Ischemic Stroke Activates Hematopoietic Bone Marrow Stem Cells

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ABSTRACT

Rationale: The mechanisms leading to an expanded neutrophil and monocyte supply after stroke are incompletely understood.

Objective: To test the hypothesis that transient middle cerebral artery occlusion (tMCAO) in mice leads to activation of hematopoietic bone marrow stem cells.

Methods and Results: Serial in vivo bioluminescence reporter gene imaging in mice with tMCAO revealed that bone marrow cell cycling peaked 4 days after stroke (p<0.05 versus pre tMCAO). FACS and cell cycle analysis showed activation of the entire hematopoietic tree, including myeloid progenitors. The cycling fraction of the most upstream hematopoietic stem cells increased from 3.34%±0.19 to 7.32±0.52 after tMCAO (p<0.05). In vivo microscopy corroborated proliferation of adoptively transferred hematopoietic progenitors in the bone marrow of mice with stroke. The hematopoietic system’s myeloid bias was reflected by increased expression of myeloid transcription factors, including PU.1 (p<0.05), and by a decline in lymphocyte precursors. In mice after tMCAO, tyrosine hydroxylase levels in sympathetic fibers and bone marrow noradrenaline levels rose (p<0.05, respectively), associated with a decrease of hematopoietic niche factors that promote stem cell quiescence. In mice with genetic deficiency of the β3 adrenergic receptor, hematopoietic stem cells did not enter the cell cycle in increased numbers after tMCAO (naive control, 3.23±0.22; tMCAO, 3.74±0.33, p=0.51).

Conclusions: Ischemic stroke activates hematopoietic stem cells via increased sympathetic tone, leading to a myeloid bias of hematopoiesis and higher bone marrow output of inflammatory Ly6C<sup>high</sup> monocytes and neutrophils.

Keywords: Bone marrow, stroke, hematopoietic stem cells, monocyte

Nonstandard Abbreviations and Acronyms:

- Adrb3: β3 adrenoreceptor
- BrdU: 5-bromo-2-deoxyuridine
- tMCAO: transient middle cerebral artery occlusion, a model of experimental stroke in mice
- HSPC: hematopoietic stem and progenitor cells
- HSC: hematopoietic stem cells
- LKS: Lin<sup>-</sup> c-Kit<sup>+</sup> Sca-1<sup>+</sup> hematopoietic stem and progenitor cell
- GMP: granulocyte macrophage progenitor
- MDP: macrophage and dendritic cell progenitor
- CLP: common lymphoid progenitor
- GFP: green fluorescent protein
INTRODUCTION

The majority of strokes result from thrombotic events leading to ischemic injury of the brain. This sterile injury to the brain triggers a profound reaction of the immune system. Microglia, which are the most numerous resident immune cells of the central nervous system, proliferate and undergo inflammatory activation. Importantly, brain ischemia also triggers a systemic immune response. While blood lymphocyte numbers decline, levels of circulating neutrophils and monocytes increase in stroke patients1,2. These myeloid cells are recruited to the brain3 where they may contribute to the brain's recovery but also to reperfusion injury. Thus, the systemic number of innate immune cells, which recent studies relate to outcomes in patients2,4,5, increases acutely after stroke. These increased levels of circulating cells may reflect demargination from tissue vascular beds or increased production. Here we tested whether increased cell production contributed to this observed phenomenon. Innate immune cells have a life span on the order of hours to a few days. The number of leukocytes in blood is limited and cell reserves in the marginal blood pool, the bone marrow and the spleen exhaust rapidly after ischemic injury. We therefore examined the source of increased innate immune cell numbers in the circulation and in the ischemic brain, and the signals that regulate leukocyte supply after stroke. We hypothesized that bone marrow hematopoietic stem cells, a source of neutrophils and monocytes in the steady state, increase activity after transient middle cerebral artery occlusion (tMCAO) in mice.

We report that tMCAO activates the hematopoietic system at its most upstream point. Shortly after brain injury, hematopoietic stem cells enter the cell cycle, giving rise to downstream myeloid progenitors and innate immune cells. Bone marrow hematopoiesis acquires a strong myeloid bias, with reduced frequency of lymphoid progenitor cells. Increased autonomic nervous system activity after stroke activates hematopoietic stem cells through modulation of the hematopoietic bone marrow niche environment, contributing to the leukocytosis observed in patients.

METHODS

A detailed method section is available online.

Animals and stroke procedure.
Adult C57BL/6 and FVB/N mice (10-12 weeks old) were obtained from Jackson Laboratories and repTOP™ mitoIRE mice were purchased from Charles River Laboratories. Adrb3-/- mice (gift from P. Frenette) and Nestin-GFP reporter mice (gift from G. Enikolopov) were bred in our facilities. Experimental stroke was induced by a transient occlusion of the middle cerebral artery (tMCAO). The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures.

In vivo staining of bone marrow vasculature and bone lining cells.
To visualize bone structures, mice were administered intravenously with OsteoSense® 750EX (4 nmol/mouse, PerkinElmer). For in vivo endothelial cells labeling, mice were given i.v. APC anti-mouse CD31 (MEC13.3), Alexa Fluor® 647 anti-mouse VE-Cadherin (BV13) and APC anti-mouse Sca-1 (D7, 2 µg/mouse in 100µl PBS).

Whole mount immunofluorescence staining of the sternum.
Sternal were harvested and processed as described previously6, sectioned longitudinally and stained with rabbit anti-mouse tyrosine hydroxylase antibody (Millipore). Samples were incubated with secondary antibody and imaged using an Olympus IV100 microscope.
Confocal microscopy.

For serial intravital microscopy of the calvarium, SLAM HSCs were FACS-sorted, labeled with DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate, Molecular Probes) and injected i.v. into non-irradiated Nestin-GFP recipient mice. The GFP signal, while not specific for mesenchymal stem cells, guided revisiting similar regions of interest in serial intravital imaging. To highlight bone architecture, OsteoSense 750EX was administered (PerkinElmer). We used rhodamine-labelled Griffonia simplicifolia lectin (RL-1102, Vector Laboratories) to outline the vasculature. In vivo imaging was performed on days 1 and 5 after the adoptive cell transfer with a confocal microscope (IV100 Olympus). Z-stacks images for each location were acquired at 2µm steps. For visualization of LKS cells in sternum, Lin−c-Kit−Sca-1− cells were FACS sorted and labeled ex-vivo with two different fluorescent dyes including CellTrackerTM CM-Dil and SP-DiOC18(3) (Molecular Probes) prior to adoptive transfer into recipient mice.

RESULTS

Bone marrow activity increases after stroke.

We used a transient middle cerebral artery occlusion model of experimental stroke (tMCAO) in mice to address the link between the bone marrow and blood monocytosis and neutrophilia seen after ischemic brain injury in patients and rodents. Because mature myeloid cells, which are recruited to the inflamed brain and contribute to stroke, originate in the bone marrow, we evaluated bone marrow myeloid cell subsets by flow cytometry following stroke induction. While infarct sizes were comparable on day 1 and day 3 after tMCAO (Figure 1A), the percentages and numbers of mature CD11b+ cells (Online Figure I and Figure 1B) including neutrophils (Figure 1C) and inflammatory Ly6Chigh monocytes (Figure 1D) significantly increased in the bone marrow when compared to naive control mice. In contrast, bone marrow Ly6Clow monocyte levels did not change. Bone marrow myeloid cell content increased somewhat, but remained significantly lower after a sham procedure that exposed mice to similar surgical trauma as tMCAO but did not induce brain ischemia (Online Figure IIA).

To investigate whether the bone marrow undergoes broad activation after experimental stroke, we took advantage of MITO-Luc mice. These transgenic mice harbor a transgene in which luciferase gene expression is driven by the activity of a Nuclear Factor-Y-dependent cyclin B2 promoter (NF-Y). This enabled us to non-invasively monitor cell proliferation in the bone marrow of living mice before and after ischemic brain injury over a two-week period. As shown in Figure 1E, bone marrow luciferase activity increased rapidly after stroke, peaked on day 4 and returned to baseline levels by day 14. Sham surgery did not lead to a significant increase of bone marrow bioluminescence on day 4 (Online Figure IIB).

Stroke accelerates myelopoiesis.

After stroke, myeloid cells, especially neutrophils and monocytes, increase their presence in circulation. These cells are also recruited to the ischemic brain. Encouraged by the observed increase of proliferation imaging signal, we therefore next focused on hematopoiesis’ contribution to increased systemic leukocyte numbers. Whole bone marrow cultures from animals with stroke gave rise to higher numbers of colonies after 7 days (Figure 1F), reflecting increased hematopoietic progenitor cell activity. To further explore the marrow’s response following tMCAO, we performed flow cytometry to examine whether the increased mature myeloid cell numbers arise from upstream myeloid progenitor’s activity. We observed a significant increase in both, relative frequencies and absolute numbers of Lin−c-Kit−Sca-1−CD16/32−CD34+granulocyte macrophage progenitors (GMP) and Lin−c-Kit−Sca-1−CD16/32−CD34+CD115+macrophage and dendritic cell progenitors (MDP) after tMCAO (Fig. 1G). After sham surgery, a
mild increase of GMP and MDP did not reach statistical significance when compared to naive controls. Their numbers remained significantly lower than what was observed after tMCAO (Online Figure IIC, D).

To determine whether myeloid progenitors increased proliferative activity in the context of stroke, mice were given i.p. injections of 5-bromo-2-deoxyuridine (BrdU) on day 3 after tMCAO. Twenty-four hours after a single dose of BrdU, the fraction of BrdU+ GMP and MDP expanded significantly in animals with stroke when compared to naive control mice (Figure 1H). To decipher whether downstream mature neutrophils and monocytes derive from the accelerated proliferation of myeloid progenitors, we next examined BrdU incorporation into these differentiated cells. Indeed, higher numbers of both neutrophils and monocytes (Figure 1I) had incorporated BrdU (Figure 1J) after stroke, indicating that they had recently emerged from cycling progenitors. Because each GMP or MDP can give rise to multiple neutrophils and monocytes, the increase in BrdU+ cells was more pronounced in differentiated leukocytes than in progenitors.

*Stroke induces a myeloid bias of hematopoiesis.*

Leukocytosis after stroke is often associated with lymphopenia. We therefore also examined behavior of lymphoid committed progenitors in the bone marrow following stroke. In contrast to the expansion of myeloid progenitor cells, flow cytometric analyses of lymphoid progenitors revealed a drastic reduction in both, the relative frequency and absolute number of Lin\(^{-}\) IL7R\(\alpha\)\(^{+}\) c-Kit\(^{int}\) Sca-1\(^{int}\) common lymphoid progenitors (CLP), starting as early as day 1 after stroke induction (Figure 2A). Early immature B cells defined as Lin\(^{-}\) B220\(^{int}\) CD93\(^{+}\) were significantly diminished in the bone marrow on day 3 after tMCAO (Figure 2B). Altogether, the divergent activity changes of myeloid and lymphoid progenitors suggest that stroke may skew hematopoiesis toward the myeloid lineage.

To further explore this hypothesis, we FACS-sorted LKS from mice 3 days after tMCAO. These cells occupy a position in the hematopoietic tree that is just upstream of the divergence of lymphopoiesis and myelopoiesis. Consistent with the activation and expansion of myeloid progenitors at the expense of lymphoid progenitors, LKS from mice with stroke expressed higher levels of NF-\(\kappa\)B as well as transcription factors involved in myeloid differentiation such as PU.1 and CEBP\(\beta\)\(^1\),\(^2\). Furthermore, expression of several myeloid specific genes, including S100A9 and S100A8 alarmins, myeloperoxidase and chitinase 3-like-3 were also increased in LKS isolated from animals with stroke (Figure 3). In contrast, expression levels of mtg16, Mcsfr, c-myb and irf8 were not altered (Online Figure III). In agreement with the severe reduction of lymphoid progenitors and despite unchanged Ikaros expression levels, IL7R\(\alpha\), which is expressed by lymphoid LKS, was significantly reduced after tMCAO, highlighting a blockade of lymphoid commitment (Figure 3).

*HSC are the upstream point of bone marrow activation after stroke.*

Since myeloid but not lymphoid progenitors increased activity after stroke, we next asked whether upstream HSPC, which give rise to these cells, also respond to ischemic brain injury. Both, relative percentages and absolute numbers of LKS (Lin\(^{-}\) c-Kit\(^{+}\) Sca-1\(^{-}\)) in the femur bone marrow were significantly elevated after stroke (Figure 4A). The frequency and absolute number of the most upstream SLAM HSC subpopulation (Lin\(^{-}\) c-Kit\(^{+}\) Sca-1\(^{-}\) CD48\(^{-}\) CD150\(^{+}\)) increased in mice with tMCAO (Figure 4A, please see Online Figure IV for expanded gating strategy). Notably, the extent of the bone marrow HSC response correlated with infarct size (Figure 4B). Sham surgery did not result in significantly increased stem cell numbers (Online Figure IIE, F). The increase in numbers of hematopoietic stem cells after stroke was corroborated by higher percentages of both LKS and SLAM HSCs in the non G0 phase of the cell cycle 24 hours after stroke, as assessed by flow cytometry after ki-67 staining (Figure 4C).
These data indicate that HSC enter the cell cycle, expand in numbers and give rise to downstream progeny after ischemic stroke.

To directly visualize the proliferation of upstream stem and progenitor cells in the bone marrow after stroke, we performed ex vivo confocal microscopy on sternal bone marrow preparations following adoptive transfer of labeled LKS. One day before tMCAO, non-irradiated recipients received an intravenous mixture of FACS-sorted LKS that were labeled with two differently colored fluorescent membrane dyes (40,000 LKS per mouse; 1:1 cell ratio). Three days after stroke and four days after adoptive cell transfer, red CM-Dil+ and green SP-DiOC18(3)+ LKS were imaged in the sternal bone marrow, using naive control recipients without stroke as controls. The number of cell clusters, i.e. equal to or more than 2 cells in direct proximity, was increased in the marrow of mice with stroke (Figure 5). Notably, we never observed clusters containing a mix of red and green cells, indicating that proximate LKS likely proliferated after seeding the marrow.

We next performed serial intravital microscopy of the skull bone marrow following intravenous adoptive transfer of 20,000 FACS-sorted DiD labeled SLAM HSCs, cells that are upstream of LKS, into Nestin-GFP recipient mice. The GFP signal, together with vascular dye, aided in relocation of the same bone marrow regions in the second imaging session. Calvaria were imaged one day before and again 3 days after stroke, enabling us to serially monitor individual HSCs. Similar to transplanted LKS, SLAM HSCs also increased in number 3 days after tMCAO (Figure 6). Collectively, these data indicate that ischemic injury of the brain triggers proliferation of the most upstream hematopoietic stem and progenitor cells, suggesting that the entire hematopoietic tree, but especially the myeloid lineage, increases proliferation and causes the blood leukocytosis observed in patients after stroke.

**Stroke increases the sympathetic tone in bone marrow.**

HSC activity is regulated by the microenvironment of the bone marrow stem cell niche. Interestingly, autonomic tone, which is increased after stroke16-19 may influence the signaling of hematopoietic niche cells20, 21. Local sympathetic nerve fibers may release norepinephrine within the bone marrow to increase cell cycling, as observed in circadian rhythms or after MI20, 21 and in mice exposed to chronic psychosocial stress22. We therefore studied whether sympathetic tone alerts bone marrow cells after stroke.

Norepinephrine content in the bone marrow of mice with tMCAO increased significantly (Figure 7A). In line with this finding, immunofluorescence staining for tyrosine hydroxylase, the rate limiting enzyme in norepinephrine synthesis, was more pronounced in whole mount sternal preparations one day after cerebral ischemic injury. The increased staining pattern followed the typical distribution of sympathetic nerve fibers along bone marrow arterioles (Figure 7B-C). Norepinephrine indirectly regulates HSC migration and proliferation by modulating stromal cell expression of several factors, including Cxcl1220, which retains HSC in position and promotes a quiescent HSC state. In whole bone marrow, Cxcl12, VCAM-1, stem cell factor, angiopoietin-1 and IL-7 mRNA fell significantly 24 hours after tMCAO (Figure 7D), indicating that after stroke the molecular interaction between stromal niche cells and hematopoietic cells is markedly altered.

Bone marrow β3-adrenergic receptor signaling activates HSC but not LKS after stroke.

Bone marrow norepinephrine acts through β3-adrenergic receptors expressed by mesenchymal stromal cells, triggering downregulation of above maintenance factors20. We attempted to measure expression of the β3-adrenergic receptor in HSC and LKS isolated by flow sorting, however, in contrast to robust expression of this receptor by niche cells22, β3-adrenergic receptor mRNA was not detectable in HSPC. To determine the role of β3-adrenergic signaling in bone marrow niche cells after stroke, we...
conducted flow cytometric cell cycle analyses in Adrb3<sup>−/−</sup> mice after tMCAO. Since HSC activation correlates with stroke size (Figure 4B), only mice with large stroke volumes (defined as ≥ 35 mm<sup>3</sup>) were used for this analysis (wild type, 71.2±21.2; Adrb3<sup>−/−</sup>, 68.5±10.3 mm<sup>3</sup>). In wild type mice, LKS and HSC increased frequencies in both G1 and S/G2/M cell cycle phases one day after tMCAO (Figure 8A,B). In contrast to wild type mice, in which the number of cycling HSC rose to 7.32% ± 0.52% after tMCAO, HSC in mice with genetic deficiency for the β<sub>3</sub> adrenergic receptor did not increase cycling (3.74% ± 0.33%, p<0.01 versus wild type tMCAO), suggesting that the sympathetic nervous system regulates HSC activation through this receptor. Downstream LKS increased cell cycle entry after stroke in Adrb3<sup>−/−</sup> mice (Figure 8C,D). 24 hours after tMCAO, infarct size was comparable in Adrb3<sup>−/−</sup> and FVB/N wild type control mice (48.0±8.8 versus 42.9±14.6, p=0.23, n = 8-14). The loss of body weight after stroke was comparable in both cohorts (Adrb3<sup>−/−</sup> 2.6±0.2g, FVB/N 2.6±1.1g, p=0.49).

Altogether, these data are consistent with the notion that after ischemic stroke, sympathetic nervous signaling acts on hematopoietic niche cells, which then alter the bone marrow microenvironment to push increased numbers of HSC into the cell cycle.

DISCUSSION

In contrast to a maturing knowledge on local inflammation in the brain and changes in circulating immune cells<sup>9, 11, 12, 23</sup>, remarkably little is known about how the bone marrow, the primary site of hematopoiesis, reacts to ischemic stroke<sup>24, 25</sup>. In the current study, we report that tMCAO in mice activates hematopoietic stem cells and downstream hematopoietic progenitors, leading to an increased output of inflammatory monocytes and neutrophils while the number of lymphocyte progenitors declined. We identify enhanced bone marrow adrenergic signaling through the β3 adrenoreceptor on niche cells as a mechanism of hematopoietic stem cell activation. As a result, niche cells altered expression of cytokines and retention factors that regulate hematopoiesis.

Cells residing in the hematopoietic niche regulate leukocyte production through a number of soluble signals and adhesion molecules. These cells include mesenchymal stem cells, perivascular cells, endothelial cells, macrophages and osteoblasts<sup>26, 27</sup>. Integration of cell-cell communication informs hematopoietic stem cells whether they should remain quiescent, proliferate, differentiate or migrate. Quiescent hematopoietic stem cells and cycling downstream progenitors may reside in different bone marrow locations<sup>28</sup>. Some reports indicate that the most upstream, quiescent hematopoietic stem cells reside close to the endostem next to arterioles ensheathed with nestin-expressing mesenchymal stem cells, whereas more downstream precursors may locate in proximity to sinusoids<sup>29</sup>. Several key niche components, including Cxcl12 (also known as SDF-1), are provided by multiple cell types altering the localization and quiescence of HSC<sup>30</sup>. During circadian oscillation of bone marrow activity<sup>20</sup>, after myocardial infarction<sup>21</sup>, and in mice exposed to chronic psychosocial stress<sup>22</sup>, the sympathetic nervous tone changes, reflected by increased bone marrow levels of norepinephrine. This neurotransmitter binds β3 receptors on niche cells, which leads to downregulation of maintenance factors in the niche<sup>20</sup>. Reduction of the quiescence signal Cxcl12 increases hematopoietic stem cell activity<sup>26, 27</sup>, in parallel to what we observed in mice with stroke. Thus, it appears that several stimuli, including circadian rhythms, ischemic injury and chronic psychosocial stress converge on a similar neuro-immunological pathway.

Cxcl12 and IL7, whose expression fell after stroke, are also essential for the development of lymphoid progenitors such as early B cell precursors and CLPs<sup>31, 32</sup>. As stem cells, lymphoid and myeloid precursors in the bone marrow may not share the same micro-environmental niches for their respective maintenance and development, it is likely that stroke has broad but differential effects on multiple hematopoietic niches.
Genetic deficiency of β3 adrenoreceptor abolished the increase in HSC cycling after tMCAO; however, downstream LKS were still activated in Adrb3−/− mice. This interesting divergence suggests that specific regulation of progenitor cell classes may occur, possibly via differences in their location. Increased sympathetic signaling may primarily affect mesenchymal cells that are located next to arterioles in quiescent niches, and therefore reach hematopoietic stem cells residing there. After activated hematopoietic stem cells translocate to positions closer to sinusoids, they may be exposed to different, potentially soluble signals, e.g. circulating danger associated molecular patterns. For instance, hematopoiesis increases in response to toll-like receptor ligands or interferons in the setting of infection. In the context of stroke, break down of the blood-brain barrier allows systemic distribution of circulating messengers including inflammatory cytokines and alarmins liberated from ischemic and necrotic brain cells, which could likewise alert the hematopoietic system. How local or circulating stimuli, including danger signals, chemokines, survival factors and pro-inflammatory cytokines may differentially impact on distinct progenitor cell subsets (i.e. myeloid versus lymphoid) and/or their respective niches in the bone marrow after stroke will be the focus of future studies. Experiments with cell-specific deletion of the β3-adrenergic receptor should further refine the cellular identity of involved niche cells.

While activation and proliferation of microglia dominate the early cellular response to stroke, break down of the blood-brain barrier also enables robust recruitment of circulating immune cells, including neutrophils and monocytes. These cells are centrally involved in wound healing, including the healing of ischemic wounds. After myocardial infarction, monocytes are essential for tissue repair but their oversupply is detrimental for healing and leads to heart failure. Macrophage depletion studies suggest comparable mechanisms for brain ischemia, as lack of these cells leads to hemorrhagic conversion of ischemic stroke. On the other end of the spectrum, the level of inflammatory monocyte subtypes in blood correlates with worse outcome in stroke patients. Thus, there may be a hypothetical "sweet spot" of immune cell activity after stroke, in which salutary functions of resident microglia and recruited myeloid cells support resolution of inflammation and regeneration of the injured brain.

Thirty percent of acute stroke patients acquire infections, especially pneumonia. While infections frequently occur after any injury, and stroke-related sequela such as paralysis may also contribute, the high incidence of post-stroke infections is accompanied by lymphocyte apoptosis. Some authors postulated the existence of a peripheral immunosuppression, reporting an impressive atrophy of the thymus and the spleen after stroke that lasted for several days thereafter, a phenotype attributed to lymphocyte apoptosis. Our data indicate that hematopoiesis increased and that the bone marrow accelerated production of innate immune cells, pointing to an important exception from the peripheral immunosuppression after stroke. The lymphoid lineage suppression we observed in the bone marrow of mice with stroke may contribute to the previously reported blood lymphopenia.

The very leukocytes that are supplied to the ischemic brain and circulate in increasing numbers after stroke are also a driving force behind complications of atherosclerosis. Monocytes give rise to plaque macrophages and foam cells, which become apoptotic and form necrotic cores. Macrophage-derived proteases digest the protective fibrous cap. Such a destabilized plaque (e.g., within the carotid artery) may rupture and lead to ischemia of the brain. We recently described that experimental myocardial infarction accelerates hematopoiesis and atherosclerosis, possibly explaining the high secondary event rates observed in patients with a first infarct. Likewise, stroke may increase the supply of neutrophils and monocytes to remote atherosclerotic plaque, thus promoting stroke reoccurrence.

In ischemic heart disease, blood leukocytosis is associated with worse outcome. There are limited data for stroke patients, possibly reflecting that leukocytes and their subsets have dual roles, including support of recovery and healing. In addition, stroke pathophysiology is heterogeneous, and patients often
suffer from infections, which can influence leukocyte levels. In 1999, the Stockholm study\textsuperscript{46} reported that when controlled for initial stroke severity, multivariate regression analysis did not show association of leukocytosis to outcome. This study included patients with hemorrhagic stroke, and CT identified infarction in 60% of the population. A more recent study, which only included patients with ischemic stroke, found an association of leukocytosis with early poor outcome\textsuperscript{5}. Another report correlated increased CD14\textsuperscript{hi} CD16\textsuperscript{lo} monocyte levels with worse 90-day outcome and higher mortality in patients after adjustment for age and symptoms on admission\textsuperscript{2}. Many preclinical studies imply that anti-inflammatory intervention may improve stroke recovery\textsuperscript{23}. However, translation of these studies into clinical therapy has proven difficult, arguing that better understanding of systemic leukocyte subset fate in the setting of stroke is warranted\textsuperscript{33}.

Our study reports that increased signaling of the sympathetic nervous system activates hematopoietic stem cell activity in the bone marrow, increasing the output of neutrophils and inflammatory Ly-6C\textsuperscript{hi} monocytes after stroke. At the present time, it is unclear whether the bone marrow’s response to stroke represents a therapeutic target to improve stroke recovery and to prevent secondary stroke. Preclinical\textsuperscript{47,49} and some early clinical\textsuperscript{52,53} data suggest that beta blocker use may be beneficial after stroke. Observed effects may or may not be related to bone marrow activity, as the affinity of most clinical beta blockers for the β3 adrenoreceptor is low. Beta blockers lower blood pressure, which likely also influences outcome and reoccurrence of stroke. In addition, bone marrow-derived immune cells may support stroke recovery. Hence, while targeting overproduction of inflammatory immune cells is worthwhile in cardiovascular disease\textsuperscript{52}, there is a compelling need to gather additional data on precisely when and how to therapeutically intervene after stroke.

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DISCLOSURES
None.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Stroke increases bone marrow progenitor activity. A, Infarct size from TTC-stained brain sections on day 1 and day 3 after stroke induced by tMCAO in C57BL/6 mice (n= 4-6 mice per group). Flow cytometric-based enumeration of (B) CD11b+ myeloid cells, (C) neutrophils and (D) monocyte subsets per femur following tMCAO (n= 4-6 mice per group, one-way ANOVA). E, Representative bioluminescence images of MITO-Luc mice monitored one day before (baseline) and after tMCAO over a 2-week period. Quantification of luciferase activity in MITO-Luc mice following tMCAO at indicated time points is shown on the right (n= 4 mice per group, one-way ANOVA). F, Bone marrow (BM) colony-forming unit (CFU) assay on day 4 after tMCAO (n= 8 mice per group, Mann-Whitney test). G, Representative gating strategy and FACS dot plots for GMP (Lin- c-kit+ Sca-1- CD16/32+ CD34+) and MDP (Lin- c-kit+ Sca-1- CD16/32+ CD34+ CD115+) in the bone marrow on day 3 after tMCAO. Bar graphs display both frequencies in whole bone marrow and absolute numbers of cells per femur (n = 6-10 mice per group, Mann-Whitney test). H, On day 3 after tMCAO, mice were given BrdU i.p (1mg) and BrdU incorporation in myeloid progenitors was analyzed 24 hours later. Representative FACS dot plots show BrdU+ GMP (upper panels) and MDP (lower panels). Bar graphs on the right display percentages of BrdU+ GMP and MDP progenitors. I, Absolute numbers of neutrophil (upper panels) and monocytes per femur (lower panels). J, Analysis of BrdU incorporation in bone marrow neutrophils and monocytes following a single injection of BrdU (n= 4-6 mice per group, Mann-Whitney test). Mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 2.** Stroke decreases activity of lymphoid progenitors in the bone marrow. A, Representative flow cytometry staining of CLP (Lin- IL7Rα c-kitint Sca-int) from bone marrow cells on day 3 after tMCAO. B, Representative staining of immature B cells (Lin- B220int CD93+) from bone marrow cell suspensions. Bar graphs show both frequencies of lymphoid progenitors in total bone marrow cells and total cell numbers per femur. n= 4-6 mice per group, one-way ANOVA. Mean ± s.e.m., *P < 0.05, **P < 0.01.

**Figure 3.** Bone marrow LKS cells exhibit a myeloid bias after stroke. Experimental stroke was induced or not in C57BL/6 mice by tMCAO and LKS from the bone marrow were sorted by FACS three days later. Changes in gene expression levels were evaluated by RT-qPCR. n = 4-5 mice per group, Mann-Whitney test. Mean ± s.e.m., *P < 0.05, **P < 0.01.

**Figure 4.** The bone marrow response after stroke occurs at the most upstream hematopoietic stem cell level and correlates with injury size. Bone marrow cell suspensions were stained for LKS and HSC on days 1 and 3 after tMCAO in C57BL/6 mice. A, Representative staining, frequencies and numbers of LKS (Lin- c-kit+ Sca-1+; upper panels) and HSC (Lin- c-kit+ Sca-1+ CD48- CD150+; lower panels) per femur. B, Correlation between frequencies and numbers of SLAM HSC with infarct sizes was evaluated from different cohorts of mice on day 3 after tMCAO. C, FACS analysis of Ki-67+ LKS and HSC cells at indicated time points after experimental stroke. n= 6 mice per group, one-way ANOVA. Mean ± s.e.m., *P < 0.05, **P < 0.01.

**Figure 5.** Confocal imaging of LKS progenitor expansion in bone marrow of mice with stroke. Mice were injected intravenously with a mixture of FACs-sorted Lin- c-kit+ Sca-1+ labeled ex-vivo with red CM-Dil and green SP-DiOC18(3) fluorescent dyes prior to stroke induction. Imaging of whole mount sternal bone marrow preparations was performed three days after tMCAO. Fluorescent signal from the bisphosphonate imaging agent Osteosense-750 is depicted in blue and outlines bone. Vascular endothelial cells were stained by intravenous injection of fluorescently labeled antibodies targeting CD31, Ve-Cadh and Sca1. Scale bar represents 200 µm (low magnification) and 50µm (high magnification). The bar graph on the right displays the number of either green (SP-DiOC18) or red (CM-Dil) clusters with ≥2
cells per sternal preparation (n=3 mice per group). Neighboring cells of mixed color were not detected (ND). Mean ± s.e.m., *P < 0.05.

**Figure 6.** Serial intravital microscopy reports increased HSC expansion in the bone marrow of mice with stroke. Mice with and without stroke were transplanted with 20,000 FACS-sorted donor Lin− c-kit+ Sca-1+ CD48− CD150− HSC labeled ex-vivo with DiD fluorescent dye. Intravital microscopy of the mouse calvarium was performed serially, one day before and again 3 days after tMCAO. Blue color represents the fluorescence signal produced by the bone imaging agent Osteosense-750; the fluorescence lectin signal stained blood vessels in red. DiD labeled hematopoietic stem cells (HSC) are shown in white. Scale bar represents 50 µm. Bar graph displays the fold increase of HSC per field of view between the two imaging sessions in both groups (n=3 mice per group). Mean ± s.e.m., *P < 0.05.

**Figure 7:** Stroke increases the sympathetic nervous activity in the bone marrow. A, One day following tMCAO, bone marrow norepinephrine content was measured by ELISA. B, Whole mount immunofluorescence staining of tyrosine hydroxylase rich nerve fibers of the sternal BM on day 1 after tMCAO. C, Bar graph shows quantification of tyrosine hydroxylase positive area per field of view. D, Quantitative real-time PCR of HSC retention/maintenance related genes in the bone marrow on day 1 after tMCAO. n= 4-6 mice per group, Mann-Whitney test. Mean ± s.e.m., *P < 0.05.

**Figure 8:** Adrenergic signaling regulates hematopoietic stem cell activation after stroke via the β3 adrenergic receptor. tMCAO was induced in wild type (A,B) and Adrb3-/- mice (C,D) and cell cycle was analyzed in LKS progenitors and hematopoietic stem cells (HSC) one day later. A,C Representative FACS dot plots of cell cycle staining in LKS (upper panels) and HSC (lower panels). B, D, Bar graphs show flow cytometric analysis of LKS and HSC in both G1 and S/G2/M phases of the cell cycle. In wild type mice, HSC and LKS entered the active cell cycle phases, whereas Adrb3-/- mice showed a lack of HSC activation after stroke. n= 4-8 mice per group, Mann-Whitney test. Mean ± s.e.m., **P < 0.01 versus naive controls of same genotype.
Novelty and Significance

What Is Known?

- Microglia and recruited blood leukocytes contribute to the innate immune response after stroke.
- Ischemic stroke leads to monocytosis and neutrophilia, inflammatory immune cells that migrate to atherosclerotic lesions as well as ischemic tissue.

What New Information Does This Article Contribute?

- The bone marrow provides increased numbers of monocytes and neutrophils after stroke through increased myelopoiesis, while lymphoid progenitors are less active.
- Indirect sympathetic signaling to bone marrow niche cells leads to activation of most upstream hematopoietic stem cells.
- Stroke increases bone marrow noradrenaline levels that alter the hematopoietic niche by signaling through β3 adrenergic receptors.

Neutrophils and monocytes are recruited from the blood stream into the acutely ischemic brain where they, together with locally activated microglia, mount the response to ischemic stroke. While it is known that systemic numbers of these inflammatory myeloid cells expand after stroke, their source was incompletely understood. Here we show, using flow cytometry and confocal microscopy of the bone marrow, that ischemic stroke activates the entire hematopoietic tree. Bioluminescence imaging revealed that proliferative bone marrow activity peaks on day 4 after ischemia. A myeloid bias of hematopoiesis accelerates production of innate immune cells while bone marrow lymphopoiesis is suppressed. After stroke, bone marrow levels of noradrenaline increase, associated with reduced Cxcl12 levels. In mice with genetic lack of β3 adrenergic receptors, the activation of hematopoietic stem cells is inhibited. These data show that increased bone marrow production of leukocytes contributes to the acute immune system activation after stroke.
Figure 3

Relative mRNA levels / β-actin

- NFκB
- PU.1
- CEBPα
- CEBPβ
- Ikaros
- S100A9
- S100A8
- Mpo
- Chil3
- Il-7R

naive control 3d post-tMCAO
Figure 8

**A**

- **LKS**
  - **isotype**
    - Ki-67: 0
    - DAPI: 95.9
  - **control**
    - Ki-67: 23.8
    - DAPI: 71.3
  - **tMCAO**
    - Ki-67: 44.3
    - DAPI: 41.1

- **HSC**
  - **isotype**
    - Ki-67: 0
    - DAPI: 97.4
  - **control**
    - Ki-67: 16.5
    - DAPI: 79.7
  - **tMCAO**
    - Ki-67: 26.6
    - DAPI: 64.9

**B**

- **% LKS**
  - **Naive control**
    - G1 phase: 50
    - S/G2/M phase: 15
  - **1d post-tMCAO**
    - G1 phase: 10
    - S/G2/M phase: 5

- **% HSC**
  - **Naive control**
    - G1 phase: 30
    - S/G2/M phase: 20
  - **1d post-tMCAO**
    - G1 phase: 10
    - S/G2/M phase: 8

**C**

- **LKS**
  - **isotype**
    - Ki-67: 0
    - DAPI: 92.5
  - **control**
    - Ki-67: 23.6
    - DAPI: 69.3
  - **tMCAO**
    - Ki-67: 39.1
    - DAPI: 37.9

- **HSC**
  - **isotype**
    - Ki-67: 0
    - DAPI: 97
  - **control**
    - Ki-67: 17.8
    - DAPI: 79.2
  - **tMCAO**
    - Ki-67: 16.6
    - DAPI: 79.3

**D**

- **% LKS**
  - **Naive control**
    - G1 phase: 30
    - S/G2/M phase: 15
  - **1d post-tMCAO**
    - G1 phase: 5
    - S/G2/M phase: 8

- **% HSC**
  - **Naive control**
    - G1 phase: 10
    - S/G2/M phase: 10
  - **1d post-tMCAO**
    - G1 phase: 10
    - S/G2/M phase: 8

**Adrb3**

- **Naive control**
- **1d post-tMCAO**
Ischemic Stroke Activates Hematopoietic Bone Marrow Stem Cells
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Supplemental Material

Ischemic stroke activates hematopoietic bone marrow stem cells

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Detailed methods

Animals and stroke procedure
Adult C57BL/6 and FVB/N mice (10-12 weeks old) were obtained from Jackson Laboratories and repTOP™ mitoIRE mice were purchased from Charles River Laboratories. Adrb3−/− mice (gift from P. Frenette) and Nestin-GFP reporter mice (gift from G. Enikolopov) were bred in our facilities. Experimental stroke was induced by a transient occlusion of the middle cerebral artery (tMCAO). Mice were anesthetized with 1.5-2% isoflurane. Temperature was kept between 36-36.5°C using a temperature control system (Harvard Apparatus). After midline neck skin incision, the right carotid artery bifurcation was dissected, and the external carotid artery (ECA) was permanently ligated. A clip was then placed on the internal carotid artery, and a temporary ligation was tied onto the common carotid artery. An arteriotomy was then performed on the ECA. A commercial silicon coated filament (7019, Doccol corp, MA, USA) was then introduced and pushed from the ECA to the ICA after removal of the clamp, until blocked in the carotid termination. The filament was left in place for forty-five minutes and gently pulled out afterwards. A permanent knot on the ECA secured the arteriotomy from bleeding, and the temporary knot on the CCA was then removed to allow complete reperfusion. Painkillers were given at the completion of the surgical procedure (buprenorphine 0.1mg/kg subcutaneously) and mice recovered in a clean cage. In additional control experiments, sham surgery was performed using similar conditions as in tMCAO but without induction of brain ischemia. Specifically, the right carotid bifurcation was dissected and the external carotid artery permanently ligated. Afterwards, the incision was sutured and the mice allowed to wake up in a clean cage. No arteriotomy was performed. The Subcommittee on Research Animal Care at Massachusetts General Hospital approved procedures.

Flow cytometry
After bone marrow harvest, single cell suspensions were obtained and total number of cells per femur were determined using a hemocytometer and Trypan Blue staining method for cell viability (Cellgro, Mediatech, Inc, VA). All antibodies used in this study were purchased from eBioscience, BioLegend or BD Biosciences. For mature myeloid cells analysis, monoclonal antibodies including anti-CD11b (M1/70), Ly6G (1A8), CD115 (AFS98), Ly6C (AL-21) were used. Neutrophils were identified as CD11b+ Ly6G+, Ly6C(low) monocytes were identified as CD11b+ CD115+ Ly6C(low) and Ly6C(high) monocytes were defined as CD11b+ CD115+Ly6C(high). For hematopoietic stem and progenitor cells analyses, cells were stained with biotin conjugated antibodies against lineage markers including B220 (RA3-6B2), CD4 (GK1.5), CD8α (53-6.7), NK1.1 (PK136), CD11b (M1/70), CD11c (N418), Gr-1 (RB6-8C5), Ter119 (TER-119) followed by streptavidin Pacific Orange™ or APC/Cy7 conjugates, and antibodies against c-Kit (2B8), Sca-1 (D7), IL7Ra (SB/199), CD93 (AA4.1), CD16/32 (2.4G2), CD34 (RAM34), CD135 (A2F10), CD48 (HM48-1) and CD150 (TC15-12F12.2). Hematopoietic stem cells (HSC) were identified as Lin− c-Kit+ Sca-1+ CD48− CD150−, LKS were identified as Lin− c-Kit− Sca-1+. Granulocyte-macrophage progenitors (GMP) were identified as Lin− c-Kit− Sca-1+ CD16/32+ CD34+ and macrophage and dendritic cell progenitors (MDP) were defined as Lin− c-Kit− Sca-1+ CD16/32+ CD34− CD115+. Common lymphoid progenitors (CLP) were identified as Lin− IL7Ra+ c-Kit+ Sca-1− and early immature B cells were defined as Lin− B220−/hi CD93+. Cell numbers per femur were calculated as total cells per femur sample multiplied by percentage of cells obtained from the appropriate FACS gates. For BrdU incorporation assays, 1 mg of BrdU was injected i.p. 24 hours before harvesting. BrdU staining was performed using the FITC BrdU flow kit (BD Biosciences) according to the manufacturer’s protocol. For cell cycle analysis, cells were stained for LKS and HSC cell surface markers as described above, fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer’s instructions. Cells were then stained with anti-Ki67 antibody (SolA15, eBioscience) or isotype control. After washing, 1μl of DAPI (FxCycle™ Violet stain, Life Technologies) was subsequently added to samples. Data acquisition was performed using LSRII Flow Cytometer (BD). Data were analyzed using with FlowJo software (Tree Star).
In vivo staining of bone marrow vasculature and bone lining cells
To visualize bone structures, mice were administered intravenously with OsteoSense® 750EX, a fluorescent in vivo bisphosphonate imaging agent (4 nmol/mouse, PerkinElmer), 24 h before ex vivo staining of sternal preparations. For in vivo endothelial cells labeling, mice were given i.v APC anti-mouse CD31 (MEC13.3), Alexa Fluor® 647 anti-mouse VE-Cadherin (BV13) and APC anti-mouse Sca-1 (D7, 2 µg/mouse in 100 µl PBS), 30 min prior to organ harvest.

Whole mount immunofluorescence staining of the sternum
Sterna were harvested and processed as described in¹. Briefly, sternal bones were transected with a surgical blade to obtain compartments of sternal BM. Each specimen was further sectioned longitudinally to expose the bone marrow. Sternal preparations were fixed with 4% paraformaldehyde in PBS in a 96-well plate at room temperature for 30 minutes. Whole mount tissues were blocked and permeabilized overnight with 20% goat serum and 0.5% Triton X-100 in PBS. After washing with 0.1% Triton X-100 in PBS (4 x 15 minutes), BM tissues were stained with rabbit anti-mouse Tyrosine Hydroxylase antibody (Millipore, 1:100) in 20% goat serum and 0.1% Triton X-100 in PBS, for 2 days at 4°C. After washing, samples were incubated for 2 hours at RT with Alexa Fluor 488 goat anti-rabbit secondary antibody (Life technologies, 1:200). After washing, whole-mount tissues were imaged using an Olympus IV100 microscope and z-stacks images acquired at 2-5µm steps were processed with ImageJ software (NIH).

Confocal microscopy
For serial intravital microscopy of the calvarium, SLAM HSCs (Lin-c-Kit+Sca-1+CD48+CD150+) were FACS-sorted using a FACSaria II cell sorter (BD) from C57BL/6 mice and labeled ex vivo with the Vybrant® DiD Cell-Labeling Solution (1,1′dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate, Molecular Probes®) according to manufacturer’s protocol. 20,000 labeled HSCs were injected i.v. into non-irradiated Nestin-GFP recipient mice. Before imaging, whole-mount tissues were stained with OsteoSense® 750EX, a fluorescent bisphosphonate imaging agent administered i.v. 24 hours prior to imaging (4 nmol/mouse, PerkinElmer). To outline the vasculature, we used rhodamine-labelled Griffonia simplicifolia lectin (RL-1102, Vector Laboratories). Lectin was injected i.v. (50µl at 2 mg/ml) immediately prior imaging. In vivo imaging was performed on days 1 and 5 after the adoptive cell transfer using a confocal microscope (IV100 Olympus). Z-stacks images for each location were acquired at 2µm steps and post-processing was performed using Image J software (NIH). For visualization of LKS cells in sternal BM, Lin-c-Kit+Sca-1+ cells were FACS sorted and labeled ex vivo with two different fluorescent dyes including CellTracker™ CM-Dil and SP-DiOC18(3) (Molecular Probes) prior to adoptive transfer into recipient mice.

TTC staining
Brains were removed and sliced on a brain matrix every millimeter. Fresh tissue sections were then soaked in a solution of 2% TTC (2,3,5-Triphenyltetrazolium chloride, Sigma, St. Louis, MO) for 15 minutes protected from light. Stroke volume was measured using ImageJ software (imagej.nih.gov) by planimetry. Indirect stroke volume was given subtracting the volume of the non-injured parenchyma of the stroke hemisphere from the volume of the contralateral hemisphere.

In vivo bioluminescence imaging
Transgenic repTOP™ mitoIRE mice were anesthetized and injected i.p. with 150 mg/kg body weight with D-Luciferin (RR Labs Inc, San Diego, CA). Five minutes later, mice were moved to the imaging chamber of the IVIS™ 100 imaging system in a supine position. A region of interest (ROI) was drawn to encompass the entire mouse and light emission was recorded every 5 min until the signal intensity passed its peak value. Signal intensities were measured in photons per second using Living Image® software (Caliper Life Sciences).
Colony-Forming cell assay
CFC assays were performed using a semi-solid cell culture medium (MethoCult® GF M3434, STEMCELL Technologies) following the manufacturer’s instructions. Bones were flushed with Iscove’s Modified Dulbecco’s Medium (Lonza) supplemented with 2% FCS and 2×10^4 whole bone marrow cells were cultured in 35 mm dishes in duplicates and incubated at 37°C, 5% CO2 in a humidified incubator. Colonies were counted after 7 days using a low magnification inverted microscope (Nikon Eclipse TE2000-S).

RNA isolation and gene expression
Total RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. For qRT-PCR, RNA was treated with DNase I and reverse-transcribed using the high capacity RNA to cDNA kit (Applied Biosystems). Runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Taqman primers and reagents (Applied Biosystems). Threshold cycle values from target genes were normalized to housekeeping genes expression including Actinb (Actb) or glyceraldehyde-3-phosphate dehydrogenase (Gapdh) using the 2^(-ΔΔCt) method.

ELISA
Quantitative measurement of norepinephrine in the bone marrow was determined using a noradrenaline sensitive ELISA Assay Kit according to manufacturer’s protocol (DLD Diagnostika, Eagle Biosciences). Femurs were harvested and immediately snap-frozen and homogenized in a catecholamine stabilizing solution (4mM sodium metabisulfite, 1mM EDTA and 0.01N hydrochloric acid, pH=7.5) prior to the procedure.

Statistics
Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc.). Results are reported as mean ± standard error of mean. For a two-group comparison, a Mann-Whitney test for nonparametric data was used. For a comparison of more than two groups, an ANOVA test, followed by a Bonferroni test for multiple comparisons, was applied.

References
Online Figure I: Bone marrow myeloid cell content after stroke

A, FACS gating strategy and representative dot plots for identification of myeloid cell subsets in the bone marrow in non ischemic controls (upper panels) and on day 3 after stroke (lower panels) induced by tMCAO in C57BL/6 mice. B, Percentages of myeloid cells (Lin- CD11b+), neutrophils (Lin- CD11b- Ly6G+ CD115-), Ly6C<sup>high</sup> monocytes (Lin- CD11b+ Ly6G- CD115+ Ly6C<sup>high</sup>) and Ly6C<sup>low</sup> monocytes (Lin- CD11b+ Ly6G- CD115+ Ly6C<sup>low</sup>) in whole bone marrow at indicated time points following stroke. n= 4-6 mice per group, one-way ANOVA. Mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.
Online Figure II: Sham surgery mildly alters myeloid content in the bone marrow.

Bone marrow cell suspensions from naive controls, sham-operated controls and mice with tMCAO (day 4) were stained for stem and progenitor cells. FACS enumeration per femur and percentages of mature myeloid cells (A), GMP (C), MDP (D), LKS (E), HSC (F) in whole bone marrow (n= 4-6 mice per group, one-way ANOVA). B. Quantification of luciferase activity in MITO-Luc mice on day 4 following sham operation and tMCAO (n= 4 mice per group, one-way ANOVA). Mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.
Online Figure III: Unchanged gene expression in LKS after stroke
Experimental stroke was induced or not in C57BL/6 mice by tMCAO and LKS from the bone marrow were sorted by FACS three days later. Gene expression levels of mtg16, Mcsfr, c-myb, gata2 and irf8 in LKS were not altered after ischemic injury of the brain as assessed by RT-qPCR. n = 4-5 mice per group.
Online Figure IV: FACS gating strategy for identification of hematopoietic stem cells in the bone marrow.
SLAM HSC (red population) are identified as lineage negative (B220− CD4− CD8− NK1.1− CD11b− CD11c− Gr-1− Ter119− IL7Rα−) c-kit+ Sca-1+ CD135+ (Flk2), CD34− CD48− CD150+. 