Chapter 5

Protocols for Identifying, Enumerating, and Assessing Mouse Eosinophils

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Abstract

Eosinophils are prominent in allergic diseases, and their effector functions are studied in numerous gene-deleted and transgenic mouse models. However, mouse eosinophils and human eosinophils are not structurally or functionally equivalent, and assays designed to evaluate the properties of human eosinophils may or may not be reliable or effective in experiments targeting their murine counterparts. In this chapter, we emphasize methods focused on detection, isolation, and functional assessment of eosinophils from mouse tissue and present a protocol that promotes the growth and differentiation of eosinophils from unselected mouse bone marrow progenitors. Overall, these protocols provide a scaffold on which the relative contributions of mouse eosinophils can be evaluated.

Key words Piece-meal degranulation (PMD), Eosinophil peroxidase (EPO), Major basic protein (MBP), Eotaxin, Ribonuclease, IL5-R**α**, CCR3

1 Introduction

Although eosinophil-related biomedical research relies substantially on the use of mouse models of human disease, mouse eosinophils and human eosinophils are actually quite different from one another. One must not assume that protocols designed to evaluate human eosinophils will be appropriate or even adequate for the study of mouse eosinophils. Some of the most prominent differences between these cells are listed in Table 1. For instance, human eosinophils from peripheral blood have characteristic bilobed nuclei and prominent refractile granules that stain vividly with acidic dyes. However, mouse eosinophil morphology is somewhat more subtle; the nucleus can take on different shapes (Fig. 1), and the granules are smaller and less refractile than those found in human eosinophils [1]. Many methods are available to stain eosinophils that distinguish them from the other granulocytes, primarily neutrophils

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Table 1
Mouse and human eosinophils are not identical

Characteristic	Human	Mouse
Nuclear morphology	Bilobed	Bilobed and donut shaped
Granule morphology	Large, refractile	Smaller, less refractile
Secretory ribonucleases	Cationic ECP; more neutral EDN	Highly divergent mEAR cluster
Galectin-10/Charcot-Leyden crystal protein	Present in high concentration	Absent; no identifiable ortholog
High affinity IgE receptor	Present; surface expression	Absent
Cell surface Siglec expression	Siglec 8	Siglec F
Degranulation	Highly responsive; multiple secretagogues	Minimally responsive; few identified secretagogues

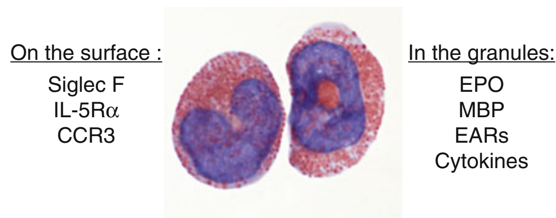

Fig. 1 Freshly isolated naïve mouse bone marrow eosinophils stained with Diff Quik (*see* Protocol 3.6). Note the two nuclear phenotypes. Also indicated are select surface markers and some contents of the granules that are stained here in *red*

and basophils [2]. The protocol provided here has been optimized so that mouse eosinophil granules stain prominently.

Eosinophils possess a few relatively specific proteins that can be used as markers for confocal microscopy or flow-cytometric-based identification. The granule major basic protein (MBP) and eosinophil peroxidase (EPO) and cell surface proteins Siglec F, CCR3, and IL-5Rα have all been used to identify mouse eosinophils. We present a protocol for intracellular staining of MBP [3] and a protocol that utilizes antibodies directed against Siglec F for the identification of eosinophils by flow cytometry. Human eosinophils degranulate readily in response to many stimuli but mouse eosinophils differ in that they do not release their granule contents under similar conditions or in response to similar secretagogues. Mouse eosinophils, similar to human eosinophils, exhibit piecemeal degranulation; therefore any one secretagogue may not stimulate

the release of all granule components equally. We have provided several protocols that assess the release of eosinophil contents. The first is an assay that detects the activity of EPO using a cell-impermeable substrate as first reported by Adamko et al. [4]. The second assay utilizes multibead cytokine assays or ELISAs to measure cytokine release. And the third method is an assay that detects the activity of the eosinophil-associated ribonucleases against a tRNA substrate as reported by Rosenberg and Domachowske [5]. Finally, we provide the protocol for a chemotaxis assay that utilizes eotaxin-2 as a stimulant.

The protocols included in this chapter are organized into three sections. The first set of protocols (3.1–3.5) focuses on isolation of source material; specifically, single cell suspensions from mouse bone marrow, spleen, lung, and bronchoalveolar lavage fluid, typically from interleukin-5 (IL-5) transgenic or antigen sensitized and challenged mice, from which eosinophils can be enumerated, isolated, and evaluated. This group also includes a protocol that describes methods to grow and differentiate eosinophils from the bone marrow of wild-type and gene-deleted mice (bmEos). The second set of protocols (3.6–3.9) is directed at evaluating the physical attributes of the eosinophils found in this source material. We provide protocols for staining eosinophils in single cell suspensions, for isolation of RNA and analysis of eosinophil gene expression by quantitative RT-PCR, protocols for analysis of immunoreactive proteins by confocal microscopy and immunohistochemical analyses and methods to detect eosinophils by flow cytometry. We also provide protocols for functional assays such as eosinophil degranulation (3.10–3.12) and chemotaxis (3.13).

2 Materials

2.1 Buffers and Reagents

1. BSA/PBS: 0.1 % or 1 % BSA (as specified) in PBS.
2. RPMI-1640 without phenol red.
3. RPMI-1640 (with phenol red).
4. Hanks' Buffered saline solution (HBSS).
5. Fetal bovine serum (FBS): heat inactivated for 1 h at 56 °C.
6. FBS/HEPES/HBSS: 1 % fetal bovine serum in HBSS with 10 mM HEPES.
7. EDTA/PBS: 10 mM EDTA in PBS.
8. Lung isolation media: RPMI-1640, 0.05 % DNase, 20 mM HEPES, 2 mMl-glutamine 100 IU penicillin and 10 μg/ml streptomycin, 60 μl of 50 mg/ml Liberase.
9. FBS/RPMI: 3 % FBS in RPMI-1640.
10. Percoll: 40 % Percoll and 70 % Percoll.

11. ACK: ammonium chloride lysis buffer.
12. OPD (*o*-phenylene-diamine): 800 μl 5 mM OPD in 4 ml 1 M Tris–HCl (pH 8.0), 5.2 ml H_2O and 1.25 μl 30 % H_2O_2.
13. SDS: 0.2 % sodium dodecyl sulfate.
14. H_2SO_4, 4 M.
15. Pertussis toxin, 100 ng/ml.
16. Platelet-activating factor (PAF): 1 mM in DMSO, store at −20 °C. Use at final concentration 1–5 μM.
17. IL-6: 20 ng/ml in 0.1 % BSA/PBS, store in single-use aliquots at −80 °C.
18. Cycloheximide, 5 μg/ml.
19. Multibead cytokine assay (Millipore, BioRad).
20. Deoxyribonuclease (DNase) I.
21. Diethyl-pyrocarbonate (DEPC)-treated water.
22. First Strand cDNA Synthesis Kit for RT-PCR.
23. Real time PCR primer probe sets (Applied Biosystems, ABI): EPO, MBP, IL-5Rα/CD125, CCR3, and GAPDH.
24. Cell permeabilization and fixation reagent (i.e. Fix and Perm (Caltag Laboratories)).
25. 4 % paraformaldehyde.
26. Methanol—ice cold.
27. DAPI—4′,6-diamidino-2-phenylindole dihydrochloride.
28. Cell differential staining reagents (i.e. Diff-Quik).
29. Cell viability stain (i.e. Live-Dead Stain (Invitrogen)).
30. Antibodies: anti-CD16/CD32, Rabbit anti-MBP (#509, provided on request from Dr. Nancy A. Lee and Dr. James J. Lee, Mayo Clinic, Scottsdale, Arizona), goat anti-rabbit IgG-Alexa Fluor 647, anti-Siglec-F, anti-CD11c, anti-CD45.
31. Base media: RPMI-1640 with 20 % FBS, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 1× NEAA, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 10 μg/ml streptomycin.
32. Chemotaxis assay media: RPMI-1640 media supplemented with 1 % Fetal Calf Serum (FCS) and 10 mM HEPES Buffer.
33. Chemoattractants such as eotaxin: suspended in chemotaxis assay media.
34. RNA stabilization buffer (i.e. RNAlater).
35. Qiagen RNeasy kit.
36. Real-time quantitative PCR buffer, such as ABI 2× Taqman reagent.

37. Phosphate buffer: 100 mM sodium phosphate in DEPC water, pH 7.4.
38. 40 mM Lanthanum nitrate in DEPC water.
39. 6 % perchloric acid in DEPC water.
40. 20 mg/ml yeast tRNA in DEPC, aliquot immediately and store in single-use aliquots at −80 °C.
41. T and B bead buffer: 0.5 % BSA and 2 mM EDTA, pH 7.2 in PBS.
42. T and B cell microbeads: CD90.2 (Thy1.2, Miltenyi) and CD45R (B220, Miltenyi).
43. RNase Assay STOP solution: 1:1 v/v mixture of 40 mM lanthanum nitrate and 6 % perchloric acid, prepare immediately prior to use.
44. Bovine ribonuclease A: 100, 1,000, and 10,000 ng/ml in PBS.
45. bmEos base media: 20 % FBS (heat inactivated), 25 mM HEPES, 100 IU/ml penicillin and 10 μg/ml streptomycin, 2 mM glutamine, 1× NEAA, 1 mM sodium pyruvate, 50 μM β-ME all in RPMI-1640.
46. Mouse Interleukin-5: (mIL-5, R&D): resuspend in 0.1 % BSA/PBS at 5 μg/ml and use at 10 ng/ml. Store in single-use aliquots at −80 °C.
47. Mouse Stem Cell Factor (mSCF, Peprotech): resuspend in 0.1 % BSA/PBS at 10 μg/ml and use at 100 ng/ml. Store in single-use aliquots at −80 °C.
48. Mouse Flt3L (PeproTech): resuspend in 0.1 % BSA/PBS at 10 μg/ml and use at 100 ng/ml. Store in single-use aliquots at −80 °C.

2.2 Supplies

1. Cytofunnels.
2. 96-well flat bottom plates.
3. Cell strainers, 40 and 70 μm.
4. HTS Transwell®-96 Permeable Support System with 5.0 μm pore polycarbonate membrane.
5. 5 ml Polystyrene Round-Bottom Tubes.
6. CS column (Miltenyi).
7. Small feeding needle (22 × 1″ Cadence, Inc.).

2.3 Equipment

1. Centrifuge.
2. Cytospin centrifuge.
3. Light microscope with 64× oil immersion objective.
4. ELISA Plate reader with 492 nm filter.
5. 37 °C, 5 % CO_2 humidified incubator.
6. BioRad BioPlex multibead plate reader.

7. Confocal microscope.
8. Flow cytometer.
9. Tissue homogenizer.
10. Real-time quantitative PCR instrument.
11. Spectrophotometer capable of reading absorbance at 260 nm.
12. Vario MACS Magnet (Miltenyi).
13. Hemacytometer.

3 Methods

3.1 Isolation of Total Bone Marrow Cells

1. This protocol is generally applicable to all strains of mice. Recovery from a wild-type naïve mouse is typically $10–20 \times 10^6$ cells with about 5 % eosinophils; this percentage will increase to 40–60 % eosinophils when using bone marrow from IL-5 transgenic mice [6].
2. Euthanize mice according to approved animal care procedures. Remove the femurs and tibias, remove the flesh from the bones, with a scalpel cut off the ends of the bones as distally as possible. Insert a 25 gauge needle into the end of the bone and flush the marrow into a 15 ml tube with a total of 12 ml of RPMI-1640 per four mouse bones.
3. Centrifuge the cells and lyse the red blood cells by suspending the pellet in 9 ml of dH_2O for 30 s and then add 1 ml of 10× PBS to adjust tonicity to normal, centrifuge and repeat two more times.
4. Suspend the cells in 10 ml of HBSS and then count the cells to determine the total cell number. Eosinophils and eosinophil progenitors from bone marrow can be enumerated by direct staining (*see* Protocol 3.6), by immunohistochemical staining (*see* Protocol 3.7), and/or by flow cytometric methods (*see* Protocol 3.8).

3.2 Isolation of Cells from the Spleen

1. The spleen of the IL-5 transgenic (tg) mouse is an excellent source of mature eosinophils. This protocol, modified from that of Aizawa et al. [7] and Shen et al. [8] provides a method for the isolation of 10^7 cells with >90 % eosinophils from spleens of IL-5tg mice.
2. Euthanize mice according to approved animal care procedures. Place the spleen in cold 1 % FBS/10 mM HEPES/HBSS until ready to process.
3. Once the isolation of single cells begins, keep cells at room temperature and use reagents brought to room temperature. Move the spleen into a petri dish or six-well plate and cut into small pieces. Place the spleen fragments in a 70 μm strainer

inserted into a 50 ml tube. Force the tissue through the mesh using the plunger of a 3 ml syringe. Rinse the strainer with 10 ml of 1 % FBS/10 mM HEPES/HBSS and repeat process using a 40 μm strainer.

4. Centrifuge the cells, resuspend the pellet in 1 ml of cold FBS/HEPES/HBSS and lyse the red blood cells with ACK lysis buffer or with 45 ml of dH_2O for 30 s followed by 5 ml of 10× PBS, centrifuge and repeat two more times.
5. After the last centrifugation, suspend the cells in 50 ml of HBSS prior to the determination of total cell number. Approximately $2–10 \times 10^7$ cells will be recovered from the spleen of a wild-type naïve mouse [9].
6. From this point, eosinophils are isolated by negative selection. B and T cells are removed via interactions with anti-CD45R and anti-CD90.2 microbeads, respectively. Centrifuge 10^7 cells at 300 × *g* for 10 min and completely remove the supernatant. Suspend cells in 90 μl of ice-cold T and B bead buffer and add 10 μl anti-CD45R/B220 and 10 μl anti-CD90.2 microbeads (*see* **Note 1**).
7. Incubate exactly 15 min at 4 °C tumbling end over end.
8. Wash the cells in 2 ml of ice-cold T and B bead buffer and pass through a 70 μm sieve and centrifuge.
9. While centrifuging, set up the CS column per manufacturer's directions. Flush the CS column with T and B bead buffer through the syringe and then elute 10–30 ml of T and B bead buffer through the column from the top. Attach a 20–25-gauge needle to act as a flow regulator (a smaller needle will yield greater purity, but will diminish the number of cells recovered).
10. After centrifuging the cells, suspend the pellet at 10^8 cells in 500 μl of buffer and apply the cells to the column.
11. Collect the unlabeled cells, which will pass through the column and wash column twice in 1 ml buffer. Then add an additional 10 ml of buffer to the column. The column will retain T and B cells and eosinophils will be unlabeled and pass through the column. Determine the number of collected, unlabeled eosinophils.
12. Eosinophils from the spleen can be assessed by direct staining (*see* Protocol 3.6), by immunohistochemical staining (*see* Protocol 3.7), and/or by flow cytometric methods (*see* Protocol 3.8). These cells can also be used for degranulation studies (*see* Protocols 3.9–3.11) and for chemotaxis assays (*see* Protocol 3.12).

3.3 Isolation of Cells from the Lung

1. This protocol is modified from that of Carlens et al. [10] and generates single cell suspensions from lung tissue for evaluation of eosinophils elicited in response to antigen sensitization

and airway challenge, or in response to systemic and/or local expression of cytokine transgenes. Approximately $2–5 \times 10^6$ cells are recovered from a pool of two wild-type naïve mouse lungs, and 10×10^6 cells from a pool of two lungs from wild-type mice sensitized and challenged with ovalbumin, as described in reference [11].

2. Euthanize mice according to approved animal care procedures. Open the chest cavity and perfuse the lungs by injecting 8 ml of 10 mM EDTA/PBS into the right ventricle of the heart to remove peripheral blood cells and then remove the lungs from the mouse. The lung will turn noticeably whiter in color as the RBCs are flushed out; good perfusion of the lungs is critical for cell recovery (i.e. poor perfusion means reduced yield).
3. Pool the lungs from two mice and mince in a petri dish using a straight razor blade.
4. Transfer lung tissue to a 50 ml beaker containing freshly prepared 15 ml of lung isolation media and add 60 μl of 50 mg/ml Liberase and stir for 30 min at 37 °C.
5. After incubation, put the contents of the beaker through a 70 μm cell strainer. Rinse the strainer two to three times with 5 ml of media with 3 % FBS and then pass the cell/media mixture through a 40 μm strainer.
6. Centrifuge the cells. Pipette off the liquid and lyse the red blood cells by suspending the pellet in 3 ml of ACK lysing buffer and incubate for 5 min at room temperature, add 10 ml of media with 3 % FBS and centrifuge cells again.
7. Suspend the pellet in 8 ml of 40 % Percoll then overlay on 3 ml of 70 % Percoll and centrifuge at $580 \times g$ for 30 min at room temperature with the rotor brake off.
8. Collect the cells at the interface and wash with 10 ml HBSS. Centrifuge cells, suspend pellet in 1 ml HBSS and count. Cell populations from the lung can be enumerated by direct staining (*see* Protocol 3.6), by immunohistochemical staining (*see* Protocol 3.7), and/or by flow cytometric methods (*see* Protocol 3.8).

3.4 Isolation of Cells from Bronchial Alveolar Lavage Fluid

1. Naïve wild-type mice have few to no eosinophils in the bronchial alveolar lavage fluid (BALF). BALF collected from wild-type BALB/c mice sensitized and challenged with ovalbumin will have up to 60 % eosinophils [11].
2. Euthanize mice according to approved animal care procedures, but keep in mind that cervical dislocation, if performed, will need to be done gently so as not to damage the trachea and surrounding tissues.
3. Place the mouse on its back with limbs fixed and soak the chest and abdomen with 70 % ethanol. Dissect to open the mouse

from the diaphragm up to the top of the neck and pin down neck skin to immobilize the head in a prone position. When dissected appropriately the trachea should be visible.

4. Put 0.8 ml of 1 % BSA/PBS in a 1 ml syringe and attach a small feeding needle. Insert feeding needle through the mouth into the trachea. Some resistance should be felt and the feeding needle should be visible in the trachea. Fill lungs with 0.7 ml of 1 % BSA/PBS. Lungs should visibly inflate; if not remove needle and try again as the solution is going into the stomach or coming back out of the mouth.
5. When the lungs inflate, carefully pull back on the syringe to remove fluid. Usually you can recover roughly 0.5 ml of BALF. Repeat this process with another 0.8 ml of 1 % BSA/PBS.
6. The total volume of BALF collected is 0.5–1.0 ml per mouse. Collect the cells by centrifugation. Cell populations from the BALF can be enumerated by direct staining (*see* Protocol 3.6), by immunohistochemical staining (*see* Protocol 3.7), and/or by flow cytometric methods (*see* Protocol 3.8).

3.5 Differentiation of Eosinophils from the Bone Marrow

1. This protocol provides a method for generating large numbers of phenotypically mature eosinophils ex vivo from unselected bone marrow progenitors [12]. We [13, 14] and others [15, 16] have used this method to generate eosinophils from a number of gene-deleted strains.
2. Prepare 500 ml of bmEos base media and add SCF and Flt3L to 50 ml of bmEos base media. Prepare concentrated stocks in 0.1 % BSA/PBS and freeze at −80 °C, thaw immediately before use and add to media at 100 ng/ml each. Use cytokine containing media within 1 week.
3. Euthanize mice and collect marrow (as per Protocol 3.1) under sterile conditions. Once all marrow has been collected, centrifuge to pellet cells.
4. Prepare sterile dH_2O and 10× PBS, lyse red blood cells by pipetting up and down two times in 9 ml of dH_2O and then immediately add 1 ml of 10× PBS to make solution isotonic.
5. Centrifuge again.
6. Lyse again if necessary, if not, suspend in 1× PBS or media for counting and preparing slides for differential count. Do not perform lysis procedure more than three times.
7. Centrifuge, remove PBS, and put into bmEos base media + SCF and Flt3L at 10^6 cells/ml.
8. On day 2 of culture, remove ½ volume of media from each culture (will contain cells), centrifuge and suspend the cells in the fresh media containing SCF and Flt3Lthat is equivalent to the volume removed, or more if it is necessary to

adjust the concentration to 10^6 cells/ml, and return all cells to the original flask. On day 2, count the cells and make slides for differential count. The cell count should remain unchanged from day 0.

9. On day 4, make IL-5 media by adding IL5 to base media at 10 ng/ml. Make 50–100 ml and use as needed within 1 week.
10. Remove media and nonadherent cells from the flask. Count the cells and prepare slides for differential staining. Centrifuge cells and remove all the SCF + Flt3L media. Replace with media containing IL-5 such that the concentration is again 10^6 cells/ml. Put all of the cells back into the original flask.
11. On day 6, remove ½ volume of media containing some nonadherent cells from each culture. Count cells and prepare slides for differential count. Centrifuge, remove media, and suspend cells in amount of volume removed or more if it is necessary to adjust the concentration to 10^6 cells/ml. Return all cells to original flask.
12. On day 8, pipette the media over the bottom of the flask to dislodge loosely adherent cells. Remove media and all nonadherent cells from the flask. Count cells, make slides for differential count
13. Centrifuge, remove ½ media, and suspend cells in amount of volume removed or more if it is necessary to adjust the concentration to 10^6 cells/ml. Move all cells into a new flask.
14. On day 10, the cells in culture should be primarily eosinophils. Count and determine eosinophil purity every other day as desired (*see* **Note 2**). Eosinophils obtained from bone marrow culture can be assessed by direct staining (*see* Protocol 3.6), by immunohistochemical staining (*see* Protocol 3.7), and/or by flow cytometric methods (*see* Protocol 3.8). These cells can also be used for the examination of RNA expression (*see* Protocol 3.9), degranulation studies (*see* Protocols 3.10 and 3.11) and for chemotaxis assays (*see* Protocol 3.12).

3.6 Slide Preparation and Cell Counts

1. After generating cells via any of the aforementioned methods, centrifuge 50,000 cells and suspend in 100 μl of 0.1 % BSA/PBS.
2. Pipette the 50,000 cells in 100 μl of 0.1 % BSA/PBS into a cytofunnel assembled with glass slide and labeled in pencil (ink will come off slide in subsequent staining steps). Centrifuge at 500 × *g* for 5 min.
3. Remove the cytofunnel from the glass slide. The cells should be visible as a round smudge.
4. Stain the slides with Diff Quik as follows: 5 min in fixative, then at least 2 min air dry; 3 min in solution 1 (xanthene dye)

followed by three quick washes in dH_2O, then 2 min air dry; 10 s in solution 2 (thiazine) followed by three washes in dH_2O.

5. Air or blot dry and cover slip.
6. Eosinophils are detected based on the pink to red staining of their specific granules and are readily visible under a 64× oil immersion objective. Eosinophils can be determined as a fraction of the total cells per high-powered field (HPF, counting 10–20) and reported as eosinophils/HPF, or as a fraction of a fixed number of total cells (such as 500) and can be reported as percent of total cells.

3.7 Immunostaining of Eosinophils with Rabbit Anti-mouse MBP for Confocal Analysis

1. This protocol uses the polyclonal rabbit anti-mouse MBP.
2. Wash 10^6 cells isolated or differentiated as described above with 3 ml of 0.1 % BSA/PBS and transfer to a 15 ml tube.
3. Fix and permeabilize cells with FIX & PERM as per manufacturer's protocol or fix with 500 μl 4 % paraformaldehyde followed by washing with 3 ml of 0.1 % BSA/PBS and then adding 500 μl ice-cold methanol to permeabilize. After fixing and permeabilizing the cells, move them to a 1.5 ml tube for staining.
4. Dilute rabbit anti-mouse MBP at 1:5,000 in 0.1 % BSA/PBS and stain cells in a total volume of 100 μl for 1 h at 4 °C. *See* **Note 3** for other antibody choices. Appropriate controls include an irrelevant primary rabbit primary antibody and a sample incubated with only the conjugated secondary antibody.
5. Wash the cells three times in 1 ml of 0.1 % BSA/PBS.
6. Dilute goat anti-rabbit IgG-Alexa Fluor 647 at 1:100 dilution 0.1 % BSA/PBS and stain cells in a total volume of 100 μl for 1 h in the dark at room temperature (*see* **Note 4**).
7. Wash the cells three times in 1 ml of 0.1 % BSA/PBS and add the nuclear stain, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 1 μg/ml and for 15 min in the dark.
8. Wash the cells three times in 1 ml of 0.1 % BSA/PBS and fix onto glass slides using cytofunnels (*see* Protocol 3.6) and coverslip (*see* **Note 5**).
9. Collect images on a confocal microscope using a 63× oil immersion objective. Fields are selected "blinded" in that the microscope is focused on the DAPI-stained nuclei and that field was imaged regardless of the presence or absence of MBP-Alexa Fluor 647 positive cells as Alexa Fluor 647 is far red and not visible to the eye. Settings are selected based on the negative controls and then are not changed when imaging the sample stained with anti-MBP and the secondary antibody.

3.8 Flow Cytometric Analysis Utilizing the Eosinophil Surface Marker Siglec F

1. Antibodies directed against the cell surface Ig-type lectin, mouse Siglec F, are generally specific and selective for mouse eosinophils, with some exceptions, as noted below.
2. Suspend cells in HBSS and put 10^6/1 ml in flow tubes, with one tube for each sample and each single color control. For one sample and four colors, there will be a total of nine tubes—unstained, Live-Dead, and a tube for each antibody, a tube for each isotype and the sample tube stained with all colors.
3. Incubate with Live-Dead reagent according to manufacturer's suggestions (*see* **Note 6**).
4. Wash cells with 3 ml of 0.1 % BSA/PBS to remove unincorporated stain.
5. Suspend cells in 0.1 % BSA/PBS at 10^6/100 μl.
6. Incubate cells with anti-Siglec F, anti-CD11c, and anti-CD45 in the presence of 0.5 μg of anti-CD16/CD32 blocking antibody in a total volume of 100 μl for 30–60 min at 4 °C in the dark (*see* **Note 7**).
7. Wash with 3 ml of 0.1 % BSA/PBS and flow samples immediately (go to **step 9**) or fix with 500 μl of 4 % formaldehyde in PBS.
8. When ready to perform flow cytometry, remove formaldehyde by washing with 3 ml of 0.1 % BSA/PBS and resuspend samples in 100 μl 0.1 % BSA/PBS.
9. Collect at least 100,000 events on the flow cytometer. Compensation may be performed on the flow cytometer using single color tubes or compensation beads. Compensation can also be performed post-collection as long as the single color controls were collected.
10. All analyses are performed on the living cells since dead cells can be autofluorescent (*see* **Note 8**) and can bind antibody nonspecifically. The data can be reported as percentage of live cells. After gating on live cells, CD45 positive cells are selected; eosinophils will be Siglec F^+ and $CD11c^-$. This method correlates well with direct counting of cells fixed to slides and stained with Diff Quik [9].

3.9 RNA Isolation and Analysis of Eosinophil Transcript Expression by QPCR

1. Quantitative RT-PCR has largely supplanted Northern blots for analysis of RNA. This is a standard protocol in use in our lab; information on several eosinophil-related primer probe sets is also included.
2. Collect tissue or cells of interest and immediately place in a tube containing cold RNAlater and store at 4 °C overnight. The amount of RNAlater used can vary; follow the manufacturer's instructions. For long-term storage move from 4 °C to −80 °C. If making RNA from purified eosinophils, proceed

directly to **step 3** without any homogenization. *See* **Note 9** for specific issues regarding isolation of RNA from eosinophil-enriched sources.

3. Thaw tissue on ice and rinse in DEPC-treated water to remove salts from the RNAlater.
4. Homogenize 30 mg tissue and isolate RNA following the Qiagen RNeasy minikit instructions. Briefly, homogenize tissue in lysis buffer containing β-mercaptoethanol, centrifuge, pass supernatant over column, wash and perform on-column DNase treatment, wash and elute RNA in RNase-free water.
5. Reverse transcribe 2 μg of RNA using a First Strand cDNA Synthesis Kit
6. One or two microliters cDNA can be used per 25 μl Taqman PCR reaction using Fam-labeled probe and primers to each gene of interest. Eosinophil genes of interest might include primer-probe sets for EPO, MBP, IL-5Rα/CD125, and CCR3.
7. All experiments include the following controls: a no reverse transcriptase, no template controls, and mouse GAPDH-VIC is used as the endogenous control (*see* **Note 10**).
8. Expression of eosinophil gene of interest (GOI) is normalized to GAPDH to account for variations in initial template concentration ($\Delta Ct = Ct_{GOI} - Ct_{GAPDH}$) and then expressed relative to data collected from control (condition or time point, $\Delta\Delta Ct = \Delta Ct_{expreimental} - \Delta Ct_{control}$). Generally data is expressed as relative fold (RQ) expression ($RQ = 2^{-\Delta\Delta Ct}$) where the data is reported relative to control time point or condition (*see* **Note 11**).

3.10 Detection of Eosinophil Peroxidase Release from Mouse Eosinophils

1. This colorimetric assay was adapted from the report of Adamko et al. [4] and measures the peroxidase activity of eosinophil peroxidase (EPO) utilizing a substrate that is not cell membrane permeable therefore only degranulated/released EPO is measured, not EPO that remains within granules, inside the cell.
2. Prepare concentrated secretogoue at 100-fold concentrations such that 1 μl used in a 100 μl assay will achieve desired final concentration. For example, two effective secretagogues for mouse eosinophils, C16 platelet-activating factor (PAF), and C16 lysoPAF can be prepared in DMSO at 1 mM and stored at −20 °C. Prior to using, thaw and dilute further in DMSO if desired.
3. Suspend cells in RPMI-1640 without phenol red at 250,000 cells/ml. Put 100 μl in each well of a 96-well plate. In addition to the wells containing the secretagogue, each plate contains a set of cells that remain untreated and a set of wells in which the cells lysed in 0.2 % sodium dodecyl sulfate in order to determine the total EPO content. Perform assay in duplicate or triplicate.

4. Add 1 μl of secretagogue or vehicle to each well as appropriate and 0.2 % SDS to a set of wells to determine total EPO content and incubate at 37 °C, 5 % CO_2 for 30 min.
5. Add 100 μl OPD reagent to each well. Monitor color change in the SDS lysed wells very closely and terminate when these wells achieve a brownish color. Terminate the reaction by adding 100 μl 4 M H_2SO_4 to each well and read at 492 nm (*see* **Note 12**).
6. Data are reported as percent of total EPO [(absorbance of stimulated sample − no treatment) × 100/total EPO from SDS-lysed cells]. All data are presented as mean ± SEM.

3.11 Detection of Cytokines Released from Eosinophils

1. Eosinophils store a number of cytokines in the granules. We have successfully used recombinant mouse IL-6 as a secretagogue to elicit the release of these cytokines from eosinophils [14]. The relatively short incubation period (1 h) and the inclusion of cycloheximide will assure that the cytokine measured is from intracellular stores and not from *de novo* synthesis.
2. Suspend cells in RPMI-1640 without phenol red at 10^6 cells/ml. Put 100 μl in each well of a 96-well plate. Perform assay in duplicate or triplicate.
3. Add 1 μl recombinant IL-6 to achieve a final concentration of 20 ng/ml. This concentration stimulates the release of IL-1β, IL-9, IL-12(p70), IFNγ, TNFα, and MCP-1/CCL2 from bone marrow-derived eosinophils [14]. Cycloheximide can be used at a final concentration of 5 μg/ml and added prior to the addition of IL-6 to inhibit *de novo* protein synthesis and to insure that you are looking at the release of stored products rather than newly synthesized and secreted productions.
4. After addition of the stimulant, incubate cells at 37 °C, 5 % CO_2 for 60 min.
5. After incubation, centrifuge plate and store the cell free supernatant in the freezer at −80 °C until assayed for cytokine content.
6. Thaw samples on ice and proceed to multibead cytokine assays following the manufacturer's (Millipore, BioRad) directions to prepare standards and run assay (*see* **Note 13**).
7. Collect data on a plate reader such as the BioPlex (BioRad) plate reader per manufacturer's suggestions.

3.12 Detection of Ribonuclease Activity

1. This method measures the ribonucleolytic activity of the eosinophil associated ribonucleases [5]. Mouse eosinophils contain several closely related eosinophil-associated ribonucleases (EARs). This assay will not determine which of these ribonucleases is released, but the combined activity can be assessed.

2. Prepare enough ribonuclease assay STOP solution (1:1 v/v mixture of 40 mM lanthanum nitrate and 6 % perchloric acid) for assay. STOP solutions should be prepared fresh and kept on ice. Perform experiment in triplicate; include a negative control (no sample or RNase) and a positive control (bovine RNase) and blank.
3. Put 300 μl $NaPO_4$ and 500 μl DEPC-dH_2O in all tubes.
4. Put up to 50 μl of solution to be tested (BALF or solution containing degranulation products) in each tube except the blank.
5. Add 500 μl STOP solution to the blank tube only.
6. Defrost tRNA (20 mg/ml yeast tRNA) solution on ice and add 10 μl of tRNA to all tubes for appropriate time (3, 5 or 10 min).
7. Once time has elapsed, add 500 μl ice-cold STOP solution to all tubes, mix, and place on ice for 10 min.
8. Precipitate the acid-insoluble undigested tRNA at 16,200 × *g* for 5 min at room temp.
9. Measure the optical density (OD) of the supernatant to assess the production of acid-soluble ribonucleotides at OD_{260}. The OD_{260} is directly proportional to the ribonuclease activity in the product (BALF, cell culture supernatant, etc.) assayed.

3.13 Chemotaxis Assay

1. This is a standard assay that can be used to evaluate the chemotactic responses of isolated mouse spleen eosinophils and/or bone marrow-derived eosinophils. The assay requires the use of a chemokinesis control in which the chemoattractant is placed in both the upper and the lower wells so that the non-directed movement of the eosinophils can also be assessed (*see* **Note 14**).
2. Remove the plastic lip from the Transwell Support System and add 100 μl of chemotaxis assay media into the feeder port of each well of the receiver plate. Incubate the Transwell Support System for at least 1 h at 37 °C in a humidified CO_2 incubator. This initial equilibrium will improve cell attachment.
3. Wash isolated eosinophils in RPMI-1640 and resuspend 10^6 cells/ml in chemotaxis assay media (*see* **Note 15**).
4. Dilutions of the chemoattractants or their solvents are prepared in assay media and kept on ice until use.
5. Transfer the insert plate to a second 96-well plate.
6. Remove the assay media from the receiver plate and add 100 μl of chemoattractant (recombinant eotaxin) or vehicle control to the wells. Perform the experiment in duplicate or triplicate and use a range of concentrations in the nanomolar range.

7. Place the insert plate back into the receiver plate. Take care not to trap air bubbles underneath the inserts. Add 100 μl of the cell suspension to each insert (10^5 cells/well). Remember to add chemoattractant to the chemokinesis control well(s). Put on the plastic lid and incubate the Transwell Support System for at least 2 h at 37 °C in a humidified CO_2 incubator.
8. Carefully remove the insert plate from the receiver plate. Transfer the cells that have migrated through the insert towards the chemoattractant into a 5 ml FACS tube and add 150 μl of PBS to each sample.
9. Enumerate the cells that migrated by counting on a flow cytometer for 30 s at high flow rate or manually with hemacytometer.
10. Migration in response to a chemoattractant is expressed as the chemotactic index, (CI = # cells migrated in response to chemoattractant/# cells migrated in response to vehicle control). Alternatively, data can be reported as percent of vehicle control.

4 Notes

1. Protocol is written for 10^7 cells. If more cells are used, then scale up proportionally. The limit for the CS column is 2×10^8 magnetically labeled beads.
2. BALB/c bone marrow cultures increase in total cell number from D4 to day 10 and continue to increase thereafter and reach approximately 90 % eosinophils or greater by day 10. C57BL/6 bone marrow cultures do not proliferate to the same extent and lag 1–2 days behind in % eosinophils, achieving 90–100 % eosinophils at day 12.
3. Mouse eosinophils express CCR3, IL-5Rα, and Siglec F which can be detected on the cell surface and also store EPO and MBP in the granules.
4. Secondary antibodies conjugated to other fluorochromes may be used for confocal analysis with the exception of PE as it photo-quenches rendering it unusable for this application.
5. The coverslip can be mounted in confocal mounting media to reduce autofluorescence and prolong detection signal. One such mounting media is ProLong (Invitrogen).
6. It is a very good idea to titrate both antibody and Live-Dead reagent as this will reduce cost and increase signal to noise ratio.
7. Siglec F is a fairly specific marker for mouse eosinophils although it is also expressed on alveolar macrophages [17]. Anti-CD11c will differentiate between eosinophils ($CD11c^-$) and alveolar macrophages ($CD11c^+$).

8. Eosinophils are autofluorescent so great care must be taken to include unstained controls and single color controls for all test antibodies. Autofluorescence seems to be the greatest problem in the FITC/Alexa Fluor 488 channel.
9. In our experience, RNA isolated from eosinophil-enriched tissues can yield poor results in the QPCR assays. This method using the RNAeasy mini kit yields RNA that is efficiently transcribed and provides good results in QPCR assay (i.e. GAPDH Ct in the low 20s). RNA isolated with other methods can be re-isolated with the Qiagen kit. If Ct values for GAPDH are not in the low 20s, the RNA should be re-isolated, re-transcribed, and the QPCR assay repeated.
10. The expression of the endogenous control transcript needs to be assessed in each experiment to make certain that its expression is not fluctuating with the experimental conditions. Endogenous controls can be run separately or multiplexed, but this should be determined empirically for each primer-probe set as multiplexing can lead to interference.
11. It is tempting to say expression of one gene is higher than another but this is not correct, as one cannot know *a priori* the relative efficiencies of a different primer-probe pairs. One can be more precise by making standard curve of GOI and the endogenous control and reporting actual copy numbers per GAPDH.
12. It may be necessary to determine the development time empirically so that the SDS lysis control will not be off scale. A final concentration of 5 μM of either C16-PAF or C16-lysoPAF stimulates the release of 40–50 % of total EPO [14].
13. The multibead assays work well with cell culture supernatants as well as BALF. They also work well with serum samples provided the standards are rehydrated according to manufacturer's directions. We have not had great success with multibead assays using plasma samples. Choose a cytokine assay method based on the amount of sample available and expense. Multibead cytokine assays represent a fast and convenient way to determine concentrations of a significant number of cytokines using a relatively small (50 μl) sample. ELISAs take longer to run and require more sample volume. In general, if more than three cytokines are to be assayed, it is probably cheaper to use a multibead kit than individual ELISAs.
14. To explore the possible contributions of the chemokinesis (in addition to chemotactic) responses of eosinophils, it may be desirable to perform checkerboard analysis. In a checkerboard analysis, the concentration of chemotactic agent is increased stepwise from zero (media alone) to highest concentration in both the top and bottom wells. If the response is due

solely or primarily to chemotaxis (not simply chemokinesis), then maximum cell migration will be observed in response to the sharpest gradient (i.e. when the cells are in media alone and the opposite chamber contains chemoattractant). In contrast, chemokinesis would be observed when the cells were immersed directly in increasing concentrations of mediator.

15. In some experiments it might be useful to cytokine-starve the cells by placing them in bmEos base media for 18 h prior to the assay. When assaying single cell suspensions from eosinophil-enriched tissues, gate on the migrated granulocyte population rather than on total cells. Migrated eosinophils can be detected with anti-Siglec F for a more specific analysis.

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