Supplementary Figure S1. Total donor chimerism does not accurately reflect HSC chimerism following unfractionated bone marrow transplantation. 6.5×10^7 unfractionated nucleated bone marrow cells from wild type female CD45.2 mice were transplanted into unirradiated wild type female CD45.1 x CD45.2 F1 recipients. Total peripheral blood and BM HSC (defined as KLS $CD135^-CD34^-$) chimerism was measured 16 weeks after transplantation.

Supplementary Figure S2. ACK2 treatment leads to marked reduction of BM cellularity and increases in osteoblast size. Humerus bones from untreated (Day 0) or ACK2-treated $RAG2^{-/-}\gamma c^{-/-}$ mice were isolated 2 or 9 days after antibody injection. Bones were fixed, decalcified, paraffin embedded, and hematoxylin and eosin stains were performed on sections. Ob, osteoblast; Er, immature erythroid colony.

Supplementary Figure S3. ACK2 treatment results in near complete yet transient HSC depletion *in vivo*. Bone marrow cellularity and HSC frequency were used to determine the number of HSCs in the bone marrow. Spleen sizes were also assessed for signs of extramedullary hematopoiesis. A) control, B) At time of ACK2 clearance (Day 9), C) One week post ACK2 clearance (Day 16). Flow cytometric profiles of HSCs in $lineage^-$ BM using 3C11, a c-kit-specific antibody that binds a distinct epitope from ACK2, as well as KLS $CD135^-CD150^+$ frequency, of which 19.5% are HSCs (*1*), are shown adjacent to each corresponding time point. Mean values +/- SEM are shown (n=3 for each time point).

Supplementary Figure S4. ACK2 treatment selectively depletes HSCs at early timepoints. Total numbers of HSCs (lin$^-$c-kit$^+$Sca-1$^+$CD135$^-$CD150$^+$), MEPs (lin$^-$c-kit$^+$Sca-1$^-$CD34$^-$FcγR$^-$), CMPs (lin$^-$c-kit$^+$Sca-1$^-$CD34^{low}FcγR^{low}), and GMPs (lin$^-$c-kit$^+$Sca-1$^+$CD34^{high}FcγR^{high}) were quantified and compared to untreated control mice. Mean values +/- SEM are shown (n=3); ** indicates p-value<0.001 as compared to the relative number of HSCs at the same time point.

Supplementary Figure S5. ACK2 administration results in depletion of HSC in both wild type and immunodeficient mice. The number of KLS CD135$^-$CD150$^+$ HSCs in both femurs and tibia of ACK2-treated and control mice was determined by flow cytometry 2 days after treatment with 1mg of ACK2. Mean values +/- SEM are shown (n=2); ** indicates p-value<0.001.

Supplementary Figure S6. Normal cell cycle status in recipient and donor HSCs after ACK2 treatment. Cell cycle profiles of untreated controls and both recipient and donor HSCs from ACK2-treated RAG2$^{-/-}\gamma c^{-/-}$ mice that had been transplanted 7 months earlier with wild type HSCs were obtained. HSCs were identified as KLS CD150$^+$CD34$^-$ cells.

Supplementary Figure S7. Multiple rounds of ACK2 treatment and HSC transplantation result in consistently high levels of donor chimerism. A) RAG2-/-γc-/- mice were conditioned with 500µg of ACK2, and transplanted seven days later with CD45.1$^+$ 5000 LT-HSCs. Mice were allowed to recover for seven days and were once again conditioned with ACK2 and transplanted seven days later with 5000 GFP$^+$ LT-

HSCs. A third round of ACK2 treatment and LT-HSC transplantation was performed using 5000 $CD45.2^{+}CD45.1^{+}$ HSCs. Peripheral blood was analyzed 24 weeks later for donor granulocyte chimerism as before. Chimerism values arising from each transplant in four separate animals are shown.

Supplementary Table S1. Modest hematological effects of ACK2 treatment. Peripheral blood was sampled from a control mouse and an animal that had been treated with ACK2 9 days beforehand. Several hematological parameters were assessed.

Supplementary Text.

Studies in unconditioned mice have demonstrated that unfractionated donor BM transplants lead to chimerism levels that are linearly proportional to the dose of transplanted cells, suggesting that "space" is not a limiting factor in BM transplants (*2*). However, these studies did not determine whether all BM cell types or only certain subsets of cells are readily displaced. To examine this issue, we transplanted 6.5×10^{7} unfractionated bone marrow cells from female CD45.2 mice into female CD45.1 x CD45.2 F1 recipients to exclude the possibility of graft rejection. This transplanted cell dose represents 12-15% of the total recipient bone marrow cellularity by one estimate (*3*), and 20-28% by another (*4*). The mean total chimerism in the peripheral blood at 16 weeks post-transplant was 11% (Fig. S1), similar to the predictions made in earlier studies (*2*). However, the mean BM HSC chimerism was only 4.5%, approximately 2.5 fold lower than the total chimerism (Fig. S1). These data demonstrate that

measurement of total chimerism leads to an overestimation of BM HSC chimerism, and that even when the graft includes transplantation-enhancing non-hematopoietic cells (*5, 6*), the majority of HSCs did not engraft.

Importantly, all mice survived the treatment with no obvious signs of distress aside from a temporary loss of coat color, as previously reported (*9, 10*). The lack of mortality in ACK2-treated mice is likely due to the mechanism of ACK2 depletion, in that mature effector blood cells are not directly affected by the treatment and are only lost gradually due to attrition. This is supported by the observation that a number of hematologic parameters in the peripheral blood are only modestly affected by ACK2 treatment (Table S1). Moreover, significant numbers of mature erythrocytes and regenerating erythroid colonies were observed in the bone marrow 9 days after ACK2 treatment (Fig. S2, bottom left panel). Upon histological examination, we found only a gradual diminishment of bone marrow cellularity (Fig. S2), accompanied by a progressive increase in the size of osteoblasts (Fig S2, right column). Additionally, both male and female mice treated with ACK2 remained fertile and had viable offspring. Thus, the side effects of antibody-mediated depletion of HSCs stand in marked contrast to lethal irradiation, which requires the early transplantation of bone marrow or hematopoietic progenitors to prevent the death of the animal (*11-14*), a procedure which in humans is accompanied by high levels of morbidity and significant mortality. Indeed, conditioning regimens carry significant health risks including infertility, prolonged thrombocytopenia, organ damage, immunosuppression and high rates of secondary malignancies (*15*). These risks are precisely the reason why many clinicians have chosen to treat SCID patients with BM transplants without cytotoxic conditioning.

The importance of SCF-mediated c-kit signaling for HSCs has been demonstrated in W/W mice, which completely lack functional c-kit expression and die *in utero* unless transplanted with normal HSCs (*7*). The inhibition of c-kit signaling apparently must be complete to deplete HSCs, since neither the partial inhibition of c-kit signaling caused by 2B8 (Fig. 2E) nor genetic mutations that lead to a partial abrogation of c-kit function lead to a substantial diminishment of HSC numbers *in vivo* (*8*).

Additionally, we assayed the effect of ACK2 on a variety of hematopoietic progenitor cells *in vivo* after ACK2 administration. At two days post-treatment, all myeloid progenitors in the bone marrow, which express similar levels of c-kit as do HSCs (*17*), began to decline; however HSCs were most dramatically impacted (Fig. S4). By nine days post-treatment, HSCs (Fig. S4), myeloid progenitors (Fig. S4), and common lymphoid progenitors (*18*) (data not shown) were severely diminished. These data support a mechanism by which progenitor cells are gradually lost due to the lack of replenishment by HSCs.

Wild-type mice as well as B cell-deficient μMT mice (*16*) also showed decreased levels of HSCs in their bone marrow post ACK2 treatment (Fig. S5). Interestingly, the recovery of HSC numbers appeared to be more rapid in B cell-sufficient or T cell-sufficient animals after ACK2 clearance (data not shown), suggesting an unexpected potential role for lymphocytes in stimulating hematopoietic recovery.

It is important to note that obtaining high numbers of HSCs has already proven to be clinically feasible, because most human HSC grafts are isolated from the peripheral blood of mobilized donors using regimens that lead to an expansion of HSCs (*19, 20*). However, transplantation of such a large bolus of cells might potentially be avoidable

since similarly high levels of donor chimerism were consistently obtained through three rounds of conditioning and transplantation of 5000 HSCs (for a total of 15,000 transplanted HSCs) (Fig. S7). The total contribution of the third transplant was consistently higher than the first two HSC transplants combined.

Materials and Methods

Mice

All animal procedures were approved by the International Animal Care and Use Committee. Recipient mice used in these studies were 4-8 weeks old recombinase activating gene 2-deficient (RAG2-/-) (*25*), RAG2-/- interleukin-2 common gamma chain-deficient (RAG2-/-γc-/-) (*26*), or μMT mice (*16*). Donor mice used were 8-12 weeks old GFP transgenic mice expressing GFP from the chicken β-actin promoter (*27*), or congenically distinguishable CD45.1 or CD45.2 C57Bl/Ka mice. All mouse strains were bred and maintained at Stanford University's Research Animal Facility.

HSC Transplantation

Bone marrow was harvested from donor mice by crushing bones, lysing red blood cells with ACK lysis buffer (150mM NH_4Cl, 1mM $KHCO_3$, and 0.1mM EDTA), and removing debris on density gradient using Histopaque 1119 (Sigma, St. Louis, MO). Bone marrow was then c-kit$^+$ enriched using CD117$^+$ microbeads (AutoMACS, Miltenyi Biotec, Auburn, CA). Cells were stained with antibodies described below and HSC were isolated by single or double FACS based on previously defined reactivity for particular cell surface markers (c-kit$^+$lineage$^-$Sca-1$^+$CD34$^-$CD150$^+$) on the BD FACS-Aria (BD Biosciences, San Jose, CA). Cells were transplanted by retro-orbital injection.

Antibodies

The following monoclonal antibodies were purified and conjugated using hybridomas maintained in our laboratory: 2C11 (anti-CD3), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), 6B2 (anti-B220), 8C5 (anti–Gr-1), M1/70 (anti–Mac-1), TER119 (anti-Ter119), A20.1.7 (anti-CD45.1), AL1-4A2 (anti-CD45.2), 2B8 (anti–c-kit), 3C11 (anti-c-kit), E13-161-7 (anti–Sca-1). Antibodies were conjugated to biotin, Pacific Blue, Pacific Orange, PE, allophycocyanin (APC), Alexa 488, Alexa 647 or Alexa 680 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The following were purchased from eBiosciences (San Diego, CA): antibodies against CD3, CD4, CD8, B220, Mac-1, Ter119, and Gr-1 conjugated to PE-Cy5; anti–c-kit and anti–Mac-1 conjugated to PE-Cy7; anti-CD135 (A2F10) conjugated to PE; anti-CD34 conjugated to FITC or biotin; and anti-B220 conjugated to APC-Cy7. Anti-CD41 conjugated to FITC, anti-CD48 conjugated to FITC, goat-anti-rat conjugated to APC, and anti-TCRß (H57-597) conjugated to APC were purchased from BD Biosciences. Anti-CD150 conjugated to Alexa 647 was purchased from Biolegend (San Diego, CA). Streptavidin conjugated to Alexa 488 and Alexa 680, and goat-anti-rat conjugated to Alexa 488 was purchased from Invitrogen. Streptavidin conjugated to Quantum Dot 605 was purchased from Invitrogen.

ACK2 Production and Purification

The ACK2 hybridoma was a generous gift of S. Nishikawa (Kyoto, Japan). The cell line was expanded and subcloned to establish an ACK2 high producing hybridoma cell line. Cells were grown in the Integra flask system (Integra Biosciences, Chur, Switzerland) and media containing antibody was collected. ACK2 was purified on an IgG purification column by binding the ACK2 to the column and eluting with 100mM Glycine and 5mM NaN_3. The eluted positive fractions (OD_{280} >0.2) were combined, dialyzed for 12 hours in PBS, and concentrated using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany). Subsequent ACK2 preparations were prepared by Bio Express (W. Lebanon, New Hampshire).

ACK2 Administration and Clearance

500µg of ACK2 was administered through retro-orbital injection to 4-8 week RAG2-/-γc-/- mice. Peripheral blood was isolated from the tail vein of these mice every other day and allowed to clot for 1 hour. Samples were centrifuged for several minutes and serum was isolated. 10,000 mast cells (*28*), a generous gift of A. Piliponsky and S. Galli, were subsequently stained with 50µl of serum, followed by goat-anti-rat IgG APC or Alexa 488, to test for ACK2 antibody presence. In addition, mast cells were stained with a known anti-c-kit antibody, 2B8 conjugated to APC as a control. These cells were analyzed on the BD FACS-Aria. To determine HSC depletion, both femurs and tibia were obtained from conditioned mice and prepared as above. Cells were counted and HSC frequency was determined on the BD FACS-Aria by gating on KLS $CD135^-CD150^+$ cells.

ACK2 Conditioning and Transplantation

500µg of ACK2 was administered intravenously to 4-8 week Rag2-/- or Rag2-/-γc-/- mice. Mice were transplanted at the time point that ACK2 was shown to no longer be present in the serum (D7 or D9 depending on preparation). HSC for transplantation were obtained from bone marrow of donor mice, which were isolated on the BD FACS-Aria by gating on c-kit^{+}lineage^{-}Sca-1^{+}CD34^{-}CD150^{+} cells.

Engraftment Analysis

Blood was obtained from the tail vein of transplanted mice at various time points. It was separated using 2% dextran at 37^{o}C for 30 min, and subsequently lysed using ACK lysis buffer (150mM NH_4Cl, 1mM $KHCO_3$, and 0.1mM EDTA) for 5 minutes. Cells were stained with antibodies described above and analyzed on the BD FACS-Aria. Donor granulocyte chimerism was determined by analyzing the percentage of Ter119^{-}CD3^{-} B220^{-}Mac1^{high}side scatter^{high} cells that were also donor^{+}. Several animals were sacrificed and HSCs were isolated similarly to donor mice in order to confirm that the HSC chimerism mimicked the granulocyte chimerism. To determine the effects of ACK2 on hematopoietic progenitors, bone marrow was isolated from mice treated with 500µg ACK2 as above. Cells were counted and progenitor frequency was determined on the BD FACS-Aria. Cell numbers were compared to those of untreated animals. HSC were gated as lin^{-}c-kit^{+}Sca-1^{+}CD135^{-}CD150^{+}, MEP were gated as lin^{-}c-kit^{+}Sca-1^{-} CD34^{-}FcγR^{-}, CMP were gated as lin^{-}c-kit^{+}Sca-1^{-}CD34^{low}FcγR^{low}, and GMP were gated as lin^{-}c-kit^{+}Sca-1^{+}CD34^{high}FcγR^{high}.

Transplantation into Irradiated Recipients

Recipient mice were treated with 950 cGy prior to transplantation. Each mouse was transplanted with the entire splenocyte population from an ACK2 mouse treated 9 days prior, 200,000 unfractionated bone marrow cells from an ACK2 mouse treated 9 days prior, or 100 donor^{+} KLS CD34^{-}CD150^{+} HSC from a primary transplanted mouse (ACK2 conditioned and transplanted with 5000 donor HSC 39 weeks prior).

In Vitro Culture

Exactly 10 HSCs (KLS CD34^{-}CD150^{+}) were clone sorted on the BD-Aria into 96-well round-bottom plates and cultured in the presence of 50 ng/ml SCF (R&D Systems) or 50 ng/ml TPO (R&D Systems) in Iscove's Modified Dulbecco's Medium (Invitrogen) with 10% fetal calf serum (Omega), 1 mM sodium pyruvate (Invitrogen), 100 μM non-essential amino acids (Invitrogen), and 50 μM β-mercaptoethanol. HSC treated with antibody received 10μg/ml of ACK2 or 2B8. Viable cells were counted each day under the microscope.

Cell Cycle Analysis

Bone marrow cells were c-kit-enriched as before and stained with anti-CD34 FITC, anti-c-kit (2B8) PE-Cy7, anti-CD150 Alexa 647, anti-Sca-1 Alexa 680, and anti-lineage PE-Cy5. Cells were then fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature, washed twice with PBS, and resuspended in PBS/ 0.2% saponin + 2μg/ml 4',6-diamidino-2-phenylindole (DAPI) prior to analysis.

Histology

Mice were treated with 500µg ACK2 and at 2 days and 9 days post treatment, the humerus was removed and placed in Bouin's fixative for >1 day. Bones were decalcified in formic acid, paraffin-embedded, sectioned, stained with hematoxylin and eosin, and mounted using xylene-based media.

Analysis of peripheral blood

Peripheral blood was collected from the tail vein and deposited directly into heparin-coated tubes. Analysis was performed by the Department of Comparative Medicine's Diagnostic Laboratory at Stanford University.

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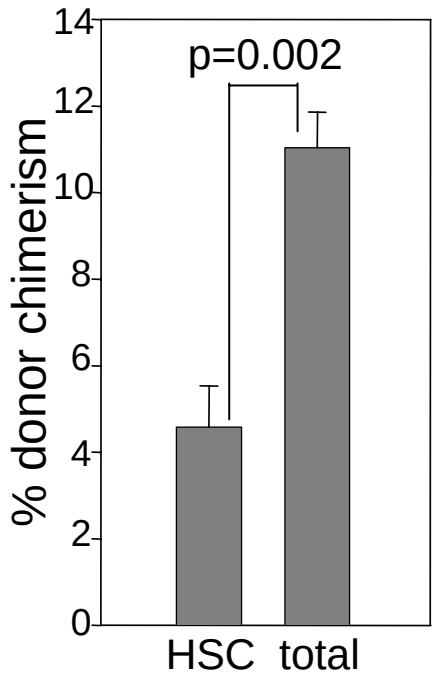
Supplemental Figure 1



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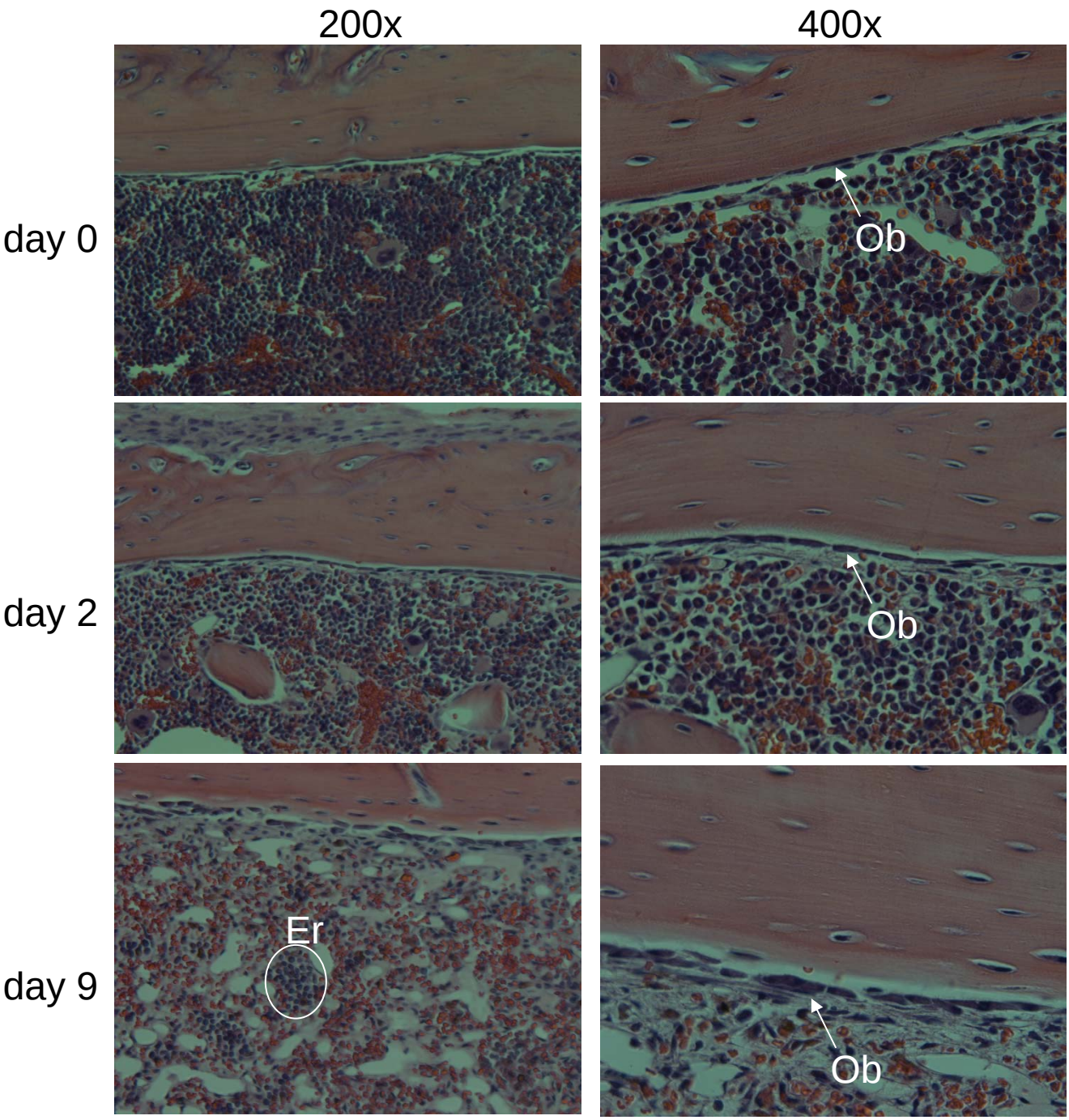

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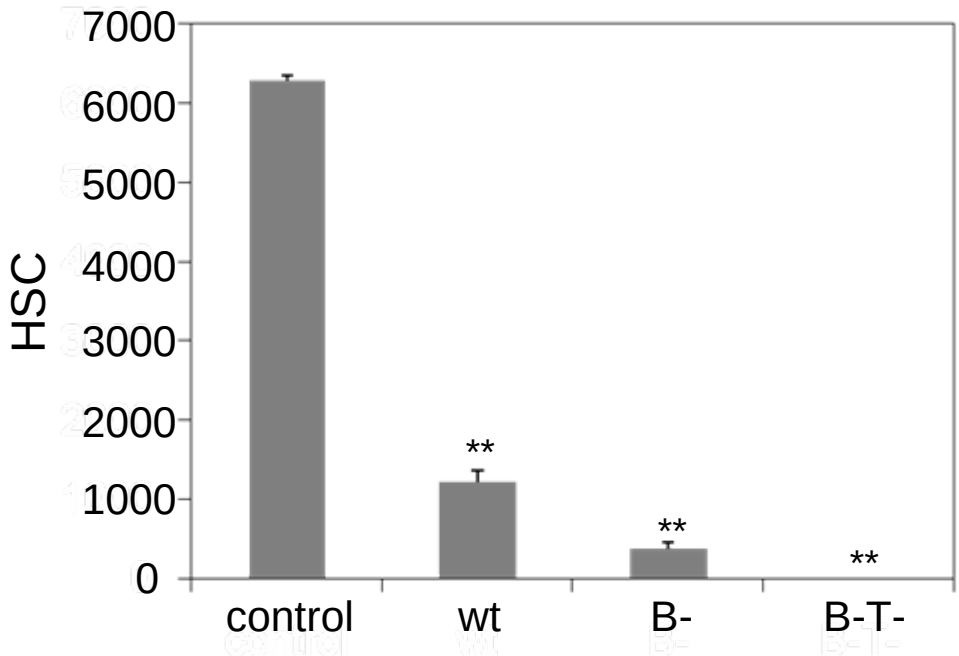

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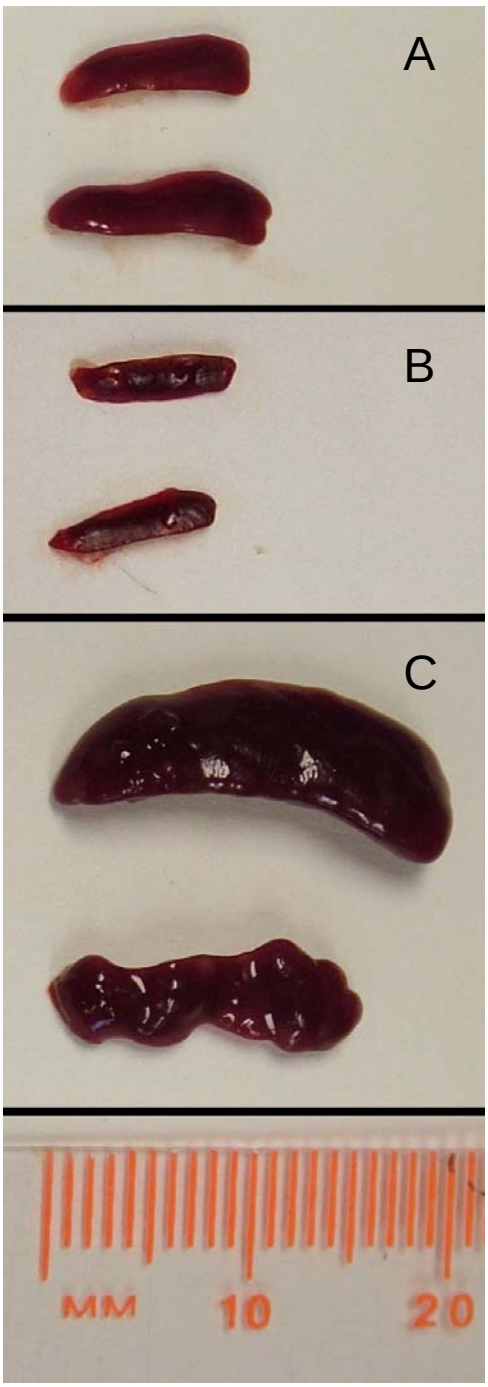

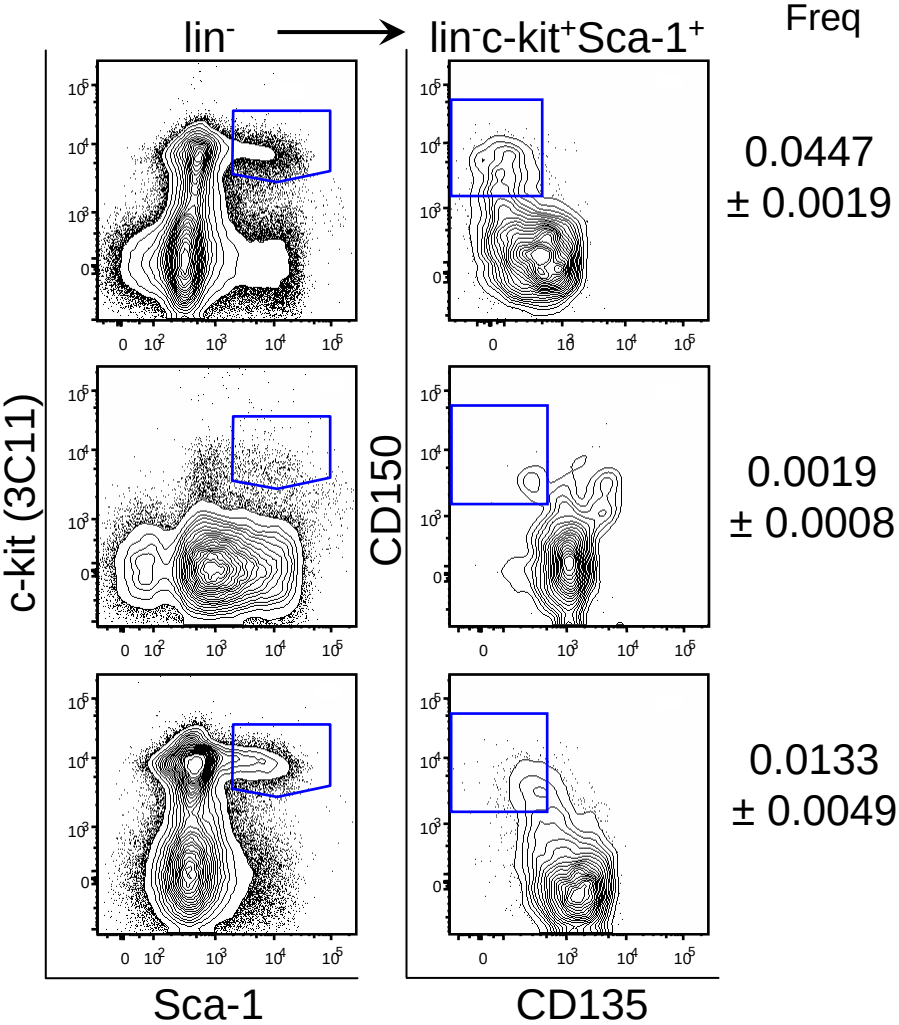

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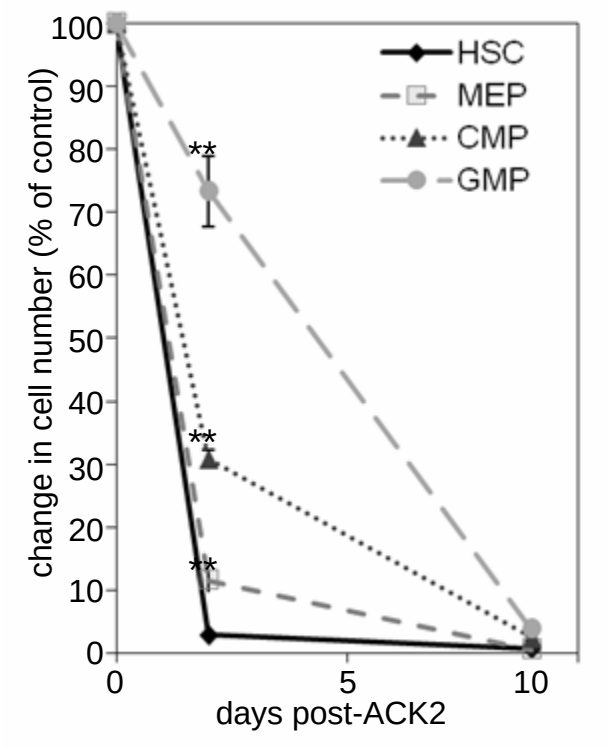

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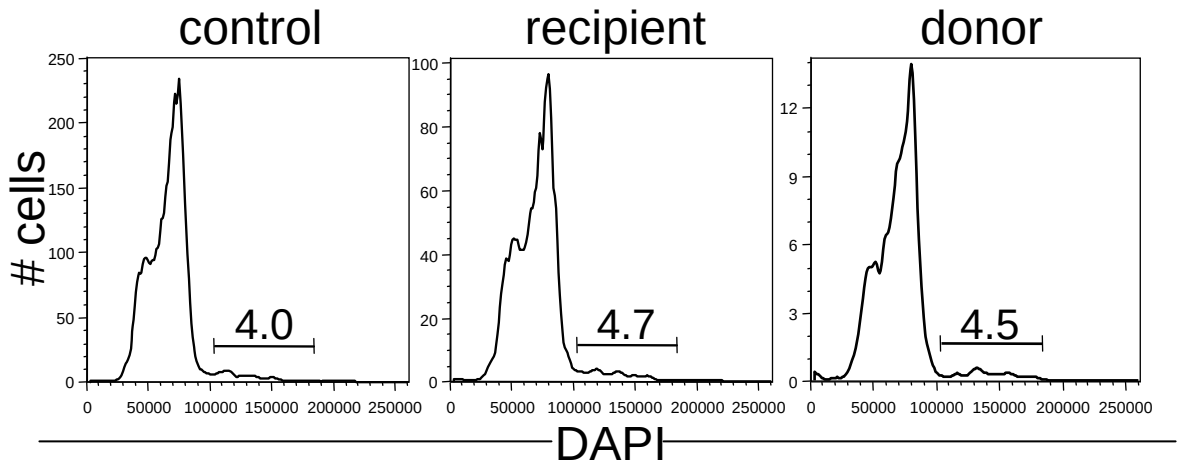

Supplemental Figure 7