Novel FACS strategy for identification of early hematopoietic progenitors including BFU-e, CFU-e and erythroid-biased MPPs

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Method Article

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Abstract

We used single-cell RNA-seq and fate potential assays to develop a novel FACS strategy that identifies erythroid-biased multipotent progenitors, and erythroid committed progenitors, directly in freshly isolated mouse bone marrow. It also identifies subsets enriched for progenitors of the basophil/ mast cell lineage or the megakaryocytic lineage. It divides Kit+CD55+ hematopoietic progenitors in mouse bone marrow into five populations, as follows: P1, highly purified CFU-e progenitors, also called Committed Erythroid Progenitors (CEPs); P2, highly enriched BFU-e progenitors, also called Early Erythroid Progenitors (EEPs); P2 also contains some CFU-e, and uncommitted progenitors with erythroid/megakaryocytic/basophil potential (EBMegPs); P3, enriched for basophil/mast cell progenitors; P4, enriched for megakaryocytic progenitors; and P5, containing EBMegPs and erythroid/megakaryocytic/ basophil-biased MPPs.

Introduction

Although CFU-e and BFU-e progenitors were defined functionally over four decades ago, their complete and pure prospective isolation from murine bone-marrow has not been possible. Our recent single-cell RNA-seq study developed a FACS strategy that achieves their isolation, as well as the isolation of a number of other early hematopoietic progenitors. In this protocol, we first enrich Kit+ cells from total mouse bone marrow, using magnetic beads. This population is then labeled with antibodies against CD55, CD150, CD105, CD41, CD49f and CD71. Kit+CD55- cells are lympho/ myeloid progenitors, whereas Kit+CD55+ cells contain erythroid/ megakaryocytic/ basophil progenitors. We subdivide Kit+CD55+ cells into 5 subsets, P1 to P5. P5 contains uncommitted progenitors biased towards the erythroid/ megakaryocytic/ basophil lineages, including MPPs. P4, P3, and P1/P2 contain megakaryocytic, basophil/ mast cell, and erythroid progenitors, respectively. Specifically, P2 contains largely BFU-e cells, whereas P1 contains CFU-e, and no other progenitors. Of note, P1 can be further sub-divided into earlier CFU-e, forming larger colonies, and later CFU-e, forming smaller colonies, by selecting for P1-CD71^{medium} and P1-CD71^{high} gates, respectively.

Reagents

Reagents Staining buffer (1X PBS; 0.2% BSA; 0.08% Glucose) Easy Sep buffer (1X PBS; 2% FBS; 1mM EDTA) Mouse Streptavidin RapidSpheres Isolation Kit (STEMCELL technologies, Cat No. 19860A) Rat Serum (STEMCELL technologies, Cat No. 13551, or equivalent) 0.5 M EDTA, pH 8.0 (Invitrogen by Life technologies, Cat No. 15575-038, or equivalent) Biotin anti-TER119 (BD Biosciences, Clone TER119, Cat No. 553672) Biotin anti-CD11b (BD Biosciences, clone M1/70, Cat No. 557395) Biotin anti-Ly-6G and Ly-6C (BD Biosciences, clone RB6-8C5, Cat No. 553125) Biotin anti-CD4 (BD Biosciences, clone RM4-5, Cat No. 553045) Biotin anti-CD8a (Ly-2) (BD Biosciences, clone 53-6.7, Cat No. 553029) Biotin anti-CD19 (BD Biosciences, clone 1D3, Cat No. 553784) Streptavidin, Alexa Fluor™ 488 conjugate (Invitrogen, Cat No. S11223) CD117- APC Cy7 (Biolegend, clone 2B8, Cat No. 105826) TER119-BUV395 (BD Biosciences, clone TER-119, Cat No. 563827) CD71- PE Cy7 (Biolegend, clone RI7217, Cat No. 113812)

CD55-AF647 \ (Biolegend, clone RIKO-3, Cat No. 131806) CD105-PE \ (Biolegend, clone MJ7/18, Cat No. 120408) CD150-BV650 \ (Biolgened, clone TC15-12F12.2, Cat No. 115931) CD41-BV605 \ (Biolegend, clone MWReg30, Cat No. 133921) CD49f – BV421 \ (Biolegend, clone GoH3, Cat No. 313624) DAPI \ (4',6-Diamidino-2-Phenylindole, Dihydrochloride) \ (invitrogen, Cat No. D1306)

Equipment

Dissection equipment, 70% ethanol in water spray, Mortar and Pestle, 2ml syringes, 26-gauge needles, 70 µm cell strainer, microcentrifuge, “The Big Easy” magnet \ (Cat No. 18001) from STEMCELL technologies \ (or equivalent)

Procedure

****1. Preparation of cell suspensions from mouse bone marrow**:** • Obtain femora and tibiae from 10 mice and remove the muscles • Cut the tip of the bones and flush the shaft with 2 to 3 ml of staining buffer or medium using a 2 ml syringe with a 26-gauge needle. • Crush the bones after flushing, using mortar & pestle to obtain any remaining cells • Pass cells through 70 µm cell strainer to remove cell clumps. Moisten filter with buffer or medium before use. • Pellet cells by centrifugation at 1400 rpm for 5 min. • Resuspend the cells in staining buffer and count. \ (approximately 2×10^9 cells are obtained from 10 wild type mice.) ****2. EasySep mouse Streptavidin RapidSpheres negative selection for Lin⁺ cells:**** • Mouse Streptavidin RapidSpheres Isolation Kit \ (Cat No. 19860A) and “The Big Easy” magnet \ (Cat No. 18001) from STEMCELL technologies are used for this selection. • For Negative selection for Easy Sep sort, cells are resuspended at 2×10^8 cells/ ml in Easy Sep buffer. • For tibia/femur of 10 mice, cells are resuspended in total volume of 10ml EasySep buffer, containing Rat Serum \ (1% final concentration) to block the Fc receptors, and a cocktail of lineage-specific antibodies, each at a final concentration of 0.005 mg/ml: of biotin lanti-TER119, anti-CD11b \ (Clone M1/70), anti-Ly-6G and Ly-6C \ (Clone RB6-8C5), anti-CD4 \ (Clone RM4-5), anti-CD8a \ (Ly-2) \ (Clone 53-6.7), anti-CD19 \ (Clone 1D3). Mix and incubate for 30 min on ice. • Vortex RapidSpheres and add to sample at 100µl/ml \ (1ml /10ml). Mix well and incubate on ice for 15 min. • Divide 10 ml total volume of sample into two 5 ml samples \ (Use 15 ml conical Falcon tubes). • Bring each sample to a 10 ml total volume with Easysep buffer, and place the tube in The Big Easy magnet for 10 minutes. Pour the supernatant into a new empty 15 ml Falcon tube. Repeat the magnet wash for higher purity. • Pellet lineage- depleted cells by centrifugation at 1400 rpm for 5 min. ****3. Staining for populations P1 to P5:**** • Wash the lineage-depleted cells with cold staining buffer and pellet by centrifugation at 1400 rpm for 5 min. • Stain for 1 hour on ice in 1ml of staining buffer containing 1% Rat Serum and the following antibodies: • 0.01 mg/mL Streptavidin Alexa Fluor 488 • 0.001 mg/mL CD117- APC Cy7 • 0.002 mg/mL TER119-BUV395 • 0.0001 mg/mL CD71- PE Cy7 • 0.01 mg/mL CD55-AF647 • 0.004 mg/mL CD105-PE • 0.004 mg/mL CD150-BV650 • 0.002 mg/mL CD41-BV605 0.0005 mg/mL CD49f – BV421 • Wash cells with cold staining buffer and resuspend in 1ml staining buffer containing DAPI and 5 mM EDTA. • Use cells for FACS analysis or for Cell sorting

Troubleshooting

- Any sorting needs to be performed at low pressure/wide nozzle conditions. E.g. 100 μ M/14 psi or 130 μ M/12 psi on a BD FACSAria II, following the gating strategy illustrated in Figure 1
- We include single-color controls and 'fluorescence minus one' controls in order adjust voltages, set compensation for spectral overlap, and place gates correctly

Figures

P1 = CFU-e P1-CD71^{med}: large CFU-e
P1-CD71^{high}: small CFU-e

P2 = BFU-e

P3= basophil/ mast cell progenitor enriched

P4= megakaryocytic progenitor enriched

P5= Uncommitted; EBMegPs;

Figure 1

FACS strategy for early erythroid/basophil/megakaryocytic hematopoietic progenitors Gating strategy illustrating derivation of populations P1 to P5. Starting with total murine bone marrow, a gate that excludes aggregates ('singlets') is first selected, followed by a viable cell gate, a Kit+Lin- gate, and a CD55+ gate. The resulting Kit+Lin-Cd55+ cells can be resolved using CD49f and CD105, into a population

CD49f^{high} CD105^{low} cells, that give rise to P3/ P4/ P5, or to CD49f^{low} CD105^{high} cells, which give rise to P1/ P2. P1 can be further resolved based on CD71 expression as indicated.