Simvastatin improves hematopoietic stem cell engraftment by preventing irradiation-induced marrow adipogenesis and radio-protecting the niche cells

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Supplemental experimental procedures

Mice and Treatments

C57BL/6J (CD45.2) and B6.SJL- $Ptprc^{a}$ $Pep3^{b}$/BoyJ (CD45.1) mice purchased from Jackson Laboratory (Bar Harbor, ME, USA), were housed and bred in the institutional experimental animal facility. 8-12 week-old mice were used in the experiments. All procedures were approved by institutional animal ethics committee (IAEC) of NCCS.

Simvastatin (or vehicle) was orally administered to the mice for 5 days/week and subcutaneously once a week (25mg/kg body weight). To study the effect of Simvastatin on engraftment, the recipients were treated with Simvastatin/vehicle for 1 week before and 3 weeks after the BM transplantation. To study the effect of Simvastatin on steady-state hematopoiesis, the donors were treated with Simvastatin/vehicle for 4 weeks.

Reconstitution and activation of Simvastatin

Simvastatin 25mg (Sigma-Aldrich Louis, MO, USA) was dissolved in 1ml of absolute ethanol (pre-warmed to 60^{0}C). 600µl of 0.6 N NaOH and 3ml of Distilled Water was added to it and the mixture was incubated at 37^{0}C for 30 minutes to convert the inactive form (lactone) to active form (β-hydroxyacid). The pH was adjusted to pH 7.6 and the volume was made up to 5ml with water. Mice were fed with Simvastatin (25mg/kg body weight) for 5 days a week, along with one sub-cutaneous injection per week. Control mice were fed with vehicle control comprising equivalent amount of ethanol, NaOH, water and pH adjusted to 7.6.[1]

Hemogram and serum cholesterol estimation

For hemogram analysis, peripheral blood (PB) was collected by retro-orbital bleeding in EDTA-coated tubes. The samples were analyzed using Automated Hematology analyzer (Cellenium 19-Trivitrom health care). For analysis of serum cholesterol, mice were fasted overnight and

peripheral blood was collected. Serum cholesterol was analyzed using Liquizyme cholesterol detection kit (Beacon Diagnostics, Gujarat, India) as per the manufacturer's instructions.

Flow Cytometric analyses of various cell types

Doublets were excluded by FSC-H X FSC-W and SSC-H X SSC-W. Single antibody stained channels were used for compensation. Appropriate isotype control antibodies were used for each antibody. All flow-qualified antibodies were purchased from BD Biosciences (San Jose,CA, USA), or eBioscience (San Diego, CA, USA), unless mentioned specifically. Specific clones of various antibodies used are listed in Table S1.

Repopulation assay:

For repopulation studies, CD45.1 and CD45.2 congenic chimera mouse model was used. To study the effect of Simvastatin-treatment of recipients on the repopulation by donor cells, CD45.2 recipients were treated with Simvastatin or vehicle for 1 week. Mice were lethally irradiated (9.5 Gy, γ irradiation Co^{60}; split doses given 4 hours apart) and infused with $5X10^5$ CD45.1 BM cells (isolated from un-manipulated donors), and Simvastatin-treatment was continued for 3 weeks post-transplant. The results of the primary screen applied showed that this dose of cells ensures survival of the mice and gives the desired level of engraftment to perform all further analyses. At 4 weeks post transplantation, the level of engraftment was found to be more than 80% in both control and Simvastatin-treated recipients. The multi-lineage repopulation by the donor cells in the recipients' peripheral blood was monitored using antibodies to CD45.1 APC (Donors' cells), CD3e PE-Cy7 (T cells), CD45R (B220) eFluor® 450 (B Cells), CD11b PE and Ly6G/6C PE (Myeloid lineage cells).

For secondary transplantations, engrafted CD45.1 donor cells were sorted from the recipients' marrow and $1X10^5$ sorted donor cells admixed with $1X10^5$ CD45.2 BM cells were infused into

the irradiated secondary recipients (CD45.2). Multi-lineage repopulation in peripheral blood was analyzed 4 weeks post transplantation.

To study the effect of Simvastatin on steady-state hematopoiesis, CD45.1 mice were treated with Simvastatin or vehicle for 4 weeks. $5X10^5$ BM cells isolated from control or Simvastatin-treated donors (CD45.1) were admixed with $2.5X10^5$ CD45.2 cells and infused into lethally irradiated (9.5 Gy) recipients (CD45.2). At 4 and 16 week post- transplantation, multi-lineage repopulation by the donor cells was assessed by flow cytometry analyses of peripheral blood as mentioned above.

Phenotypic characterization of different bone marrow cells

BM cells were isolated by chopping the bones into pieces and treating them with collagenase (Type IA, Sigma, 1mg/ml) for 45 minutes. The RBCs were lysed and the cell suspension was passed through strainer (40μm) to remove clumps. The cells were immuno-stained for analysis of various populations. The samples were acquired on Canto II™ or ARIA II / III (SORP) flow cytometer (BD Bioscience) and analyzed using FACS Diva (V6.1.3) software (BD Bioscience).

HSC subsets and progenitors

For characterization of donor-derived HSCs in the recipients bone marrow, the BM cells were stained with biotin-labeled lineage cocktail (BD Biosciences), followed by streptavidin- PerCp-Cy5.5, CD45.1 eFluor® 450, Sca-1 PE, c-Kit PE-Cy7, Flt3 APC. The HSCs were identified as lineage negative (lin^-) $Flt3^-Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) cells in the $CD45.1^+$ gated population.

For characterization of SLAM HSCs, the cells were stained with biotin-labeled lineage cocktail followed by streptavidin APC-Cy7, Sca-1 PE, c-Kit PE-Cy7, CD150 APC and CD48 eFluor® 450. SLAM HSCs were characterized as Lin^- $Sca1^+$ $c\text{-}Kit^+$ $CD150^+$ $CD48^-$.

For analysis of long-term- and short-term-HSCs (LT-HSCs and ST-HSCs) and various hematopoietic progenitors, the cells were stained with biotin-labeled lineage cocktail, followed by streptavidin PerCp-Cy5.5, Sca-1 PE-Cy7, c-Kit FITC, CD34 eFluor® 450, Flt3 (CD135) PE, FcγRII/III (CD16/32) APC, IL7R (CD127) APC-eFluor® 780. Gating strategy and identification of different population is depicted in Figure S3D. [2-5]

BM niche cells

The BM cells were stained with biotin-labeled anti-Sca-1 antibody followed by staining with streptavidinAPC-Cy7, CD45.1/CD45.2 eFluor® 450, Ter119 PB, CD31 FITC, CD51 PE and PDGFRα (CD140a) APC.

$CD45^-$ $Ter119^-$ population was gated and different niche cells present in this population were identified as follows; (Figure S2B) [6, 7]

Endothelial cells: $CD45^-$ $Ter119^-$ $CD31^+$ $Sca\text{-}1^+$

Osteoblast precursor MSCs: $CD45^-Ter119^-CD31^-Sca\text{-}1^+CD51^+$

Osteoblasts: $CD45^-Ter119^-CD31^-Sca\text{-}1^-CD51^+$

$PDGFR\alpha^+$ MSCs: $CD45^-Ter119^-CD31^-Sca\text{-}1^+CD140a^+$

For enumeration of $Nestin^+$ MSCs, the cells were first stained with purified anti-Nestin antibody (Sigma) followed by anti-rabbit Alexa fluor 647(Molecular probes), CD45.1/ CD45.2 eFluor® 450, Ter119 PB. $Nestin^+$ MSCs were identified as $CD45^-$ $Ter119^-Nestin^+$ cells. [8]

For enumeration of BM endothelial precursor cells (EPCs), the BM cells were stained with biotin-labeled lineage cocktail followed by streptavidin APC-Cy7, Sca-1 PE, c-Kit PE-Cy7, VEGFR2 APC, VE-Cadherin FITC, and CD34 eFluor® 450. EPCs were characterized as Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ $CD34^+$ $VEGFR2^+$ $VE\text{-}Cadherin^+$ cells.

Cell Cycle analysis

Cells were stained with Hoechst 33342 and Pyronin Y (Sigma), followed by staining with biotin lineage cocktail. They were further stained with streptavidin Alexa fluor 488, Sca-1 PE-Cy7 and c-Kit APC. The LSK cells were gated and their cell cycle status was analyzed.

Colony Forming Unit (CFU) assay

$1X10^4$ BM cells were suspended in Methocult medium (Stem cell Tech, Vancouver, CA) supplemented with mouse-specific recombinant growth factors namely, IL-3 (10ng/ml), SCF (10 ng/ml), IL-6 (10ng/ml) and EPO (2U/ml) (Peprotech, Rocky Hill, NJ, USA) and plated in 35 mm low adherence dishes (Stem Cell Tech). The plates were incubated at 37^0C in 5% CO_2 in a humidified atmosphere. After 10 days, colonies formed were counted under inverted phase contrast microscope (Olympus). Colonies belonging to Burst-forming Unit-Erythroid (BFU-E), Granulocyte-monocyte colony forming units (CFU-GM) and Granulocyte-Erythroid-monocyte-megakaryocyte (CFU-GEMM) were scored using standard morphological criteria.

CFU-fibroblast (CFU-F) assay:

BM cells ($1X10^6$) were suspended in IMDM supplemented with 20% FBS, and seeded in 60mm dishes. The dishes were incubated at 37°C/5% CO_2 for 12 days without disturbing. The non-adherent cells were washed and the adherent colonies were fixed with 10% buffered formaldehyde. Plates were stained with 0.1% crystal violet and the colonies were counted under an inverted microscope (Olympus).

Culture of Lineage negative cells with Simvastatin:

Bone marrow-derived lin^{-ve} cells were isolated by incubating BM cells with biotin-conjugated antibody cocktail (Lineage cocktail, BD Biosciences) followed by streptavidin-conjugated paramagnetic beads (Dynal). Unbound cells were collected. This lin^{-ve} fraction is enriched with

HSCs. Lin^{-ve} cells were cultured with or without simvastatin (1µM) in IMDM supplemented with 20% FBS and growth factors (mIL-3 10ng/ml, mIL-6 25ng/ml, and mSCF 25ng/ml; Peprotech, Rocky Hill, NJ, USA) for 7 days. Cells were harvested and analyzed for various HSC subsets by flow cytometry.[9]

BM histology

Femurs were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Sections of 4µm thickness were cut and stained with hematoxylin and eosin (H&E).

Cell line

M210B4, a murine bone marrow-derived mesenchymal stromal cell line was used as a model system in some of the experiments. The cell line was originally purchased from ATCC and was maintained in RPMI supplemented with 10% FBS.

Radioprotection:

M210B4 cells were dosed with γ- irradiation (3.5Gy). The total protein was extracted in RIPA lysis buffer. Radio-protection was assessed in terms of Rho kinase inhibition, by western blot analysis of cell lysates using antibodies to myosin light chain-2 (MLC-2) and phosho-MLC-2 (Cell Signaling; Danvers, MA, USA).

Western Blot analysis:

The protein concentration of the cell lysates was estimated using micro BCA protein assay kit (Pierce, Thermo scientific; Asheville, NC, USA). Equal protein aliquots were separated on an SDS-PAGE gel and then electrophoretically transferred to PVDF membrane. The membranes were incubated for 1 hr in blocking buffer (5% BSA in TBST) [50 mM Tris, 150 mM NaCl, 0.1% Tween 20] and then probed with the respective primary antibodies overnight at 4°C [Myosin light chain (MLC), p-MLC]. Blots were washed with TBST and incubated with an

appropriate secondary HRP- conjugated antibody (Cell Signaling Technology). Immuno-reactive conjugates were detected using LumiGlow substrate (Cell Signaling Technology) according to the manufacturer's instructions. Densitometric analysis was performed using Image J software (NIH) and the values were normalized either with the input native proteins.

DNA Damage Repair

DNA Damage Repair (DDR) response was determined by immuno-fluorescence staining of irradiated cells as described below.

Immuno-fluorescence:

M210B4 cells were grown on glass and dosed with γ- irradiation (3.5Gy). The cells were fixed with freshly prepared 1% buffered paraformaldehyde and were briefly permeabilized with 0.1%Triton X-100/PBS. The cells were blocked with 1% BSA/PBS and stained with primary antibody to p-ATM-Ser1981 (1:50) (Upstate, Milipore; MA, USA) and secondary donkey anti-mouse antibody tagged with Cy3 (1:50) (Chemicon, Milipore; MA, USA) antibody. The coverslips were mounted on thin glass slides in a mounting medium supplemented with an anti-fade agent. Images were acquired on confocal laser-scanning microscope (Carl Zeiss, Jena, Germany, LSM 510 META; 63X/oil/1.4NA objective) using AIM 4.2 software. Mean Fluorescence Intensity (MFI) was measured using Image J software. Nuclei in 10 independent non-overlapping fields were scored.

Gene expression analysis

Cells were lysed in lysis/binding buffer and mRNA was isolated using the Dynabeads® mRNA DIRECT™ Purification Kit (Invitrogen) according to manufacturer's instructions. The isolated mRNA was reverse transcribed using Superscript III ^{RT} enzyme (Invitrogen). Samples without reverse transcriptase were used to detect genomic DNA contamination.Primers were designed

using Primer-3 software V0.4.0. (http://prodo.wi.mit.edu/primer3/).Primer sequences are listed in Table S2. Quantitative PCRs were performed using the Platinum® SYBR® Green qPCRSuperMix-UDG (Invitrogen) and analyzed on ABI 7500 fast system (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized with the GAPDH. Relative gene expression was calculated as ($2^{\Delta Ct} \times 100\%$).

STATISTICS

Statistical analysis was performed using one-way repeated measures analysis of variance (One-Way RM ANOVA) using Sigma Stat software (Systat Software, Inc). $p<0.05$ was considered significant. Results are expressed as mean value ± SEM.

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Table S1: Specific clones of the antibodies used

No.	Antibody	Specific clone / Cat No
1	CD45.1	A20
2	CD45.2	104
3	CD3e	145-2C11
4	CD45R (B220)	RA3-6B2
5	CD11b	M1/70
6	Ly6G/6C	RB6-8C5
7	Sca-1	D7
8	c-Kit	2B8
9	Flt3	A2F10
10	CD150	mShad150
11	CD48	HM48-1
12	CD34	RAM 34
13	Fcγ RII/III (CD16/32)	93
14	IL7R	A7R34
15	Ter119	TER119
16	CD31	390
17	CD51	RMV-7
18	PDGF Rα	APA5
19	Nestin	N5413 (Catalog No.)
20	VEGFR2	Avas12a1
21	VE-Cadherin	BV13
22	MLC-2	3672 (Catalog No.)
23	phospho MLC-2 (Ser19)	3671 (Catalog No.)
24	phospho ATM Ser1981	10H11.E12

Table S1: List of specific clones of the antibodies used in the experiments.

Table S2: Primer sequences used for qRT-PCR

No.	Mouse specific primers used	Gene ID		Primer Sequence (5’ - 3’)
1	Angiopoietin 1	NM_001286062.1	F	CTTCAAGGCTTGGTTTCTCG
			R	TCCATGAGCTCCAGTTGTTG
2	Gapdh	NM_001287514.1	F	ACTGCCACCCAGAAGACTGT
			R	CCATGCCAGTGAGCTTCC
3	Jagged-1	NM_013822.5	F	GAGGTACCTGCGTGGTCAAT
			R	CCGATACCAGTTGTCTCCGT
4	Runx-2	NM_001146038.2	F	TGACACTGCCACCTCTGACT
			R	ATGAAATGCTTGGGAACTGC
5	Ppar-gamma	NM_001204201.1	F	AGACAACGGACAAATCACCA
			R	GAAACTGGCACCCTTGAAAA
6	Sdf 1 alpha	NM_021704.3	F	GCTCTGCATCAGTGACGG
			R	ATCTGAAGGGCACAGTTT
7	Vegf-A	NM_001025250.3	F	GGAGTACCCCGACGAGATAG
			R	CTATGTGCTGGCTTTGGTGA
8	Thpo	NM_001289896.1	F	CAGGTCCCCAGTCCAAATCT
			R	GGCTTGGAGAAGGAGGAAGT

Table S2: List of the gene-specific primer sequences used for quantification of mRNA by qRT-PCR analyses.

Legends for Supplemental figures

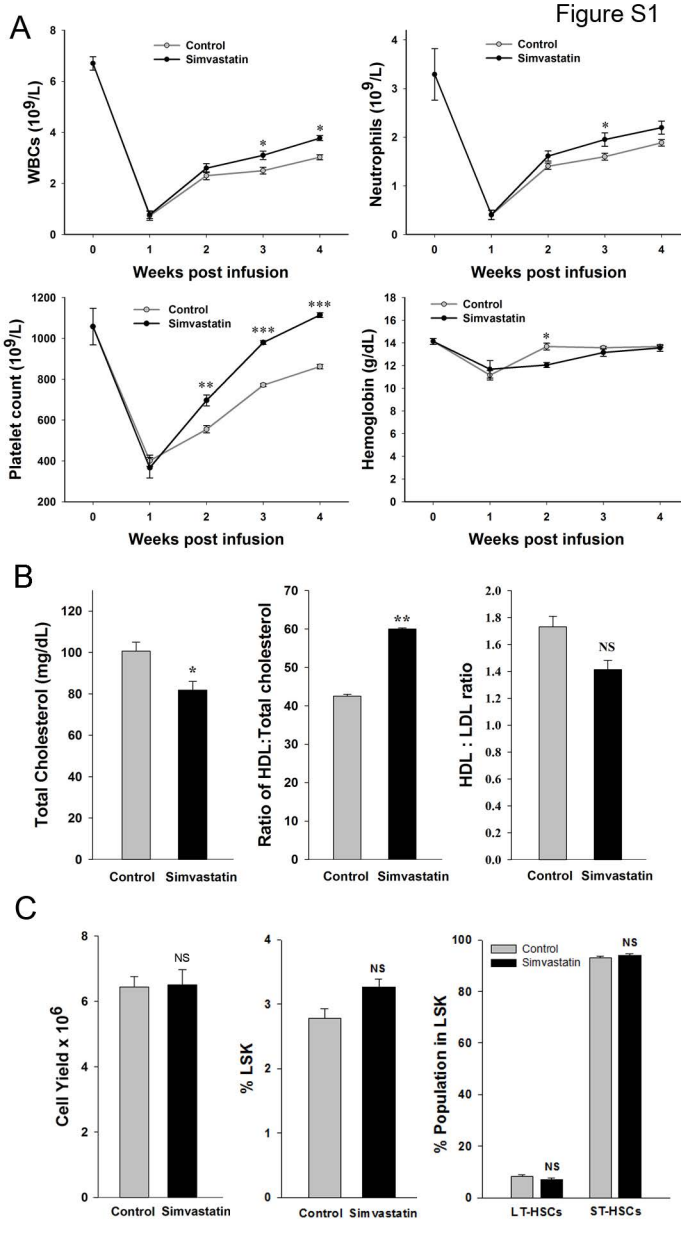
Online Supplementary Figure S1.Simvastatin-treated recipients show better post-transplant hematopoietic recovery. Related to Figure 1. (A) Analyses of total white blood cells (WBCs), neutrophil, platelets and haemoglobin content in the peripheral blood of recipients done at weekly interval post-transplant. **(B)** Serum cholesterol analyses of the recipients done at 3 weeks post-transplant are depicted. Blood samples of 6 mice per group were analysed. **(C)** Simvastatin does not have a direct effect on HSC proliferation. Lin^- cells were cultured with Simvastatin (1μM) for 7 days. Total cells harvested and HSC analysis are graphically depicted. Data are represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, ***$p<0.001$. NS - not significant.

Online Supplementary Figure S2. Simvastatin radio-protects niche cells. Related to Figure 2. (A) Haematoxylin and Eosin (H&E) stained bone sections of control and Simvastatin-treated recipients showing higher density of trabeculae (yellow arrows) and blood vessels (Red arrows) in the bones of Simvastatin-treated mice. Bar represents 100μm.**(B)** Gating strategy used for flow cytometric analyses of different niche cells in the bone marrow namely, endothelial cells, osteoblasts, osteo-precursor MSCs, PDGFR-α^+ MSCs and $Nestin^+$ MSCs. **(C)** Gating strategy used for flow cytometric analyses of bone marrow EPCs.**(D)** Immuno-staining of M210B4 stromal cells with antibody to p-ATM (Ser 1981). The Simvastatin-treated cells dosed with γ-irradiation show stronger expression of p-ATM-Ser1981 in their nuclei compared to the control irradiated cells. Right panel depicts 2.5D analyses of the fluorescence intensity generated by AIM 4.2 software.

Online Supplementary Figure S3. Simvastatin boosts the stem cell number at steady-state condition by targeting the HSC niche. Related to Figure 3. (A) Simvastatin does not affect marrow cellularity.Absolute numbers of hematopoietic cells per femur and tibia is shown. N= 6. Data are shown as mean ± SEM. NS = not significant. **(B)** Haematoxylin and

Eosin (H&E)-stained sections of control and Simvastatin-treated mice. Overall marrow cellularity (marked by square boxes) in both the groups is comparable. Bar represents 100 μm. **(C)** Simvastatin-treatment does not affect total WBC count (left panel) and hemoglobin levels (right panel) in mice. Platelet count in Simvastatin-treated mice was significantly high (middle panel). N = 6. * $p < 0.05$. **(D)** Gating strategy used to enumerate various lineage-committed progenitors in the marrow of control and Simvastatin-treated mice. Progenitors belonging to multi-potential progenitors (MPP), Megakaryocyte-Erythrocyte progenitors (MEP), common myeloid progenitors (CMP), Granulocyte-monocyte progenitor (GMP) and Common lymphoid progenitors (CLP) were enumerated. **(E)** Data show that Simvastatin treatment does not skew the hematopoiesis towards any specific lineage. **(F)** Representative flow panel of cell cycle analysis of gated LSK population in marrow cells collected from control and Simvastatin-treated mice. Right panel shows flow data in quantitative terms. N= 6. * $p < 0.05$. **(G)** Gating strategy used to analyse engraftment by donor cells isolated from control and Simvastatin-treated mice, in the recipients' peripheral blood. Engrafted donor cells (CD45.1) were further analyzed for frequency of lymphoid and myeloid cells present in them (Right panel). N=4, 8-10 mice/group/experiment. **(H)** Colony-forming-Unit-Fibroblast (CFU-F) assay performed on the marrow cells isolated from control and Simvastatin-treated mice. N=3. NS = not significant.

Figure S1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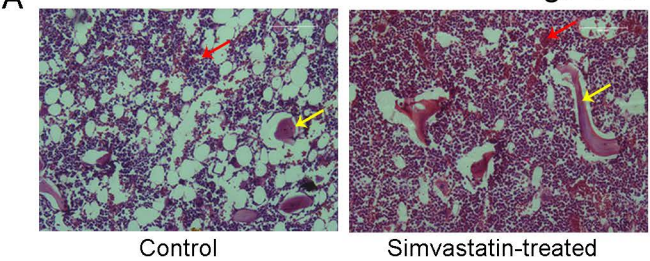
Neutrophils (10^9/L)
Platelet count (10^9/L)
Hemoglobin (g/dL)
Control
Simvastatin
Weeks post infusion
B
Total Cholesterol (mg/dL)
Ratio of HDL:Total cholesterol
HDL : LDL ratio
NS
C
Cell Yield x 10^6
% LSK
% Population in LSK
LT-HSCs
ST-HSCs

Figure S2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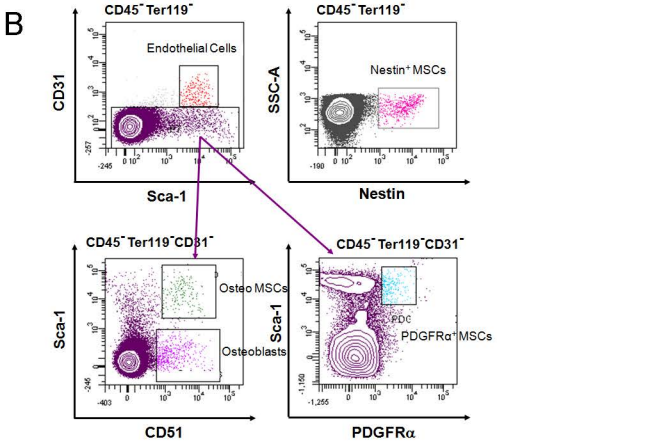
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Endothelial Cells
CD31
Sca-1
CD45⁻ Ter119⁻
Nestin⁺ MSCs
SSC-A
Nestin
CD45⁻ Ter119⁻CD31⁻
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Sca-1
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PDC
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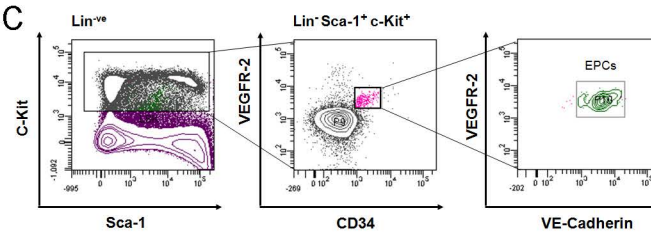
Sca-1
PDGFRα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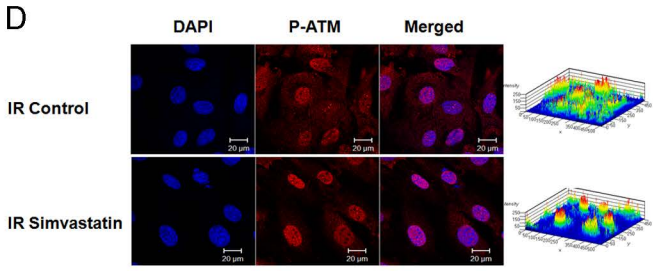
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Merged
IR Control
IR Simvastatin
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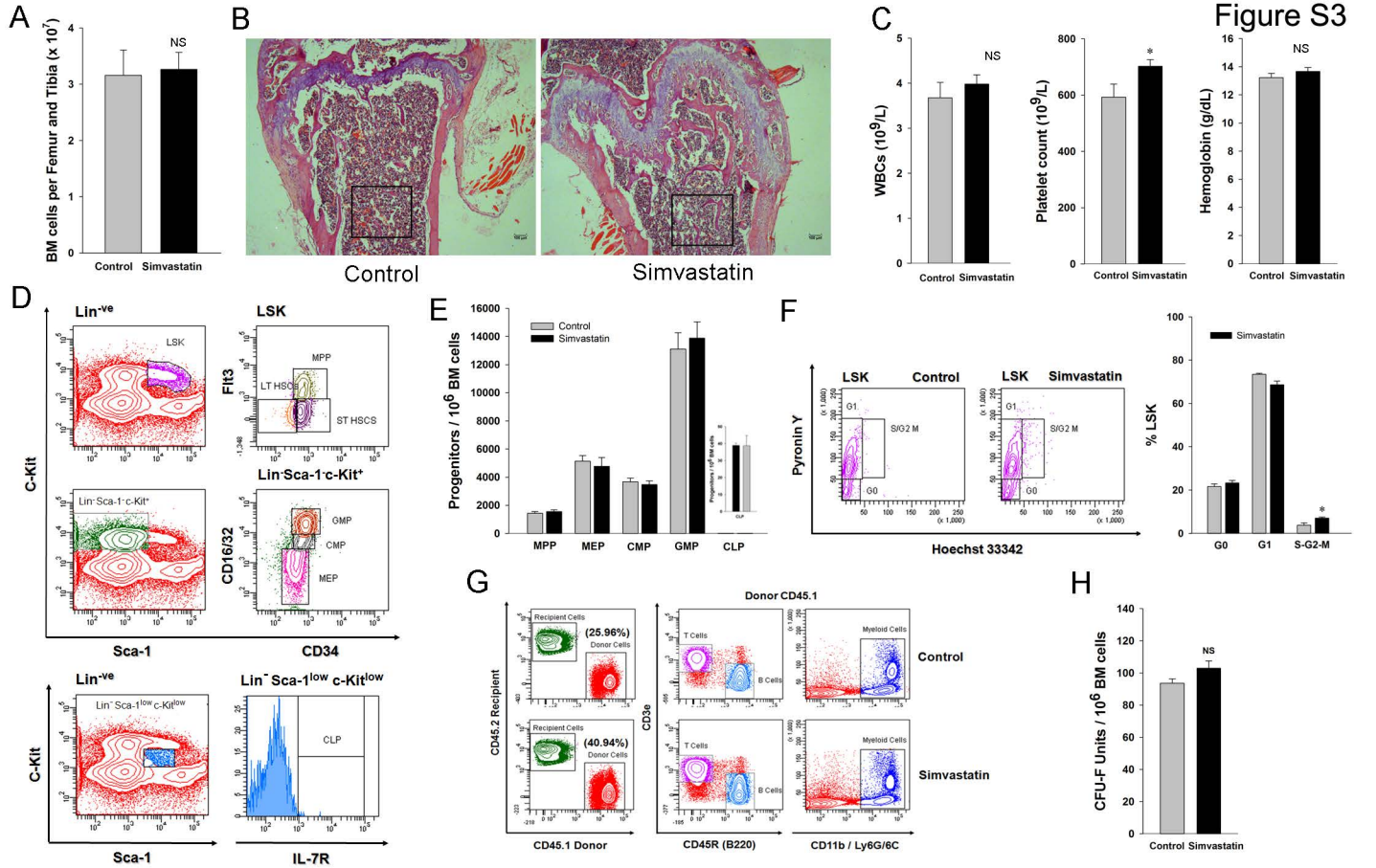
Figure S3


A
BM cells per Femur and Tibia (x 10^7)
NS
Control
Simvastatin
B
Control
Simvastatin
C
WBCs (10^9/L)
Platelet count (10^9/L)
Hemoglobin (g/dL)
NS
*
NS
Control
Simvastatin
D
Lin-ve
LSK
MPP
LT HSC
ST HSCS
Flt3
Lin-Sca-1-c-Kit+
GMP
CMP
MEP
CD16/32
C-Kit
Sca-1
CD34
Lin- Sca-1low c-Kitlow
CLP
IL-7R
E
Control
Simvastatin
Progenitors / 10^6 BM cells
MPP
MEP
CMP
GMP
CLP
F
LSK Control
LSK Simvastatin
G1
S/G2 M
G0
Pyronin Y
Hoechst 33342
% LSK
G0
G1
S-G2-M
G
Donor CD45.1
Recipient Cells
(25.96%)
Donor Cells
(40.94%)
T Cells
B Cells
Myeloid Cells
Control
Simvastatin
CD45.2 Recipient
CD45.1 Donor
CD3e
CD45R (B220)
CD11b / Ly6G/6C
H
CFU-F Units / 10^6 BM cells
NS
Control
Simvastatin