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Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals. C57BL/6 (CD45.2 Ly5.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), Taconic (Hudson, NY) or Charles River Laboratories (Wilmington, MA). Transgenic mice (C57BL/6 background) expressing enhanced green fluorescent protein (eGFP) under the β-actin promoter were generated as described (Wright et al., 2001a). Congenic CD45.1 Ly5.1 (B6.SJL-*Ptprca Pep3*^{b}/BoyJ) and lymphotoxin α-deficient mice (B6.129S2-Lta^{tm1Dch}/J) were from The Jackson Laboratory. Rag2$^{-/-}$γc$^{-/-}$ mice (C57BL/10), and nude mice (B6.Cg/JBomTac-Foxn1^{nu}N3), as well as age- and sex-matched wild-type mice were from Taconic. Mice were housed in a specific pathogen-free, viral antibody-free barrier facility and were used at 5-10 weeks of age in accordance with guidelines of the animal committees of the CBR Institute for Biomedical Research and Harvard Medical School (Boston, Massachusetts).

Reagents. FTY720 was a gift from V. Brinkmann (Novartis, Basel, Switzerland). 1-[4-(1,2,3,4-Tetrahydroxybutyl)-1H-imidazol-2-yl]ethanone (THI) was obtained from Albany Molecular Research, Inc. (North Syracuse, NY). PTX was from Calbiochem (San Diego, CA), and SEW2871 was purchased from Cayman Chemical (Ann Arbor, MI).

Thoracic duct cannulation. For TD cannulation, mice received olive oil 30 min prior to cannulation (200 μl per gavage) to facilitate visualization of the lymph vessels. The animals were then anesthetized with xylazine (10mg/kg, Phoenix Pharmaceutical, St. Joseph, MO) and ketamine HCl (50mg/kg, Fort Dodge Animal Health, Fort Dodge, IA). A polyethylene catheter (PE-10, VWR, Houston, TX) was inserted into the right jugular vein for continuous infusion (1 ml/hr) of Ringer's lactate (Abbott Laboratories, North Chicago, IL) containing 1U/ml heparine (American Pharmaceutical partners, Los Angeles, CA). Using a Zeiss OPMI 1 FC dissecting microscope (Carl Zeiss Meditec, Dublin, CA), the TD was exposed through a left subcostal incision. Silastic® silicon tubing (0.012" I.D., Dow Corning, Midland, USA) was flushed with heparinised (50 U/ml) phosphate-buffered saline (DPBS, Mediatech, Herndon, VA), inserted into the cisterna chyli through a ~ 0.3mm incision and fixed with isobutyl cyanoacrylate monomer (Nexaband®, Abbott Laboratories). The remaining part of the tubing was exteriorized through the posterior abdominal wall. Subsequently, the abdominal incision was closed using a 6-0 nonabsorbable running suture (Sofsilk, Tyco Healthcare Group, Norwalk, CO). Lymph was collected from awake, unrestrained mice for 12 h in Myelocult medium (StemCell Technologies, Vancouver, BC, Canada) at 4°C. Where

indicated, mice were treated with pertussis toxin (50 μg/kg iv, 2 h prior to cannulation), FTY720 (1mg/kg ip, 6 h prior to cannulation), THI (drinking water with 10g/L glucose plus 50μg/ml THI for 3 days prior to cannulation), or SEW2871 (20mg/kg 6h prior to cannulation (Wei et al., 2005)).

Parabiosis. For parabiosis, 6- to 8-week-old sex- and age-matched C57BL/6 WT and β-actin/eGFP mice were anesthetized by intraperitoneal injection of xylazine and ketamine HCl. The lateral aspects of both animals were shaved, matching skin incisions were made from the olecranon to the knee joint and the subcutaneous fascia was bluntly dissected. Olecranons and knees were joined with a single 6-0 suture, and the dorsal and ventral skins were approximated by staples (Stoelting, Wood Dale, IL) or continuous 6-0 sutures. As previously reported, parabiosis surgery does not lead to enhanced HSPC mobilization (Wright et al., 2001b). In addition, we did not see significant differences between the frequency of CFU-Cs in the lymph of parabionts (5.4 ± 1.8 CFU-Cs per ml lymph) as compared to mice without parabiosis surgery (4.9 ± 0.8 CFU-Cs per ml lymph), suggesting that parabiosis surgery does not lead to enhanced HSPC mobilization or trafficking across the TD. Where indicated, mice were surgically separated and wounds were surgically closed prior to collection of TD lymph.

Colony-forming unit assays. MNCs from various tissues or sorted Lin^+, $Lin^-c\text{-}Kit^-$ or $Lin^-c\text{-}Kit^+$ cells isolated from TD lymph, BM and PBL were assessed for CFU-C content by culturing 6 x 10^6 cells in methylcellulose-containing MethoCult M3434 medium (StemCell Technologies, Vancouver, Canada) with 10 μg/mL recombinant human (rh) insulin, 200 μg/mL human transferrin (Iron - Saturated), 50 ng/mL recombinant murine (rm) SCF, 10 ng/mL, rmIL-3, 10 ng/mL, rhIL-6, 3 units/mL rhErythropoietin supplemented with amphotericin B (1μg/ml, GIBCO, Grand Island, NY) according to the manufacturer's protocols. Where indicated, MNCs were isolated from the lymph, PB or BM of parabiotic mice or from β-actin-eGFP BM chimeras (20 weeks after reconstitution). The donor mice were treated with PTX (50 μg/kg iv, 2h prior to cannulation), FTY720 (1mg/kg ip, 6h prior to TD cannulation), SEW2871 (20mg/kg 6h prior to cannulation), or THI (drinking water with 10g/L glucose plus 50 μg/ml THI for 3 days prior to cannulation). To define the effect of FTY720 on cytokine-induced HSPCs mobilization, mice received daily injections of FTY720 (1mg/kg ip) and G-CSF (daily injection of 0.5mg/kg, i.p.) or vehicle, respectively, for 6 days prior to blood sampling.

To detect HSPCs within extramedullary tissues, parabiotic mice or β-actin/eGFP BM chimeras were exsanguinised and perfused with 40 ml PBS containing 5 mM EDTA (Sigma-Aldrich, St. Louis, MO) via the right and left ventricle to remove any residual blood. Both lungs, the heart, liver, both kidneys, the entire small intestine, brain, the skin of both ears and the lower limb muscles were harvested and cut into 0.5 mm pieces. The tissue samples were incubated overnight in Myelocult medium (StemCell Technologies) at 37°C to allow emigration of MNCs. Emigrated MNCs were then isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and cultured in MethoCult M3434 medium (StemCell Technologies) as described above. This method was chosen to reduce contamination of the mononuclear cell preparation with non-hematopoietic, parenchymal cells. To detect cells with CFU-C capacity within LN, spleen and BM we

prepared single cell suspensions by gentle mechanical disintegration of the tissues through the nylon mesh of a 40μm cell strainer (BD Falcon, BD Biosciences). In case of the spleen and bone marrow, red blood cell lysis was performed prior to plating of mononuclear cells in methylcellulose.

Flow cytometric analysis. Lineage staining used a cocktail of biotinylated anti-mouse antibodies to Mac-1 (CD11b), Gr-1 (Ly-6G and Ly-6C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), CD19, CD11c and B220 (CD45R; all Abs were from BD Biosciences, Franklin Lakes, NJ). For detection and phenotypic characterization of HSPCs we used lineage staining and Peridinin-chlorophyll-protein Complex (PerCP)-conjugated streptavidin, phycoerythrin (PE)-labelled anti-mouse antibodies to CD45.2, CD44, IL-7Rα (clone SB/199), CD34, CXCR4, CD44, PSGL-1, LFA-1, integrin α4 and α5, allophycocyanin (APC)-labelled anti-c-Kit (CD117) monoclonal antibody (mAb), and fluorescein isothiocyanate (FITC)-tagged anti-Sca-1 (Ly-6A/E) mAb (all from BD). For staining of mature leukocytes subsets we used anti-mouse mAb to Gr-1 (PE), TCR-β (APC), CD8a (PE), CD19 (PE), and B220 (APC), CD45.1 (FITC) and CD45.2 (PerCP) (all from BD). All samples were analyzed on a FACScalibur flow cytometer.

Homing assay. Sorted Lin^- ($2x10^5$) TD cells labelled for 15 min at 37°C with 10mM CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine; Molecular Probes-Invitrogen, Carlsbad, CA) or β-actin/$eGFP^+$ Lin^- TD cells were injected into the tail vein of irradiated (1300 rad) or non-irradiated recipients, respectively. Mice were killed after 24 h and single-cell suspensions from spleens and BM were analyzed by flow cytometry for the presence of labeled cells.

Two-photon intravital microscopy (2P-IVM) of mouse BM. 2 x 10^5 β-actin/eGFP or CMTMR-labelled Lin^- TD cells were injected into the tail vein of unconditioned mice or mice that had been lethally irradiated (1300 rad) 24 h previously. Twelve or 24 h after transfer, recipients were anaesthetized, high molecular weight (2 MDa) TRITC-dextran (Molecular Probes-Invitrogen) was injected iv as a blood plasma marker, and skull BM was visualized by 2P-IVM as described (Mazo et al., 2005). Images were processed using Volocity software (Improvision Inc., Lexington, MA).

PCR analyses. BM-derived $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells were sorted using a high speed MoFlo cytometer (DakoCytomation, Glostrup, Denmark). The expression of S1P receptor mRNA was assessed with the use of the following forward and reverse primer pairs: $S1P_1$ (pair A), 5´-GCCGACAGCAGCAAGATG-3` and 5´-TAGAGGGCGAGGTTGAGTGAG-3`; $S1P_1$ (pair B), 5´-ACTACACAACGGGAGCAACAG-3` and 5´-GATGGAAAGCAGGAGCAGAG-3`; $S1P_2$, 5'-CTCACTGCTCAATCCTGTCATC-3' and 5'-TTCACATTTTCCCTTCAGACC-3'; $S1P_3$ 5'-TTCCCGACTGCTCTACCATC-3' and 5'-CCAACAGGCAATGAACACAC-3'; $S1P_4$ 5'-TGCGGGTGGCTGAGAGTG-3'and 5'-TAGGATGAGGGCGAAGACC-3'; $S1P_5$ 5'-CCGCTGTTTCTCTTGCTATTAC-3'and 5'-GTTGGAGGAGTCTTGGTTGC-3'.

Quantitative PCR. Mature lymphoid ($B220^+CD19^+$ B cells, $CD8^+TCR\text{-}\beta^+$ and $CD4^+TCR\text{-}\beta^+$ T cells) cells, two distinct subsets of BM-derived HSPCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (enriched in HSCs) and $Lin^-c\text{-}Kit^+Sca\text{-}1^-$ cells (enriched in GMP/CMP)), as well as TD MNC were stained with appropriate mAbs and sorted using a FACSAria cell

sorter (BD Biosciences). Sorted Lin^- or $Lin^-c\text{-}Kit^+$ TD-derived cells were incubated with medium containing stem cell factor (R&D, 20 ng/ml), Flt-3 ligand (R&D, 100 ng/ml) in the absence or presence of LPS (Sigma, 10 μg/ml) and CD14-Fc (R&D, 1 μg/ml) for 12 h at 37°C and 5% CO_2. Thereafter, RNA was isolated from sorted cells and cDNA was prepared using Reverse-iT MAX 1st Strand Synthesis kit (Abgene, Surrey, UK) for quantitative PCR on an ABI 7300 sequence detection instrument (Applied Biosystems) using Taqman Gene expression assays Mm00514644_m1 ($S1P_1$/Edg-1) and Mm99999915_g1 (GAPDH) and the Taqman Fast Universal Master Mix (all from Applied Biosystems). The amount of $S1P_1$ mRNA was determined using the ΔΔCT method and is expressed as relative amount of LPS-treated cells normalized to GAPDH. The myeloid cell line E2a/Pbx1 was used to determine myeloid $S1P_1$ mRNA expression and served as reference (Sykes and Kamps, 2001).

Chemotaxis Assay. Sorted Lin^- BM-derived cells (10^5 cells per well) or CD4+ T cells (10^6 cells per well) were tested for transmigration across uncoated transwell filters with 5 μm diameter pores (Corning Costar Corp.) in response to S1P (100 nM, Sigma-Aldrich) or CXCL12/SDF-1α (50 nM, PeproTech), or medium in the bottom chamber. After 3 h, transmigrated cells were harvested and enumerated by flow cytometry using standard techniques. Where indicated, transmigrated and input Lin^- cells were stained using anti-c-Kit and anti-Sca-1 mAbs to specifically address the migration of HSPC subsets as described (Wright et al., 2002).

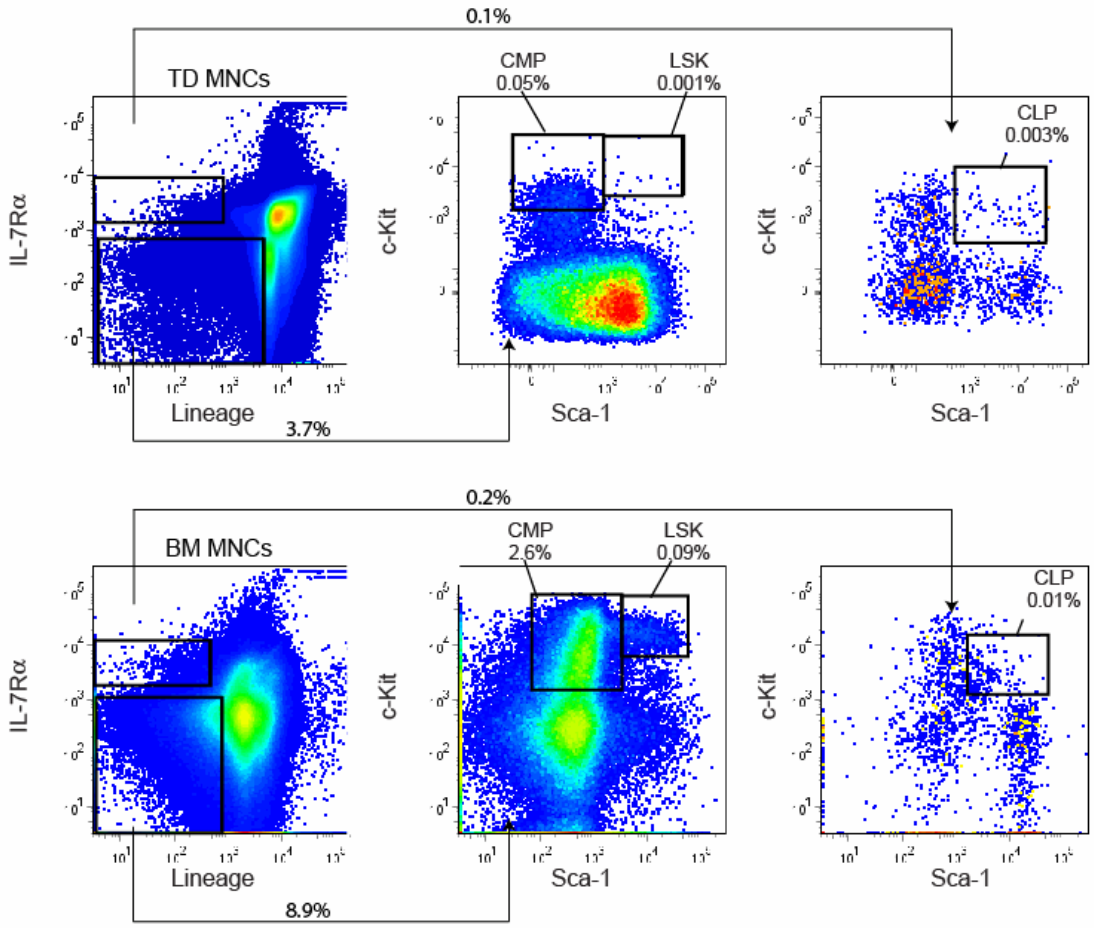
SUPPLEMENTAL TABLE

Antigen	Positive LSK cells [%]	MFI LSK [AU]	MFI Lin^+ MNCs [AU]
CD45	100.0	1464.94	1516.67
CXCR4	82.35	96.49	15.43
PSGL-1	94.12	899.85	443.18
α4 integrin	99.50	355.93	380.38
α5 integrin	91.18	370.93	23.09
LFA-1	94.44	1018.57	491.24
CD44	88.08	488.78	503.91
Isotype-IgG	0.65	29.48	8.34

Expression of CD45 and trafficking molecules by TD-derived Lin^- c-Kit^+Sca-1^+ (LSK) cells. The percentage of positive LSK cells and the mean fluorescence intensity of all LSK cells are indicated. LSK cells stained with irrelevant isotype-matched IgG served as controls. For comparison the MFI of Lin^+ MNCs is given for each staining in the right column. Data representative of 2-3 experiments for each antigen.

SUPPLEMENTAL FIGURES

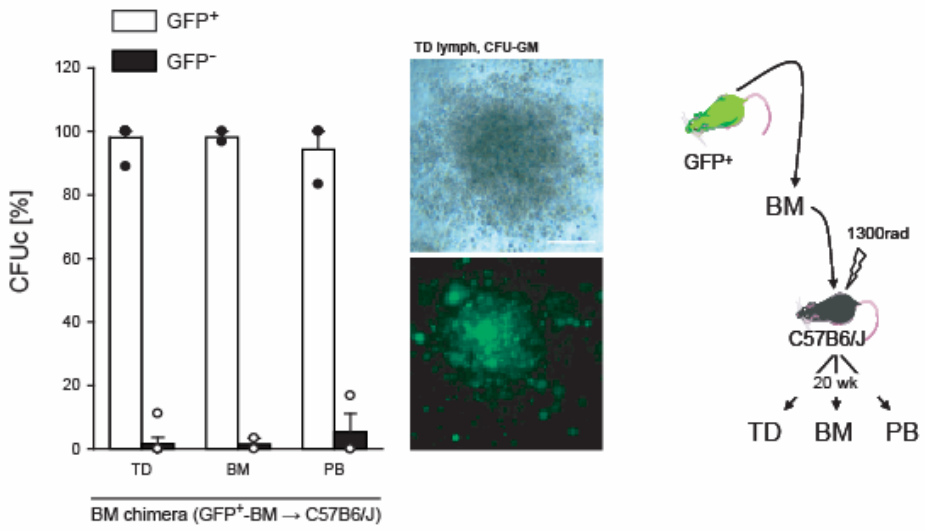
suppl. Figure 1



Suppl. Figure 1 Multiple HSPC subsets travel in the lymph. FACS analysis of TD lymph (above) and BM cells (below). The cells that stained in the negative to low range for lineage markers (Mac-1 (CD11b), Gr-1 (Ly-6G and Ly-6C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), CD19, CD11c and B220, left panels) were subdivided into IL-7Rα positive (right panels) and negative fractions (middle panels). Sca-1 and c-Kit profiles of each fraction showed the presence of Lin$^-$ c-Kit$^+$Sca-1$^+$ (LSK) and Lin$^-$ c-Kit$^+$Sca-1$^-$ population among IL-7Rα$^-$ cells, resembling the phenotype of primitive bone marrow (BM) hematopoietic stem cells and committed common myeloid progenitor cells (CMP), respectively. The population of IL-7Rα$^+$ cells includes a small but stable population of Lin$^-$ c-Kit$^+$Sca-1$^+$ cells, resembling the phenotype of committed BM common lymphoid progenitor cells (CLP). Numbers show the frequency of gated cells among total MNCs.

suppl. Figure 2

A



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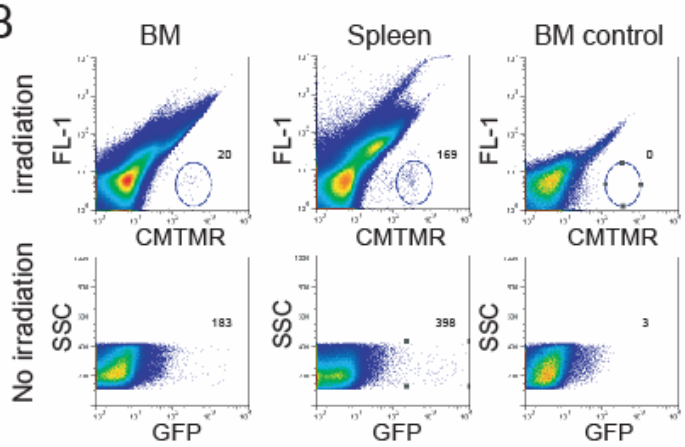
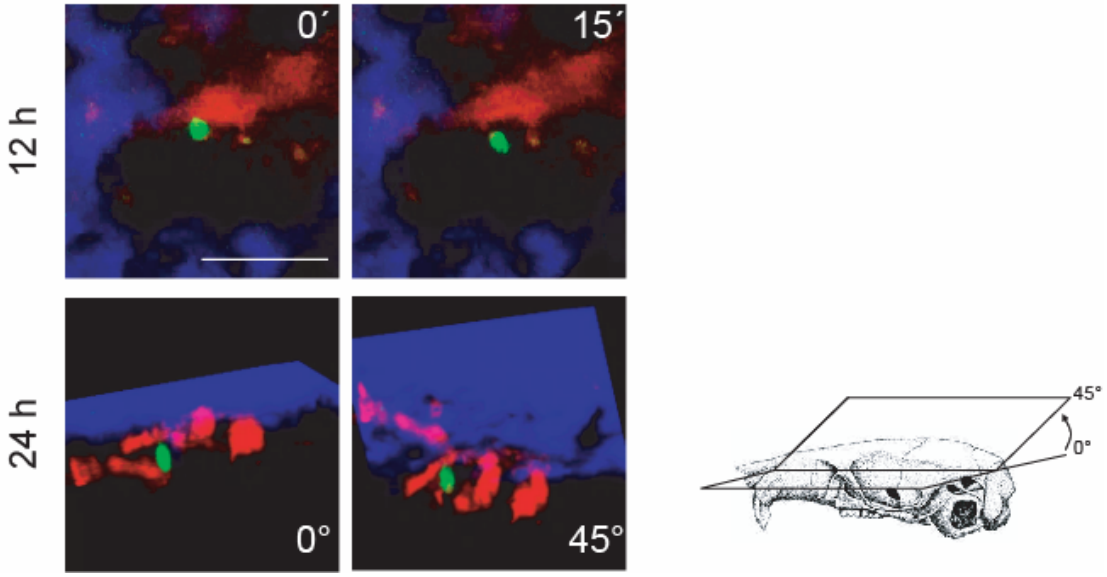
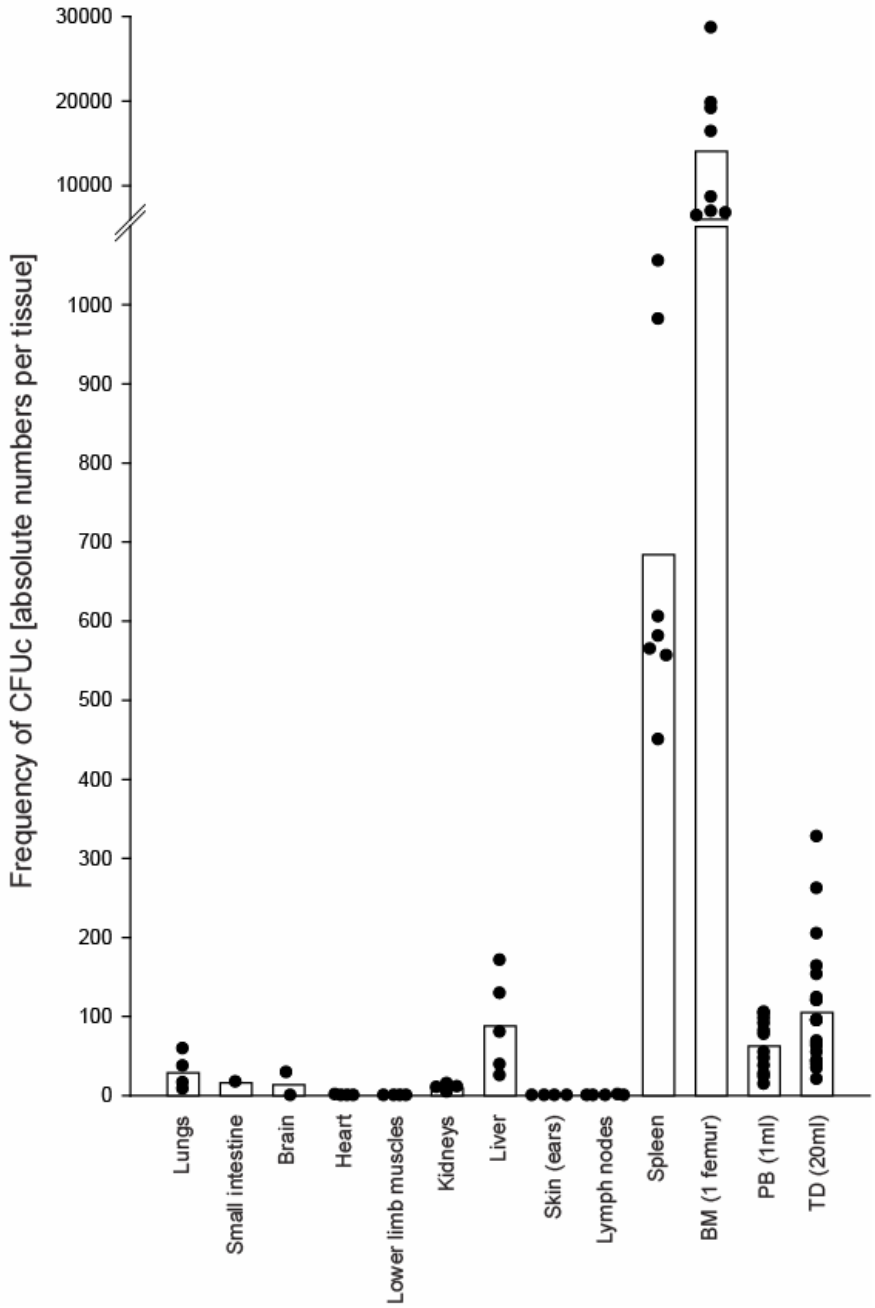


C



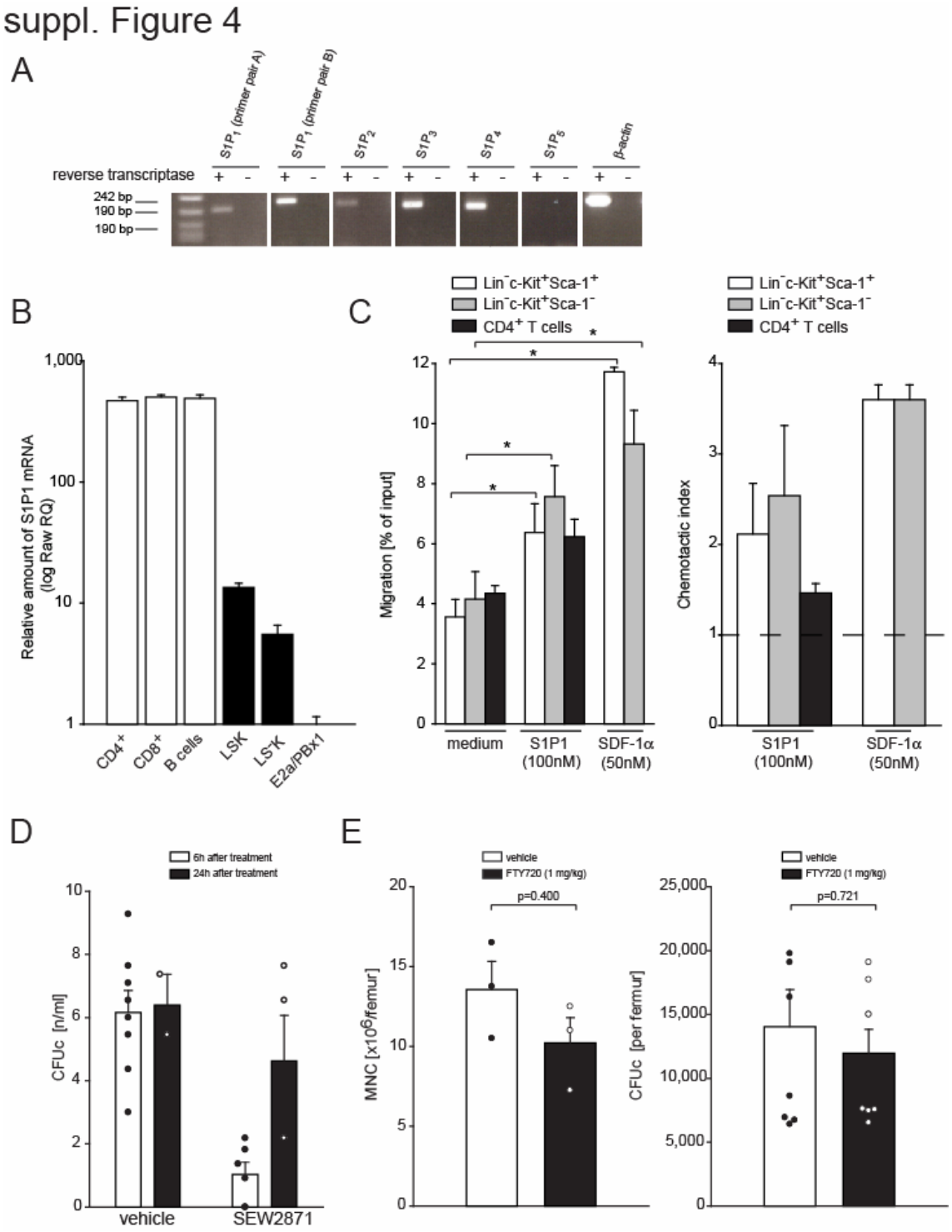
Suppl. Figure 2 Lymph-borne clonogenic cells are bone marrow-derived and have bone marrow homing capacity. A, Relative frequencies of GFP^- and GFP^+ CFU-Cs in thoracic duct lymph (TD), bone marrow (BM) and peripheral blood (PB) of wildtype (WT) mice 20 weeks after lethal irradiation and reconstitution with 5 x 10^7 BM cells from GFP transgenic donors (schematic protocol is shown on the right). Error bars show SEM. Micrographs (middle) depict representative brightfield (top) and fluorescence (bottom) images of CFU-Cs. The scale bar represents 50 μm. **B,** CMTMR-tagged (top row) or GFP^+ (bottom row) Lin^- TD cells were injected into lethally irradiated or non-irradiated mice, respectively. Homing to BM (left panels) and spleen (middle panels) was determined by harvesting tissues 24h after adoptive transfer and analyzing single-cell suspensions using FACS. Numbers of CMTMR-tagged or GFP^+ cells per 10^6 acquired MNCs are indicated. BM obtained from untreated mice is shown as a control (right panels). **C,** GFP^+ Lin^- TD cells were injected into WT recipients and visualized after 12h (top) or 24h (bottom) in skull BM cavities using 2P-IVM. The top micrographs show the same field of view in 15 min intervals documenting extravascular displacement of a homed GFP^+ donor cell (green). The bottom panels show a 3D-projection of the same volume of tissue at two different angles. The optical planes are indicated in the schematic diagram of a mouse skull (right). Blue, autofluorescence and second harmonic signal from bone; red, BM vessels filled with TRITC-dextran; green, GFP^+ donor cells. See also **supplemental movies 1-3**. The scale bar represents 50 μm.

suppl. Figure 3



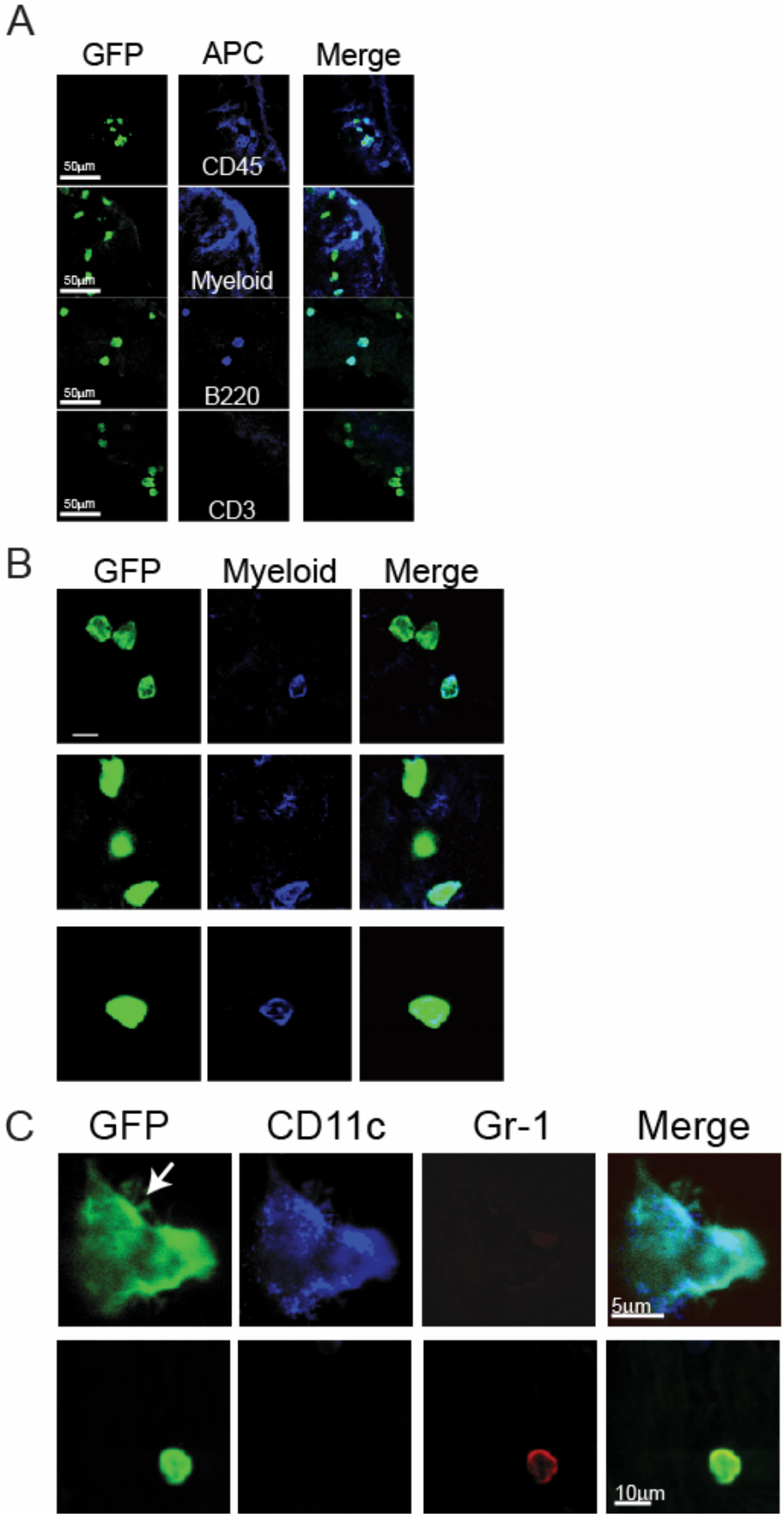
Suppl. Figure 3 Frequency of CFU-Cs in various normal mouse tissues. After rigorous removal of intravascular blood cells (see **methods**), MNCs were isolated from the lung, the entire small intestine, the brain (cerebrum and cerebellum), the heart, the bilateral lower limb muscles, both kidneys, the entire liver, skin (from both ears), lymph nodes (pooled cervical, axillary, brachial, mesenteric, and inguinal), and the spleen. As a reference, CFU-C assays were performed using BM MNCs isolated from one femur and MNCs obtained from 1ml peripheral blood (PB), or from 20ml lymph, which is the amount of lymph that passes the thoracic duct (TD) within 24h. Absolute numbers of

CFU-Cs recovered from the indicated specimens are shown. Results obtained from individual mice are shown as filled circles.



Suppl. Figure 4 S1P and S1P receptors guide the recirculation of HSPCs. A, S1P receptor mRNA expression in sorted Lin^- c-Kit^+ $Sca\text{-}1^+$ (LSK) BM cells (for sequence of primers see Materials and Methods). An intron spanning (primer A) or a non-intron spanning (primer B) set of primers was used to determine $S1P_1$ mRNA expression. **B,** Quantitative PCR analysis of $S1P_1$ mRNA expression by mature cells ($CD4^+$ and $CD8^+$ T cells, $B220^+$ B cells, E2a/Pbx1 myeloid cells), and the immature HSPC subsets Lin^- c-$Kit^+Sca\text{-}1^+$ (LSK) and Lin^-c-$Kit^+Sca\text{-}1^+$ (LS^-K). The amount of $S1P_1$ mRNA is expressed as relative amount of E2a/Pbx1 myeloid cells normalized to GAPDH. Experiments were performed in quadruplicate, *$P<0.05$. C, $CD4^+$ T cells, Lin^-c-$Kit^+Sca\text{-}1^+$ (LSK) and Lin^-c-$Kit^+Sca\text{-}1^-$ (LS^-K) BM-derived cells were tested for their ability to migrate to 100nM S1P or to 50nM CXCL12/SDF-1α. Migration to the lower chamber was determined by flow cytometry as described (Wright et al., 2002) and is given as percentage of the number of input cells (left panel). The chemotactic index (right panel) was calculated as the number of migrated cells in chemokine-containing wells divided by the number of migrated cells in wells containing medium alone. *P<0.05, n=5-8 experiments per group. **D,** Absolute frequency of CFUc in TD lymph of mice treated with vehicle or the $S1P_1$ antagonist SEW2871 6h or 24h after treatment. **E,** BM MNCs were collected from femura of mice 6 h after treatment with vehicle or FTY720. Left panels, total number of MNCs per femur, right panels, CFU-C per femur. Results obtained from individual mice are shown as filled circles**.** Error bars show SEM.

suppl. Figure 5



Suppl. Figure 5 HSPC-enriched TD cells give rise to myeloid effector cells in peripheral tissues. 10^5 $Lin^-CD45.2^+GFP^+$ **(A, B)** or 10^4 $Lin^-c\text{-}Kit^+CD45.2^+$ GFP^+ **(C)** were obtained from TD MNCs by cell sorting and implanted under the left kidney capsule of $CD45.1^+$ recipients in the absence (**A, B**) or presence (**C**) of LPS. **A,** Expression of CD45, myeloid lineage markers (CD11c, CD11b, F4/80, Gr-1), B220, or CD3 after implantation of GFP^+Lin^- TD-derived cells in the kidney was assessed using the respective APC-labelled antibodies. **B,** Representative images of myeloid GFP^+ cells in kidneys injected with Lin^-GFP^+ cells without LPS. **C,** Representative images showing the expression of Gr-1, GFP, or CD11c by TD-derived cells in the kidney. Arrow indicates dendrites of a $CD11c^+$ cell.

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