Sirolimus augments hematopoietic stem and progenitor cell regeneration following hematopoietic insults

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Abstract
The role of mammalian target of rapamycin and its suppressor sirolimus in the regulation of hematopoietic stem and progenitor cells (HSPCs) is controversial. We show here that sirolimus enhanced regeneration of HSPCs in mice exposed to sublethal total body irradiation (TBI) and other regenerative stressors. Sorted Lin^-CD150^ bone marrow cells from sirolimus-treated TBI mice had increased expression of c-Kit and other hematopoietic genes. HSPCs from sirolimus-treated TBI mice were functionally competent when tested by competitive engraftment in vivo. Postradiation regeneration of HSPCs in mice treated with sirolimus was accompanied by decreased \(\gamma\)-H2AX levels detected by flow cytometry and increased expression of DNA repair genes by quantitative polymerase chain reaction. Reduction of cell death and DNA damage post-radiation by sirolimus was associated with enhanced clearance of cellular reactive oxygen species (ROS) in HSPCs. Increased HSPC recovery with sirolimus was also observed in mice injected with hematotoxic agents, busulfan and 5-fluorouracil. In contrast, sirolimus showed no effect on HSPCs in normal mice at steady state, but stimulated HSPC expansion in mice carrying the Wv mutation at the c-Kit locus. In human to mouse xenotransplantation, sirolimus enhanced engraftment of irradiated human CD34^+ cells. In summary, our results are consistent with sirolimus' acceleration of HSPC recovery in response to hematopoietic stress, associated with reduced DNA damage and ROS. Sirolimus might have clinical application for the treatment and prevention of hematopoietic injury.

KEYWORDS
DNA damage, hematopoietic stem and progenitor cells, hematopoietic stress

1 INTRODUCTION

Hematopoietic stem and progenitor cells (HSPCs) are a rare population of precursor cells with capacity for self-renewal and multilineage differentiation. In the bone marrow (BM), HSPCs maintain blood cell homeostasis and respond to regenerative stress. Exposure to irradiation or hematotoxic chemicals, such as busulfan and 5-fluorouracil, damages HSPCs and decreases blood cell production.\textsuperscript{1-4} Effective countermeasures to stimulate HSPC regeneration, reverse hematopoietic damage, and restore normal blood production are obviously useful in these clinical circumstances.

Sirolimus is a product of the bacterium \textit{Streptomyces hygroscopicus}, first isolated from a soil sample collected on Rapa Nui (Easter Island).\textsuperscript{5-7} Also referred to as rapamune and rapamycin, sirolimus is approved for...
clinical use to prevent organ transplant rejection and to treat the disease lymphangioleiomyomatosis8,9,10 and has also been tested in a broad range of clinical settings, such as nonmyeloablative BM transplantation of sickle cell disease11,12 and allogeneic HSPC transplantation for graft-versus-host disease.13-16 Sirolimus acts directly to inhibit mTOR complex 1 (mTORC1) and indirectly to suppress mTORC2 signaling to regulate cellular metabolism, survival, and growth.17

Sirolimus’ wide biological effects also include life-extension in genetically heterogeneous mice.18 Mechanisms of sirolimus’ antiaging effects have been investigated in model organisms such as Drosophila melanogaster,19 yeast,20 and worms.21 The effects of sirolimus and mTOR on hematopoiesis and HSPCs have also been studied under various experimental settings. In a mouse model of tuberous sclerosis complex gene deletion with constitutive mTOR activation, young animals exhibited accelerated HSPC aging with increased expression of p16, p19, p21, and reduced engraftment potential, all restored by sirolimus administration.22 Sirolimus suppressed mTOR activity and enhanced HSPC engraftment after exposure of mouse and human cells ex vivo.23 Similarly, after expansion of mouse c-Kit"Sca-1"Lin" (KS) cells, sirolimus mitigated mTOR activation, suppressed HSPC senescence, promoted HSPC expansion, and enhanced long-term HSPC engraftment.24 These observations all suggest a negative role of mTOR in hematopoiesis and a positive role of sirolimus in suppressing mTOR activity so as to enhance HSPC function. In contrast, mTOR activity was reported to be a requirement for the expansion of human CD34’ hematopoietic progenitor cells,25 and deletion of mTOR complex 1 (mTORC1) and regulatory-associated protein of mTOR (Raptor) in mouse HSPCs caused pancytopenia, splenomegaly and monocytoid cell accumulation,26 suggesting a positive role of mTOR in the maintenance of normal hematopoiesis. Thus, the involvement of mTOR and sirolimus in the regulation of hematopoiesis and HSPC function is not entirely clear.

We previously reported sirolimus as effective treatment of immune-mediated BM failure in mice,27 and demonstrated that attenuation of marrow destruction and protection of hematopoiesis by sirolimus were mainly due to depletion of clonogenic T cells and preservation of immunosuppressive regulatory T cells. In the current study, we tested sirolimus’ direct effects on HSPC regeneration under conditions of nonimmune-mediated hematopoietic stresses, induced by sublethal irradiation and by myeloablative drugs busulfan and 5-fluorouracil. In these models of hematopoietic injury, sirolimus enhanced HSPC regeneration. BM cells from sirolimus-treated total body irradiation (TBI) mice were functionally competent to engraft lethally irradiated recipients. Furthermore, sirolimus-mediated HSPC regeneration after hematopoietic injury was associated with reduced DNA damage and reactive oxygen species (ROS).

2 METHODS

2.1 Animals, irradiation, and treatment with sirolimus

Inbred C57BL/6 (B6), congenic B6.SJL-Ptprc<sup>−</sup>Pep3<sup>−</sup>/Boy (CD45.1), hybrid (BALB/cBy × B6F1) (CByB6F1), heterozygous C57BL/6J-Kit<sup>−</sup>W<sup>−</sup>v<sup>−</sup> (B6-Kit<sup>−</sup>W<sup>−</sup>v<sup>−</sup>), and NOD.Cg-Prkdcscid Il2rgtm1Wjl/Sz (NSG) mice were all originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and were bred and maintained in National Institutes of Health animal facilities under standard care and nutrition. All animal studies were approved by the Animal Care and Use Committee at the National Heart, Lung, and Blood Institute. B6, CByB6F1, or B6-Kit<sup>−</sup>W<sup>−</sup>v<sup>−</sup> mutant mice received TBI from a cesium gamma source (J. L. Shepherd & Associates, Glandale, California) at a dose rate of 90 rads/min.

Irradiated animals were treated with sirolimus for 2 weeks starting at 1 hour after irradiation or left untreated as controls. Sirolimus (rapamycin) was obtained from LC Laboratories (Woburn, Massachusetts), dissolved in pure ethyl alcohol (The Warner-Graham Company, Cockeysville, Maryland) at 50 mg/mL, and stored in aliquots at −30°C. Right before injection, sirolimus was diluted to 200 μg/mL with a 5% PEG-400 (poly ethylene glycol MW 400, Sigma-Aldrich, St. Louis, Missouri) and 5% Tween 80 (Sigma-Aldrich) diluted, filtered through 0.22-μm Millex<sup>−</sup>G<sub>−</sub>C. Stock busulfan was diluted with water 1:5 right before injection to get a working solution of 1 mg/mL, filtered through 0.22-μm Millex<sup>−</sup>G<sub>−</sub>S syringe filter, and i.p. injected into B6 mice at 150 mg/kg. Three injections were performed on days 0, 15, and 29, respectively. Some 5-fluorouracil-treated animals were then received sirolimus (2 mg/kg) with five daily injections/week for 2 weeks starting 3 days after the last 5-fluorouracil injection. Animals were bled and analyzed 3 days after the last sirolimus injection. Mice treated with 5-fluorouracil without sirolimus injection were used as controls.

Busulfan (Sigma-Aldrich) was dissolved in acetone at 6 mg/mL and stored at −20°C. Stock busulfan was diluted with water 1:5 right before injection to get a working solution of 1 mg/mL, filtered through 0.22-μm Millex<sup>−</sup>G<sub>−</sub>S syringe filter, and injected i.p. into B6 mice at 10 mg/kg at three injections per week for a total of 10 injections per animal. Some busulfan-injected animals were then received sirolimus (2 mg/kg) with five daily injections/week for 2 weeks starting 3 days
after the last busulfan injection. Animals were bled and analyzed 3 days after the last sirolimus injection. Mice treated with busulfan without sirolimus injection were used as controls.

## 2.3 | Blood and BM cell counts, cell staining, and flow cytometry

Blood was collected from the retro-orbital sinus into ethylenediaminetetraacetic acid (EDTA)-added Eppendorf tubes. Complete blood count (CBC) was performed by a HemaVet 950 analyzer (Drew Scientific, Inc., Waterbury, Connecticut). After mouse euthanasia by CO2, BM cells were extracted from tibiae and femurs, filtered through 95-μM nylon mesh, and counted by a Vi-Cell counter (Beckman Coulter, Miami, Florida). Total BM counts were calculated as cell numbers from two tibiae and femurs × four as previously described. BM cells were stained with antibody mixtures on ice for 30 minutes in fetal bovine serum (FBS)-supplemented RPMI 1640 (Life Technologies) and acquired using BD FACSCanto II and BD LSRFortessa flow cytometers operated by FACSDiva software (Becton Dickson, San Diego, California). Stained BM cells from TBI and sirolimus-treated mice were analyzed for the proportions of HSPCs.

Monoclonal antibodies for murine CD3 (clone 145-2C11), CD4 (clone GK 1.5), CD8 (clone 53-6.72), CD11b (clone M1/70), CD25 (clone 7D4), CD45R (clone RA3-6B2), CD45.1 (clone A20), CD45.2 (clone 104), CD48 (clone HM48-1), CD117 (c-Kit, clone 2B8), CD150 (clone TC15-12F12.2), erythroid cells (clone Ter119), granulocytes (Gr1/Ly6-G, clone RB6-8C5), stem cell antigen 1 (Sca1, clone TC15-12F12.2), and γ-H2AX were from Biolegend (San Diego, California). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanin 5 (PE-Cy5), PE-cyanin 7 (PE-Cy7), allophycocyanin (APC), or brilliant violet 421 (BV421).

## 2.4 | Colony-forming cell assays

BM mononuclear cells from various treatment groups in the three hematopoietic stress models were mixed in semisolid methylcellulose media containing interleukin (IL)-3, IL-6, stem cell factor (SCF) and erythropoietin (EPO; STEMCELL Technologies, Inc., Vancouver, British Columbia, Canada) at 3 × 10^4 cells/plate. BM cell cultures were grown at 37°C with 5% CO2. Colonies were counted at day 10.

## 2.5 | Competitive repopulation

BM cells extracted from B6 mice that received 5Gy TBI without or with 13 daily injections of sirolimus were used as donors, while BM cells from congenic B6-CD45.1 mice were used as competitors. BM cells from each donor were diluted in IMDM, mixed with BM cells from B6-CD45.1 competitor at 5:1 ratio, and were injected into lethally irradiated (11 Gy TBI using the same ^137cesium gamma source) B6-CD45.1 recipients with each recipient receiving 10^6 donor and 2 × 10^5 competitor BM cells. Recipient mice were bled once every month for 7 months to measure donor HSPC contribution to the peripheral hematopoietic compartment via CD45.1/CD45.2 allelic difference by flow cytometry.

## 2.6 | Gene expression array

Total RNA was isolated from sorted Lin^−/CD150^+ CD117^+^ BM cells with the RNeasy Kit (Qiagen, Valencia, California), according to the manufacturer’s instructions. Then RNA was digested with RNase free DNase I (Qiagen) and assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). First strand cDNA was synthesized with 200 ng of total RNA using RT^2^ First Strand Kit (Qiagen). Quantitative analysis of mRNA expression of hematopoiesis-related genes or DNA damage signaling-related genes was performed using mouse PCR arrays (Qiagen), according to the manufacturer’s instructions.

## 2.7 | Cell death, reactive oxygen species, and DNA damage post-irradiation

B6 mice were irradiated at 5 Gy, then euthanized, and BM cells were cultured in IMDM supplemented with 10% FBS, 10 ng/mL of IL-3, FMS-like tyrosine kinase 3 ligand (FLT3L), and SCF in the presence of sirolimus for 3 days or without sirolimus as controls. By flow cytometry, BM cells double negative for both annexin V and 7-aminoactinomycin D (7-AAD) were recognized as live cells; ROS levels were reflected by the fluorescence intensity of cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Life Technologies); and DNA damage was measured by intracellular γ-H2AX levels in KSL BM cells.

## 2.8 | Xenotransplant model

Human granulocyte colony-stimulating factor (G-CSF)-mobilized CD34^+^ cells (kindly provided by Dr Andre Larochelle at National Heart, Lung, and Blood Institute [NHLBI]/National Institutes of Health [NIH]) underwent 5-Gy irradiation, then were transplanted (2 × 10^5^ human CD34^+^ cells/mouse) into NSG mice that had been irradiated at 3 Gy. Transplanted mice were treated with or without sirolimus (2 mg/kg/d five daily injections/week for 2 weeks). The percentage of human CD45^+^ cells vs total cells in the BM at 3 weeks were counted as engraftment of human CD34^+^ cells by flow cytometry.

## 2.9 | Statistics

Data obtained from CBC, cell counting, and flow cytometry were analyzed by unpaired t test, variance analyses, and multiple comparisons using GraphPad Prism statistical software. Data from competitive repopulation assay with multiple time observations of the same recipients were analyzed using JMP statistical discovery software with a mixed model of repeated measures analyses to test overall donor effects. Data are presented as means with standard errors. Statistical significance was declared at P < .05.
3 | RESULTS

3.1 | Sirolimus augmented HSPC regeneration in response to radiation stress

In our first model of hematopoietic stress, B6 mice were subjected to TBI of 5 Gy, then treated with sirolimus. TBI mice without sirolimus administration were controls (Figure 1A). At day 14 there were no significant changes in white blood cells, red blood cells, platelets or total BM cells between experimental and control groups (Figure 1B), but we observed two to four times higher percentages and total numbers of HSPCs including KSL, KSLCD150⁺ (KSLS), and KSLCD48⁻ (KSLSS) cells in sirolimus-treated mice (Figure 1C). When BM cells were cultured for 10 days in methylcellulose, there were two to three times...
more granulocyte colony-forming units (CFU-G), megakaryocytic CFU (CFU-M), granulocyte/macrophage CFU (CFU-GM), and total colony-forming cells (CFCs) in sirolimus-treated TBI mice than in TBI controls (Figure 1D).

We compared expression of genes related to hematopoiesis in Lin-CD150+ BM cells from TBI mice with or without sirolimus treatment. Sirolimus upregulated expression of genes important for hematopoietic regulation: Cd34, ETS variant 6 (Etv6), and c-Kit, along with down regulation of genes with defined roles in immune function: Blnk, Pax5, TNF superfamily member 11 (Tnfsf11), Cd2, Cd8, and Jag1 (Figure 1E).

To confirm HSPC functionality, we transplanted BM cells from sirolimus-treated TBI mice and from TBI control donors (CD45.2) into lethally irradiated recipients in a standard competitive repopulation assay, using B6-CD45.1 mice as competitors and recipients (Figure 2A). There was higher engraftment from sirolimus-treated TBI donors relative to TBI controls, in recipient peripheral blood cells at different time points (Figure 2B). In examining the effect of sirolimus on hematopoietic lineage differentiation, we found that BM cells from either sirolimus or TBI donors had similar low levels of contribution to CD3+ T cells in the recipient peripheral blood (Figure 2C). However, BM cells from sirolimus donors had significantly higher levels of contribution to CD45R+ B cells and CD11b+ myeloid cells in recipient peripheral blood relative to BM cells from untreated TBI donors (Figure 2C), with the effect being consistently observed over time, indicating that sirolimus augmented hematopoietic lineage commitment favoring B cells and myeloid cells but not T cells. When recipient animals were euthanized at 7 months after transplant, we recovered similar numbers of total BM cells, but significantly higher total KSL cells and donor-originated KSL cells from recipients of sirolimus-treated TBI donors than from recipients of TBI control donors (Figure 2D). Thus, sirolimus augmented donor BM cell engraftment of recipient mature blood cells and increased donor-derived KSL cell recovery in recipient BM cells. These data demonstrate that HSPCs expanded by sirolimus were functionally competent.

3.2 | Sirolimus may preserve HSPCs by decreasing DNA damage and cell death through clearance of reactive oxygen species

Radiation is a classic inducer of DNA damage, and the mTORC1 pathway is implicated in the DNA damage response and repair system; therefore we hypothesized that sirolimus might reduce DNA damage and cell death induced by irradiation. To test our hypothesis, we irradiated B6 mice, then treated them with sirolimus for 3 days. Intracellular levels of γ–H2AX, a biomarker of DNA double-strand breaks, were evaluated by flow cytometry. Sirolimus-treated mice showed decreased γ–H2AX in BM KSL cells compared to untreated TBI mice (Figure 3A). To further investigate the molecular changes in sirolimus-

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induced HSPC regeneration, BM Lin−c-Kit+ cells were sorted and then their transcriptomes interrogated for genes in DNA damage signaling pathway. Compared with TBI control mice, cells from sirolimus-treated mice showed higher expression of Abl1, Ddit3, Mlh3, Bax, Rad17, Fancg, Mif, and Mgmt, but decreased expression of Mcph1, Terf1, and Xrcc3 (Figure 3B). All of the upregulated genes are related to DNA damage response; half of them (Abl1, Mlh3, Fancg, and Mgmt) are also implicated in DNA repair.

To further investigate the protective effects of sirolimus on cell death, we irradiated and euthanized B6 mice, then cultured their BM cells with or without sirolimus for 3 days. BM live cells double negative for annexin V and 7AAD were significantly higher in sirolimus-treated BM cells by flow cytometry. We did not observe greater proportions of KSL cells in sirolimus-treated BM cells compared with untreated BM cells probably due to the short period of culture, but we did see a large increase of c-Kit+ cells in Lin− population (Figure 3C). There was a significant decrease of ROS detected by H2DCFDA in the c-Kit+Lin− cells treated by sirolimus relative to the same population from untreated BM cells, suggesting that reduction of ROS might be one mechanism of sirolimus action in decreasing DNA damage and cell death.

**FIGURE 3** Sirolimus reduces cell death and DNA damage in HSPCs following irradiation by decreasing ROS. A, γ-H2AX expression in BM KSL cells from TBI (N = 3) or SIR (N = 4) mice detected by flow cytometry. B6 mice were irradiated at 5 Gy, then were treated with sirolimus for 3 days (2 mg/kg/day, i.p., SIR, N = 4) or left untreated as controls (TBI, N = 3). BM cells were extracted for intracellular γ-H2AX detection or were sorted for gene expression. **P < .01. B, Expression of genes related to DNA repair in BM c-Kit+Lin− cells induced by sirolimus following irradiation. Each column in heatmap represents pooled c-Kit+Lin− cells from the same group. Red color represents high expression, blue low expression. Genes with more than twofold change compared with TBI controls are shown. C, Cell death in BM cells and ROS levels in c-Kit+Lin− cells post irradiation. B6 mice were irradiated at 5 Gy, then were euthanized, and BM cells were cultured with sirolimus for 3 days (100 ng/mL, SIR, N = 3) or without sirolimus as controls (TBI, N = 3). In the representative histogram of ROS, blue line represents SIR, red line TBI control, and gray line unstained negative control. BM, bone marrow; CFU, colony-forming unit; CFC, colony-forming cells; HSPCs, hematopoietic stem and progenitor cells; i.p., intraperitoneal; KSL, c-Kit+Sca-1−Lin−; ROS, reactive oxygen species; TBI, total body irradiation.
FIGURE 4  Sirolimus stimulates HSPC regeneration following 5-FU-induced hematopoietic injury. A, B6 mice were injected with 5-FU at 150 mg/kg (i.p.) on days 0, 15, and 29, respectively, then were treated with 10 sirolimus injections (SIR, 2 mg/kg/d, i.p., N = 10) in 2 weeks starting 2 days after the last 5-FU injection or were left untreated as controls (5-FU, N = 10). B, Complete blood counts (WBCs, RBCs, and platelets) and total BM cell recovery following sirolimus treatment post 5-FU injection. C, Proportions and total numbers of KSL, and KSLS cells in the BM from SIR-treated and untreated mice. D, Proportions and total numbers of c-Kit+ cells in Lin− and total BM cell populations. E, Frequencies of CFU-GM, CFU-G and total CFC in SIR-treated mice (N = 5) and 5-FU control mice (N = 5). **P < .01; ***P < .001. 5-FU, 5-fluorouracil; BM, bone marrow; CFU, colony-forming unit; CFC, colony-forming cells; HSPCs, hematopoietic stem and progenitor cells; i.p., intraperitoneal; KSL, c-Kit+Sca-1+Lin−; KSLS, KSLCD150+.
Sirolimus stimulates HSPC regeneration in response to busulfan-induced hematopoietic depression. A, B6 mice received 10 i.p. injections of busulfan (BSF) at 10 mg/kg three times per week, then were treated with 10 injections of sirolimus (SIR, 2 mg/kg/d, N = 10) in 2 weeks starting 3 days after the last BSF injection or were left untreated as controls (BSF, N = 10). B, Complete blood counts (WBCs, RBCs, and platelets) and total BM cell recovery following sirolimus treatment post BSF injection. C, Proportions and total numbers of KSL, and KSLS cells in the BM from SIR-treated animals and BSF control mice. D, Proportions and total numbers of c-Kit+ cells in Lin− and total BM cell populations. E, Frequencies of CFU-G, CFU-M, CFU-GM, and total CFC in the BM cells from SIR-treated-BSF mice and BSF control mice. *P < .05; **P < .01. BM, bone marrow; CFU, colony-forming unit; CFC, colony-forming cells; HSPCs, hematopoietic stem and progenitor cells; i.p., intraperitoneal; KSL, c-Kit+Sca-1−Lin−; KSLS, KSLCD150+.
3.3 | HSPC restoration by sirolimus after 5-fluorouracil and busulfan administration

We sought to generalize sirolimus' regenerative effects on HSPCs to other stressors of hematopoiesis. To this end, B6 mice were injected with 5-fluorouracil or busulfan, then left untreated as controls or treated with sirolimus at 2 mg/kg/d i.p. for 10 days in 2 weeks (Figures 4A and 5A). Sirolimus increased HSPC recovery after both forms of myeloablation, similar to improvement after TBI stress. In these models of chemical injury, there was no apparent effect on peripheral blood red blood cells (RBCs), white blood cells (WBCs), and platelets and on total BM cells (sirolimus appeared to decrease WBCs in busulfan mice; Figures 4B and 5B). However, sirolimus significantly increased the percentages and total numbers of BM KSL and KSLS cells, relative to 5-fluorouracil (Figure 4C) and busulfan (Figure 5C) control mice. Sirolimus increased c-Kit expression in the Lin− cells population but not in total BM cells in 5-fluorouracil-treated mice (Figure 4D), and increased c-Kit expression in both Lin− and total BM population in busulfan mice (Figure 5D). When cultured in vitro, BM cells from sirolimus-treated donors formed more CFU-G, CFU-M, CFU-GM, and total CFCs, relative to BM cells from busulfan (Figure 5E) control animals, but there was no increase of colonies in sirolimus-treated 5-fluorouracil group (Figure 4E). Thus, sirolimus also appeared to stimulate HSPC regeneration following BM injury in two separate chemotoxic models.

3.4 | Limited sirolimus effects on normal HSPCs

We next examined whether sirolimus could produce similar effects in normal mice at steady state, absent hematopoietic insult. We first injected sirolimus into normal CByB6F1 mice: sirolimus induced a decline in total BM cells with no change in WBCs and insignificant declines in RBCs or platelets when compared with untreated mice (Figure S1A). There were increases in the proportions and total numbers of KSL and KLDC150+ cells in sirolimus-treated mice, but these changes were not statistically significant (Figure S1B). In two separate experiments, treating normal B6 mice with sirolimus reduced WBCs significantly but there were only insignificant decreases in RBCs and BM cells without change in platelets (Figure S1C). Again, there were only slight increases in the proportions and total numbers of KSL and KSLS cells following sirolimus treatment in B6 mice (Figure S1D), and sirolimus did not affect the frequency of CFCs (Figure S1E). Thus, sirolimus exerted limited to no effect on HSPC production under steady state without hematopoietic stress under our testing conditions.

**Figure 6** Sirolimus stimulates HSPC expansion in B6 mice carrying c-Kit mutation. Heterozygous B6-Wv mice carrying c-Kit mutation were untreated (CON, N = 7) or were injected with 2 mg/kg/d of sirolimus (SIR, N = 8) for 13 days. Mice were bled and analyzed at day 14. A, Peripheral blood RBC, WBC, and platelet counts and total BM cells in B6-Wv mice treated with or without sirolimus. B, Proportions and total numbers of KSL and KLDC150+ cells in the BM of B6-Wv mice treated with or without sirolimus. C, Proportions and total numbers of c-Kit-expressing Lin− and total BM cells in sirolimus-treated B6-Wv mice. *P < .05; ***P < .001; ****P < .0001. BM, bone marrow; HSPCs, hematopoietic stem and progenitor cells; KSL, KLDC150+.
3.5 | Sirolimus-mediated HSPC regeneration is associated with c-Kit up-regulation

From both transcriptome and cell surface protein analyses, we observed upregulation of c-Kit by sirolimus at both the RNA and protein levels. Modulation of c-Kit expression is mechanistically meaningful since c-Kit is the receptor kinase for SCF, and the SCF/c-Kit interaction signals HSPC proliferation and differentiation. To test if expansion of c-Kit+ population by sirolimus was due to upregulated expression in cells that normally expressed c-Kit, we irradiated B6 mice at 5 Gy, sorted out c-Kit+Lin− and c-Kit−Lin− BM cells, and then cultured both cell fractions in vitro for 3 days, with or without sirolimus. Whole BM cells showed expansion of c-Kit+Lin− cells (Figure S2A), while there was no expansion of c-Kit+Lin− cells within the c-Kit− cell population (Figure S2B), and c-Kit+ cells retained c-Kit expression (Figure S2C). Thus, sirolimus did not convert c-Kit−Lin− cells into c-Kit+ cells, and increased c-Kit expression was due to expansion of existing c-Kit+ cells.

To further examine the effect of sirolimus on HSPCs with c-Kit defects, we took advantage of B6 mice with a spontaneous Wv mutation at the c-Kit locus (B6-Wv mice; heterozygous B6-Wv mice were derived from normal B6 mice). Treatment of B6-Wv mice with sirolimus did not alter peripheral blood cell composition but increased total BM cellularity (Figure 6A). Sirolimus significantly increased the proportions and total numbers of KSL and KSLS cells when compared to control B6-Wv mice (Figure 6B), and sirolimus increased c-Kit expression of both Lin− and total BM cells (Figure 6C).

We questioned if both mechanisms of reducing DNA damage and modulating c-Kit expression were necessary for sirolimus' effect on murine HSPCs. We irradiated c-Kit mutant mice and treated them with sirolimus. Sirolimus decreased total BM cells and white blood cell count but did not affect red blood cell and platelet numbers (Figure S3A). In contrast to unirradiated c-Kit mutant mice (Figure 6), sirolimus decreased total KSL cells (Figure S3B) with no change in c-Kit+Lin− cells (Figure S3C). However, we observed a significant increase in proportion and total number of KSLS in sirolimus-treated c-Kit mutant mice when compared with untreated control mice (Figure S3D), suggesting expansion of more primitive long-term HSCs by sirolimus despite the c-Kit defect under conditions of DNA damage.

3.6 | Sirolimus increases engraftment of injured human HSPCs in a xenotransplant model

To test if sirolimus had similar preservative effects on human HSPCs, we irradiated human CD34+ cells and then injected them into NSG mice. At 3 weeks after transplantation, control recipient mice unexposed to sirolimus showed almost no engraftment of irradiated human CD45+ cells in BM. In contrast, recipient mice that had received sirolimus for 10 days showed significantly higher engraftment of human CD45+ cells in BM (Figure 7). These observations suggest that sirolimus improved regeneration not only of murine HSPCs but also human HSPCs after radiation-induced injury.

4 | DISCUSSION

Gamma-irradiation causes BM suppression and hematopoietic cell damage. Prophylaxis with sirolimus augmented hematopoietic recovery when B6 mice were later exposed to TBI.30,31 These studies suggested a preventive benefit of sirolimus, and it is unknown whether sirolimus might be used to enhance HSPC recovery after hematopoietic damage. Here we show that sirolimus augmented HSPC regeneration when treatment was initiated after marrow injury had occurred. Specifically, following hematopoietic insults sirolimus treatment not only increased the number of BM cells carrying KSL cell markers but increased donor BM cell reconstitution and production of mature blood cells (historically, some have reported discordance between HSPC phenotype and function22,28). In our experiments, sirolimus mediated a gain in cells with HSPC phenotype and HSPC engraftment functionality. Sirolimus also mitigated hematopoietic
injury induced by 5-fluourouracil and busulfan, indicating that the effects of sirolimus stimulating HSPC regeneration extend to diverse insults.

Our observations demonstrate positive effects of sirolimus in promoting HSPC regeneration when the hematopoietic system is damaged by external insults. Notable in our current results is the variable response in mature blood cells following sirolimus treatment: sirolimus caused declines in WBCs and total BM cells in some experiments with no change or with even increased RBCs and platelets in others. We speculate that fine tuning of mTOR signaling in different cell populations may regulate the fate of HSPCs. Indeed, seemingly paradoxical effects of sirolimus have been noted by others, as suppression of mTORC1/2 may regulate the fate of HSPCs, and regeneration of HSPCs with sirolimus occurred in one study markedly inhibited the growth of hematopoietic progenitor cells but enhanced the generation of hematopoietic stem cells.

We observed consistent sirolimus-mediated c-Kit up-regulation in BM hematopoietic cells, especially HSPCs, following irradiation or chemical hematopoietic injury. More than 80 years ago mutations of BM hematopoietic cells, especially HSPCs, following irradiation or a chemical agent. A number of genes besides c-Kit were also upregulated in function, consistent with sirolimus’ properties as an immunosuppressive in vivo engraftment assay might provide a definitive answer.

We speculated that hematopoietic injury (or a constitutive Wv mutation at the c-Kit locus) provided the stimulus for sirolimus to upregulate c-Kit and induce HSPC regeneration. When both c-Kit defects and DNA damage coexisted, sirolimus promoted expansion of a more specific subset of KSL also bearing CD150, an important marker of murine HSCs. Sirolimus exerted little effect on HSPCs in normal mice without hematopoietic injury but produced significant effect on HSPC expansion in c-Kit-Wv mice that carry a missense mutation in the kinase domain of the c-Kit coding sequence. We speculate that hematopoietic injury (or a constitutive Wv mutation at the c-Kit locus) provided the stimulus for sirolimus to upregulate c-Kit and induce HSPC regeneration. When both c-Kit defects and DNA damage coexisted, sirolimus promoted expansion of a more specific subset of KSL also bearing CD150, an important marker of murine HSCs. Sirolimus increased KSL and KSLs cells but not CFCs in 5-fluourouracil-treated mice. It is possible that sirolimus stimulates regeneration of specific HSPC subsets, such as more primitive long-term HSCs, that are not associated with the myeloid-progenitor CFC assay. A quantitative in vivo engraftment assay might provide a definitive answer.

The genes downregulated by sirolimus were all related to immune function, consistent with sirolimus’ properties as an immunosuppressive agent. A number of genes besides c-Kit were also upregulated in Lin^-CD150^- cells in response to sirolimus treatment following sublethal irradiation. Among them were Cd34, a standard marker for human HSPC enrichment and murine progenitor cells, and Etv6, which is involved in hematopoietic regulation under physiological and pathological conditions.

mTOR, the central modulator of cellular homeostasis, has been implicated in the maintenance of genome integrity. The mTORC1 pathway interacts with DNA damage response and repair systems. Mechanistically, extracellular growth signals or loss of intrinsic tumor suppressor Lkb1 hyperactivates the mTORC1-S6K signaling and decreases RNF168 expression, resulting in defects in DNA damage response. As the DNA damage response involves DNA repair, cell cycle regulation, apoptosis, and autophagy, sirolimus may impact multiple features of this response. Other groups have found that inhibition of the mTOR pathway by sirolimus decreased DNA damage by rescuing TIN2 expression, leading to telomere shelterin complex stabilization in kidney transplanted patients. Furthermore, sirolimus decreased DNA damage and enhanced cell growth of Werner Syndrome RecQ Like Helicase (WRN)-deficient human fibroblasts by enhanced autophagy. We demonstrated that sirolimus appeared to reduce cell death and DNA damage in HSPCs and increase expression of genes of DNA damage response signaling pathways in HSPCs. Reducing intracellular ROS levels might be one of the mechanisms that sirolimus acts through to protect HSPCs from cell death and DNA damage after irradiation. Our results are consistent with Lin et al’s report that sirolimus protected HSPCs from DNA damage by activating autophagy to remove excessive ROS and to reduce cell apoptosis when introduced before irradiation. Thus, sirolimus has both protective and therapeutic effects on irradiated HSPCs. We observed only insignificant increases in the proportion, and limited to no increase in total number of HSPC when normal CByB6F1 and B6 animals were exposed to sirolimus, further implicating stress-induced DNA damage as a mechanism of action. However, our data differ from Magee et al, in which sirolimus induced a twofold expansion of HSCs at steady state. We speculate that differences in sirolimus dose and treatment schedule (2 mg/kg/d for 10 days in 2 weeks vs 4 mg/kg/d for 7 days) and potential differences in the general housing environment (food, gut microbiota diversity) might be relevant to the interpretation of these discordant observations.

The clinical utility of sirolimus has been mainly in immunosuppression, reducing host immunity in the context of tissue transplantation, and suppressing donor immune attack on host cells in the prevention of graft-versus-host disease. Our current observations may help to extend sirolimus’ therapeutic utility beyond immunosuppression, as sirolimus acted on HSPCs to stimulate c-Kit and downstream signals, leading to increased HSPC self-renewal and engraftment. This functional property may have clinical applications in treating radiation injury, chemotherapy-induced cytopenias, and diseases due to hematopoietic injury.

5 | CONCLUSION

Our results are consistent with sirolimus’ acceleration of HSPC recovery in response to hematopoietic stress, associated with reduced ROS and DNA damage. Sirolimus might have clinical application for the treatment and prevention of hematopoietic injury.
SIROLIMUS AUGMENTS HEMATOPOIETIC STEM AND PROGENITOR CELL REGENERATION

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CONFLICT OF INTEREST
X.F. declared employment and intellectual property rights with NHLBI/NIH. The other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
X.F., J.C.: designed research, performed experiments, analyzed data, and wrote the paper; Z.L., M.H., W.S., Z.W., K.B., J.K.: performed experiments and collected data; N.S.Y.: designed the research and wrote the paper.

DATA AVAILABILITY STATEMENT
The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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