# Inactivating Mast Cell Function Promotes Steady-State and Regenerative Hematopoiesis

Julianne N.P. Smith[1], Jordan Campanelli[1], Brittany Cordova[1], Alyssia Broncano[1], Kelsey F. Christo[1], Stanton L. Gerson[1,2], Sanford D. Markowitz[1,2], Amar B. Desai[1]

[1]Department of Medicine, and Case Comprehensive Cancer Center Case Western Reserve University, Cleveland OH 44106 USA

[2]University Hospitals Seidman Cancer Center, Cleveland OH 44106 US

*Correspondence:

Amar B. Desai

Department of Medicine

Case Western Reserve University

Abd10@case.edu

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**Abstract:**

Deeper understanding of the cellular and molecular pathways regulating hematopoiesis is critical to maximize the therapeutic potential of hematopoietic stem cells (HSCs) in curative procedures including hematopoietic stem cell transplantation (HST). We have recently identified mast cells (MCs) as therapeutically-targetable components of the HSC niche. Here, we demonstrate that mice lacking MCs display peripheral neutrophilia, expansion of bone marrow (BM) HSC populations, resistance to repeated 5-fluorouracil (5-FU) administration, and a BM genetic signature primed for hematopoietic proliferation. MC deficiency functionally altered both the hematopoietic and the stromal compartment of the BM as hematopoietic reconstitution was accelerated in wildtype mice that received MC deficient BM and in MC deficient recipients that received wildtype BM. Finally, we demonstrate that mice treated at steady state with the MC stabilizing agent ketotifen exhibit increased BM cellularity as well as expansion of phenotypic HSC populations. This work provides novel mechanistic rationale to explore mast cells as a target to enhance human BM transplants. Additionally, the potential of repurposing FDA approved mast cell targeting therapies to promote hematopoietic regeneration may provide well-tolerated treatment strategies at a fraction of the cost and development time.

**Introduction:**

Hematopoietic stem cells are tightly regulated through both external microenvironmental factors in the hematopoietic niche, and through internal factors that maintain genomic stability and homeostatic quiescence to minimize replicative stress(3,4). While several immune cell populations have been demonstrated to play a role in regulating HSC fitness and proliferative capacity including mesenchymal stem cells(5), endothelial cells (6), and macrophages (7,8), to date few studies have implicated a role for mast cells, nor for histamine receptor signaling in HSC homeostasis.

Mast cells (MCs) are myeloid cells present in connective tissue tasked with regulating several physiologic processes and serving as a primary line of defense to induce vasodilation, recruit inflammatory cells, and modulate angiogenesis following external insults. MCs are morphologically distinguished by the presence of secretory granules that store inflammatory mediators and which undergo degranulation upon activation to secrete these factors into the extracellular space (9-11). Histamine release following degranulation has been widely studied therapeutically due to its critical role in downstream responses, as well as the ability to target signaling via histamine receptors H1R and H2R, and more recently H3R and H4R. Traditional H1R antagonists are used to treat allergic reactions and insomnia, and act by binding H1 receptors in mast cells, smooth muscle cells, and endothelial cells and blocking activation of phospholipase C to reduce the rapid hypersensitivity response following allergic stimulus (12). H2R antagonists bind to H2R on the basolateral surface of gastric parietal cells and reduce production and secretion of gastric acid. Thus, these molecules are widely used to treat gastroesophageal reflux disease, heartburn, and gastric ulcers(13). Additionally, evidence suggests that H4R may be a viable target to modulate hematopoiesis (14). A number of MC targeting therapeutics have been FDA approved for conditions including allergies, gastrointestinal reflux, motion sickness, and asthma. Thus, these classes of compounds which include antihistamines, mast cell stabilizers, and leukotriene inhibitors have a well-established safety profile and limited off-target toxicity (15,16).

Here we demonstrate a novel phenotype by which mast cells regulate hematopoiesis and hematopoietic output, and provide evidence that therapeutic targeting of mast cells may promote hematopoietic regeneration following transplantation and in hematologic disease characterized by impaired hematopoietic function.

## Results:

### Mast cell deficient *Kit$^{W\text{-}sh}$* mice display peripheral neutrophilia and BM HSPC expansion

We characterized the hematopoietic compartments of the commercially available mast cell deficient mouse model (Sash c-Kit$^{w\text{-}sh}$, Jackson Labs Stock #030764). The *Kit$^{W\text{-}sh}$* mutation is an inversion in regulatory elements upstream of the c-kit element, which results in the loss of mast-cell populations shortly after birth (1) (2). Our data demonstrate these mice display a ~2.4- and a ~1.4-fold increase in peripheral blood neutrophils and platelets, respectively (**Figure 1A**, and a ~1.4-fold increase in total BM cellularity (**Figure 1B)**. Immunophenotypic analysis of the BM revealed significant increases in multiple progenitor cell populations including megakaryocyte progenitors (MkP), granulocyte-macrophage progenitors (GMP), myeloerythroid progenitors (MEP), and others (**Figure 1C, and Supplemental Figure 2)**. Additionally we observed a ~1.4-fold increase in the lineage (-), Sca-1(+), cKit(+) (LSK) HSPC population and a ~2-fold increase in the CD150(+),Endoglin(+) HSC population (**Figure 1E)**. We additionally characterized spleens of c-Kit$^{w\text{-}sh}$ mice and found that consistent with previously described findings (2), these mice displayed splenic extramedullary hematopoiesis as indicated by significant increases in splenic HSPCs and CFU potential (**Supplemental Figure 3**). In spite of the increases in stem and progenitor cells, megakaryocyte lineage cells, and certain myeloid cell types, we found no impact of MC deficiency on lymphoid cell types (Supp. Fig. 4). Taken together, these data demonstrate significantly enhanced hematopoietic output following loss of mast cells. We next assessed the capacity of *Kit$^{W\text{-}sh}$* mice to recover following repeated 5-Fluorouracil (5-FU) administration. 5-FU is commonly used to induce genotoxic stress for hematopoietic recovery studies (17,18), thus we hypothesized that the enhanced hematopoietic capacity of *Kit$^{W\text{-}sh}$* mice would result in increased resistance to weekly 150 mg/kg 5-FU administrations. Indeed, we observed that genetic loss of mast cells resulted in a significant survival extension compared to littermate control (LC) mice, indicating that the increased phenotypic HSCs and progenitors are functional and confer protection against hematopoietic injury (**Figure 1F**).

**The *Kit$^{W\text{-}sh}$* BM microenvironment is primed for hematopoietic proliferation**

We performed RNA-sequencing on whole BM from *Kit$^{W\text{-}sh}$* vs. LC mice and identified a signature consistent with a capacity for enhanced hematopoietic proliferation and mobilization, driven by significant increases in genes including Hopx(19), Fgf2 (20), *Pgf (21)*, Cxcr2 (22), coupled with decreases in Osm (23), Ptgs2 (24), and Stc1 (25), suggesting that the expansion of HSC pools and recovery following 5-FU was in part due to an enhanced capacity for HSC proliferation (**Figure 2)**.

**The *Kit$^{W\text{-}sh}$* BM microenvironment promotes hematopoietic recovery following transplantation**

To determine the functional effects of the observed gene expression data in Figure 2, we performed transplantation studies in which LC donor bone marrow was transplanted either into LC or c-Kit$^{w\text{-}sh}$ recipient mice. At Day 20 following transplant we sacrificed mice and found 1.2-fold higher BM cellularity in c-Kit$^{w\text{-}sh}$ recipients compared to LC controls, 1.6-fold greater LSKs and multipotent progenitors (MPPs), and trends towards increased total long-term HSC (LT-HSC; 1.76-fold) and short-term HSC (ST-HSC; 1.93 fold) populations (**Figure 3)**. This data suggests that even after myeloablation, the *Kit$^{W\text{-}sh}$* microenvironment is more favorable to hematopoietic engraftment and thus peripheral reconstitution following HST compared to LC counterparts.

**Pharmacologic mast cell stabilization results in BM progenitor cell expansion at steady-state**

To determine whether the hematopoietic phenotype observed in the *Kit$^{W\text{-}sh}$* mice could be phenocopied pharmacologically, we treated mice for 5 days with 10 mg/kg BID of the mast cell stabilizing agent and H1R inverse agonist ketotifen. While additional studies will be required to identify optimal dosing strategies, we observed significant increases in BM cellularity (1.23 fold), LSK (1.52 fold), ST-HSC (1.57 fold, as marked by LSK CD48(-) CD150(-)), and HSCs (1.54 fold, as marked by LSK (CD48(-) CD150(+)) (**Figure 4B)**. In contrast to our observations with MC

deficient mice, five days of mast cell stabilization with ketotifen did not impact peripheral blood cell populations (**Supplemental Figure 5**).

**Discussion:**

According to the Center for International Blood and Marrow Transplant Research Institute, approximately ~25,000 HST are performed each year in the United States to treated conditions including multiple myeloma, non-Hodgkin's lymphoma, acute myeloid leukemia, myelodysplastic syndromes, and acute lymphocytic leukemia. While HST is curative in many settings and capable of significantly extending survival in others, the severe complications associated with the procedure have limited its utility and narrowed the potential patient pool(26,27). By deepening our understanding of factors regulating hematopoiesis and hematopoietic stem cell function, we have enormous potential to expand the utility of HST.

In this work we have begun to characterize a novel phenotype by which mast cells regulate hematopoiesis and hematopoietic output, that may have clinical implications for HST. Using the mast cell deficient $Kit^{W\text{-}sh}$ mouse model (1,2) we have demonstrated that lack of mast cells results in peripheral neutrophilia, expansion of BM HSC populations, resistance to repeated 5-FU administration, and a BM genetic signature primed for hematopoietic proliferation. We also utilize chimeric transplantation studies to demonstrate that the $Kit^{W\text{-}sh}$ microenvironment appears more fit to promote HSC engraftment following HST, as evidenced by an accelerated recovery compared to WT recipients following transplantation. We have also demonstrated that mice treated at steady state with the mast cell stabilizing agent ketotifen exhibit increased BM cellularity as well as expansion of phenotypic HSC populations.

In addition to elucidating a novel mechanism by which mast cells and mast cell mediators regulate hematopoiesis, this work is significant because repurposing FDA approved therapies for alternative disease indications has multiple upsides from a safety, a timeline, and a financial

perspective. These upsides include a reduced development time of between 5-7 years, a higher rate of approval for the alternative indication, a significant reduction in development costs (by anywhere from 40-70%), and having access to a wealth of existing data from the extensive FDA approval process the compounds have already passed through(28,29). Thus, the potential of repurposing FDA approved mast cell targeting therapies to promote hematopoietic regeneration can deliver much needed novel therapies to the clinic at a fraction of the cost and time to development (**Figure 5**).

This work may also have implications in additional age-associated hematologic diseases that pose a large public health burden and that are characterized by dysfunctional hematopoietic output, resulting in anemia, bone marrow failure, or persistent thrombocytosis. The potential to utilize mast cell targeting agents to increase hematopoietic output may be a novel therapeutic strategy. In addition to contributing to a deeper understanding of the mechanisms regulating normal and regenerative hematopoiesis, this work provides a foundation for and a novel mechanistic rationale to explore mast cells as a target to enhance human BM transplants.

## Methods:

**Animals:** Animals were housed in the AAALAC accredited facilities of the CWRU School of Medicine. Husbandry and experimental procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC) in accordance with approved IACUC protocols 2019-0065. C-Kit$^{w\text{-}sh}$ mice were purchased from The Jackson Laboratory. Littermate wild-type control animals were used as comparator animals for all studies and designated “LC”, and a combination of male and female mice, aged 8-12 weeks old, were used for all studies. Mice were housed in standard microisolator cages and maintained on a

defined, irradiated diet and autoclaved water. All animals were observed daily for signs of illness.

**Complete Blood Count Analysis:** Peripheral blood was collected into Microtainer EDTA tubes (Becton-Dickinson) by submandibular cheek puncture. Blood counts were analyzed using a Hemavet 950 FS hematology analyzer.

**5-FU Administration**: C-Kit$^{w\text{-}sh}$ and LC mice were treated with 150mg/kg of 5-FU (Sigma, F6627) every seven days. Mice were monitored daily and sacrificed when they exhibited >25% weight loss or became moribund. Animal survival was monitored throughout this study.

**RNA Extraction and Quantitative PCR:** Total BM RNA was extracted using the RNeasy MiniKit (QIAGEN) with on-column DNase treatment, according to the manufacturer's protocol. For the RNA sequencing analysis described in Figure 2, total RNA was sent to Novogene for sequencing of 12G raw data per sample on the NovaSeq 6000 PE150.

**Bone Marrow Transplantation:** Mice were exposed to 10Gy total body irradiation from a cesium source. 16-18hrs later, mice received 1e6 whole bone marrow cells by retroorbital injection. Recipients were monitored daily for signs of distress or illness, and sacrificed on Day 20 to assess bone marrow and spleen cellularity and cytometric assessment of cell populations.

**Ketotifen Administration:** Ketotifen fumarate was purchased from TCI America and dissolved in PBS. Mice were administered 10mg/kg ketotifen via IP injection on a BID schedule (am and pm administrations). Mice were sacrificed 2 hours following the 9$^{th}$ dose to characterize HSPCs to collect total BM RNA for subsequent RT-PCR analysis, and to collect BM to serve as donor cells for HST compared to PBS treated controls.

**Quantification of HSPCs and Splenic Cell Types:** Bone marrow cells were obtained by flushing hindlimb bones and splenocytes were obtained by mincing spleens. Cellularity was measured following red blood cell lysis. Cells were stained with antibodies against CD45R/B220

(RA3-6B2), CD11b (M1/70), CD3e (500A2), Ly-6G and Ly6C (RB6-8C5), TER-119 (TER-119), Ly-6A/E (D7), CD117 (2B8), F4/80 (Cl:A3-1), CD61 (2C9.G2), Fcer1 alpha (MAR-1), CD45.1 (A20), and CD45.2 (104) and data was acquired on an LSRII flow cytometer (BD Biosciences). Analysis was performed on FlowJo software (TreeStar).

**Statistical Analysis:** All values were tabulated graphically with error bars corresponding to standard error of the means. Analysis was performed using GraphPad Prism software. Unpaired two-tailed Student's t-test was used to compare groups, unless otherwise noted.

## Acknowledgments

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## Conflict of Interest Disclosures:

The authors have no conflicts of interest to declare.

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# Figure 1: Mast cell deficient *Kit*$^{W\text{-}sh}$ "Sash" mice display peripheral neutrophilia and BM HSPC expansion

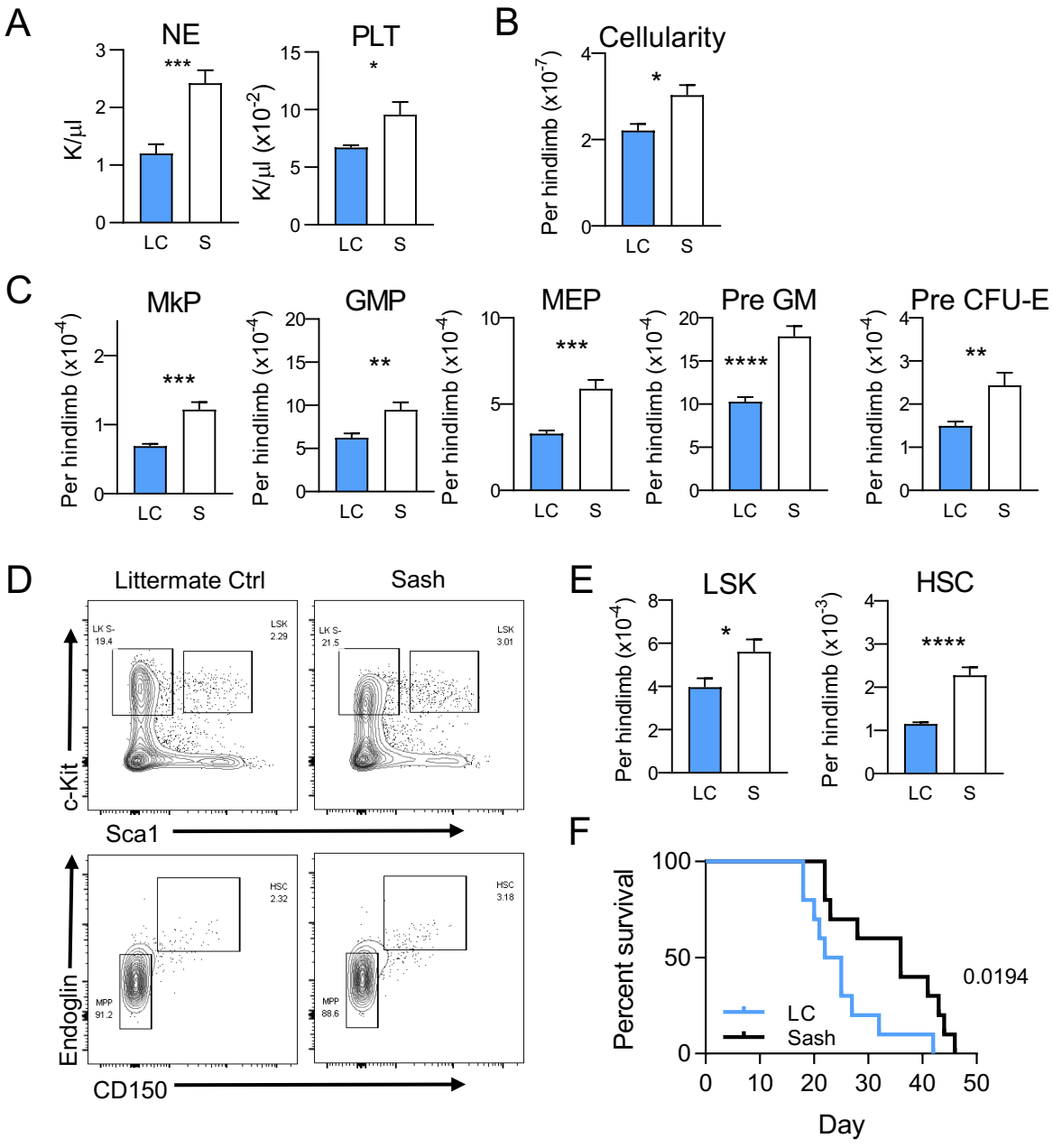


**Figure 1: Mast cell deficient *Kit*$^{W\text{-}sh}$ "Sash" mice display peripheral neutrophilia and BM HSPC expansion. A.** Complete blood count analysis of circulating neutrophils (NE) and platelets (PLT) in steady-state littermate control (LC) and Sash (S) mice. N = 10 mice/group. **B.** Quantification of total nucleated bone marrow cells flushed per hindlimb of LC and Sash mice. N = 9 mice/group. **C.** Immunophenotypic enumeration of the following progenitor cell types (analyzed from bone marrow Lineage- c-Kit+ Sca1- cells): megakaryocyte progenitors (MkP), granulocyte-macrophage progenitors (GMP), myeloerythroid progenitors (MEP), pre-granulocyte-macrophage progenitor (Pre GM), and pre-colony forming unit-erythroid (Pre CFU-E), per hindlimb. N = 9 mice/group. **D.** Representative flow cytometric plots depicting c-Kit and Sca1 staining on Lineage- bone marrow cells (top), and CD150 and Endoglin staining on Lineage- c-Kit+ Sca1+ (LSK) cells (bottom) from littermate control and Sash mice. **E.** Immunophenotypic quantification of LSK, and hematopoietic stem cells (HSC) per hindlimb. N = 9 mice/group. **F.** Overall survival time of LC and Sash mice following weekly 5-Fluorouracil challenge. N = 10 mice/group. Statistical analysis performed by Student's t-test, except when LC vs. Sash variances were significantly different, in which case non-parametric testing by Mann-Whitney was performed, and in F, where a Log-rank (Mantel-Cox) test was used.

# Figure 2: The *Kit*$^{W\text{-}sh}$ “Sash” BM microenvironment is primed for hematopoietic proliferation

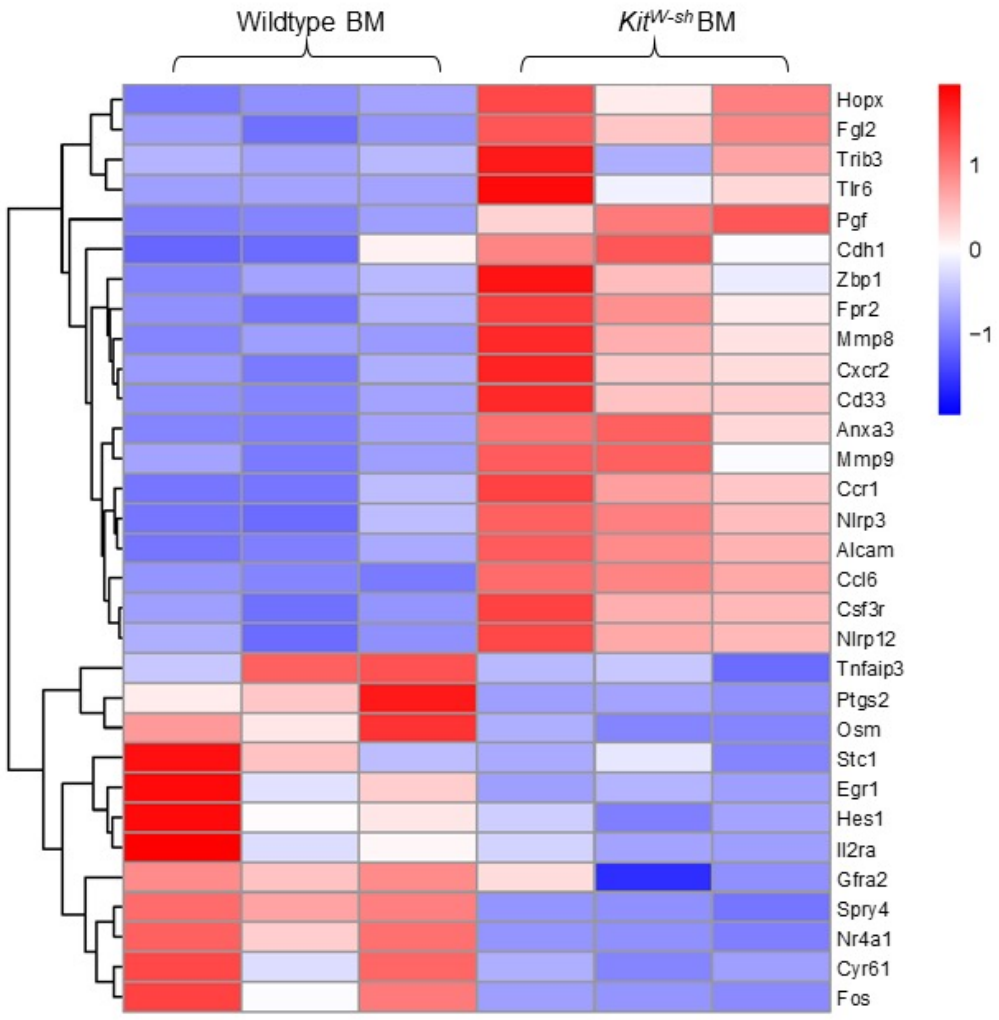


**Figure 2: The *Kit*$^{W\text{-}sh}$ “Sash” BM microenvironment is primed for hematopoietic proliferation**
Heat map representing color-coded expression levels of differentially expressed genes for biological triplicate samples of LC and *Kit*$^{W\text{-}sh}$ “Sash” total BM.

# Figure 3: The *Kit*$^{W\text{-}sh}$ "Sash" BM microenvironment promotes hematopoietic recovery following transplantation

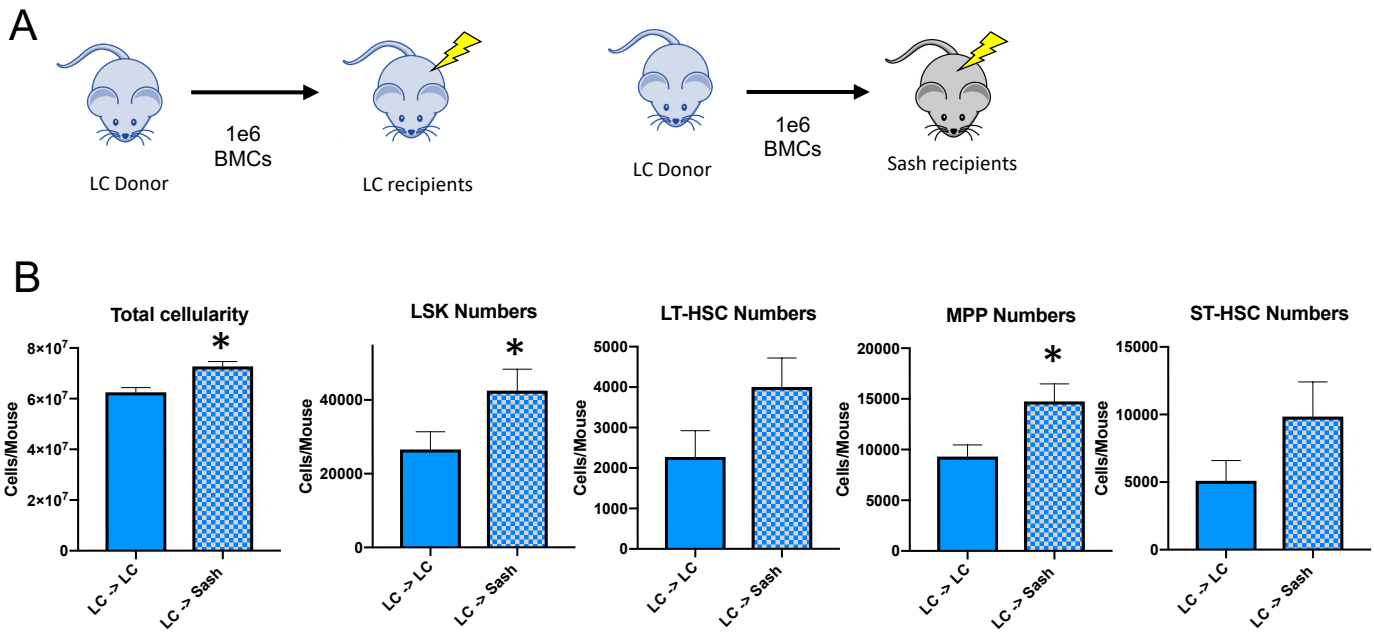


**Figure 3: The *Kit*$^{W\text{-}sh}$ "Sash" BM microenvironment promotes hematopoietic recovery following transplantation**
**A.** Schematic depicting HST of 1e6 total BM cells (either LC or "Sash") into lethally irradiated recipients (either LC or "Sash").
**B.** BM cellularity and quantification of lineage$^-$ c-Kit$^+$ Sca-1$^+$ (LSK), LSK (CD48(-), CD150(+) (LT-HSC), LSK CD48(+), CD150(-) (MPP), and LSK CD48(-),CD150(-) (ST-HSC), cells per mouse of recipients 20 days post-transplant, N = 19-22 mice per group. *$P < 0.05$, student's t-test.

# Figure 4: Pharmacologic mast cell stabilization results in BM progenitor cell expansion at steady-state

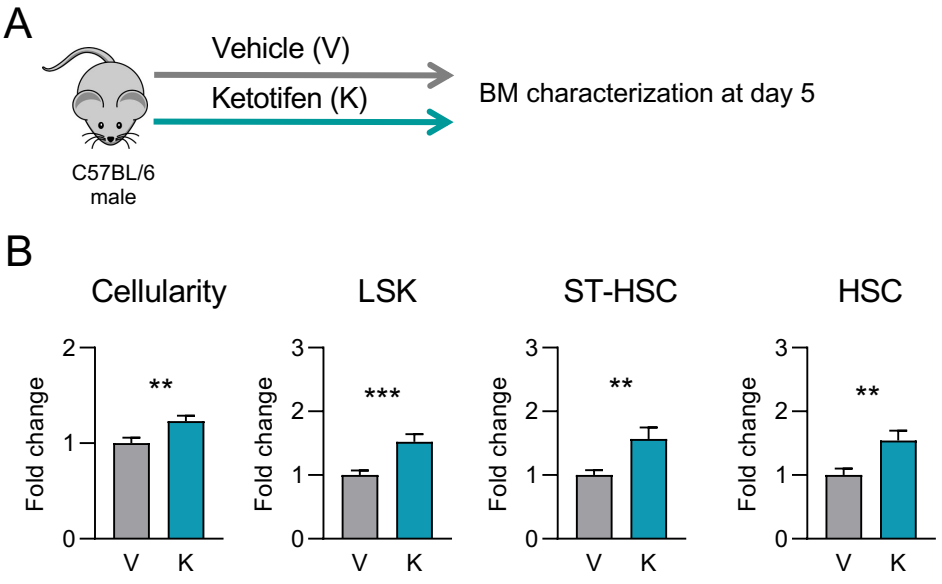


**Figure 4: Pharmacologic mast cell stabilization results in BM progenitor cell expansion at steady-state. A.** Schematic depicting 5 day (9 dose)10mg/kg b.i.d. Ketotifen administration to steady-state mice. **B.** Fold change in the number of total nucleated cells, Lineage- c-Kit+ Sca1- (LSK) cells, LSK CD48- CD150+ cells (phenotypic HSCs), and LSK CD48- CD150- cells (phenotypic ST-HSCs) per hindlimb in Veh- and Keto-treated mice. N = 10 mice/group.

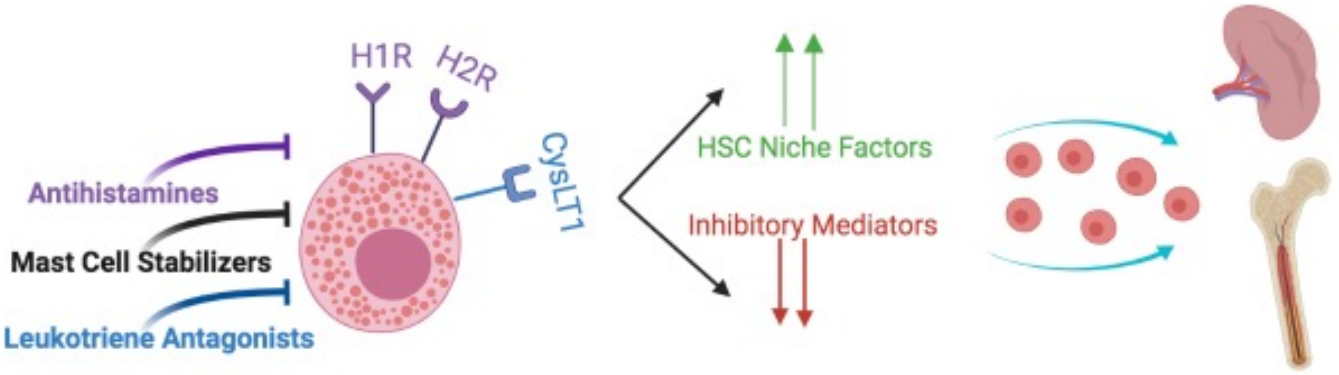


**Figure 5: Proposed therapeutic strategy to target mast cells to promote hematopoiesis. Figure created with BioRender.com.**