**SUPPLEMENTARY MATERIAL**

# *High-resolution phenotypic analysis of the mouse hematopoietic hierarchy using spectral cytometry – from stem cell subsets to early progenitor compartments*

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## Supplementary Figures

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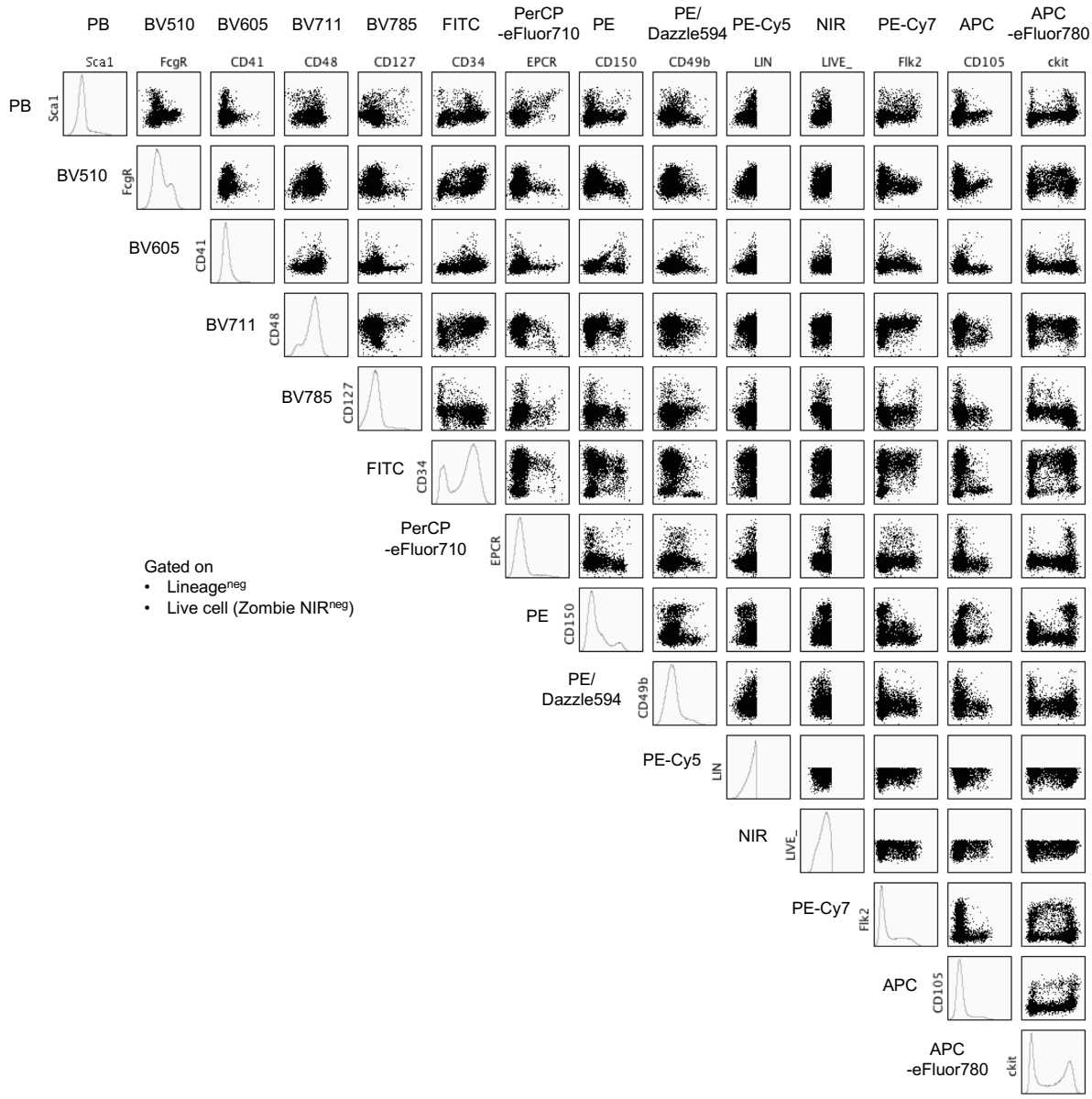
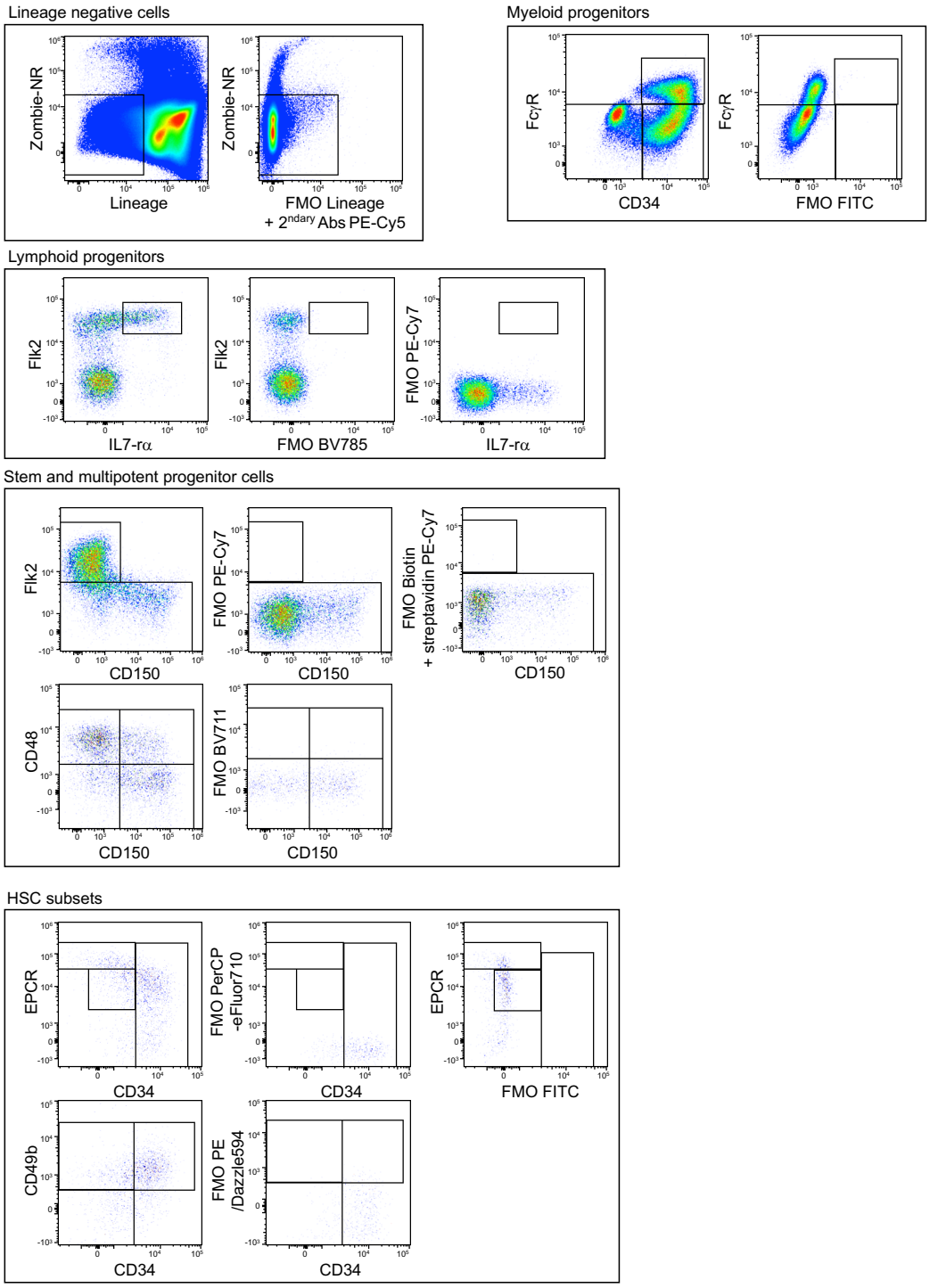
**Supplementary Table 5.** ***Number of hematopoietic stem and progenitor cells at different mouse ages.***

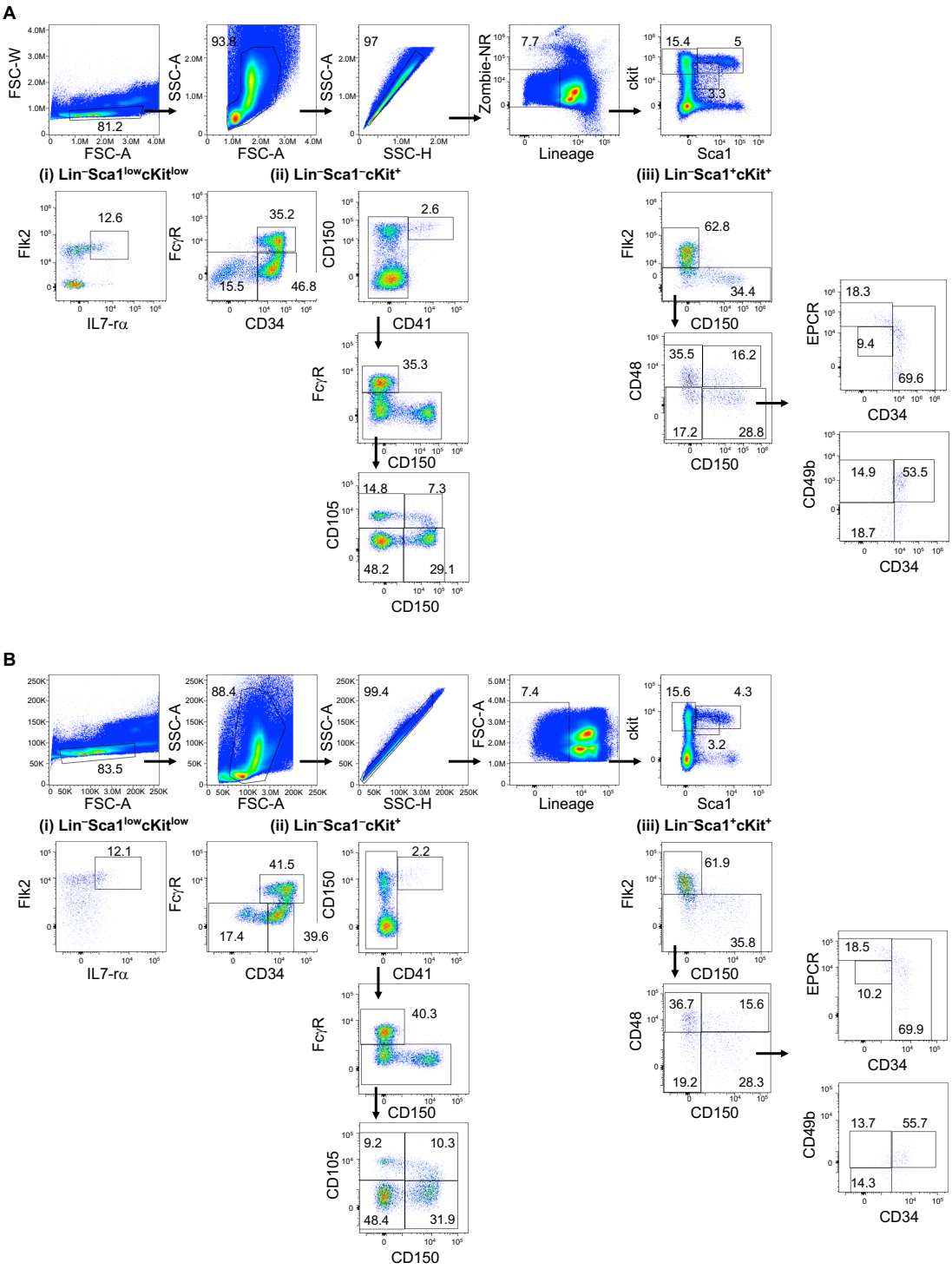



**Supplementary Figure 1.** ***Validation of unmixing accuracy in 14-color panel.***
NxN plot displays every parameter versus every other parameter to assess unmixing accuracy of the fluorochromes in the 14-color panel. Plots were gated on Live Lin$^{neg}$ BM cells from an 8-week-old wild-type mouse with gating exclusion of off-scale antibody aggregates.



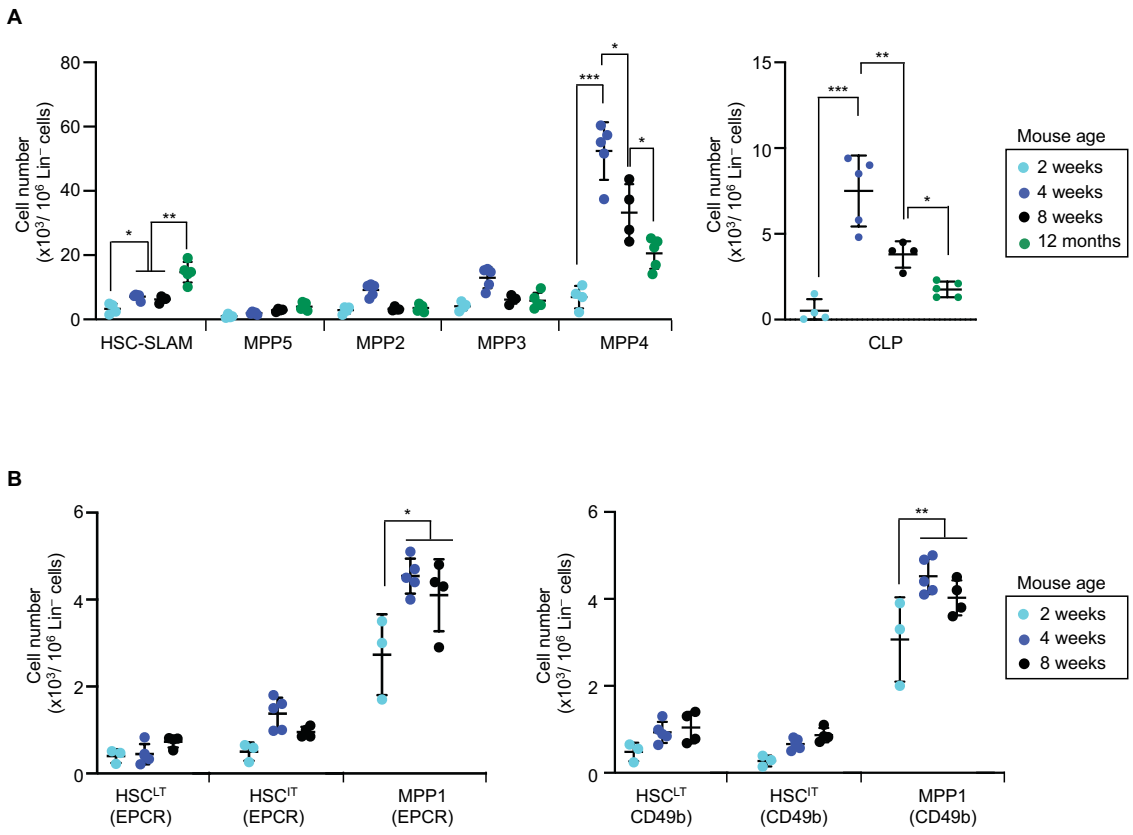

**Supplementary Figure 2.** ***Validation of the gating strategy using fluorescence-minus-one (FMO) controls.***

Background staining and negative/positive boundaries were determined using FMO controls for the definition of (i) lineage negative cells, (ii) myeloid progenitors, (iii) lymphoid progenitors, (iv) stem and multipotent progenitor cells and (v) HSC subsets.

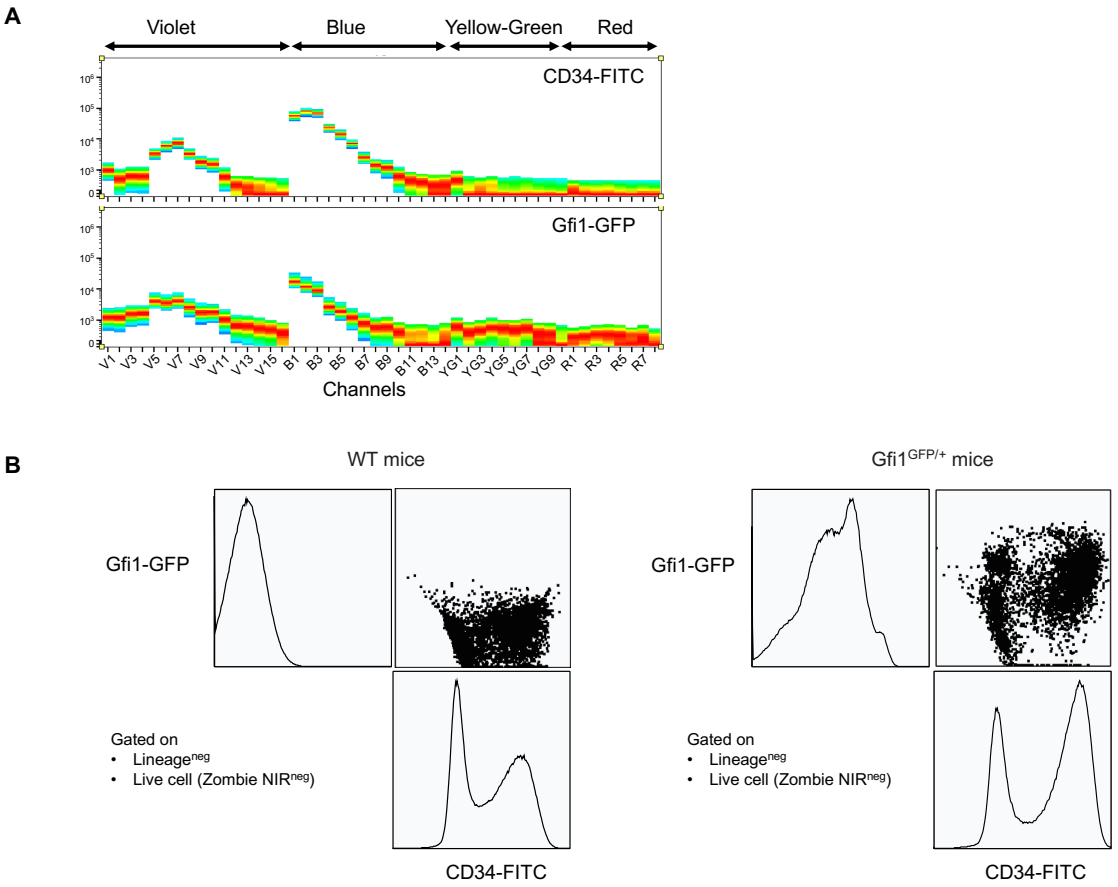



**Supplementary Figure 3.** ***Side-by-side comparison of the panel on spectral and "conventional" polychromatic flow cytometer.***

Representative flow cytometry plots of the 14-fluorescent parameter flow analysis of 8-week-old murine bone marrow (BM) cells on spectral flow cytometer (**A**) and "conventional" polychromatic flow cytometer (**B**). Figure is representative of 3 independent experiments performed side-by-side on the two cytometers.




**Supplementary Figure 4.** ***Changes in hematopoietic stem and progenitor cells at different ages.*** **(A)** Absolute numbers ± SD of HSPC populations per $10^6$ live $Lin^-$ cells in the BM of 2-, 4-, 8- and 52-week-old mice. **(B)** Absolute numbers ± SD of HSC subsets, defined by EPCR (left panel) and CD49b (right panel) expression, per $10^6$ live $Lin^-$ cells in the BM of 2-, 4-, and 8-week-old mice.




**Supplementary Figure 5.** ***Validation of unmixing accuracy between GFP and FITC.***
**(A)** Spectral histograms of CD34-FITC and Gfi1-GFP detected on the Aurora flow cytometer. **(B)** NxN plot of Lin$^{neg}$Live BM cells from 8-week-old wild-type and Gfi1-GFP reporter mice that assesses unmixing accuracy between FITC and GFP in the 14-fluorescent parameter panel.

**SUPPLEMENTARY TABLE 1:**
***Reagents used for the 14-color panel and reference controls.***

| | Name | Alternative name | Clone | Flurochrome | Source | Catalog number | Dilution | Reference Control Dilution |
|---|---|---|---|---|---|---|---|---|
| **#1** | Ter119 | Ly-76 | TER119 | Purified rat Ab | BioLegend | 116202 | 1/400 | |
| | Mac1 | CD11b | M1/70 | Purified rat Ab | BioLegend | 101202 | 1/800 | |
| | Gr1 | Ly-6C | RB6-8C5 | Purified rat Ab | BioLegend | 108402 | 1/800 | |
| | B220 | CD45R | RA-3-6B2 | Purified rat Ab | BioLegend | 103202 | 1/400 | 1/50 |
| | CD5 | | 53-7.3 | Purified rat Ab | BioLegend | 100602 | 1/800 | |
| | CD3 | | 17A2 | Purified rat Ab | BioLegend | 100202 | 1/200 | |
| | CD4 | | GK1.5 | Purified rat Ab | BioLegend | 100402 | 1/800 | |
| | CD8 | | 53-6.7 | Purified rat Ab | BioLegend | 100702 | 1/800 | |
| | Goat Anti-Rat | | F(ab')2-IgG | PE-Cy5 | Invitrogen | A10691 | 1/400 | 1/50 |
| | | | | | | | | |
| **#2** | cKit | CD117 | 2B8 | APC-eFluor780 | eBioscience | 47-1171-82 | 1/400 | 1/50 |
| **#3** | Sca1 | Ly-6a/e | D7 | Pacific Blue | Biolegend | 108120 | 1/400 | 1/50 |
| **#4** | Flk2 | CD135 | A2F10 | Biotin | eBioscience | 13-1351-85 | 1/400 | 1/50 |
| | Streptavidin | | | PE-Cy7 | BioLegend | 405206 | 1/400 | 1/50 |
| **#5** | CD48 | SLAMF2 | HM48-1 | BV711 | Biolegend | 103439 | 1/400* | 1/50 |
| **#6** | CD150 | SLAM | TC15-12F12.2 | PE | Biolegend | 115904 | 1/400 | 1/50 |
| **#7** | CD34 | Mucosialin | RAM34 | FITC | eBioscience | 11-0341-85 | 1/25 | 1/50 |
| **#8** | CD127 | IL-7Ra | A7R34 | BV785 | Biolegend | 135037 | 1/100 | 1/50 |
| **#9** | Fc$\gamma$R | CD16/32 | 93 | BV510 | Biolegend | 101333 | 1/400 | 1/50 |
| **#10** | CD49b | Itg$\alpha$II | DX5 | PE/Dazzle594 | Biolegend | 108923 | 1/200 | 1/50 |
| **#11** | EPCR | CD201 | 1560 | PerCP-eFluor710 | eBioscience | 46-2012-80 | 1/200 | 1/50 |
| **#12** | CD105 | Endoglin | MJ7/18 | APC | Biolegend | 120413 | 1/400* | 1/50 |
| **#13** | CD41 | Itg$\alpha$IIb | MWReg30 | BV605 | Biolegend | 133921 | 1/400 | 1/50 |
| | | | | | | | | |
| | | | | | | | | |
| **#14** | LIVE/DEAD | | | Zombie NIR | BioLegend | 423105 | 1/200 | 100µL/test |
| | | | | | | | | |
| | UltraComp ebeads | | | | Invitrogen | 01-2222-42 | | ~50µL/test |

* Antibody used at a concentration 1/100 for LSRFortessa “conventional” flow cytometer

**SUPPLEMENTARY TABLE 2**
***LSRFortessa instrument configuration.***

| Laser wave length (nm) | Laser power (mW) | Dichroic filters (nm) | Bandpass filters (nm) | Fluorochromes |
|---|---|---|---|---|
| 355 | | 505LP | 525/50 | - |
| | | 450LP | 450/50 | - |
| | | | | |
| 405 | 50 | 750LP | 780/60 | BV785 |
| | | 690LP | 710/50 | BV711 |
| | | 635LP | 670/30 | - |
| | | 600LP | 610/20 | BV605 |
| | | 505LP | 525/50 | BV510 |
| | | - | 450/50 | Pacific Blue |
| | | | | |
| 488 | 50 | 685LP | 695/40 | PerCP-eFluor710 |
| | | 505LP | 530/30 | FITC |
| | | | | |
| 561 | 50 | 750LP | 780/60 | PE-Cy7 |
| | | 685LP | 710/50 | - |
| | | 635LP | 660/20 | PE-Cy5 / PI |
| | | 600LP | 610/20 | PE/Dazzle594 |
| | | - | 586/15 | PE |
| | | | | |
| 640 | 40 | 750LP | 780/60 | APC-eFluor780 |
| | | 690LP | 710/50 | - |
| | | - | 670/14 | APC |

**SUPPLEMENTARY TABLE 3:**

***Mean percentage (frequency of parent) and standard deviation (SD) for all gates presented in Figure 2A (n=7).***

| | Lin$^-$ | LK | LS$^{low}$K$^{low}$ | LSK | LSK Flk2$^-$ |
|---|---|---|---|---|---|
| **Mean** | 7.0 | 14.6 | 2.2 | 4.4 | 34.5 |
| **SD** | 2.2 | 6.0 | 0.8 | 1.8 | 6.8 |

Lymphoid and myeloid progenitors

| | CLP | CMP | GMP | MEP |
|---|---|---|---|---|
| **Mean** | 10.8 | 57.1 | 23.9 | 17 |
| **SD** | 5.0 | 6.2 | 3.9 | 4.0 |

Myeloid progenitors

| | Pre-GM | GMP | Pre MegE | MKP | CFU-E | Pre CFU-E |
|---|---|---|---|---|---|---|
| **Mean** | 43.3 | 22.8 | 33.0 | 4.18 | 12.6 | 10.7 |
| **SD** | 3.9 | 4.4 | 3.3 | 0.6 | 5.0 | 2.3 |

Stem and multipotent progenitor cells

| | HSC-SLAM | MPP5 | MPP2 | MPP3 | MPP4 |
|---|---|---|---|---|---|
| **Mean** | 32.1 | 14.2 | 19.7 | 32.2 | 62.9 |
| **SD** | 4.3 | 2.8 | 3.6 | 4.9 | 6.8 |

HSC subsets

| | HSC$^{LT}$ (EPCR) | HSC$^{IT}$ (EPCR) | MPP1 EPCR |
|---|---|---|---|
| **Mean** | 15.2 | 17.0 | 60.1 |
| **SD** | 5.8 | 1.1 | 6.9 |

| HSCL$^T$ (CD49b) | HSC$^{IT}$ (CD49b) | MPP1 (CD49b) |
|---|---|---|
| 12.1 | 20.7 | 56.0 |
| 1.6 | 6.8 | 4.2 |

**SUPPLEMENTARY TABLE 4:**

***Mean percentage (frequency of parent) and standard deviation (SD) for all gates presented in Figure 3.***

2-week-old mice (n=4)

Stem and multipotent progenitor cells

| | HSC-SLAM | MPP5 | MPP2 | MPP3 | MPP4 |
|---|---|---|---|---|---|
| **Mean** | 27 | 7.34 | 27.5 | 37.5 | 33.1 |
| **SD** | 3.9 | 1.8 | 2.4 | 3.9 | 19.1 |

HSC Subsets

| | $HSC^{LT}$ (EPCR) | $HSC^{IT}$ (EPCR) | MPP1 EPCR | $HSCL^{T}$ (CD49b) | $HSC^{IT}$ (CD49b) | MPP1 (CD49b) |
|---|---|---|---|---|---|---|
| **Mean** | 9.61 | 8.15 | 79.9 | 8.96 | 11.7 | 69.5 |
| **SD** | 4.8 | 1.1 | 4.5 | 2.9 | 3.7 | 3.1 |

| | $CD34^{-}CD41^{+}$ | $CD34^{-}CD41^{-}$ | $CD34^{+}CD41^{-}$ | $CD34^{-}$ $CD105^{+}$ | $CD34^{-}$ $CD105^{-}$ | $CD34^{+}$ $CD105^{-}$ |
|---|---|---|---|---|---|---|
| **Mean** | 0.22 | 12 | 85.3 | 3.52 | 16.6 | 71.1 |
| **SD** | 0.2 | 4.9 | 3.6 | 1.7 | 3.1 | 7.0 |

4-week-old mice (n=6)

Stem and multipotent progenitor cells

| | HSC-SLAM | MPP5 | MPP2 | MPP3 | MPP4 |
|---|---|---|---|---|---|
| **Mean** | 32.1 | 14.2 | 19.7 | 32.2 | 62.9 |
| **SD** | 4.3 | 2.8 | 3.6 | 4.9 | 6.8 |

HSC Subsets

| | $HSC^{LT}$ (EPCR) | $HSC^{IT}$ (EPCR) | MPP1 EPCR | $HSCL^{T}$ (CD49b) | $HSC^{IT}$ (CD49b) | MPP1 (CD49b) |
|---|---|---|---|---|---|---|
| **Mean** | 15.2 | 17 | 60.1 | 12.1 | 20.7 | 56 |
| **SD** | 5.8 | 1.1 | 6.9 | 1.6 | 6.8 | 4.2 |

| | $CD34^{-}CD41^{+}$ | $CD34^{-}CD41^{-}$ | $CD34^{+}CD41^{-}$ | $CD34^{-}$ $CD105^{+}$ | $CD34^{-}$ $CD105^{-}$ | $CD34^{+}$ $CD105^{-}$ |
|---|---|---|---|---|---|---|
| **Mean** | 1.3 | 12.9 | 81.4 | 6.4 | 13 | 65.6 |
| **SD** | 2.3 | 4.8 | 11.4 | 1.42 | 6.72 | 5.4 |

8-week-old mice (n=7)

See SUPPLEMENTARY TABLE 3:

12-month-old mice (n=5)

Stem and multipotent progenitor cells

| | HSC-SLAM | MPP5 | MPP2 | MPP3 | MPP4 |
|---|---|---|---|---|---|
| **Mean** | 52.4 | 14.2 | 12.5 | 20.2 | 41.6 |
| **SD** | 6.9 | 2.6 | 2.4 | 5.0 | 7.8 |

HSC Subsets

| | $HSC^{LT}$ (EPCR) | $HSC^{IT}$ (EPCR) | MPP1 EPCR | $HSCL^{T}$ (CD49b) | $HSC^{IT}$ (CD49b) | MPP1 (CD49b) |
|---|---|---|---|---|---|---|
| **Mean** | 23.3 | 18.5 | 47.8 | 25.8 | 20.8 | 26.6 |
| **SD** | 11.0 | 7.7 | 22.6 | 8.3 | 21.4 | 4.8 |

| | $CD34^{-}CD41^{+}$ | $CD34^{-}CD41^{-}$ | $CD34^{+}CD41^{-}$ | $CD34^{-}$ $CD105^{+}$ | $CD34^{-}$ $CD105^{-}$ | $CD34^{+}$ $CD105^{-}$ |
|---|---|---|---|---|---|---|
| **Mean** | 53.7 | 19.3 | 26.2 | 57.5 | 13.3 | 17.3 |
| **SD** | 10.9 | 1.4 | 7.8 | 2.3 | 7.0 | 3.4 |

**SUPPLEMENTARY TABLE 5:**

***Mean number and standard deviation (SD) of hematopoietic stem and progenitor cells (per $10^6$ live $Lin^-$ cells) presented in Figure 3.***

| | 2-week-old (n=3) | 4-week-old (n=5) | 8-week-old (n=4) | 12-month-old (n=5) |
|---|---|---|---|---|
| HSC-SLAM | 3,900 ± 1,323 | 7,080 ± 896 | 6,225 ± 960 | 14,680 ± 3,211 |
| MPP5 | 1,170 ± 565 | 1,940 ± 371 | 2,850 ± 420 | 3,980 ± 1,160 |
| MPP2 | 3,400 ± 608 | 9,180 ± 1,973 | 3,275 ± 568 | 3,540 ± 1,097 |
| MPP3 | 4,633 ±907 | 12,980 ± 3,289 | 6,175 ± 1,276 | 5,820 ± 2,473 |
| MPP4 | 6,600 ± 4,214 | 52,400 ± 8,978 | 33,250 ± 8,833 | 20,580 ± 4,810 |
| | | | | |
| CLP | 187 ± 190 | 6,900 ± 2934 | 3,550 ± 1,256 | 1,760 ± 456 |
| | | | | |
| CMP | 43,433 ± 8,886 | 99,140 ± 23,126 | 102,625 ± 6,342 | 98,860 ± 24,422 |
| GMP | 35,400 ± 11,781 | 53,660 ± 23,315 | 47,375 ±14,602 | 59,780 ± 25,060 |
| MEP | 21,900 ± 10,290 | 49,500 ± 21,675 | 29,025 ± 12,928 | 22,300 ± 6,403 |
| | | | | |
| Pre-GM | 19,733 ± 2,040 | 54,620 ± 10,621 | 53,550 ± 2,538 | 52,860 ± 17,204 |
| GMP | 34,867 ± 11,152 | 49,820 ± 22, 119 | 45,750 ± 14,160 | 54,100 ± 25,068 |
| Mk-Ery | 22,733 ± 6,191 | 50,240 ± 13,220 | 44,600 ± 4,600 | 29,100 ± 5,436 |
| Pre-CFUe | 5,667 ± 1604 | 17,720 ± 6,926 | 11,150 ± 2,869 | 9,520 ± 3,571 |
| CFU-E | 15,100 ± 9,060 | 21,960 ± 11, 283 | 13,650 ± 8,812 | 12,960 ± 4,193 |
| | | | | |
| $HSC^{LT}$ (EPCR) | 400 ± 157 | 446 ± 223 | 730 ± 134 | n.a.* |
| $HSC^{IT}$ (EPCR) | 500 ± 210 | 1,376 ±369 | 950 ± 122 | n.a.* |
| MPP1 (EPCR) | 2,733 ± 929 | 4,540 ±404 | 4,100 ± 829 | n.a.* |
| | | | | |
| $HSC^{LT}$ (CD49b) | 480 ± 214 | 932 ± 242 | 1,040 ± 363 | n.a.* |
| $HSC^{IT}$ (CD49b) | 273 ± 125 | 662 ±129 | 868 ± 165 | n.a.* |
| MPP1 (CD49b) | 3,066 ± 971 | 4,520 ± 409 | 4,025 ± 403 | n.a.* |
| | | | | |

* n.a.: not applicable due to the shift in marker expression at this age