/EPB ϵ are shown in Table 22F.5.1. Additional primers for the isolation of human neutrophil gene probes can be found in Cowland and Borregaard (1999). Primers for real time RT-PCR of several important mouse neutrophil-expressed genes are shown in Table 22F.5.2

Preparation of cytoplasmic RNA from tissue culture cells using TRI reagent—

Cells are washed with ice-cold PBS, pelleted by centrifugation, and lysed in TRI reagent, typically using 1×10^7 cells per ml of TRI reagent. Lysis occurs immediately, but care must be taken to thoroughly mix the reagent with the cells to ensure complete lysis. The lysis mixture can be stored at -70°C , or further prepared for RNA isolation by the addition of chloroform. Centrifugation of the chloroform/TRI reagent mixture yields a bottom organic phase containing DNA and protein, and an aqueous phase containing RNA. The aqueous phase is then removed, and RNA is recovered by precipitation with isopropyl alcohol.

Materials— 1×10^7 cells/ml cell samples

PBS (APPENDIX 2A), ice cold

TRI reagent (Molecular Research Center, also available from Invitrogen as TRIzol)

Chloroform

Isopropyl alcohol

80% ethanol (prepared with DEPC-treated water)

DEPC-treated water (APPENDIX 2A)

1.5-ml RNase-free microcentrifuge tubes

70°C heating block

CAUTION: TRI reagent is a strong corrosive and causes burns. When handling TRI, use gloves and eye protection.

1. Collect cells by centrifuging in 15 ml conical tubes for 5 min at $250 \times g$, room temperature. Wash cells by resuspending the pellet in 5 ml of ice-cold PBS, centrifuging 5 min at $250 \times g$, 4°C , and decant the supernatant.
2. Resuspend pellet in TRI reagent and rapidly lyse cells by pipetting the mixture up and down multiple times to yield a homogeneous solution; transfer the lysed cell mixture into RNase-free 1.5 ml microcentrifuge tubes.

If the number of cells is too high, the solution will become very viscous and will prevent clean separation of organic and aqueous phases during the subsequent steps. If this occurs, double the volume of TRI reagent. This mixture can be safely stored at -70°C for future extraction.

This protocol is designed for a small-scale RNA extraction, where 1×10^7 cells are lysed in 1 ml of TRI reagent. To obtain larger quantities of RNA, the procedure can be scaled up and performed in 15- or 50-ml conical tubes (e.g., Falcon). For smaller numbers of cells, the protocol can also be reduced, e.g. 5×10^6 cells can be lysed with 0.5 mL of TRI reagent and then extracted with half of the volumes described below.

3. Add 200 μl of chloroform per 1 ml of TRI reagent mixture. Rapidly shake the tube (do not vortex) for 15 sec, and let the mixture sit 3 to 5 min at room temperature.
4. Centrifuge 15 min at $12,000 \times g$, 4°C , to separate aqueous and organic phases. Extract the top aqueous phase and place into a new microcentrifuge tube.
5. Add an equal volume of isopropyl alcohol (generally 500 to 600 μl per 1 ml TRI reagent/cell mixture), mix gently by inversion and incubate 10 min at room temperature.

The sample may be stored at -70°C at this stage, but warm the mixture up to room temperature prior to centrifugation.

6. Centrifuge the RNA/alcohol mixture for 15 min at $12,000 \times g$, 4°C (a small white pellet should form on the bottom of the tube). Decant the liquid from each tube being careful not to dislodge the pellet.
7. Wash the pellet by slowly adding 500 μl of 75% ethanol, centrifuging 5 min at $12,000 \times g$, 4°C , and decanting the 75% ethanol.
8. Let the RNA pellet air dry in a flow hood until the pellet becomes clear.

If the resuspension solution is added to a white pellet, the RNA may be difficult to dissolve; to prevent this, do not dry RNA by centrifugation under vacuum.

9. Add 30 to 50 μl of DEPC-treated water to the pellet, warm 5 min in a 70°C dry heating block, and vortex the sample to ensure thorough resuspension of the RNA.

For the small-scale procedure (e.g. 1×10^7 cells total), yields of RNA typically range from 50 to 200 μg of total RNA.

Analysis of total RNA by real time RT-PCR—Once total RNA has been extracted, cDNA is first generated using reverse transcriptase and then real time PCR is performed to quantify expression levels of the mouse myeloid-expressed genes listed in Table 22.F.2. The parameters described below were designed for use of a BioRad iCycler, therefore different equipment may require adjustments to annealing temperatures.

Materials—RNA extractions from cell samples

SuperScript™ III Reverse Transcriptase kit (Invitrogen #18080-093)

0.5-ml microfuge tubes (VWR #89000-010), autoclaved

65°C, 50°C, 70°C and 37°C water baths

cDNA samples

SsoAdvanced™ SYBR® Green Supermix (Bio-Rad #172-5261)

2.5 pmol/μl Forward Primer

2.5 pmol/μl Reverse Primer

iCycler with MyiQ™ single color Real-Time PCR detection system (Bio-Rad)

96-well PCR plates (e.g. Fisherbrand #14-230-232)

Domed 8-strip caps (e.g. Bio-Rad #TCS-0801)

Generation of cDNA

1. Assay the concentrations of RNA samples on spectrophotometer (e.g.: NANODROP-2000 spectrophotometer, Thermo Scientific)
2. Prepare the RNA/primer mixture for each sample by adding the following to each tube:.

5μg total RNA

1μl 50M oligo(dT)₂₀

1μl 10mM dNTP mix

3. Adjust the volume to 13 μl by adding sterile distilled water.
4. Heat the mixture at 65°C for 5 min and incubate on ice for at least 1 min.
5. Collect the contents of the tubes by brief centrifugation and then add the following master mix to each tube:

4μl 5× First-Strand Buffer

1μl 0.1M DTT

1μl RNaseOUT™ Recombinant Rnase

1µl SuperScript™ III RT (200 units/µl)

6. Mix the contents in each tube by pipetting gently up and down and incubate at 50°C for 60 min.
7. Inactivate the reaction mixture by heating at 70°C for 15 min and chill on ice for at least 1 min. Collect the contents of the tubes by brief centrifugation.
8. Add 1µl of 2U/µl *E. coli* RNase H and incubate at 37°C for 20 min to remove the RNA complementary to cDNA.

Real-Time PCR

1. Prepare 25 µL of amplification reaction mixture for each sample in triplicates to perform real-time PCR analysis in 96-well PCR plates:
 - 12.5µl 2× SsoAdvanced™ SYBR® Green Supermix
 - 2µl 2.5 pmol/µl Forward Primer
 - 2µl 2.5 pmol/µl Reverse Primer
 - 7.5µl sterile distilled water
 - 1µl cDNA

Too great a primer concentration may promote mispriming and accumulation of non-specific product. Too low a primer concentration may cause the PCR reaction to reach an early plateau that may affect C_T values.
2. Set up the negative controls similarly for each set of primers with no cDNA.
3. Analyze the gene expression levels in iCycler with MyiQ™ single color Real-Time PCR detection system (Bio-Rad) using following conditions:
 - Step 1: 50°C × 2 min (1 cycle)
 - Step 2: 95°C × 10 min (1 cycle)
 - Step 3 (40 cycles): 95°C × 30 sec, 55.4°C × 30 sec, 72°C × 45 sec
 - Step 4: 72°C × 10 min

Annealing temperatures may vary for different primer sets. If testing multiple cDNAs for expression levels in a single assay, use the lowest annealing temperature among the primer sets. However, if non-specific amplification occurs or primer-dimers form, the amplifications will need to be performed as separate assays.
4. Setup the melt curve analysis to identify any non-specific amplifications or primer-dimers using following conditions: step 5: 95°C × 2 min (1 cycle), step 6: 95°C × 15 sec (140 cycle) with temperature decrements of 0.5°C/cycle and lastly, upon completion set the reaction to hold indefinitely at 4°C.

Set the Real-Time detection system to monitor the extension cycle of step 3 (i.e. $72^{\circ}\text{C} \times 45 \text{ sec}$) for analysis of gene expression levels and at step 6 of melt curve to identify any non-specific amplifications and primer dimers.

5. Compute the expression levels of all the genes relative to the expression levels of reference gene, using the comparative cycle threshold method ($\Delta\text{-C}_T$).

Reagents and Solutions

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

ATRA stock solution, 10 mM—Dissolve 3 mg of ATRA per 1 ml of 100% ethanol overnight in a 37°C water bath with constant agitation. Aliquot 1-ml stock solution into amber-colored tubes and store protected from light up to 1 year at -20°C . For induction of cell lines or progenitors, this stock solution is added directly to growth media at the recommended final concentrations.

Cell labeling buffer—Add to PBS (APPENDIX 2A):

2% fetal bovine serum

5 mM $\text{Na}_2\text{-EDTA}$, pH 8.0

0.1% sodium azide

Store up to 6 months at 4°C

Commentary

Background Information

Myeloid differentiation: A variety of techniques that utilize different combinations of cytokines have been developed to induce the differentiation of stem cell progenitors towards multiple lineages. One inherent limitation of this process is that, due to the promiscuous effects of many cytokines, it is difficult to induce the differentiation of a specific lineage from multipotent progenitors. However, several reports have demonstrated that specific combinations of cytokines can generate a particular lineage of interest. In the context of myeloid differentiation, a report described by Berliner et al. (1995) provides a convenient means to generate promyelocytes from $\text{CD}34^{+}$ cells, which can be subsequently induced towards terminally differentiated neutrophils, as described in Basic Protocol 1. The advantage of this protocol is that reasonable numbers of differentiated cells can be generated, such that gene expression profiles and expression of cell surface markers can be obtained. Basic Protocol 2 describes ex vivo differentiation of mouse bone marrow, initially isolated from mouse femurs and tibiae and then depleted of lineage-specific cells via use of a cocktail of biotinylated monoclonal antibodies to lineage-specific markers and streptavidin-conjugated magnetic nanoparticles; incubation of the labeled cells in a strong magnetic field removes lineage-specific cells, thereby leaving a population enriched in hematopoietic progenitors.

An alternative approach is also provided in which colonies of granulocytes are generated in a semi-solid medium, such as methylcellulose, as described in the Alternate Protocol. This technique, based on previous studies such as those performed by Douer et al. (2000) and Tocci et al. (1996), is limited by the small number of cells generated, and therefore, is primarily useful for a qualitative assessment of neutrophil vs. macrophage differentiation from a population of progenitors. Once colonies form, cells can be “plucked” from the methylcellulose, using a pipet, for further analysis.

In addition to protocols for generating neutrophils from either CD34+ human progenitors or lineage depleted mouse progenitors, this unit also provides a protocol for generating monocytes/macrophages from enriched population of myeloid progenitors. Although this protocol begins with a population of progenitors that includes granulocytes, further induction of the progenitors with a combination of GM-CSF and M-CSF, and then finally just M-CSF produces a homogenous population of primarily macrophages (see Fig. 22.F.2).

A technique not described in this unit involves the differentiation of human embryonic stem cells or induced pluripotent stem cells, using a stromal cell line that supports granulopoiesis, such as that recently described by Lieber et al. (2004), or more recently by using mouse OP9 bone marrow stromal cells as feeder cells (Choi et al, 2012) ; inductions with cytokines are similar to protocols presented here.

Myeloid cell lines: While in vitro differentiation of stem cell progenitors provides access to multiple developmental stages during myeloid development, the usefulness of this system is limited: the process results in a heterogeneous population of cells, and the limited number of progeny can make genetic manipulations and gene profile analyses difficult. A commonly used alternative is provided by myeloid cell lines, which can be grown in great numbers and can be genetically modified to express exogenous genes of interest. Basic Protocol 3 describes the induction of leukemic, factor-independent NB-4 and HL-60 cell lines, both of which can be induced towards morphologically mature neutrophils. HL-60 cells, although originally described as promyelocytic, were isolated from a patient with AML-M2 and are currently described as myeloblastic (Dalton et al., 1988). NB-4 cells, isolated from a patient with acute promyelocytic leukemia, carry the common t(15,17) translocation and show distinct promyelocytic characteristics (e.g., AML-M3, Lanotte et al., 1991). A disadvantage of these cell lines is that they lack the expression of secondary granule protein genes (Lanotte et al., 1991; Graubert et al., 1993; Khanna-Gupta et al., 1994), thus studies to examine the expression of late genes upregulated during terminal neutrophil differentiation cannot be performed with these cells. Despite this issue, both cell lines can also be induced toward mature macrophages; in the Alternative Protocol, this induction procedure is described for both HL-60 and NB4 cell lines.

Basic Protocols 4 and 5 outline the differentiation of two useful factor-dependent mouse myeloid models. 32Dcl3 cells are myeloblastic and are induced to terminally differentiate by replacing IL-3 in the growth medium with G-CSF, as described in Basic Protocol 3. These cells therefore reflect developmental changes induced by the activation of G-CSF and downstream signaling pathways such as the STAT-3 pathway. Furthermore, they express multiple neutrophil-specific genes (Graubert et al., 1993; Lawson et al., 1998a). These cells

do pose one problem in that many cells undergo apoptosis during the initial phase of differentiation. Basic Protocol 4 describes the differentiation of the EML cell line. EML cells were generated from bone marrow and are arrested at an early multipotent progenitor stage due to the ectopic expression of a dominant negative retinoic acid receptor (Tsai et al., 1994). This developmental block is overcome by inducing the cells with a combination of IL-3 plus ATRA, and subsequent selection in GM-CSF leads to the generation of an early promyelocytic cell line, EPRO cells. EPRO cells are then terminally differentiated by induction with ATRA in the presence of GM-CSF, and the resulting cells have been shown to express multiple secondary granule protein genes (Lawson et al., 1998b). A related promyelocytic cell line, MPRO, is also available, which undergoes rapid differentiation into mature neutrophils when induced with ATRA and also demonstrates neutrophil-specific gene expression.

Finally, Basic Protocol 5 describes two cell lines, each derived from bone marrow that was transfected with an expression vector that drives the expression of the estrogen-binding domain of the estrogen receptor fused to Hoxb8, a protein well-characterized for its role in arresting myeloid maturation (Wang et al, 2006). Maintenance of these cells in β -estradiol and selection in either stem cell factor (SCF) or granulocyte macrophage colony stimulating factor (GM-CSF) yields homogenous populations of pro-neutrophils and pro-macrophages, respectively (see Wang et al, 2006). Either cell line can be induced by removal of β -estradiol from their maintenance medium, and cell surface markers or gene expression profile changes are similar to those observed in the EML/EPRO model (see Figure 22.F.3).

Myeloid characteristics: Support Protocols 2 and 3 describe straightforward procedures for assessing the myeloid characteristics of differentiating cell populations. Support Protocol 2 is simple to perform, however, it requires access to flow cytometry or other cell imaging systems. As an alternative, gene expression profiles can be used via northern blot analysis, RT-PCR or quantitative RT-PCR, as described in Support Protocol 3. Results from these assays are displayed in Figs. 22.F.3 and 22.F.4.

Critical Parameters and Troubleshooting—The key to differentiating the progenitor cell lines is to maintain a cell density in a range of $3\text{--}10 \times 10^5$ cells/ml, and to refresh the medium every 2 to 3 days. For HL-60 cells, several reports have indicated that induction with DMSO or ATRA alone induces neutrophil differentiation, but a combination of both factors appears to induce rapid development (Le Cabec et al., 1997). Induction of NB4 cells requires only ATRA. Either cell line can also be induced toward macrophages with either phorbol myristate acetate or vitamin D3, although most reports indicate a combination is best for inducing NB4 cells. For EML cells, strict adherence to the passage regimen and maintenance of a cell density between 0.5×10^5 cells/ml and 6×10^5 cells/ml is critical. During the primary induction of EML cells with IL-3 plus ATRA in the presence of SCF, a significant increase in proliferation rate will be observed. Therefore, a starting concentration of no more than 5×10^5 cells/ml is recommended. Subsequent selection of EPRO cells in GM-CSF will be accompanied by a significant amount of cell death, therefore, the selection should be performed with an initially high concentration of cells, such as $6\text{--}8 \times 10^5$ cells/ml. For both ER-Hoxb8 progenitor lines, use of either defined or certified FBS is recommended,

as these cells tend to be more sensitive to impurities that may be present in standard (e.g. qualified) FBS.

The degree of differentiation for cell lines can be affected by the age of the culture. If a sharp decrease in differentiation rate or increased apoptosis prior to differentiation is observed, a fresh culture should be obtained.

During assessment of morphologic maturation, care should be taken to not overload the slides with cells during cytopins. Generally, $1-2 \times 10^4$ cells per slide is recommended. Diluting the cells in a solution of PBS plus 0.1% bovine serum albumin can also be used to help keep cells separated during centrifugation.

Primer design and amplicon consideration for real-time PCR: A successful real-time PCR reaction requires the primers to efficiently amplify a specific, typically short region of DNA. In order to ensure efficient amplification, the following parameters should be considered when designing primer sets:

- Design primers such that the amplicon size is between 75-200 bps. The amplicon size must be at least 75 bps to easily distinguish it from any primer-dimers that might form. Shorter amplicon sizes amplify with greater efficiency because they are more likely to be denatured during the 95°C step of PCR. This allows maximum availability of the denatured templates and therefore the primers can compete and bind more efficiently to their targets. Also, since the extension rate of polymerase enzyme is 30-100 bases per second, polymerization times as short as 10-15 seconds should be sufficient to replicate the shorter amplicon sizes.
- Maintain the GC content of primers at approximately 50-60% and terminate each with 1-2 guanines or cytosines at 3' ends, which will help to avoid non-specific priming.
- The annealing temperature (T_m) of primers should be between 55-65°C. The T_m difference between the primers set (i.e. forward and reverse primer for a single target gene) should not be more than 1-2°C.
- Most efficient reverse transcription reactions will generate cDNAs up to 10kb in length, therefore primers should be designed to target longer genes no less than 1 kb distal from 3' ends. Primers should also be designed to target exons to avoid any amplification from contaminating genomic DNA. Avoid amplification of any secondary structure if possible. Use programs such as mfold (available at <http://www.bioinfo.rpi.edu/applications/mfold/>) to identify if the amplicon is likely to form any secondary structure at the annealing temperature.
- Check the sequence of forward and reverse primers to avoid formation of primer-dimers.
- Finally, verify the specificity of primers and the amplicon using tools such as the Basic Local Alignment Search Tool (available at <http://www.ncbi.nlm.nih.gov/blast/>)

Anticipated Results—In the CD34⁺ model, myeloperoxidase (MPO) is observed from the onset, with levels increasing during the first 24 to 48 hr of induction with SCF and IL-3. Levels of MPO will then sharply increase upon G-CSF induction, and remain high until the end stages of neutrophil maturation, where levels begin to decrease. Neutrophil elastase is observed in myeloblasts and early promyelocytes, with levels remaining high throughout differentiation. By comparison, MPO is highly expressed in NB-4, HL-60, EPRO, and 32Dcl3 cells, with decreased expression in each cell line upon terminal differentiation. Lactoferrin is expressed in later stages of differentiation in CD34⁺ cells and lineage-depleted cells (day 6 to 7), but is rapidly upregulated in EPRO and MPRO in response to ATRA, in 32Dcl3 cells in response to G-CSF, and in SCF ER-Hoxb8 cells upon β -estradiol withdrawal (see Fig. 22F.5.4, and Subramanian et al, 2012, for additional expression results in EML/EPRO cells); no expression is observed in NB-4 or HL-60 cell lines. Changes in the expression of cell surface markers can vary in different cell lines/models. For example, Mac-1 is low or absent in all of the cell lines prior to induction, but increased expression should be rapidly observed during differentiation (see Fig. 22F.5.3, and Lawson et al., 1998b for additional results in EML/EPRO cells). By comparison, Gr-1 is essentially lacking in undifferentiated bone marrow progenitors and uninduced SCF ER-Hoxb8, but is upregulated as either model is induced toward mature neutrophils. EML cells also lack Gr-1 expression, and levels increase in EPRO cells, but expression does not significantly increase as they mature into neutrophils. Finally, F4/80 exhibits significant upregulation in models of macrophage differentiation.

Time Considerations—To induce differentiation with each cell line, an overnight incubation of cells diluted to $3\text{--}5 \times 10^5$ cells/ml is required prior to the addition of inducing agents. The time to set up each induction process requires <1 hr, except in the case of 32Dcl3 cells, which requires two wash steps to remove IL-3 prior to G-CSF induction. For cell surface analysis, the entire procedure takes ~2 hr, not including the time for flow cytometry/cell imaging. Assessment of gene expression profiles by real time RT-PCR can be performed in 3 to 4 hr, but may require pilot experiments to optimize amplification protocols and primer designs, whereas northern blots can take up to 2 days to generate and analyze.

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Ex vivo differentiated mouse bone marrow **Mouse cell lines** **Human cell lines** **Figure 22F.5.1.**

Models of neutrophil maturation. Bone marrow is depleted of lineage-specific cells or purified for CD34⁺ expression, which can be expanded in SCF plus IL-3 to yield a population of multipotent progenitors; further induction with SCF/IL-3 plus G-CSF produces promyelocytes and myelocytes, followed by terminal differentiation in G-CSF alone to mature neutrophils. SCF-dependent EML cells are multipotent mouse progenitors that can be induced towards early promyelocytes in response to a combination of ATRA plus IL-3. GM-CSF-dependent EPRO cells (derived from the induced EML cells) can then be induced with ATRA to terminally differentiate into mature neutrophils (shown are stained EML cells, EPRO cells and induced EPRO cells). 32Dcl3 cells are mouse myeloblasts that mature into neutrophils when induced with G-CSF. SCF ER-Hoxb8 cells are also myeloblasts that terminally differentiate upon removal of b-estradiol from their growth medium. Two human models, NB-4 and HL-60, are leukemic cell lines that can be induced with ATRA to mature neutrophils (shown are stained uninduced HL-60 cells and those induced for 5 days in 1 μ M ATRA plus 1.25% DMSO).

Ex vivo differentiated mouse bone marrow

Mouse cell line

Human cell lines

Figure 22F.2.

Models of macrophage maturation. Lineage depleted bone marrow is expanded in SCF plus IL-3 to yield a population of multipotent progenitors, which are then be induced toward monocytes and macrophages with GM-CSF plus M-CSF followed by M-CSF alone; the induction yields a population highly enriched in mature macrophages. The mouse cell line GM-CSF ER-Hoxb8 can be induced toward mature macrophages upon removal of β -estradiol from their growth medium. Both human myeloid models NB-4 and HL-60 can be induced with PMA and/or Vitamin D3 toward morphologically mature macrophages, although HL-60 cells are more efficient as bipotent myeloblasts (shown are stained uninduced HL-60 cells vs. PMA-induced HL-60 cells after 5 days).

Figure 22F.3.

Cells surface marker expression profiles of mouse myeloid models. Cells isolated either prior to induction or after incubation with the inducing agent(s) were labeled with either anti-Gr1 and anti-Mac-1 (for neutrophil differentiation) or anti-F4/80 and anti-Mac-1 (for macrophage differentiation). Antibodies conjugated with a fluorescence marker or fluorochrome-conjugated secondary antibodies were used for detection. Depicted are histograms of the numbers of cells that were labeled with each antibody; isotype controls are shown in each graph as dotted lines. All analyses were performed with a Cellometer (Nexcelom) using antibodies described in Support Protocol 2.

Figure 22F.5.4.

Neutrophil gene expression profiles of murine progenitor cells during terminal differentiation. (**A-C**) Northern analyses indicate changes in the expression of lactoferrin (LF) or C/EBP ϵ in (**A**) EPRO cells induced with 10 μ M ATRA, (**B**) 32Dcl3 cells induced with 100 ng/ml G-CSF in the absence of IL-3, and (**C**) bone marrow cells induced ex vivo in different combinations of cytokines. In **A-C**, 10 μ g of total RNA was electrophoresed and blotted, then hybridized with the indicated probes. Shown on the bottom of each figure are pictures of the ethidium bromide (EtBr)-stained gels prior to transfer to nitrocellulose. (**D-F**) Real-time RT-PCR analyses demonstrate changes in the expression of neutrophil-expressed genes lactoferrin (LF), neutrophil gelatinase (NGal), and Mac-1. Depicted in each of **D-F** are fold changes in comparison to levels prior to induction of each cell type. Data shown are average levels $\pm$ standard deviations from three independent experiments.

Table 22F.5.1
Primers for RT-PCR of Human Neutrophil Genes

Gene ^a	Sense Primer	Antisense Primer
NG	5'-AGGGCGTCGTGGTTCCA-3'	5'-GCCCCTCAGTGAAGCGG-3'
ND	5'-GCAAGCTCAGCAGCAGAAATG-3'	5'-TCTCTGGTCACCCTGCCTAG-3'
LF	5'-CCAGGGCGATGCAGTCC-3'	5'-CCAACCTGTGTCGCCTG-3'
C/EBP ϵ	5'-CCCACGGGACCTACTACGAG-3'	5'-GAGGTTGCGGAGGGTGTCTA-3'

^aNG, neutrophil gelatinase; ND, neutrophil defensin; LF, lactoferrin.

Table 22F.5.2
Primers for real time RT-PCR of Mouse Neutrophil Genes

Gene ^a	Sense Primer	Antisense Primer
mS18	5'-GGCGGAGATATGCTCATGTG-3'	5'-GTCTGGGATCTTGTACTGTCGT-3'
NG	5'- GAGACGGGTATCCCTTCGAC-3'	5'- TGACATGGGGCACCATTGAG -3'
LF	5'- TGAGGCCCTTGGACTCTGT -3'	5'- CACCCACTTTTCTCATCTCGTTC -3'
Mac-1	5'- ATGGACGCTGATGGCAATACC -3'	5'- TCCCCATTACGTCTCCCA -3'

^a mS18, mouse S18 ribosomal RNA; NG, neutrophil gelatinase; LF, lactoferrin; Mac-1, CD11b.