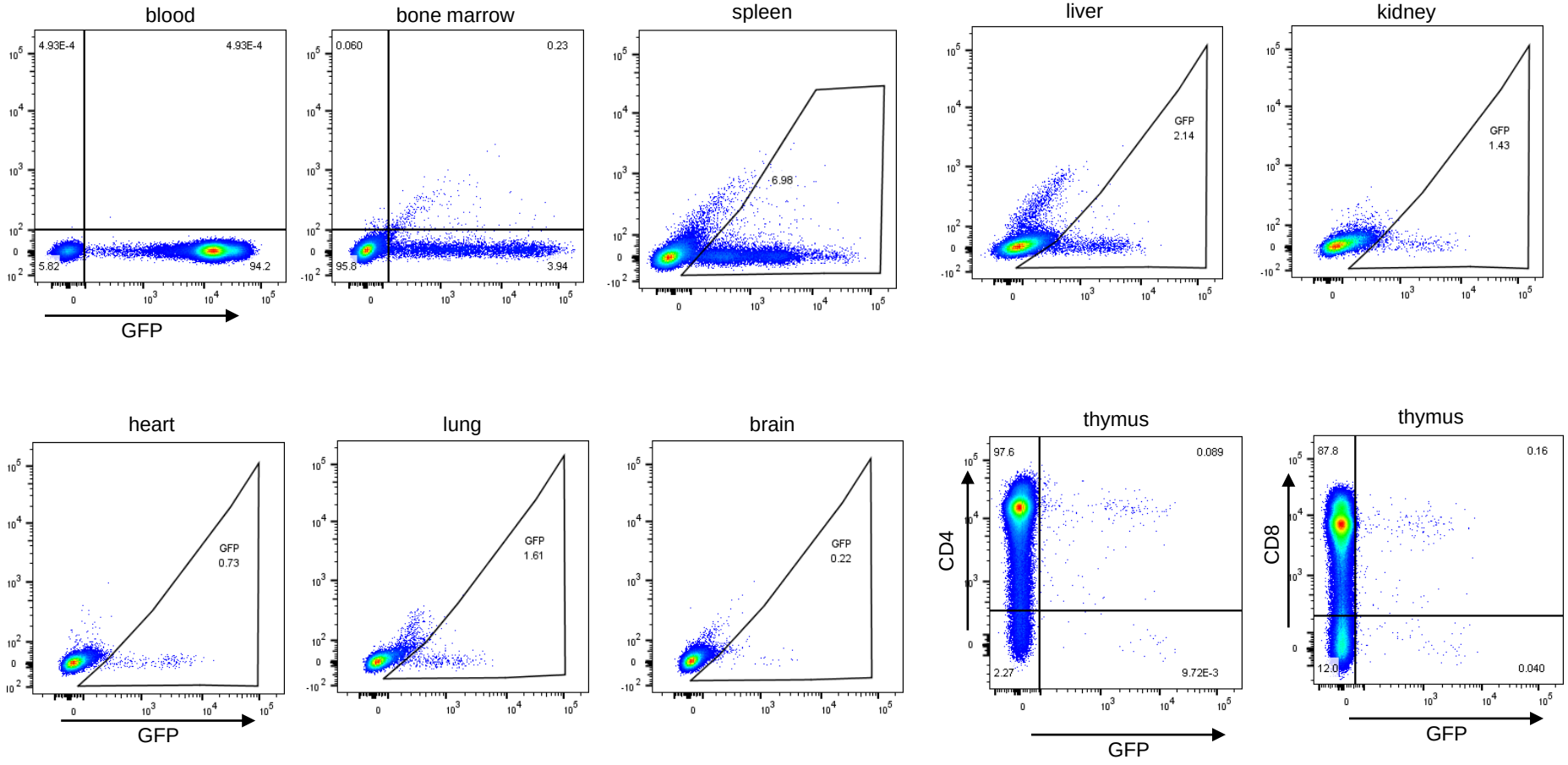
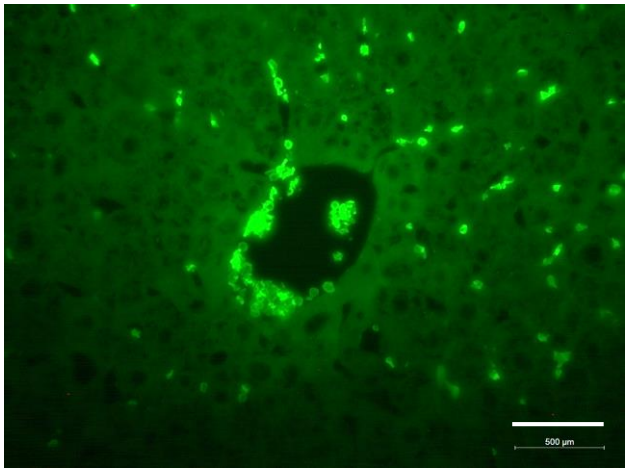
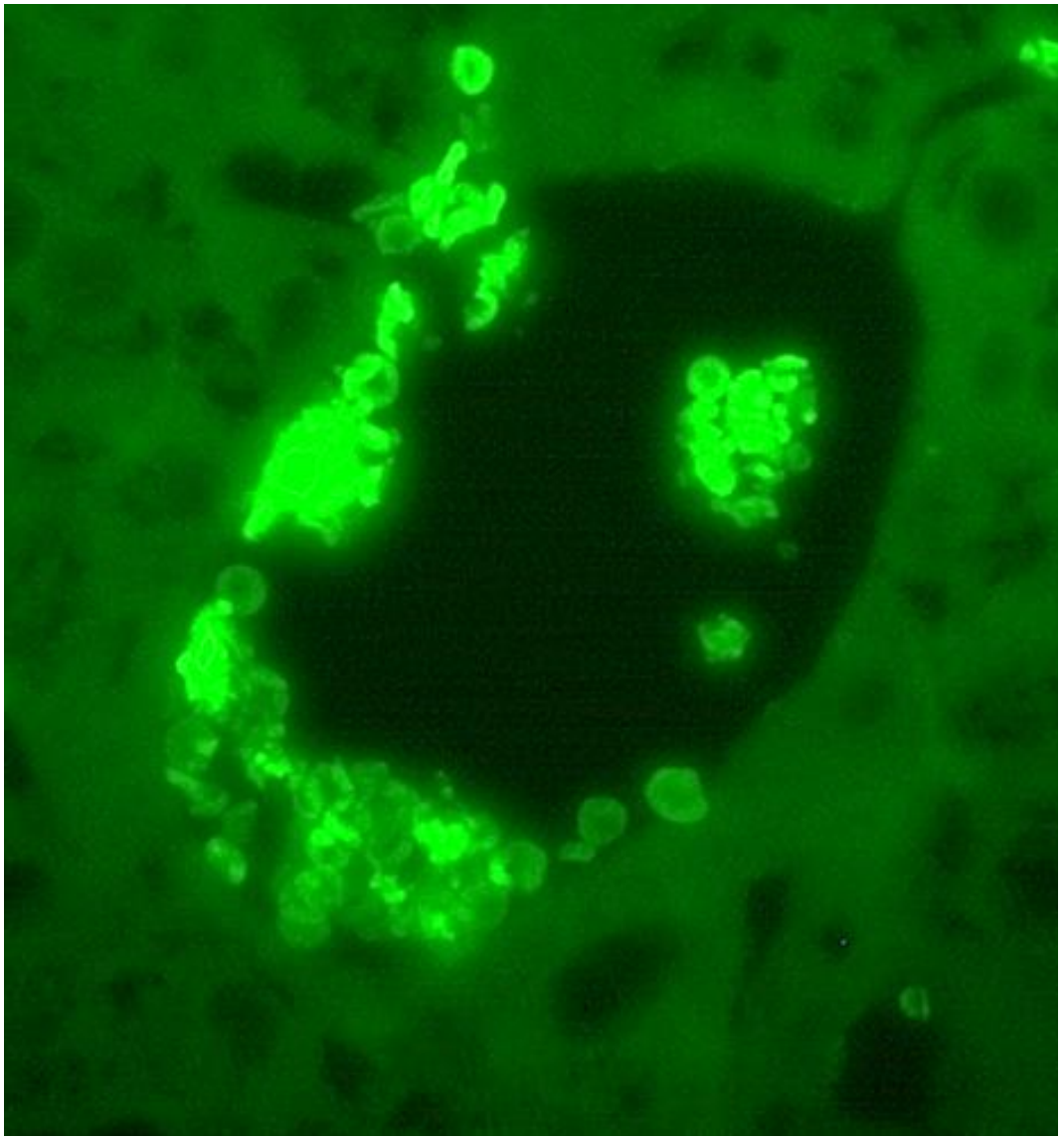
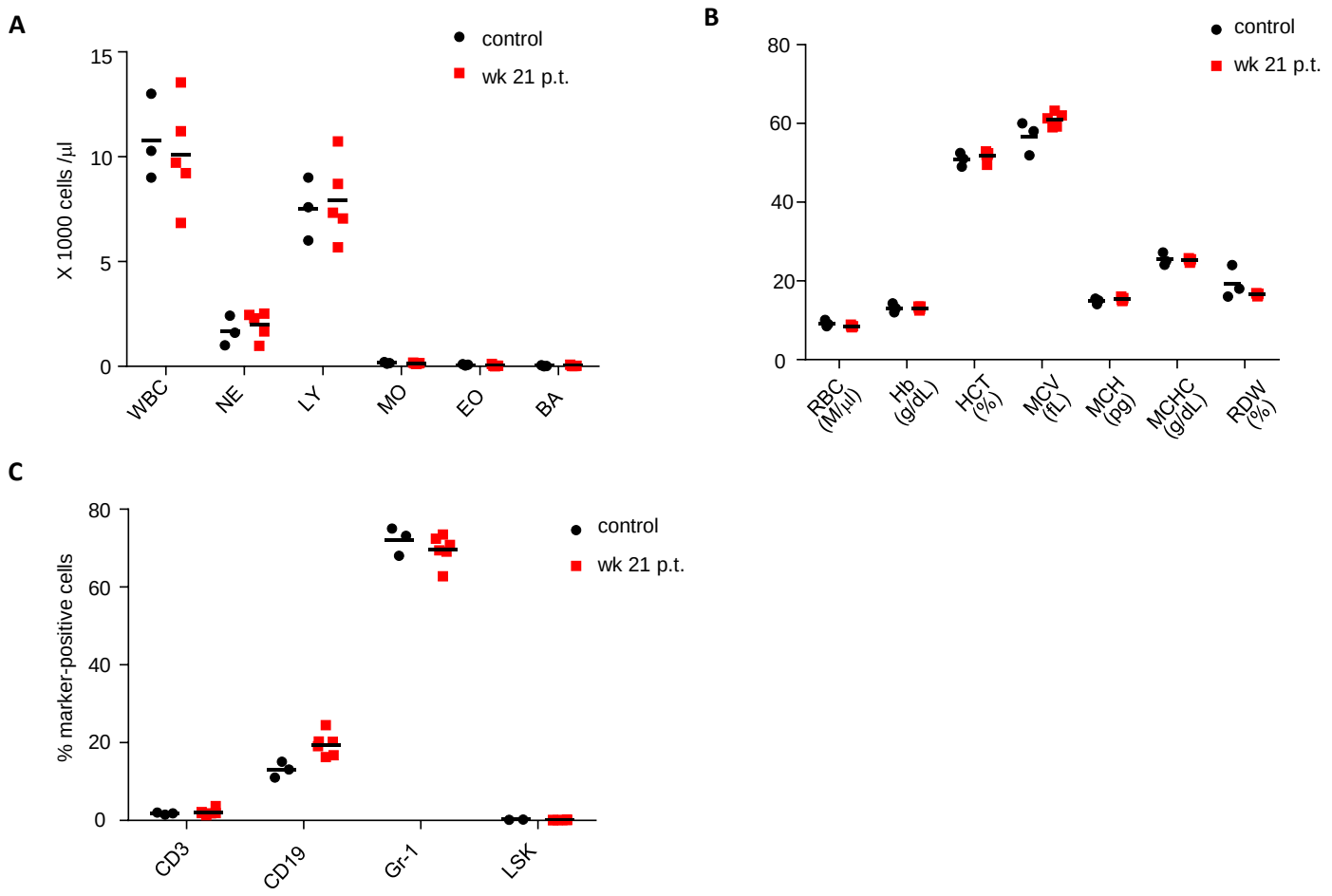



**Supplementary Figure S1. Representative flow cytometry plots of GFP positive cells in different tissues (week 21 post *in vivo* HSC transduction).**

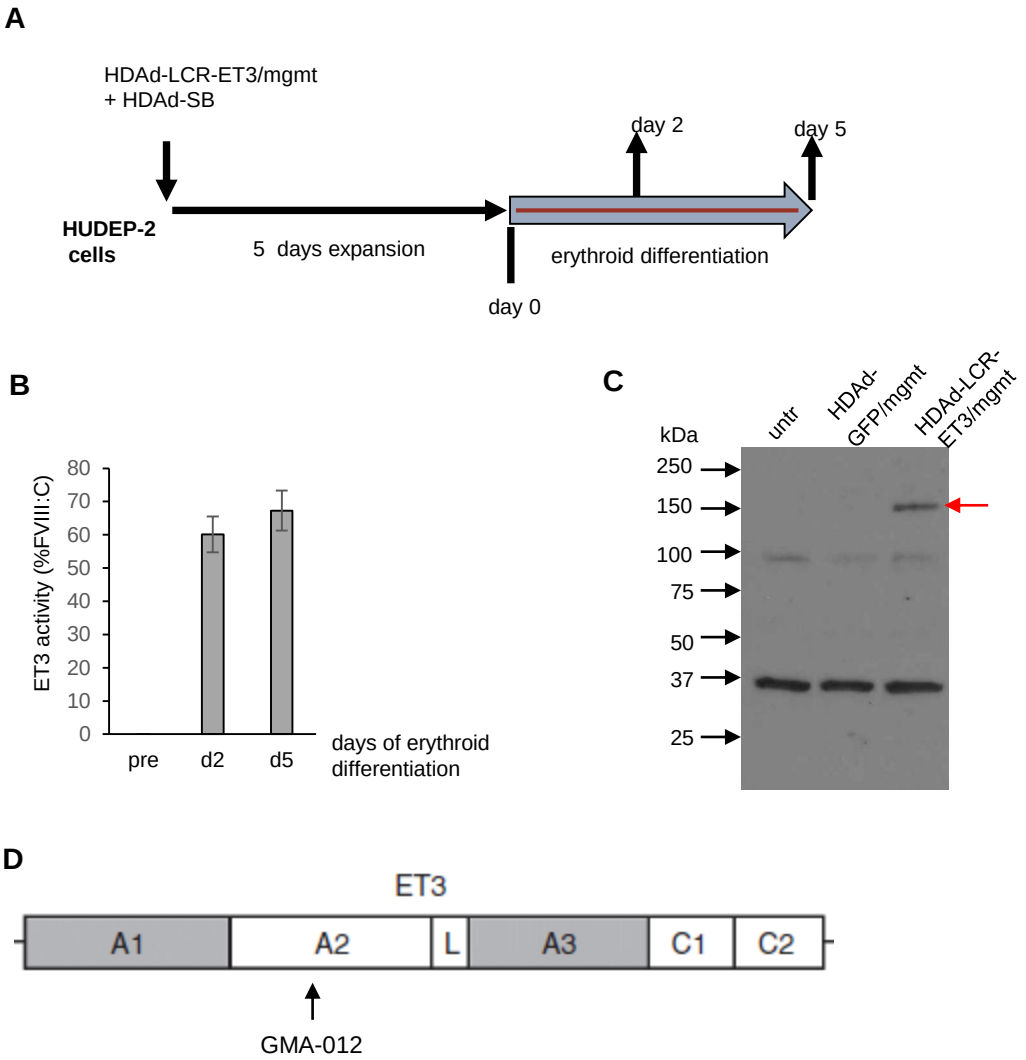


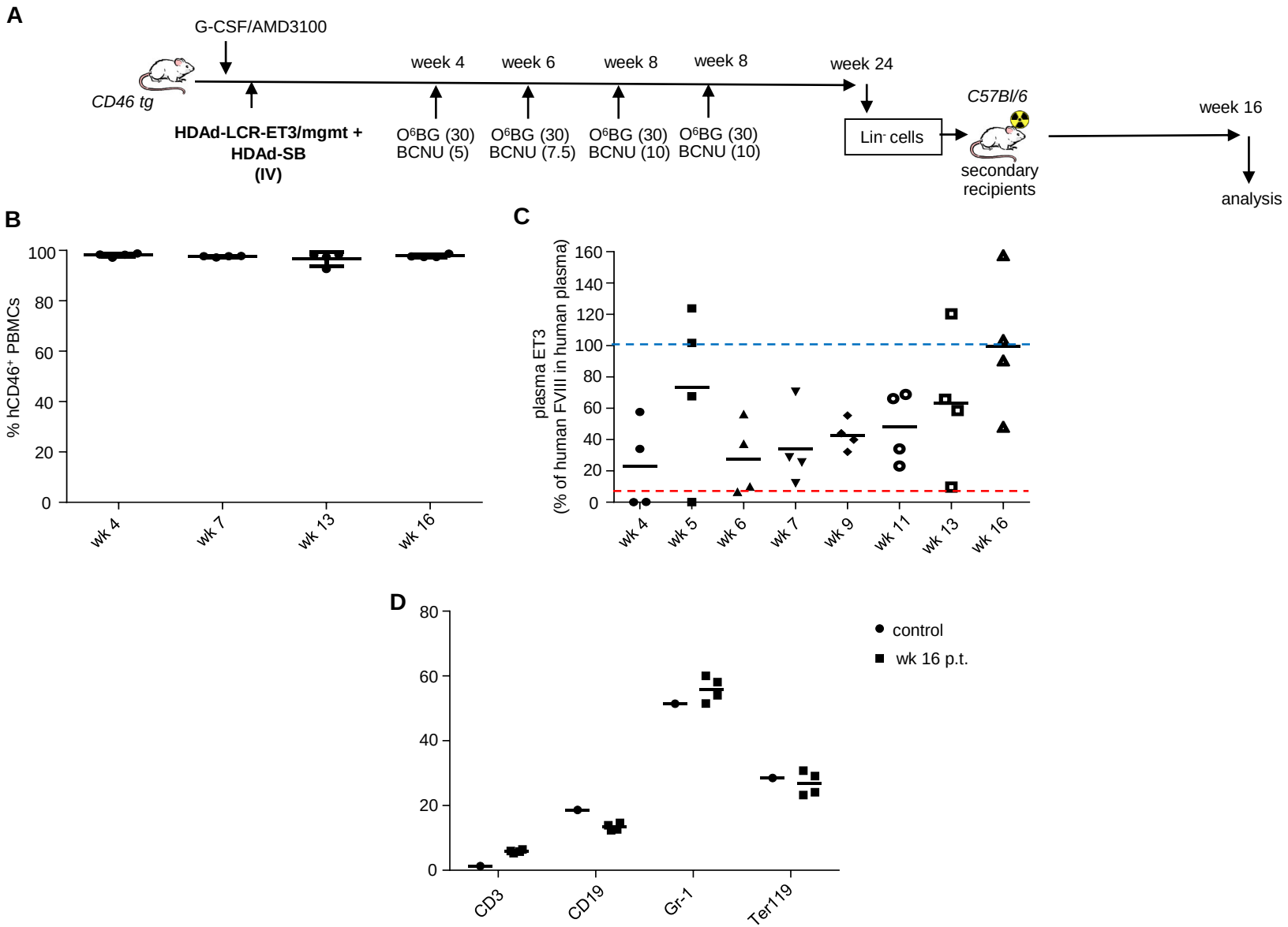
**Supplementary Figure S2. Representative GFP fluorescence on a liver section (week 21 post *in vivo* HSC transduction).** The scale bars are 500μm. Erythrocytes are visible in a large venous vessel and blood vessels in portal triads.



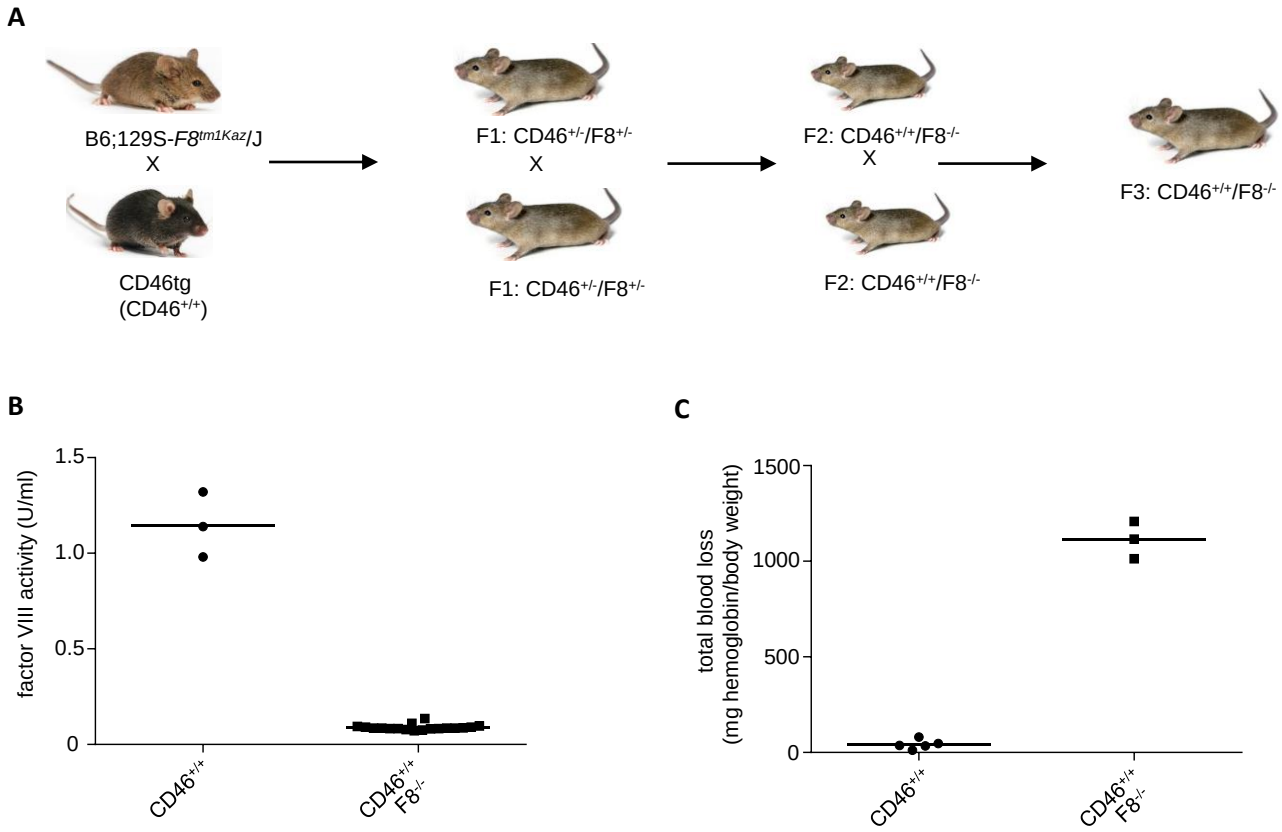

**Supplementary Figure S3. Hematology in mice after in vivo HSC transduction with HDAd-LCR-GFP (week 21 post *in vivo* HSC transduction). A)** Blood cell counts in CD46-tg mice before treatment and at week 24 after in vivo HSC transduction. **B)** Hematological parameters. RBC: red blood cells, Hb: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width. The differences between the two groups were not significant. **C)** Composition of bone marrow cell fractions.



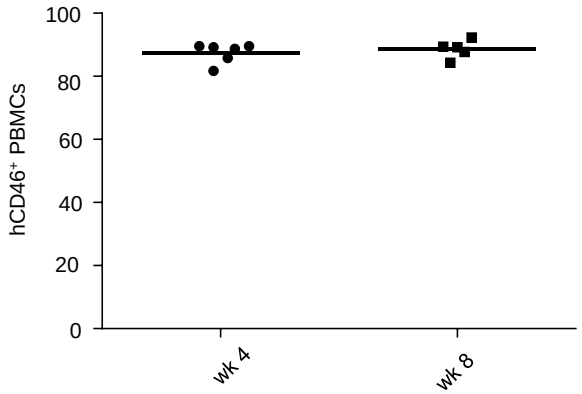

**Supplementary Figure S4. ET3 expression from immortalized erythroid progenitor HUDEP-2 cells derived from CD34⁺ cord blood cells. A)** Experimental design. HUDEP-2 cells were infected with HDAd-LCR-ET3/mgmt + HDAd-SB (1:1) at a total MOI of 2000 vp/cell. After 5 days of expansion, cells were incubated with erythroid differentiation medium. Supernatant and cell aliquots were collected at d2 and day 5 of erythroid differentiation (ED). **B)** ET3 activity measured in the supernatant by Coamatic FVIII assay before transduction ("pre") and at day 2 and 5 of ED. **C)** Cell lysates were analyzed by Western blot using an antibody against the A2 region. The specific ET3 band appears around 168 kDa. Controls include untransduced cells ("untr.") and HDAd-GFP/mgmt transduced cells. **D)** Structure of ET3 and region that is recognized by monoclonal antibody GMA-012.



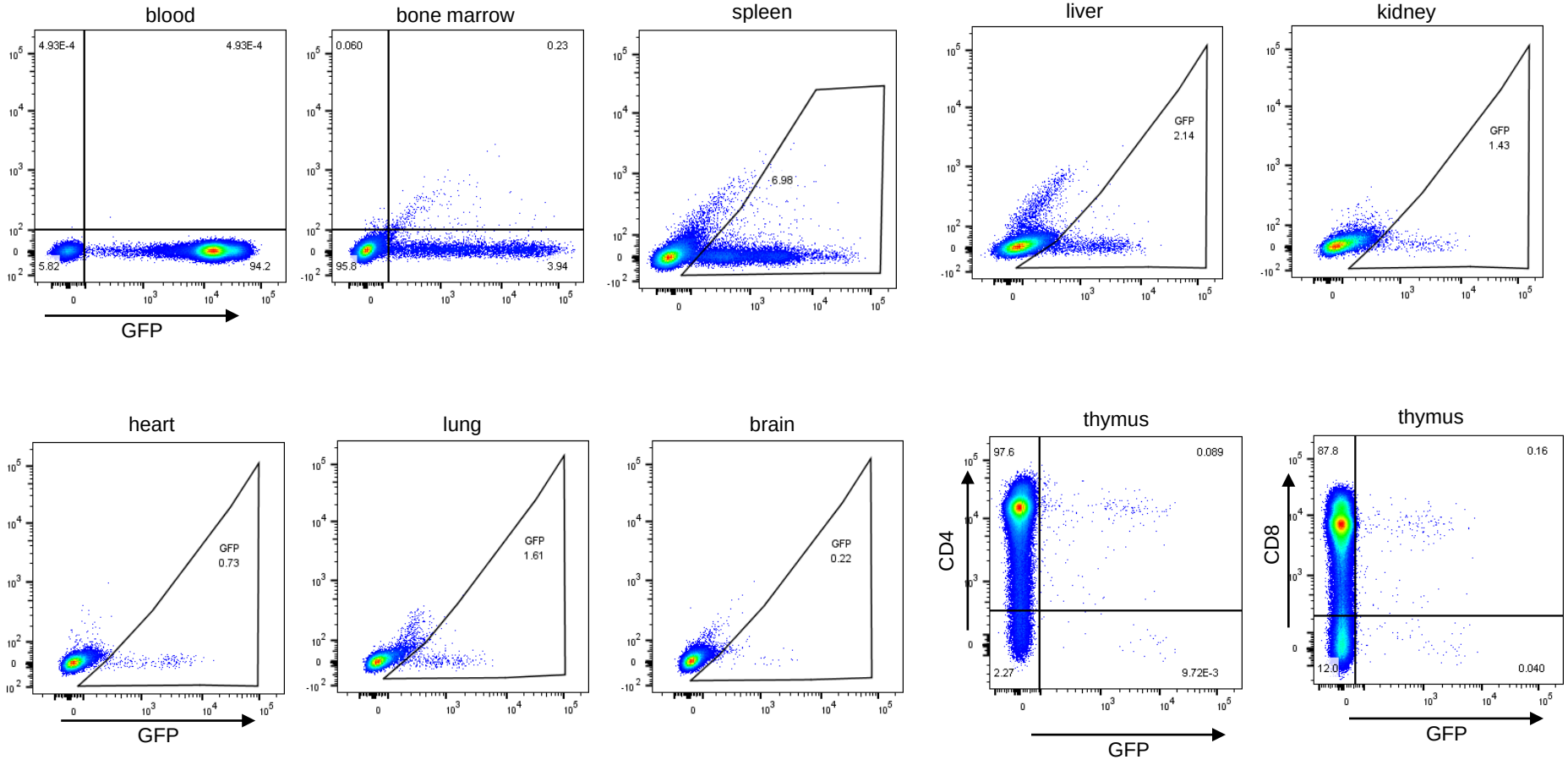

**Supplementary Figure S5. Secondary HSC transplant recipients from the study shown in Fig.2. A)** Bone marrow Lin$^-$ cells collected at week 24 after *in vivo* HSC transduction were transplanted into lethally irradiated C57Bl/6 mice, which were then kept alive without side effects for 16 weeks. **B)** Engraftment of transplanted cells based on human CD46 expression on PBMCs measured by flow cytometry. Each symbol is an individual animal. Notably, transduced donor cells expressed CD46, while recipient C57Bl/6 mice did not. **C)** Plasma concentrations of ET3 measured by ELISA. Each symbol is an individual animal. The values are expressed relative to FVIII levels in human plasma. 100% (=2180 ng/ml) would therefore correspond to 1U/ml. The dotted red line indicates 5% of physiological levels, which is considered curative for hemophilia A. **D)** Percentage of lineage-positive cells in the bone marrow in untreated C57Bl/6 mice and mice at week 16 after transplantation. The difference between the two groups is not significant.




**Supplementary Figure S6. Generation and characterization of CD46$^{+/+}$ hemophiliac mice. A)** Breeding scheme. CD46$^{+/+}$/F8$^{-/-}$ were generated through three rounds of back-crossing. **B)** ET3 activity measured in plasma of "healthy" CD46$^{+/+}$ and hemophilia CD46$^{+/+}$/F8$^{-/-}$ mice. **C)** Bleeding after tail clipping. Blood was collected in PBS over 45 min and the hemoglobin amount was determined and expressed as mg normalized to body weight.




**Supplementary Figure S7. Engraftment of ex vivo HDAd-LCR-ET3/mgmt + HDAd-SB-transduced Lin- cells.** Notably, transduced donor cells express CD46, while recipient $F8^{-/-}$ mice do not.

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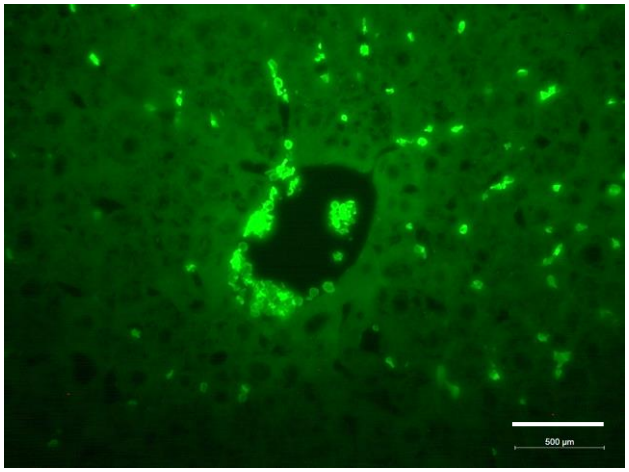


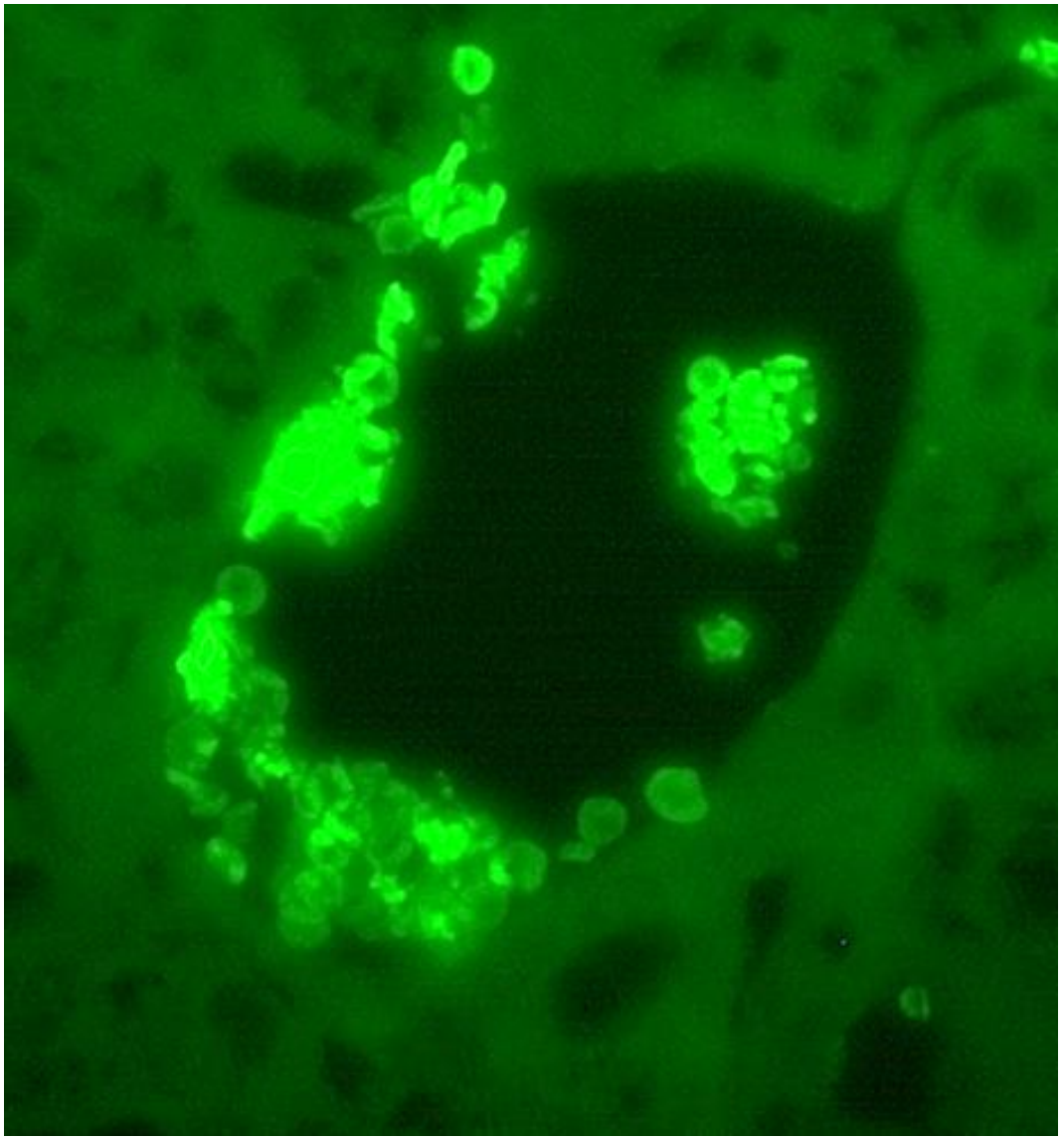

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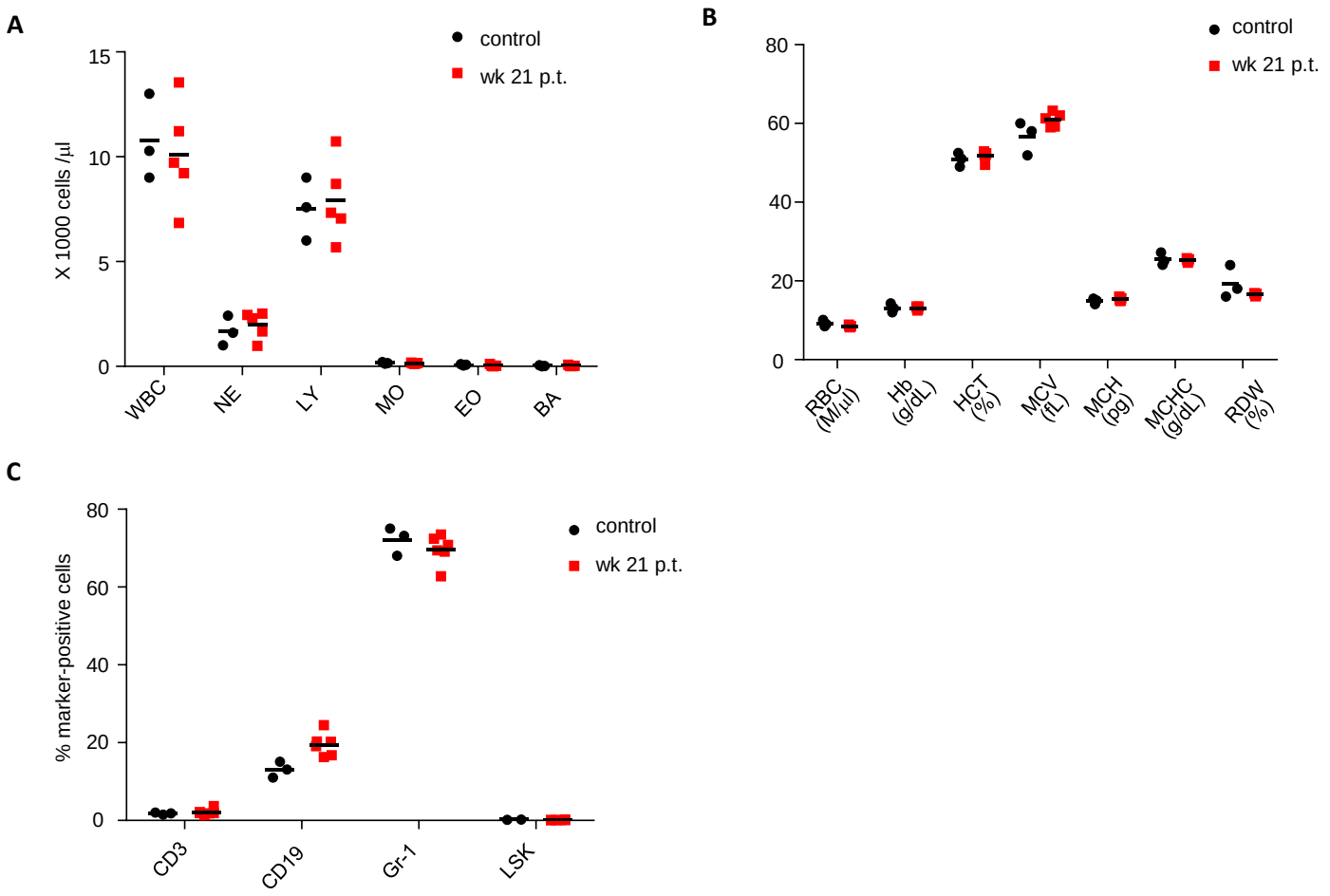


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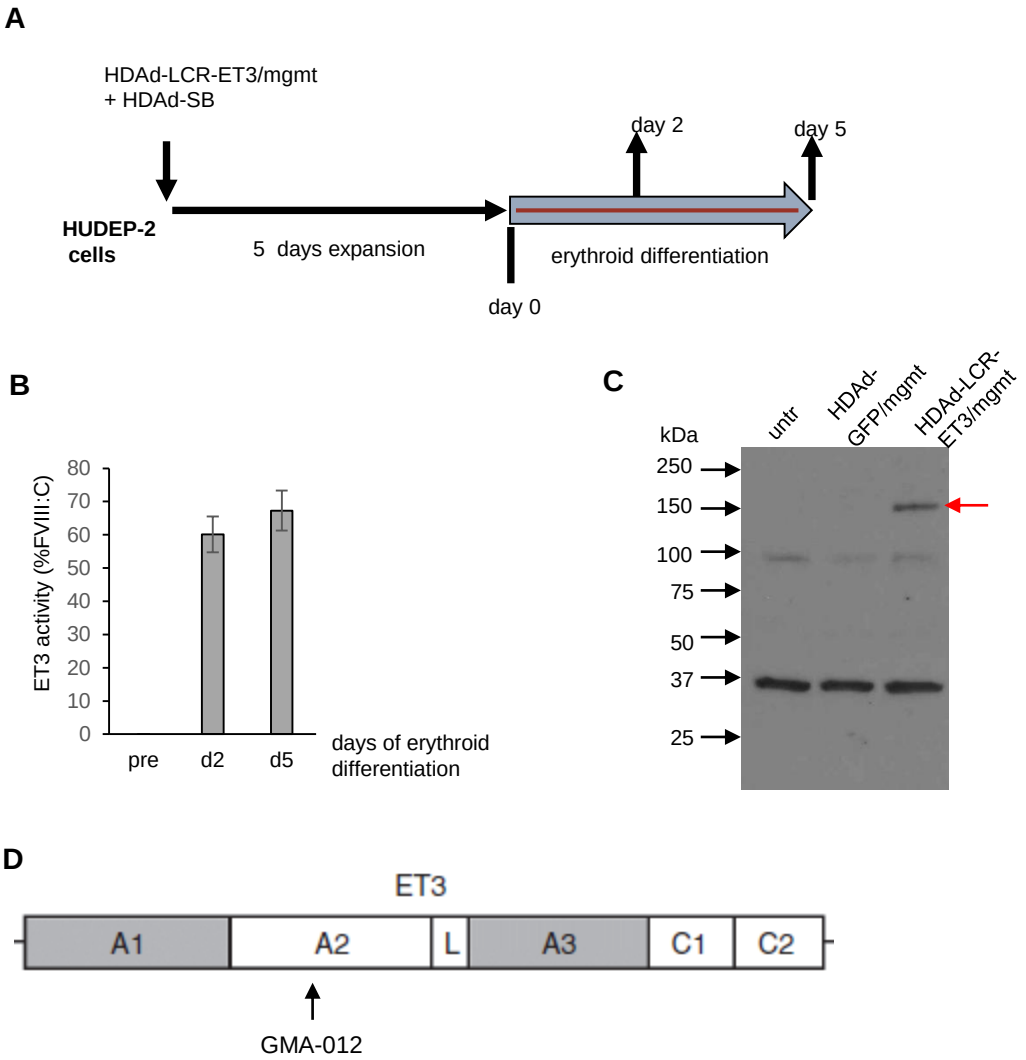


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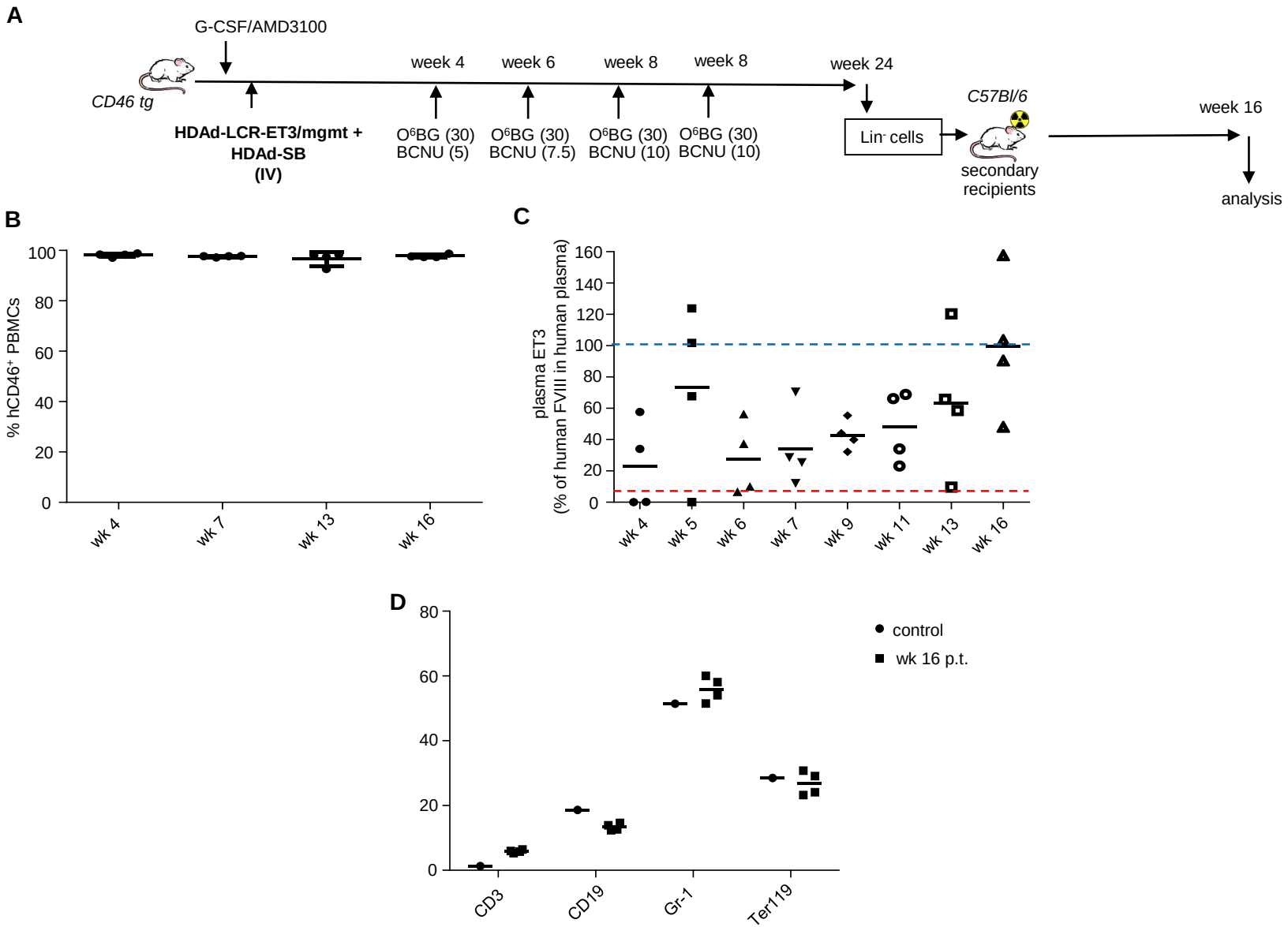


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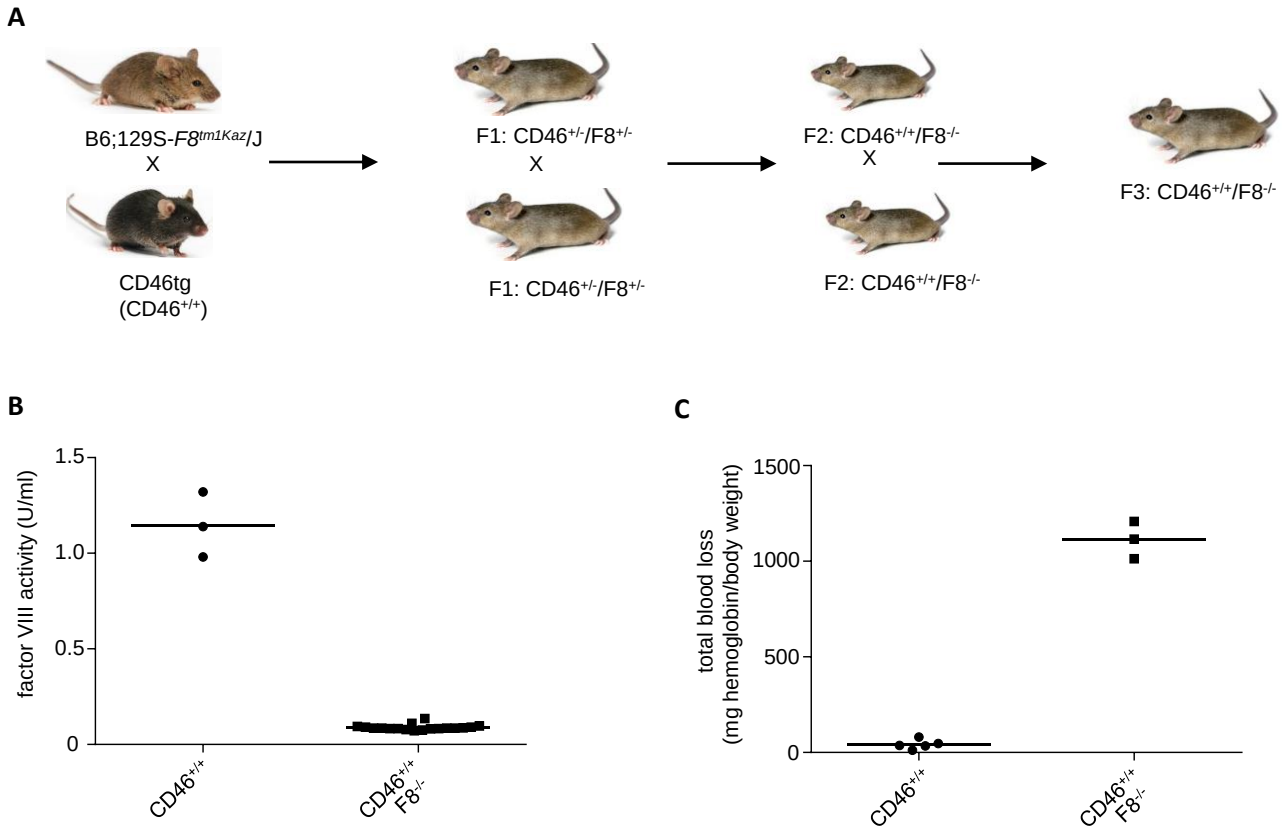


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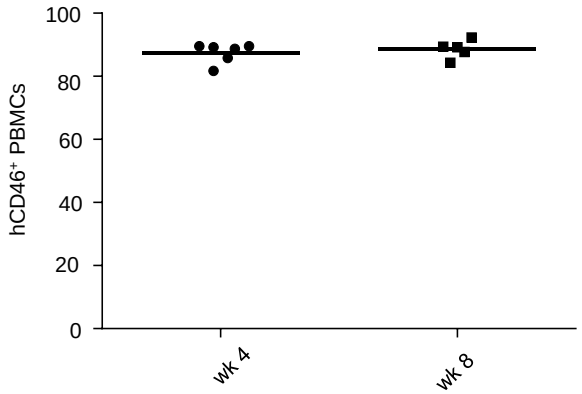


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**Supplementary Methods**

***Generation of HDAd vectors:*** HDAd-LCR-ET3/mgmt: Human β-globin 3'UTR was amplified from 293 cell genomic DNA with the following primers (Forward 5'-GATTAGCGGCCGCCACCCCACCAGTGCAGGCTGCCTATCAGAAAGT-3' and reverse 5'-GTCAAGCTTTCTAGGTATTGAATAAGAAAAATGAAGTTAAGGTGGTTGATGGTAACACTATGCT-3') and inserted into NotI/Hind3 site of the plasmid ReNeo-HP47 [14] (ReNeo-ET3-3'βUTR). The ET3-3'βUTR fragment was amplified from ReNeo-ET3-3'βUTR with primers (Forward 5'-GACTATCGATTGCTAGCTTCGAACTCGAGCCACCATGCAGCTAGAGCTCTCCACCTGTGTCTTT-3' and reverse 5'-GTCAATCGATAAGCTTTCTAGGTATTGAATAAGAAAAATGAAGTTAAGGTGGTTGATGGTAACACTATGC-3') and inserted into BstBI/ClaI sites of pBS-μLCR [5] (pBS- μLCR-ET3-3'βUTR). The EF1α-mgmt(p140k)-pA-cHS4 fragment was amplified from pBS-μLCR-γ-globin-mgmt[5] with primers (Forward 5'-CTAGAAAGCTTATCGACCCCATCCTCACTGACTCCGTCCTGGAGTT-3' and reverse 5'-TTAAGATATCACTAGTTGAGTAATTCATACAAAAGGACTCGC-3') and inserted into ClaI/SpeI sites of pBS-μLCR-ET3-3'βUTR by In-fusion HD cloning kit (Clontech) (pBS- μLCR-ET3-mgmt). The expression cassette μLCR-ET3-3'βUTR-cHS4-mgmt was released by PacI and inserted into plasmid pWEHCA-μLCR-γ-globin-mgmt-FRT2 plasmid [5] by replacing the γ-globin-mgmt cassette (pWEHCA-μLCR-ET3-mgmt-FRT2). The transposon contained ET3 and mgmt genes. The resulting plasmids were packaged into phages using Gigapack III plus packaging Extract (Agilent Genomics) and propagated.
HDAd-LCR-GFP/mgmt: To generate the μLCR-GFP-mgmt transposon vector, the ET3 sequence plasmid pBS- μLCR-ET3-mgmt was replaced by GFP (pBS- μLCR-GFP-mgmt), and the cloning strategy described above was followed resulting in pWEHCA- μLCR-GFP-mgmt.

***Animal studies.*** All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. The University of Washington is an Association for the Assessment and Accreditation of Laboratory Animal Care International (AALAC)–accredited research institution and all live animal work conducted at this university is in accordance with the Office of Laboratory Animal Welfare (OLAW) Public Health Assurance (PHS) policy, USDA Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals and the University of Washington's Institutional Animal Care and Use Committee (IACUC) policies. The studies were approved by the University of Washington IACUC (Protocol No. 3108-01). Mice were housed in specific-pathogen-free facilities.

***Isolation of lineage-depleted (Lin$^-$) bone marrow cells:*** For the depletion of lineage-committed cells, the mouse lineage cell depletion kit (Miltenyi Biotec, San Diego, CA) was used according to the manufacturer's instructions.

***Colony forming unit assay.*** A total of 2500 Lin$^-$ cells were plated in triplicates in ColonyGEL 1202 mouse complete medium (ReachBio, Seattle WA) and incubated for 12 days at 37 °C in 5 % $CO_2$ and maximum humidity. Colonies were enumerated using a Leica MS 5 dissection microscope (Leica Microsystems).

***Real-time reverse transcription PCR for ET3 mRNA:*** Total RNA was extracted from 50-100 μL blood by using TrIzol™ reagent (Thermo Fisher Scientific) following the manufacturer's phenol-chloroform extraction method and reverse transcribed to generate cDNA using the Quantitect reverse transcription kit from Qiagen. Potential genomic DNA contamination was eliminated by treatment of the RNA samples with gDNA wipe-out reagents provided in the kit. Comparative real-time PCR was performed using Power SYBR Green PCR master mix (Applied Biosystems) and run on a StepOnePlus real-time PCR system (Applied Biosystems). The following primer pairs were used: mouse RPL10 (house-keeping) forward, 5'-

TGAAGACATGGTTGCTGAGAAG-3', and reverse, 5'-GAACGATTTGGTAGGGTATAGGAG-3'; ET3 forward, 5'-ATGGCCATCAGTGGACTCTC-3', and reverse, 5'- ATCCTCAGGGCAATCTGGT-3'.
ET3 mRNA in tissues: Before tissue harvest, blood was flushed from the circulation with PBS using the heart as a pump. Mouse tissues (brain, lung, liver, kidney, spleen, heart and thymus) were collected, and smashed through a 70 μm cell strainer using the plunger from a 5ml syringe. Single cell suspensions were washed and treated with BD Pharm Lyse™ lysing buffer (BD Biosciences) to lyse erythrocytes. Total RNA was extracted from ~1 x $10^6$ cells and analyzed as described above.

***Measurement of vector copy number:*** Total DNA from bone marrow cells was extracted using the Quick-DNA miniprep kit (Zymo Research). Viral DNA extracted from HDAd-FVIII(ET3)/mgmt virus was serially diluted and used for a standard curve. qPCR was conducted in triplicate using the power SYBR Green PCR master mix on a StepOnePlus real-time PCR system (Applied Biosystems). 9.6 ng DNA (9600 pg/6 pg/cell = ~1600 cells) was used for a 10 μL reaction. The following primer pairs were used: ET3 forward, 5'-ATGGCCATCAGTGGACTCTC -3', and reverse, 5'- ATCCTCAGGGCAATCTGGT -3'.

***ELISA for plasma ET3:*** Greiner Microlon 96-well plates were incubated overnight at 4 °C with 25 μl of capture antibody (clone GMA-8011, Green Mountain, Burlington, VT) in coating buffer (0.1 M sodium-carbonate buffer pH9.6) to a final concentration of 1.5 μg/ml. The coated plate was washed twice with washing buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05 % Tween-80, 0.05 % sodium azide, pH7.4), and blocked with blocking buffer (washing buffer with 2% BSA) for at least two hours at 37 °C. All plasma samples and standards (recombinant ET3) were diluted in dilution buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 0.05% Tween-80), added to the 96-well plate, and incubated for 2 hours at 37°C. The plate was then washed three times with washing buffer and incubated with 1 μg/ml factor VIII detection antibody (clone GMA-8016, Green Mountain) labeled with biotin (Mix-n-Stain™ Biotin Antibody Labeling Kit, Sigma) for one hour at room temperature. The plate was washed two times, incubated with streptavidin-conjugated horse-radish peroxidase (Pierce, cat. #21140) for one hour at 37 °C, and developed with 1-Step Ultra TMB-ELISA substrate (Thermo Scientific). The absorbance was measured at 450 nm

**Chromogenic activity assay for plasma ET3:** Mouse blood was collected by submandibular bleeding and mixed with 10% (v/v) of 0.109 M sodium citrate. Plasma was obtained by centrifugation at 1500 x g for 15 minutes at room temperature. The plasma fractions were either used fresh or stored at -80 °C. The ET3 activity in mouse plasma were measured by Chromogenix Coamatic assay (Diapharma, West Chester, OH) following the manufacturer's instructions. Calibrator plasma prepared from a pool of citrated human plasma collected from a minimum of 20 donors was purchased from Affinity Biologicals (Ancaster, Ontario, Canada), and used to generate the standard curve. Plasma from normal hCD46 transgenic mice has a similar activity as the calibrator plasma (~1 IU/ml). ET3 activity in untreated hemophilia A (B6;129S-$F8^{tm1Kaz}$/J) mice was undetectable.

***ET3 Western blot:*** Blood was collected by submandibular bleeding. Plasma was separated by centrifugation at 1500 x g for 15 minutes at room temperature. Total blood cells or bone marrow cells were washed three times with ice cold PBS, resuspended in 1 ml lysis buffer (300 mM NaCl, 50 mM Tris pH7.5, 1 % Triton X-100, protease inhibitor), vortexed for 1 min, and incubated on ice for 30 minutes. The lysate was then precleared by pre-incubation with 50 μl protein A Dynabeads (Invitrogen) at 4 °C for 30 minutes with rotation. To prepare the bead-antibody conjugate, 50 μl protein A Dynabeads were incubated with 1 μg anti-Factor VIII antibody (clone GMA-012, Green Mountain) and 1-2 μg of anti-Factor VIII polyclonal antibody (ab61370, Abcam) in 200 μl PBS with 0.05 % (v/v) Tween-20 (PBS-T) buffer, at room temperature for 20 minutes. The antibody conjugated beads were then washed with PBS-T buffer twice and incubated with precleared lysate at 4°C overnight with rotation. Then, the beads were washed

five times with 200 µl PBS-T buffer and eluted with elution buffer (15 µl 50 mM glycine pH2.8, 15 µl Laemmli buffer with fresh β-mercapto-ethanol) by incubation at 70 °C for 10 minutes. Eluted proteins were separated by polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The blot was blocked in blocking buffer (TBS-3 % milk) overnight, and then incubated with anti-FVIII monoclonal antibody (clone GMA-012, Green Mountain). To visualize binding, the blot was incubated with goat anti-mouse immunoglobin G (IgG)-horseradish peroxidase (HRP) (BD Pharmingen) and developed with ECL Prime Western Blotting Detection Reagent (Amersham).

***Vector copy number in single colonies:*** Lin$^-$ bone marrow cells were isolated from total mouse bone marrow cells. 750 Lin$^-$ cells per 35 mm dish were plated in ColonyGELTM 1202 Mouse Complete Medium (Reachbio, Seattle). Fifteen days later, well-spaced colonies were aspirated carefully with a pipette tip and washed with 1 ml of PBS. The cell pellets were incubated with 10 µl proteinase K (Thermo Fisher) in lysis buffer (50 mM KCl, 50 mM Tris-HCl (pH8.0), 2.5 mM EDTA, 0.45 % NP-40, 0.45 % Tween-20) at 55 °C overnight, followed by 10 min at 95 °C. Samples were diluted to 100-200 µl and 4.8 µl DNA was used in a 10 µl reaction. Transgene-specific primers (see ET3 primer above) were used to measure integrated vector copy number. Mouse *GAPDH* (forward, 5'-TTCCATCCTCCAGAAACCAG-3', and reverse, 5'-GTTCTTCTCGGGCAAAAATG-3') was used as a single copy control gene (2 copies/cell). Transgene-specific signals were normalized to *GAPDH* (taken as 2 copies per cell, which allowed us to calculate the cell number in the given DNA preparation). Mouse bone marrow DNA was serially diluted and served as standard curve.

***GFP immunofluorescence analysis on tissue slides:*** Tissues were fixed in formalin overnight (o/n) and subsequently incubated for 2 hours in PBS and 5 %, 10 %, and 20 % sucrose. Tissues were then embedded in optimal cutting temperature compound (OCT) and sectioned (6 µm). Sections were incubated with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence microphotographs were taken at room temperature on a Leica DMLB microscope (Leica, Wetzlar, Germany), with a Leica DFC300FX digital camera and Leica Application Suite (v.2.4.1) R1 (Leica Microsystems, Heerbrugg, Switzerland). No specific feature within images shown in was enhanced, obscured, moved, removed, or introduced.

***Hematological analyses:*** Blood samples were collected into EDTA-coated tubes, and analysis was performed on a HemaVet 950FS (Drew Scientific). Peripheral blood smears were prepared and stained with May-Grünwald/Giemsa (Merck) for 5 minutes.

***Flow cytometry:*** Cells were resuspended at $1 \times 10^6$ cells/100 µL in FACS buffer (PBS supplemented with 1 % heat-inactivated FBS) and incubated with FcR blocking reagent (Miltenyi Biotech, Auburn CA) for ten minutes on ice. Next the staining antibody solution was added in 100 µL per $10^6$ cells and incubated on ice for 30 minutes in the dark. After incubation, cells were washed once in FACS buffer. For secondary staining the staining step was repeated with a secondary staining solution. After the wash, cells were resuspended in FACS buffer and analyzed using a LSRII flow cytometer (BD Biosciences, San Jose, CA). Debris was excluded using a forward scatter-area and sideward scatter-area gate. Single cells were then gated using a forward scatter-height and forward scatter-width gate. Flow cytometry data were then analyzed using FlowJo (version 10.0.8, FlowJo, LLC). For flow analysis of LSK cells, cells were stained with biotin-conjugated lineage detection cocktail (Miltenyi Biotec, San Diego, CA) and antibodies against c-Kit and Sca-1 as well as APC-conjugated streptavidin. Other antibodies from eBioscience (San Diego, CA) included anti-mouse LY-6A/E (Sca-1)-PE-Cyanine7 (clone D7), anti-mouse CD117 (c-Kit)-PE (Clone 2B8), anti-mouse CD3-APC (clone 17A2), anti-mouse CD19-PE-Cyanine7 (clone eBio1D3), and anti-mouse Ly-66

(Gr-1)-PE, (clone RB6-8C5). Other antibodies from Miltenyi Biotec included anti-human CD46-APC (clone: REA312). Anti-mouse Ter-119-APC (clone: Ter-119) was from Biolegend (San Diego, CA).