Supplemental Methods

Flow cytometry

Monoclonal antibodies used for mouse depletion/sorting studies: CD3 Pe-Cy5 (clone 145-2c11), CD3 APC (clone 145-2c11), CD4 Pe-Cy5 (clone GK1.5), CD4 PerCyP-Cy5.5 (clone RM4.5), CD8 Pe-Cy5 (clone 53-6.7), CD8 APC-Cy7 (5H10), CD19 Pe-Cy7 (clone 1D3), Mac-1 Pe-Cy5 (clone M1/70), Mac-1 Alexa Fluor (clone M1/70), Gr-1 Pe-Cy5 (clone RB6-8C5), Gr-1 PB (clone RB6-8C5), NK1.1 PE (clone PK136), c-Kit APC-Cy-7 (clone 2B8), Sca1 Pe-Cy7 (clone D7), CD48 APC (clone HM48-1), CD150 PB (clone TC15-12 F 12.2), CD16/32 PE (clone 93), CD34 FITC (clone RAM34), CD127 PB (clone A7R34), Flt3 PE (clone A2F10).

Monoclonal antibodies used for chimerism analyses: CD45.1 FITC (clone A20), CD45.2 PB (clone 104), CD3 APC-Cy7 (clone 145-2c11), CD19 APC (clone 1D3), Gr-1 PE (clone RB6-8C5), Mac-1 Pe-Cy7 (clone M1/70), Ter-119 Pe-Cy5 (clone TER-119), NK1.1 Alexa Fluor (clone PK136).

Monoclonal antibodies used for *in vitro* human HSC-sorting experiments: CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, CD235a (all in Pe-Cy7), CD38 PE-Cy7 (clone HIT2), CD90 PE (clone PR13), CD34 APC (clone 563), CD45RA Pac Blue (clone HI100).

Ki67 intracellular staining

For the Ki67 analyses cells were harvested and lineage depleted as described in the main methods. Around 2.5 x 10^6 cells were stained with antibodies that mark the different HSPC subsets for 20 min on ice. Zombie Aqua fixable viability dye (BioLegend, Cat. # 423101) was added to the

staining combination. Stained cells were washed with FACS buffer, fixed with Cytofix/Cytoperm buffer (BD Biosciences) for 1h at 4°C and washed twice with 1X PermWash buffer (BD Biosciences). Cells were then stained with Ki67 FITC antibody (clone B56, BD Biosciences) or isotype control in concentration 1/10, incubated for 1h at RT and washed twice with 1X PermWash buffer. Analysis was performed on BD FACS Aria II.

Bromodeoxyuridine (BrdU) *in vivo* incorporation assay

BrdU (10mg/ml) was purchased from BD Biosciences (Cat. # 550891) and 100 µl (equal to 1 mg) was injected IP into mice every 6 hours for total of 24 hours as previously described (Passegué et al. *JEM*, Vol. 202, No 11, 2005). Mice were euthanized after the last BrdU administration and both legs were harvested, processed and lineage depleted as described above. Around 2.5 x 10^6 cells were stained with cell-surface markers and incubated for 20 min on ice protected from light.

Cells were then washed with FACS buffer, resuspended in 100 µl BD Cytofix/Cytoperm Buffer per sample and incubated on ice for 30 min. Cells were washed with 1ml BD Perm/Wash buffer, resuspended in 100 µl BD Cytoperm Plus Buffer per sample and left 10 min on ice. Cells were washed again with 1 ml BD Perm/Wash buffer and re-fixated with 100 µl µl BD Cytofix/Cytoperm Buffer for 5 min on ice. Wash step was repeated, cells were resuspended in 100 µl of DNase (1mg/ml) and incubated for 1 h at 37 °. After wash step was repeated, cells were resuspended into 50 µl of Perm/Wash buffer, cells were incubated anti-BrdU mAb (clone Bu20a) for 20 min at RT. Before analysis 20 µl of 7-AAD was added to each sample.

Hematoxylin and Eosin (H&E) staining of bone marrow sections

Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow as follows: mouse femurs were formalin fixed, embedded in paraffin, and sectioned at 4μm. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems).

***In vitro* cell culture, imaging and viability assay**

Fibronectin coated 96-well flat-bottom plates were used for the murine experiments. Briefly bovine plasma fibronectin (Sigma-Aldrich, F1141) was reconstituted in 0.1% BSA + PBS w/o Ca^{++} and Mg^{++} to a final concentration of 5μg/ml and added to each well in volume 50μl. After 1h the fibronectin solution was aspirated from each well and the plate was left for an additional 1h at RT to dry. $Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD48^{-}$ cells from the BM of B6 mice were sorted by FACS. After sorting, the cells were spun down at 0.5 x g for 5 min and the cell pellet was reconstituted in HSC media, containing serum-free StemSpan media (STEMCELL Technologies), 10 ng/mL recombinant murine SCF (PeproTech) and 100 ng/mL recombinant murine thrombopoietin (PeproTech). 100 μl of cells in HSC media were plated to a density of 2000-3000 cells/well and left overnight at 37°C, 5% CO_2 to recover from sorting. For the human HSC, *in vitro* assay frozen cord blood or bone marrow CD34+ cells were purchased from STEMCELL Technologies and thawed in warm RPMI media, containing 10% FBS. 3000-4000 $Lin^{-}CD34^{+}CD38^{-}$ were directly sorted into 96-well plates, coated with human fibronectin (Corning® BioCoat™, Cat. # 354409), containing 100 μl of serum-free StemSpan media, supplemented with 10 ng/mL human

recombinant SCF (PeproTech), 100 ng/mL human recombinant TPO (PeproTech) and 40 µg/ml human LDL (StemCell Technologies, Cat. # 02698). Plates were left overnight at 37°C, 5% CO_2.

AZA was reconstituted in HSC media and added as 2X concentration (100µl/well) to final AZA concentrations 0.1, 0.5 and 1 µg/ml at baseline and 24h of cell culture. Cell imaging and cell counting was performed every 6 h by using the ImagExpress Pico automated imaging system, equipped with environmental control (5% CO_2, 85% humidity and 37°C). After 48 h cells were stained with the EarlyTox Live/Dead Assay Kit (Molecular Devices) as per manufacturer instruction for 20 min followed by staining with 10µM Hoechst 33342 nuclear dye for another 20 min at RT in the dark. Cells were imaged using ImageXpress Pico. Hoechst staining was used to identify total cells, Calcein AM to identify live cells and Ethidium homodimer-III (EthD-III) to identify death cells. Data analysis was performed on CellReporterXpress software (ImageXpress).

Whole blood transfusion

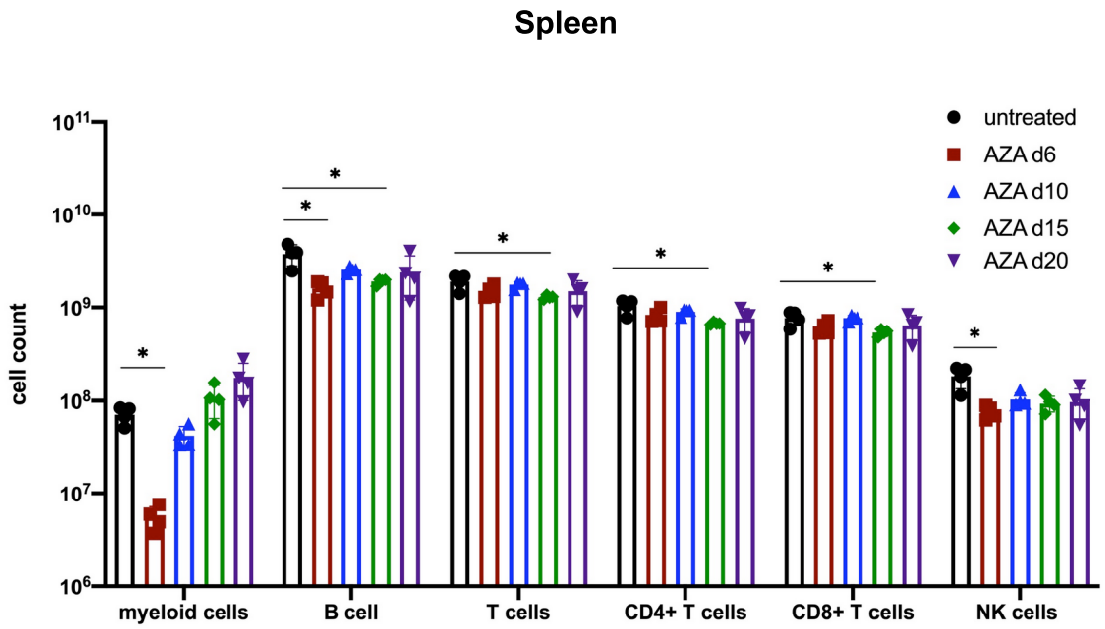
B6. Rag2cγc$^{-/-}$ mice were euthanized and 500-700 µl whole blood per mouse was collected via cardiac puncture into heparinized syringes and transferred into eppendorf tubes, containing 2 µl of 0.2 % Heparine solution (STEMCELL Technology). 100 µl of whole blood was immediately administrated via retroorbital injection in recipient mouse.

Complete blood cell count

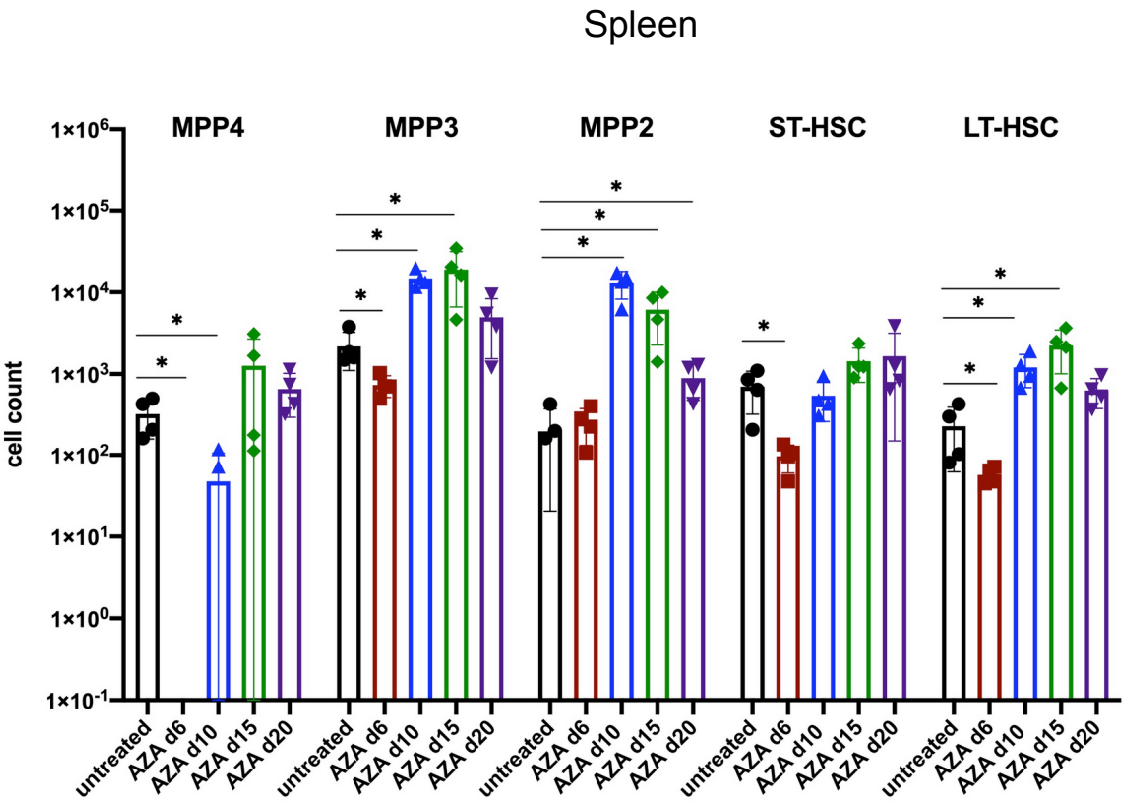
20 µl of whole blood was collected via the tail vein and complete blood counts (CBC) were conducted using Heska HemaTrue Veterinary Hematology Analyzer (Heska). For more detailed CBC analyses, including reticulocyte counts and manual differentiation, 150 µl of whole blood

was collected and processed in the Animal Diagnostic Laboratory in the Veterinary Service Center at Stanford University. Automated hematology was performed using the Sysmex XT-2000iV analyzer system. Blood smears were made for all CBC samples and reviewed by a medical technologist. Manual differentials were performed as indicated by species and automated analysis. Flagged abnormalities were additionally reviewed by a board-certified veterinary clinical pathologist.

Supplemental Figure 1



Supplemental Figure 2



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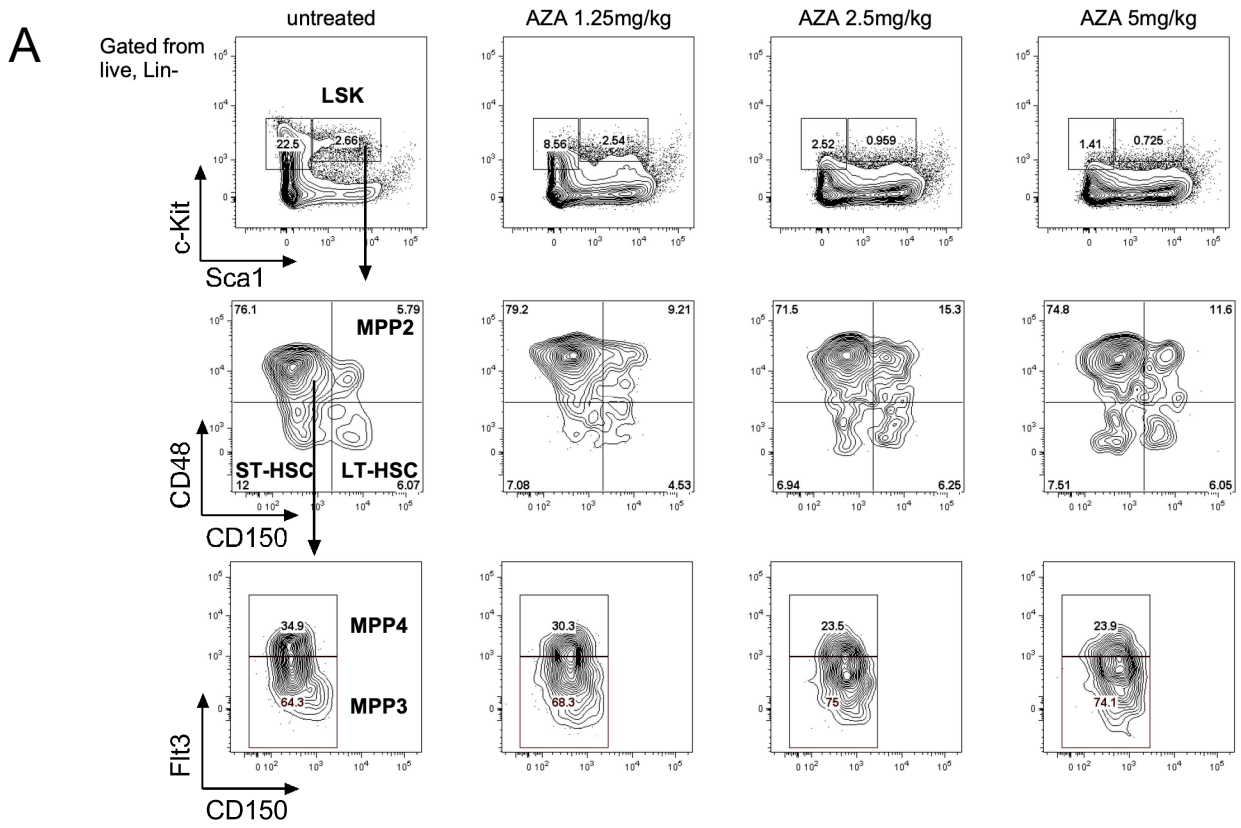

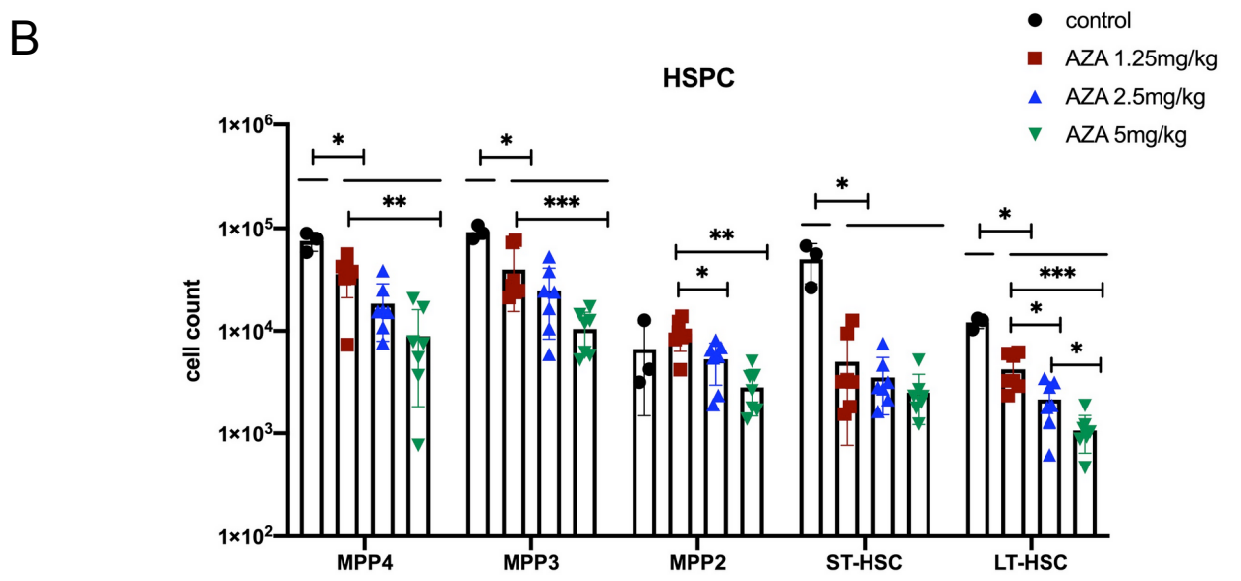

Supplemental Figure 4

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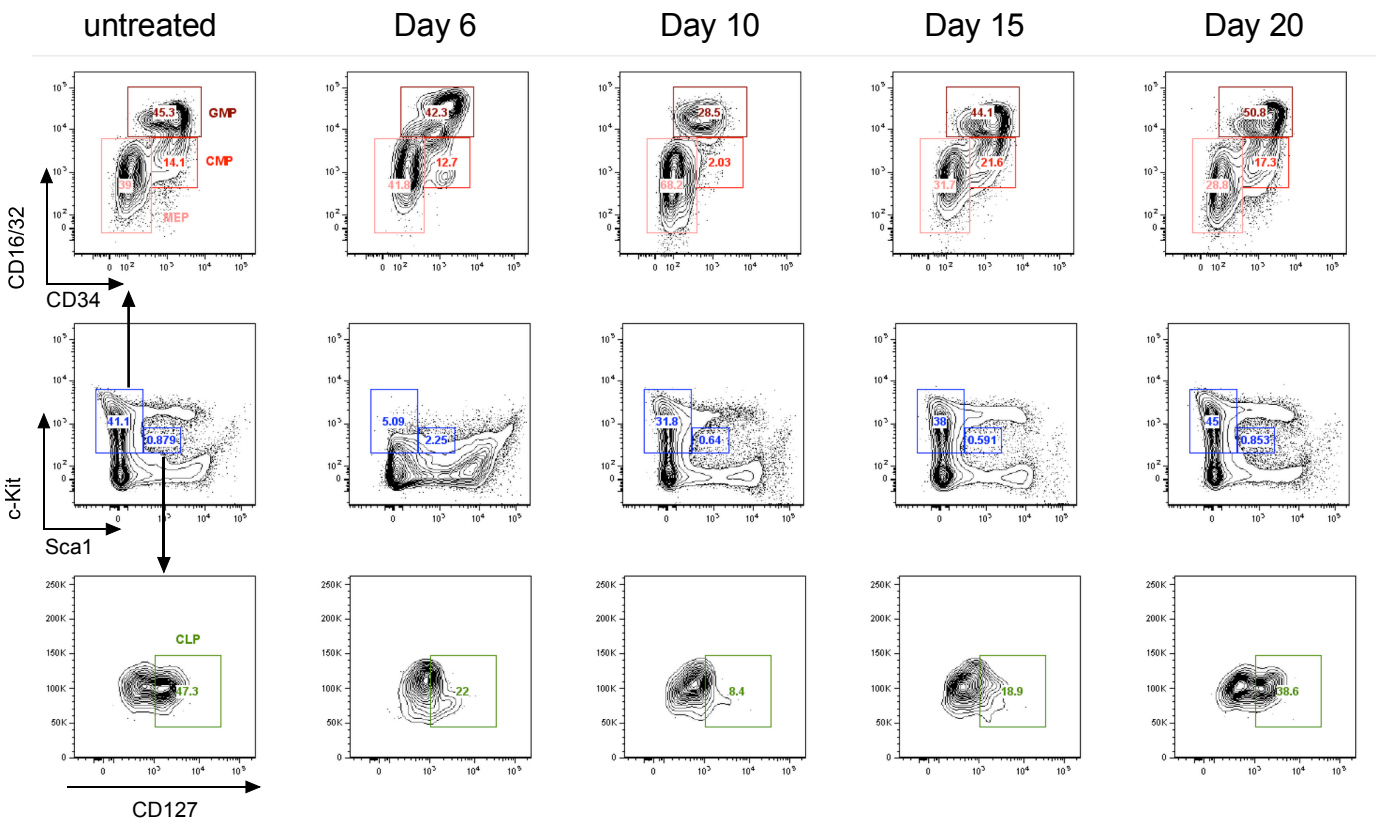

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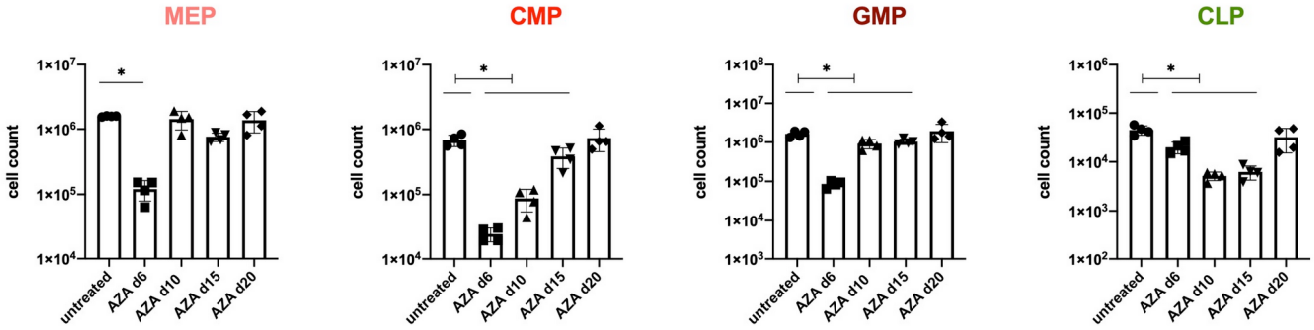

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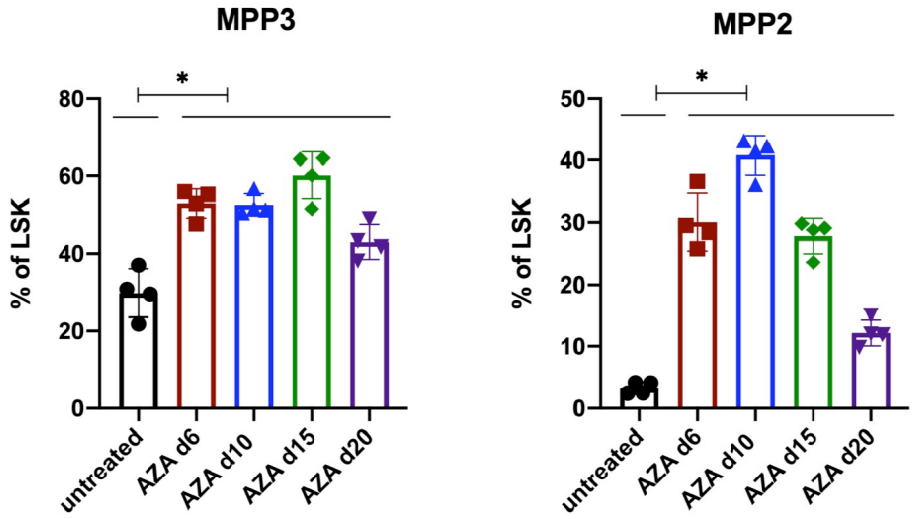

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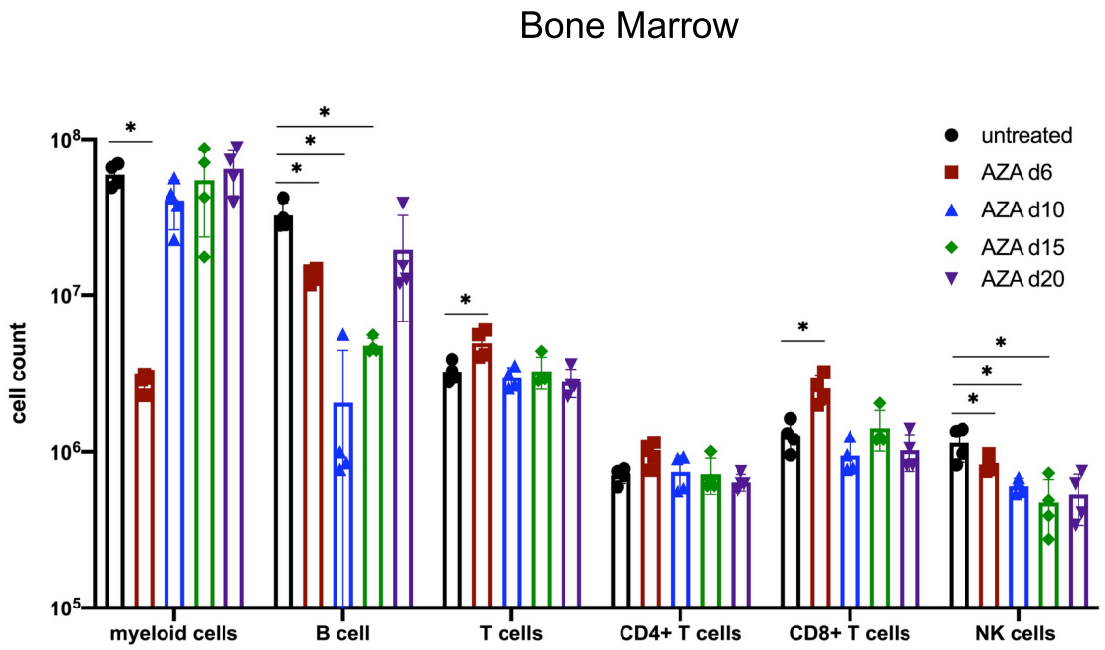
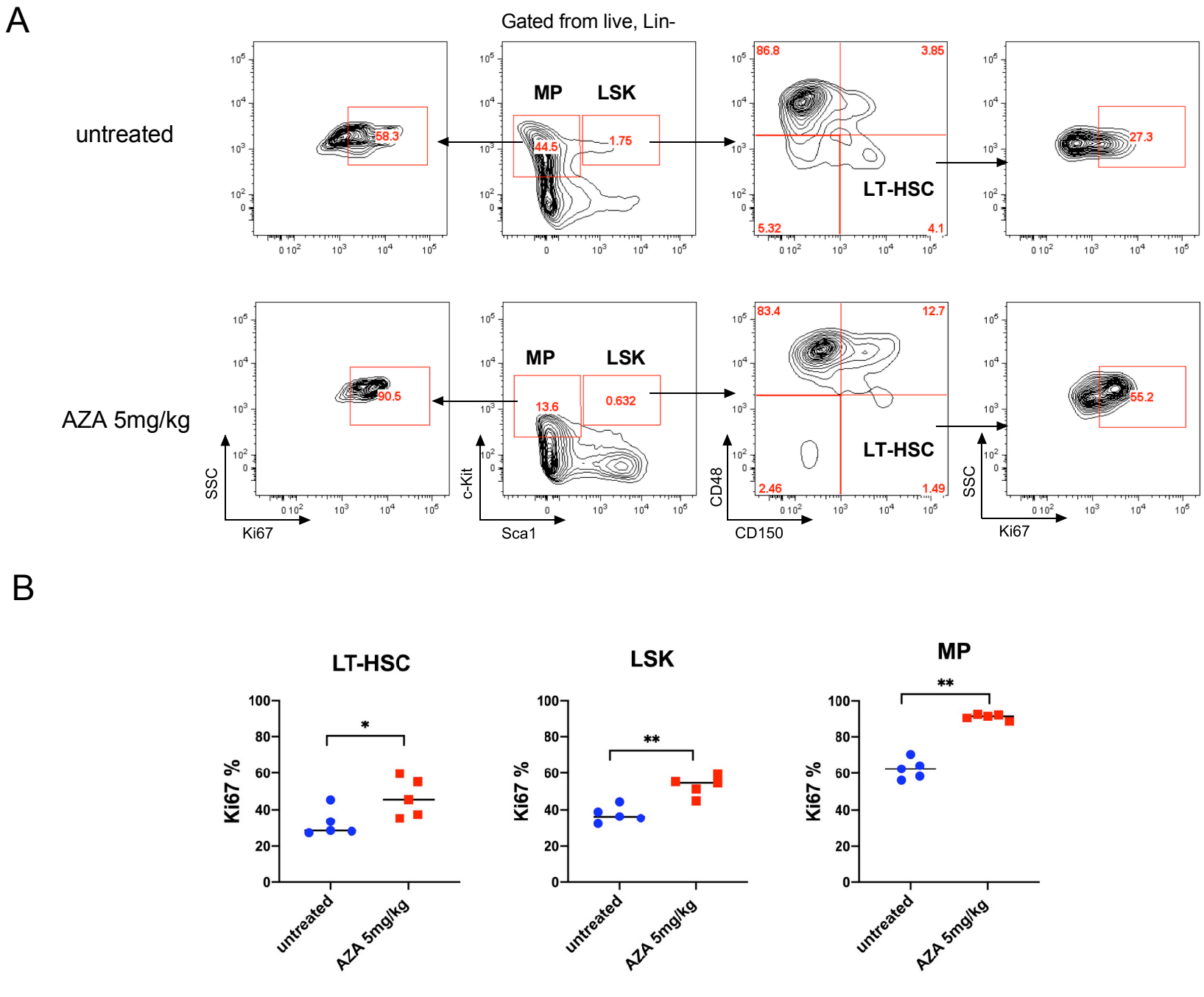


Supplemental Figure 7



Supplemental Figure 8

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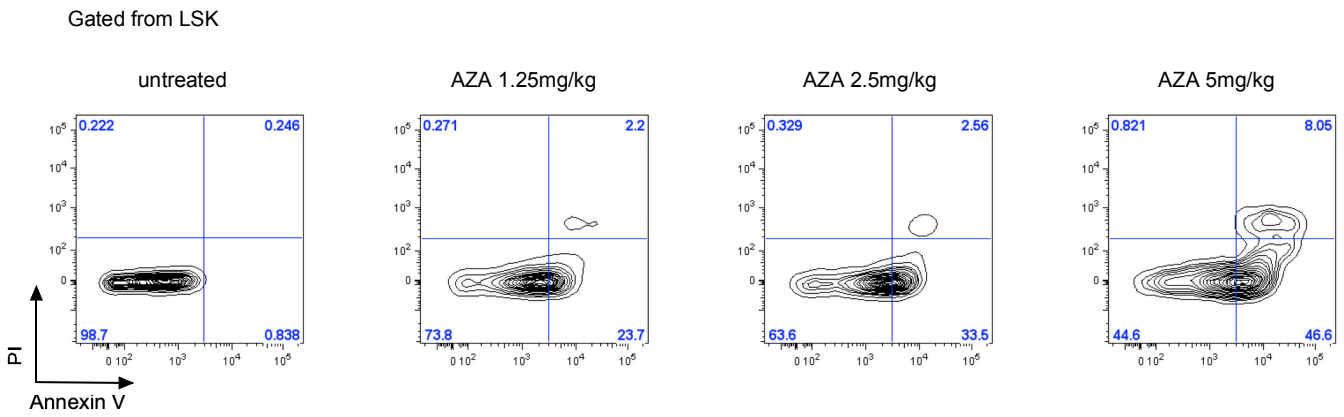

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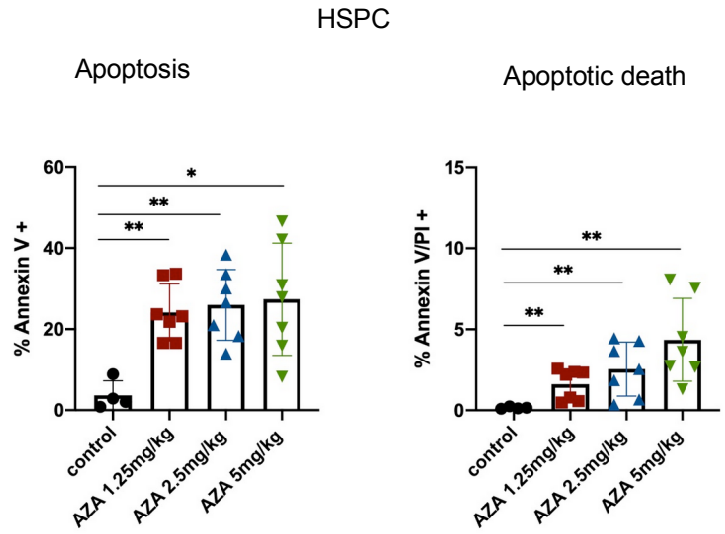

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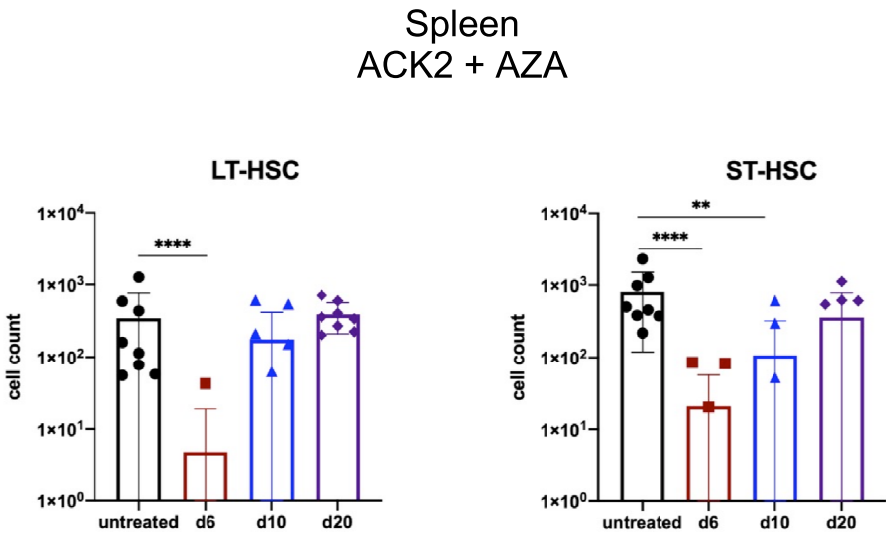

Supplemental Figure 10

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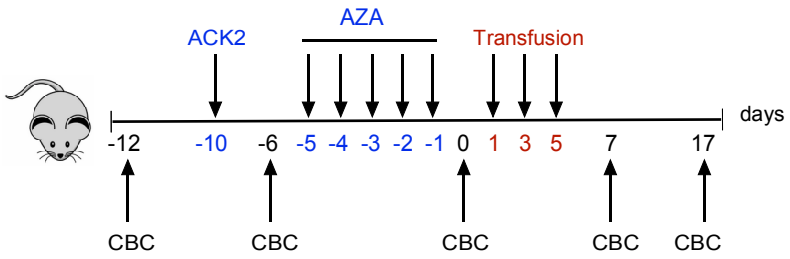

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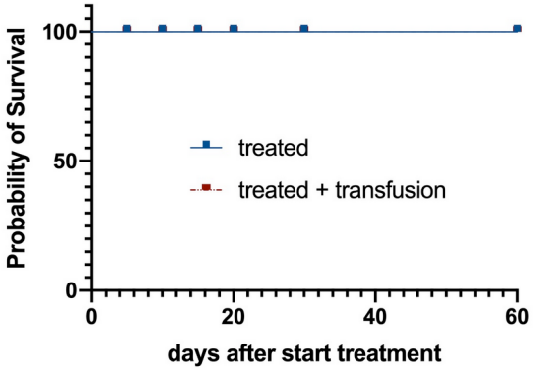

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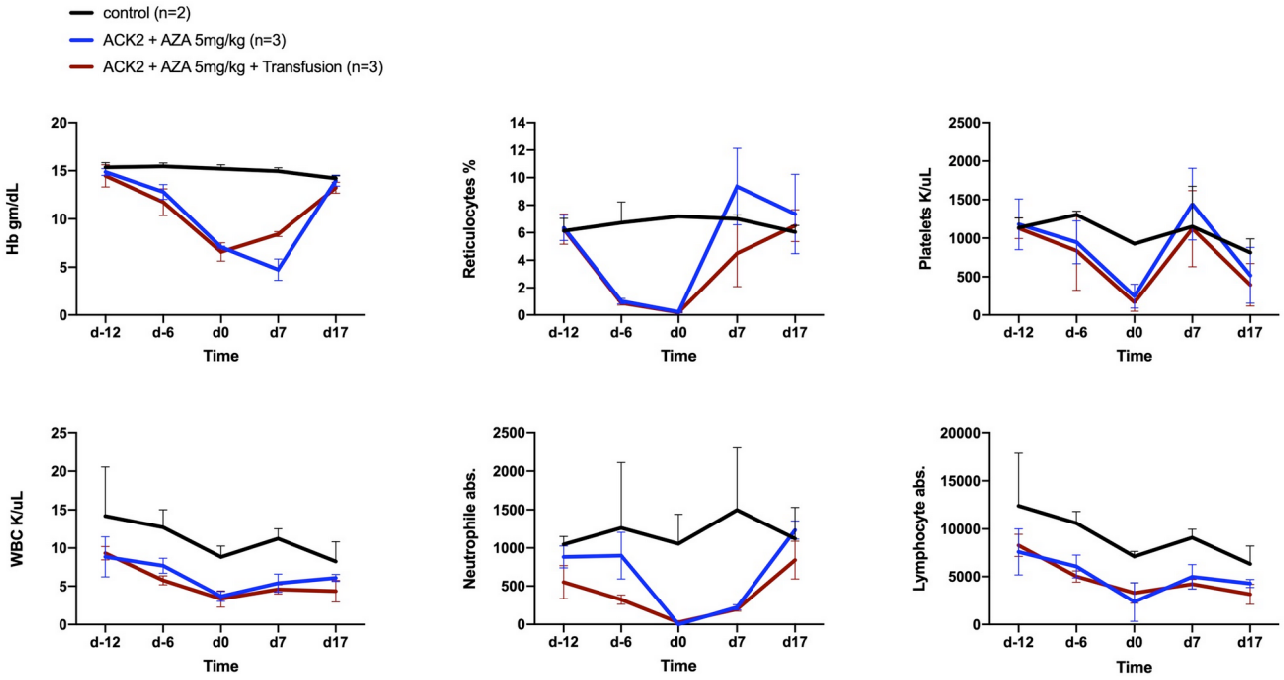

Supplemental Figure 11

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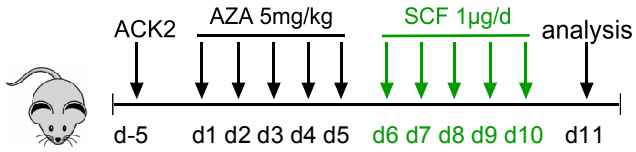

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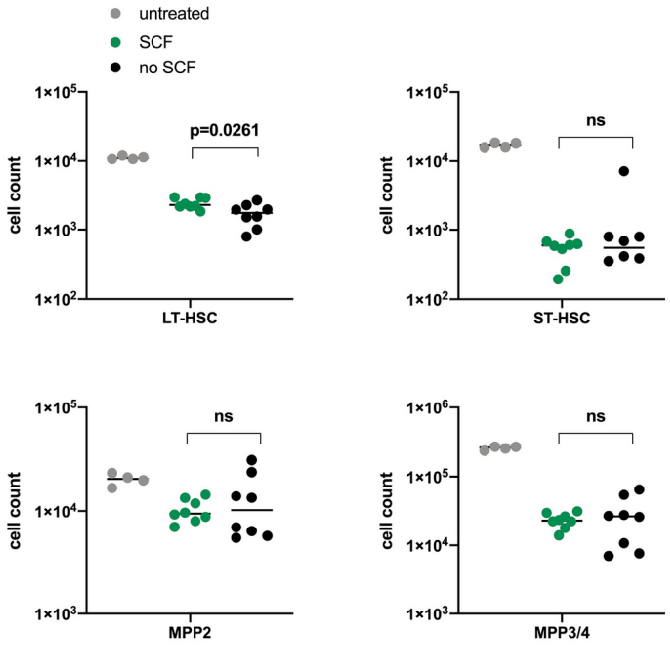

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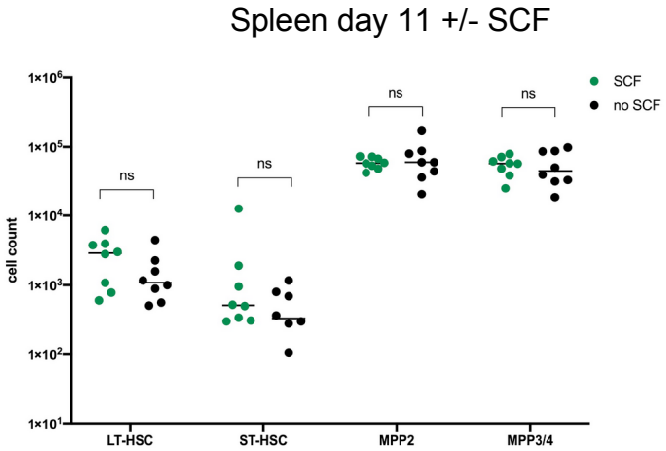
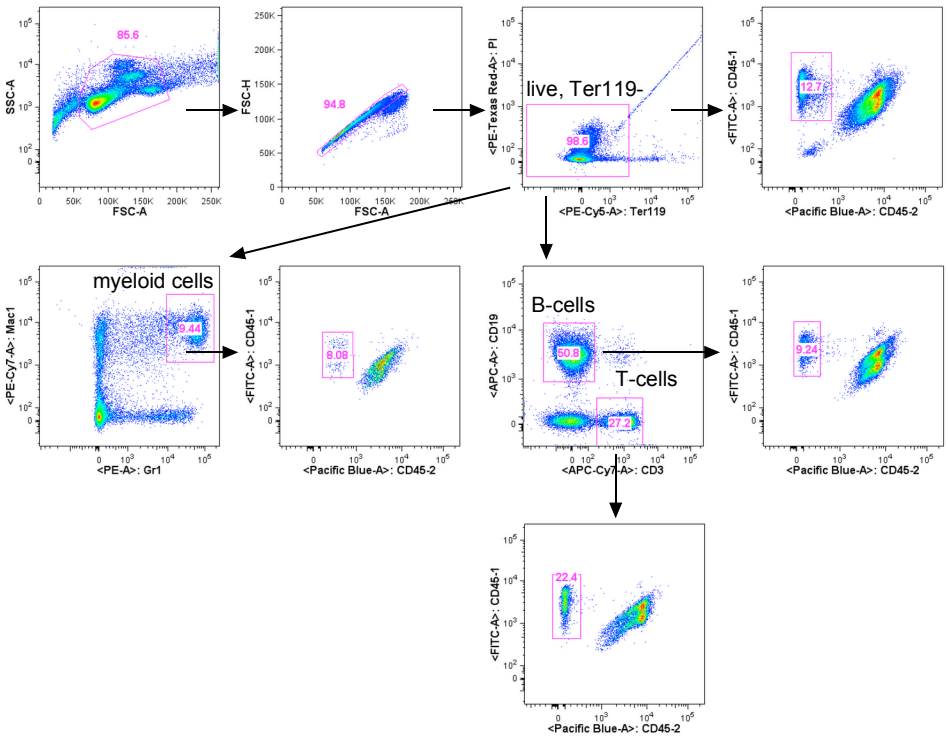


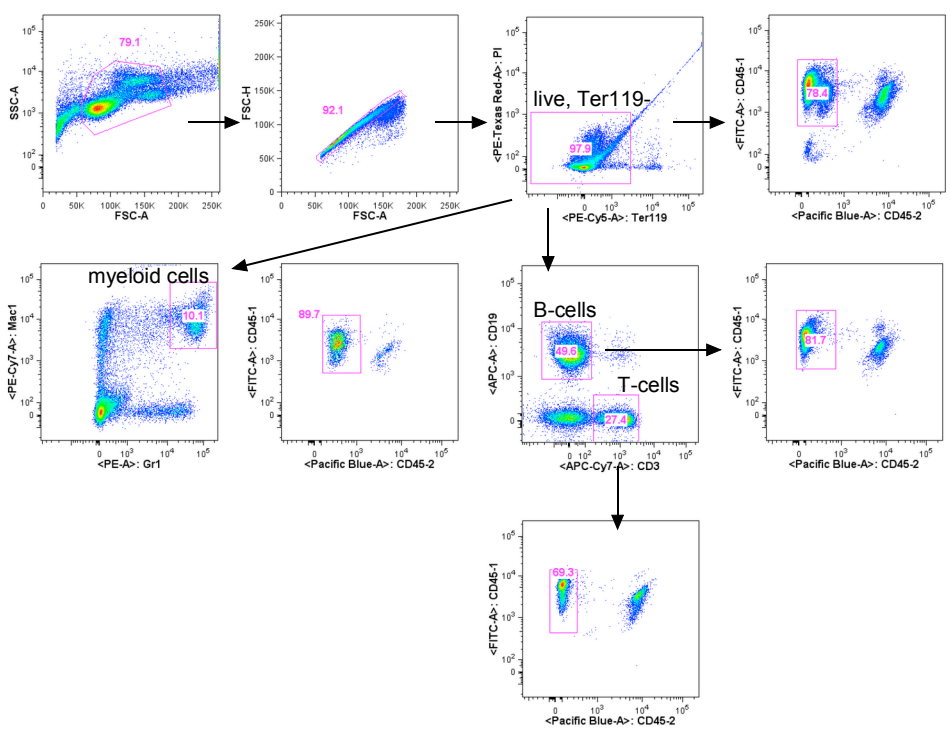
Supplemental Figure 12

PB chimerism at 24 weeks

ACK2 + AZA 2.5mg/kg



ACK2 + AZA 5mg/kg



Supplemental Figures

Supplemental Figure 1. Depletion kinetics of mature myeloid and lymphoid cells in the spleen on days 6, 10, 15 and 20 following treatment with AZA at a dose of 5mg/kg/d for 5 days. Myeloid cells ($Gr1^+$ $Mac1^+$), B cells ($CD19^+CD3^-$), T cells ($CD3^+CD19^-$), NK cells ($NK1.1^+CD3^-$), CD4 cells ($CD3^+CD4^+$), CD8 cells ($CD3^+CD8^+$). Data represent mean ± SD (n = 4 per group per timepoint). * $P < .05$.

Supplemental Figure 2. Absolute number of the different HSPC compartments in the spleen at day 6, 10, 15 and 20 following treatment with AZA at a dose 5mg/kg/d for 5 days as compared to untreated control mice. Multipotent progenitors (MPP); MPP4: $Lin^-Sca1^+c\text{-}Kit^+$[LSK]$CD150^-CD48^+Flt3^+$; MPP3: $LSKCD150^-CD48^+Flt3^-$; MPP2: $LSKCD150^+CD48^+$; Short-term HSC (ST-HSC): $LSKCD150^-CD48^-$; long-term HSC (LT-HSC): $LSKCD150^+CD48^-$. Data represent mean ± SD (n = 4 per group per timepoint). * $P < .05$.

Supplemental Figure 3. (A) Representative flow cytometry plots of HSPC compartment in the BM of untreated controls and mice treated with three different AZA doses (1.25, 2.5 and 5mg/kg/d) for 3 days. Analysis was performed 24h after the last AZA administration. Gated from lineage negative (Lin^-) live cells. **(B)** Absolute numbers of different HSPC compartments in the BM of mice following treatment with AZA 1.25, 2.5 and 5mg/kg/d for 3 days as compared with untreated controls. Data were pooled from 2 independent experiments and represent mean ± SD (n=3 for the untreated group and n=7 for the treated groups). *$P < 0.05$; **$P < .01$; ***$P < .001$.

Supplemental Figure 4. (A) Representative flow cytometric analysis showing gating strategy for the myeloid and lymphoid progenitors in the BM of mice treated with AZA at a dose of 5mg/kg/d for 5 days at day 6, 10, 15 and 20 after start of AZA as compared to untreated controls. Myeloid progenitors (MP) are gated from live, $Lin^-Sca1^-c\text{-}Kit^+$ cells and common lymphoid progenitors (CLP) are gated from live, $Lin^-Sca1^{dim}c\text{-}Kit^{dim}$ cells. Megakaryocyte-erythrocyte progenitors (MEP), granulocyte-monocyte progenitors (GMP), common myeloid progenitors (CMP). **(B)** Absolute number of MEP, CMP, GMP and CLP in the BM at day 6, 10, 15 and 20 after start AZA as compared to untreated control mice. Data represent mean ± SD (n = 4 per group per timepoint). * $P < .05$.

Supplemental Figure 5. BM MPP3 and MPP2 as % of LSK at day 6, 10, 15 and 20 after start of AZA at a dose 5mg/kg/d for 5 days as compared to untreated control mice. Data represent mean ± SD (n = 4 per group per timepoint). * $P < .05$.

Supplemental Figure 6. Depletion kinetics of mature myeloid and lymphoid cells in the BM on days 6, 10, 15 and 20 after start of AZA at a dose of 5mg/kg/d for 5 days. Myeloid cells ($Gr1^+$ $Mac1^+$), B cells ($CD19^+CD3^-$), T cells ($CD3^+CD19^-$), NK cells ($NK1.1^+CD3^-$), CD4 cells ($CD3^+CD4^+$), CD8 cells ($CD3^+CD8^+$). Data represent mean ± SD (n = 4 per group per timepoint). * $P < .05$.

Supplemental Figure 7. (A) Representative flow cytometry plots of Ki67 intracellular protein expression in LT-HSC, LSK and MP in untreated mice and after 3 days of AZA 5mg/kg/d (gated from live, Lin^- cells). (B) Ki67 intracellular protein expression (%) in LT-HSC, LSK and MP following AZA 5mg/kg/d for 3 days as compared to untreated controls (n=4 per group); * $P < .05$, ** $P < .01$

Supplemental Figure 8. (A) Representative flow cytometry plots of Annexin V/PI staining (gated from live, $Lin^-Sca1^+c\text{-}Kit^+$ cells) in untreated controls and mice treated with three different AZA doses (1.25, 2.5 and 5mg/kg/d) for 3 days. Analysis was performed 24 h after the last AZA administration. **(B)** Annexin V+ (left) and Annexin V/PI+ (right) cells shown as % from LSK in the three different dose groups as compared to untreated controls. Data were pooled from 2 independent experiments and represent mean ± SD (n = 4 in the control group and n = 7 in the AZA groups). *$P < .05$; **$P < .01$.

Supplemental Figure 9. Absolute cell counts of LT-HSC and ST-HSC in the spleen of untreated controls and mice treated with ACK2 in combination with AZA at 6, 10 and 20 days after start of AZA treatment at a dose 5mg/kg/d for 5 days; Data were pooled from two independent experiments and represent mean ± SD (n = 8-9 per group per timepoint). **$P <$ .001; ****$P < .0001$.

Supplemental Figure 10. Transient myelosuppression with complete autologous recovery of peripheral blood parameters following treatment of ACK2+AZA. **(A)** Experimental schema.

ACK2 was administrated 5 days prior start AZA; AZA was given for 5 days at a dose 5mg/kg/d; a transfusion group received 100 µl of whole blood from B6. Rag2cγc$^{-/-}$ mice every second day (cumulative x3 transfusion) after discontinuation of AZA; complete blood cell counts (CBC) were collected before the start of ACK2, before the start AZA and on days 0, 7 and 17. Mice were assessed twice weekly for signs of morbidity and mortality for 2 months. **(B)** Kaplan-Meier survival curve showing 100 % survival in treated transfused and non-transfused mice. **(C)** CBC at different timepoints following ACK2+AZA treatment and ACK2+AZA+Transfusion treatment as compared to untreated controls.

Supplemental Figure 11.

(A) Treatment schema. C57BL/6 mice were treated with recombinant murine stem cell factor (SCF) by intraperitoneal injection following treatment with ACK2+AZA. ACK2 at a dose of 500 µg was administered intravenously 5 days prior to the start of treatment with AZA. AZA was given at a dose of 5mg/kg daily by intraperitoneal injection for 5 consecutive days. Exogenous recombinant murine SCF was administered at a dose of 1µg/d for 5 consecutive days, starting 24h after the last AZA-dose. Mice were sacrificed at day 11 after the first dose of AZA and BM from both legs and spleen were analyzed for HSC-depletion by flow cytometry. **(B)** Absolute cell counts of LT-HSC, ST-HSC, MPP2 and MPP3/4 in the BM following treatment with ACK2+AZA (d11), with or without the addition of recombinant murine SCF. Data were pooled from 2 independent experiments and represent mean ± SD (n=8 per group for the treated groups (with or w/o SCF) and n=4 for the untreated (control) group); ns = nonsignificant. **(C)** Absolute numbers of LT-HSC, ST-HSC, MPP2 and MPP3/4 in the spleen following treatment with ACK2+AZA with or without the addition of recombinant SCF. The treatment scheme for this experiment is presented in main Figure 3G. Analysis was performed on day 11 post start of treatment with AZA at 5mg/kg. Data were pooled from 2 independent experiments and represent mean ± SD (n = 8 per group); ns = non-significant.

Supplemental Figure 12. Gating strategy for assessment of total, myeloid, B- and T-cell donor chimerism. Representative flow cytometry plots of peripheral blood donor chimerism at 24 weeks in recipient mice conditioned with either ACK2 + AZA 2.5mg/kg or ACK2 + AZA 5mg/kg and transplanted with 20 x 10^6 WBM cells.