Chapter 9

Fate-Mapping of Hematopoietic Stem Cell-Derived Macrophages

Katharina Mauel and Elvira Mass

Abstract

Macrophages are cells of the innate immune system, which contribute to the maintenance of tissue homeostasis and form the first line of defense against pathogens. Tissue-resident macrophages that originate from erythro-myeloid-progenitors in the yolk sac colonize the organs early during development and self-maintain in most organs throughout adulthood. Under homeostatic and pathological conditions, circulating monocytes infiltrate the tissue, where they differentiate into macrophages. However, particularly upon inflammation, phenotyping of these distinct macrophage populations using surface markers or antibody stainings is insufficient as their phenotypes converge, at least transiently. A well-established method for the developmental origin of different cell types is the use of in vivo fate-mapping models, where a fluorescent reporter will be expressed under the control of a cell type-specific promoter. Here, we describe the *Cxcr4*^{CreERT2}*; Rosa26*$^{LSL\text{-}tdTomato}$ mouse fate-mapping model, which labels hematopoietic stem cells and, thus, also monocytes and monocyte-derived macrophages while most tissue-resident macrophages are not targeted.

Key words Macrophages, Development, Ontogeny, Fate-mapping, Bone marrow, Hematopoietic stem cells, Monocytes, Spleen, Cxcr4

1 Introduction

Macrophages are innate immune cells that monitor pathogens, engulf and digest microbes, and cell debris; thus, they are vital for tissue homeostasis [1]. Most immune cells are derived from hematopoietic stem cells (HSC), as is the case for monocyte-derived macrophages. A decade ago, the prevalent understanding of macrophage biology was revised through the discovery of tissue-resident macrophages (TRM) that arise from erythro-myeloid-progenitors (EMP) in the yolk sac, before the onset of definitive hematopoiesis [2]. These HSC-independent macrophages colonize the organs and self-maintain in the adult tissue through local proliferation mainly independent of circulating HSC-derived cells [3, 4].

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To better understand the function of TRM in homeostasis and immunity in adult individuals, it is crucial to consider the ontogeny of these unique cells. With the help of fate-mapping experiments and parabiosis studies [5], it was described that some TRMs exclusively originate from yolk sac-derived precursors; that includes especially microglia in the brain and epidermal Langerhans cells in the skin [6]. Other organs are engrafted by a small portion of HSC-derived circulating monocytes in an age-related manner; that includes, e.g., red pulp macrophages in the spleen [7, 8] and peritoneal macrophages [9]. During adulthood, the primary function of TRMs is to surveil the microenvironment and induce inflammation in case of homeostatic disbalances [10], as well as to later induce tissue repair and healing. Under pathological conditions, the niche of TRMs in the respective organ can be severely altered and TRM numbers diminished, giving space for HSC-derived monocytes to infiltrate the empty niche. Due to their different developmental origin, these cells are—at least during the first few days—phenotypically and functionally different and are often characterized by a more inflammatory phenotype [11]. However, the phenotypes of TRMs and monocyte-derived macrophages may converge with time, especially when the latter become long-lived as well. Therefore, it is important to address the ontogeny of tissue macrophages in health and disease, which play a crucial role in the maintenance and re-establishment of tissue homeostasis.

A standard technique to study the developmental origin of different macrophage populations is the use of fate-mapping models. Upon the modification of two genetic loci, a fluorescent reporter will be expressed by the cell type of interest. First, a genetic locus needs to be identified that is specifically active in either HSC- or EMP-derived macrophages. Here we focus on the *Cxcr4* gene, which is expressed by long-term HSC [12]. On one allele, the recombinase enzyme Cre is inserted under the control of the *Cxcr4* promoter, which induces the specificity of the model toward the HSC-lineage. Into the ubiquitously expressed *Rosa26* genetic locus a fluorescent reporter gene is inserted [13]. Common reporters are the green fluorescent protein (GFP) or red fluorescent proteins (tdTomato); the latter is applied in this chapter. The genetic locus is further modified by the insertion of a Stop-codon in front of the fluorescent reporter. This Stop-codon is flanked by a *loxP*-sequence, which is the genetic sequence that is specifically recognized by the Cre-recombinase enzyme [14]. Taken together, in our example the Cre-recombinase is specifically expressed under to control of the *Cxcr4* promoter, which is active in HSC. Therefore, only in cell originating from that lineage, the Stop-codon is excised and the sequence encoding the fluorescent reporter tdTomato is accessible for the polymerase and can be synthesized. Here, we will describe the $Cxcr4^{CreERT2}$; $Rosa26^{LSL\text{-}tdTomato}$ mouse model and the spleen as an exemplary tissue to show how to label HSCs efficiently, providing an inducible fate-mapping model to permanently label all HSC-derived macrophages.

2 Materials

2.1 Mouse Model

Here we use heterozygous *Cxcr4*^{CreERT} [12] and homozygous *Rosa26*$^{LSL\text{-}tdTomato}$ (Ai14; JAX Strain #: 007914) mice to generate *Cxcr4*^{CreERT2}; *Rosa26*$^{LSL\text{-}tdTomato}$ mice where Cre activation is induced at 4 weeks of age with tamoxifen. Tamoxifen injections can occur also at a later time point; make sure to include a four-week wash-out period before the start of your experiment. Please refer to Chapters 2 and 8 in this book for further fate-mapping models of distinct hematopoietic waves.

2.2 Consumables

1. Scissors (standard, blunt-end).
2. Forceps (standard).
3. 15 mL tubes.
4. 50 mL tubes.
5. 1.5 mL and 2 mL microcentrifuge tubes.
6. 96-well plate, U-shaped.
7. 3.5 cm dish (alternatively 6-well plate).
8. FACS tubes.
9. Cell strainer (70 and 100 μm).
10. 1 mL syringe with 26G needle.
11. 10 mL syringe with 26G needle.
12. Metal plunger.
13. Heparin-coated tube.
14. Blood lancet.
15. Aluminum foil.

2.3 Buffers and Solutions

1. Tamoxifen stock solution: 10 mg/mL tamoxifen (Roth, T5648) in 10% (v/v) molecular grade Ethanol and 90% (v/v) corn oil (Sigma, C8267). Prepare by weighing 100 mg of tamoxifen under sterile conditions (*see* **Note 1**) in a 15 mL centrifuge tube and add 1 mL of pure Ethanol (100%). Vortex and put the suspension in a 42 °C water bath. Vortex regularly, until the tamoxifen is properly dissolved. Then add 9 mL of corn oil under sterile conditions and mix to obtain a homogeneous solution. Store at 4 °C not more than 4–6 weeks (protected from light). Each mouse will be injected with 100 μL for 5 days (*see* **Note 2**).
2. Antibody solution to validate reporter expression in monocytes: Visualization of tdTomato expression in HSC-derived monocytes via CD11b-APC (clone: M1/70, 1 μg/mL) and Ly6C-PE/Cy7 (clone: HK1.4, 1 μg/mL) staining.

3. Anesthesia: 100 mg/mL ketamin, 20 mg/mL xylazine in sterile 0.9% NaCl (*see* **Note 3**)
4. 1x Phosphate-buffered saline (PBS)
5. FACS buffer: 0.5% bovine serum albumin (BSA) and 2 mM Ethylenediaminetetraacetic acid (EDTA) in 1x PBS. Filter buffer through a 0.2 μm filter, e.g. using a bottle top vacuum filter. Store at 4 °C.
6. Digestion mix: 0.2 mg/mL DNase, 0.5 mg/mL Collagenase D, 2.4 mg/mL Dispase and 3% fetal calve serum (FCS, *see* **Note 4**) in 1x PBS. Calculate the desired volume of digestion mix (500 μL/sample; *see* **Note 5**). Keep on ice until used for tissue digestion.
7. Red-blood-cell (RBC) lysis buffer: 155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA in dH20. pH 7.1–7.4.
8. Blocking solution: 1% (v/v) anti-mouse CD16/32 (e.g. clone 93 from Biolegend), 2% (v/v) of rat serum in FACS buffer. Calculate the desired volume of blocking solution (50 μL/sample; *see* **Note 6**).
9. Antibody mix: Calculate antibody mixes based on the number of your samples (*see* **Note 7**). Fill a 1.5 mL tube with the calculated amount of FACS buffer. Add antibodies (*see* Table 1 for staining of the spleen) into a microcentrifuge tube and mix by pipetting.
10. Live/dead staining: 5 μg/mL Hoechst33258 in FACS buffer.

2.4 Equipment

1. Water bath.
2. Flow cytometer.
3. Procedure platform with needles (to fix mouse).
4. Centrifuge for FACS tubes and plates.
5. Thermomixer.

Table 1
Antibody panel for spleen tissue

First antibody mix	Antigen	Conjugate	Concentration [μg/mL]	Clone
	Ly6G	biotin	5	1A8
	TCRbeta	biotin	5	H57–597
	CD19	biotin	5	6D5
	Nkp46	biotin	5	29A1.4
Second antibody mix	Streptavidin	BV785	1	
	F4/80	APC/Cy7	0.5	BM8
	CD45	BUV805	0.25	30-F11
	CD11b	BUV661	1	M1/70
	CD64	PerCP/Cy5.5	4	X54–5/7.1

3 Methods

3.1 Tamoxifen Administration and Labeling Efficiency Control

1. Let the tamoxifen solution adhere to room temperature. Take up required volume with a 1 mL syringe that has the needle attached (*see* **Note 8**).
2. Inject each mouse intraperitoneally (i.p.) with 100 μL of tamoxifen for five consecutive days. To prevent infections, inject into alternating positions every day (left, middle, and right side of the abdomen) (*see* **Note 9**).
3. Control successful induction of the reporter expression by collecting a small blood sample from the mouse in a heparin-coated tube (for example via tail vein puncture—depending on your animal permit).
4. Add collected blood (10–50 μL) to a microcentrifuge tube, which contains 500 μL of RBC-lysis buffer.
5. Mix and incubate for 5 min on ice.
6. Add 500 μL FACS buffer.
7. Centrifuge at 400 g for 5 min at 4 °C.
8. Discard supernatant and dissolve in 50 μL blocking solution.
9. Incubate 10 min on ice and spin samples at 400 g, 5 min, 4 °C
10. Discard supernatant and resuspend pellet in 25 μL blood staining solution (*see* Subheading 2.3. Antibody solution to validate reporter expression in monocytes).
11. Incubate 30 min on ice, add 200 μL FACS-buffer and spin samples at 400 g, 5 min, 4 °C.
12. Discard supernatant and resuspend pellet in 100 μL FACS-buffer.
13. Transfer cells through a 70 μm strainer into a FACS tube and add an equal volume of 5 μg/mL Hoechst.
14. Measure tdTomato signal in monocytes ($CD11b^+Ly6C^+$) with a flow cytometer.
15. In case of successful expression of the fluorescent reporter, the mice can now be treated (depending on your experimental setup, for example with an infectious agent) or analyzed at the time-point of interest after labeling-induction with tamoxifen.

3.2 Preparation of Cell Suspension from Adult Mouse Spleen for Flow Cytometry Analysis

Depending on the scientific question, different organs might be of interest for flow cytometry analysis. Here, we show an example of how to prepare a cell suspension from adult mouse spleens suitable for flow cytometry analysis. Other organs may require a slight adaptation of the protocol to produce a single-cell suspension.

3.2.1 Isolation of Mouse Spleen for Flow-Cytometry Analysis

1. On the day of the experiment, inject the mouse *i.p.* with anesthesia. When the mouse is deeply asleep (*see* **Note 10**) fix it on a procedure platform with needles, with its back toward the platform.
2. Open the peritoneal cavity and the diaphragm. Grab the sternum with forceps and open the rib cage using scissors to expose the heart (try not to cut any large blood vessels).
3. From the heart, blood can be collected with a 1 mL syringe and 26 G needle that was flushed with EDTA solution and collected in a heparin tube. It is possible to collect 500 μL or more from an adult mouse.
4. To perfuse the mouse, gently fix the heart with forceps. Then make a small cut on top of the right ventricle (blood will come out). Have a 10 mL syringe prepared with PBS and a 26 G needle and immediately insert the needle into the left ventricle. Gently press the plunger to perfuse the mouse (*see* **Note 11**). An indicator of a successful perfusion is the liver, which will become light flesh colored when the blood is flushed out.
5. Collect the spleen by holding the pancreas with forceps and carefully cut off the spleen from the white pancreatic tissue. Place the spleen into a 3.5 cm dish with PBS on ice.
6. Dissect a piece of the tissue (30–40 mg), which is used to prepare a single-cell suspension for flow cytometry.

3.2.2 Cell Suspension Preparation

1. Transfer 30–40 mg of the spleen tissue into a 2 mL microcentrifuge tube and add 500 μL of digestion mix.
2. Cut the spleen within the tube with blunt scissors to obtain small pieces.
3. Incubate the spleen in digestion mix at 37 °C for 30 min while shaking at 1000 rpm.
4. Place the tubes on ice and have 50 mL centrifuge tubes prepared with 100 μm strainers. Wet the strainer with approximately 3 mL FACS buffer, which is collected in the 50 mL centrifuge tubes underneath.
5. Gently pipette the digested spleen suspension up and down and add it on top of the 100 μm strainer. Mesh tissue pieces with a metal plunger. Flush with additional 2–3 mL of FACS buffer and collect all flow-through in the centrifuge tube underneath.
6. Spin the sample tubes for 7 minutes, 400 g, 4 °C.
7. For red blood cell lysis, resuspend the pellet in 1 mL of RBC-lysis buffer and incubate for 5 min on ice. Then add FACS buffer (approximately 7 mL), invert the tube five times and spin (7 min, 400 g, 4 °C).
8. Remove the supernatant

3.3 Staining of Cell Suspension with Antibodies Conjugated with Fluorochromes

1. Add 50 μL of blocking solution into each tube and resuspend the pellet by pipetting.
2. Incubate for 10 min on ice.
3. Transfer the cell suspension into the 96-well plate and top up with 100 μL FACS buffer.
4. Spin down the 96-well plate for 5 min, 400 g, 4 °C.
5. Remove the supernatant by flipping the plate over the sink and tapping it on a tissue.
6. Add 50 μL of the first antibody mix and resuspend each pellet by pipetting.
7. Incubate on ice for 30 min.
8. Add 200 μL FACS buffer.
9. Repeat **steps 4–5**.
10. Add 50 μL of the second antibody mix and resuspend the pellet by pipetting.
11. Incubate on ice for 30 min in dark (cover the plate with aluminum foil).
12. Add 200 μL FACS buffer.
13. Repeat **steps 4–5**.
14. Resuspend the cell pellet in 100 μL of FACS buffer.

3.4 Flow Cytometry

1. Filter the samples into FACS tubes through a 70 μm strainer.
2. Add an equal volume (100 μL) of 5 μg/mL Hoechst to the sample.
3. Vortex and measure the sample (*see* **Note 12**).

3.5 Data Analysis

1. Export FCS files from your flow cytometer and import them in FlowJo for data analysis.
2. After compensation, discriminate different splenocytes and examine the cell-fate specific labeling with tdTomato using the gating strategy in Fig. 1.

4 Notes

1. Weigh a closed centrifuge tube on the scale, and note the weight. Under the sterile workbench, fill it with tamoxifen powder, close the centrifuge tube, and weigh it again to obtain the exact weight of the tamoxifen powder.
2. If you have larger or smaller cohorts of mice, adjust the amount of tamoxifen solution you need.
3. Always check your animal permit for the concentrations and volumes that are allowed to be injected.

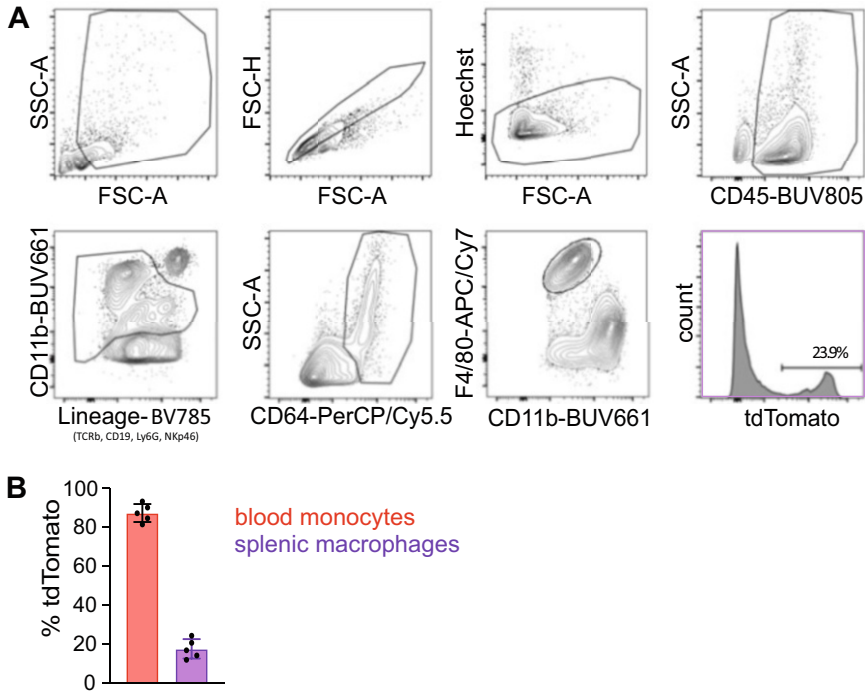

Fig. 1 Fate-mapping of monocytes and monocyte-derived macrophages using the inducible *Cxcr4*^{CreERT2}; *Rosa26*$^{LSL\text{-}tdTomato}$ model. (**a**) Gating strategy for red pulp macrophages (**c**) Quantification of cell-specific labeling with tdTomato

4. Frozen FCS has to be thawed and heat-inactivated for 30 min at 56 °C. Then aliquots can be stored at −20 °C.
5. To obtain 1 mL of digestion mix, 2.4 mg Dispase is measured with a fine scale, transferred into a 15 mL centrifuge tube and dissolved in 966 µL of PBS. 2 µL of a 100 mg/mL DNase stock solution is added as well as 2 µL of a 0.5 g/mL Collagenase D stock solution. Upon addition of 30 µL FCS, the solution is pre-warmed to 37 °C just before usage. Digestion mix has to be prepared fresh for every experimental day.
6. To obtain a 50 µL blocking solution, 2 µL rat-serum and 0.5 µL Fc-block are dissolved in 47.5 µL FACS-buffer.
7. It is essential to keep in mind, that the indicated dilutions are just an approximation, based on the used lot number. It is important to titrate your antibodies to find the ideal dilution factor for your specific batch.
8. Taking up tamoxifen through the needle ensures that no undissolved particles are taken up and injected.
9. Four weeks after tamoxifen administration, all systemic residues should be eliminated in the mouse. This is important since tamoxifen can have an impact on cellular function.

10. Check for reflexes by pinching the mouse with your fingers or forceps between the toes.
11. Blood will come out of the cut in the upper left ventricle. If the needle is not injected correctly, the lung will inflate and liquid will come out of the mouse nose.
12. In case the machine measures more than 10,000 events/second, dilute the sample with FACS buffer.

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