Supplementary information

Material and methods

Transplantation (BMT)

For the short-term radioprotection model, recipient mice (CD45.2+) were lethally irradiated (950Gy), and were transplanted with $0.5x10^6$ unfractionated donor bone marrow cells (CD45.1+) by retro-orbital injection (as described in[1]). For the monocyte lineage tracing experiments, recipient *Cx3cr1*$^{YFPCreER;tdTomato}$ recipient mice were first treated with tamoxifen (75mg/kg mouse in corn oil) injected i.p. daily for 5 days prior to transplant. Tamoxifen treated mice were then irradiated and transplanted with CD45.1+ donor bone marrow cells as described above. When serving as donors, *Cx3cr1*$^{YFPCreER;tdTomato}$ donor bone marrow cells were isolated from mice treated with tamoxifen (75mg/kg mouse in corn oil) injected i.p. daily for 5 days prior transplant. Donor cells from tamoxifen treated mice were transplanted into CD45.1+ recipient mice as described above. In *Ccl2* mutant and *Ccr2* mutant transplant, donors and recipients were as specified. In labeled monocyte chasing, monocytes were purified from td-tomato mice. td-tomato+ labeled monocytes were co-transplanted with non-fluorescent unfractionated bone marrow cells supportive cells at a 1:1 ratio, total of $0.5x10^6$ donor cells, into non-fluorescent lethally irradiated recipient mice.

RBC labeling CFSE and transfusion of labeled erythrocytes

Whole blood was obtained from C57BL/6 control mice and labeled with CFSE (LifeTechnology) following the manufacturer's instructions. 150-200μL blood was removed from recipient mice before transfusion with labeled RBCs. In BMT, BM cells were mixed with labeled RBCs and injected into mice simultaneously.

In vitro aged RBC treatment

RBCs were freshly collected in an EDTA-coated tube and aged for 16 hours 37°C in aging buffer (10mM Hepes, 140mM NaCl, 0.1%BSA, pH 7.4) containing calcium (2.5mM) and Ca^{2+} ionophore (0.5μM, A23187). Whole spleen cells were plated for 5 hours and then supernatant was removed. Adherent layer was washed by PBS and treated with aged RBCs for 3 hours. At the end of the treatment, cells were harvest for mRNA.

Phenylhydrazine (PHZ) treatment to induce acute hemolytic anemia

Mice were injected with a single dose of phenylhydrazine (100 mg/kg mouse) i.p.[1]. For

experiments using *Cx3cr1*$^{YFPCreER;tdTomato}$ mice for monocyte lineage tracing, mice were first injected with tamoxifen 75 mg/kg mouse in corn oil) for 5 days prior PHZ treatment.

Immunofluorescence

Spleens were fixed in 4% paraformaldehyde. The Animal Diagnostic Laboratory (Pennsylvania State University) prepared the tissues, embedded them in paraffin and cut sections of spleen tissue. Unstained paraffin spleen tissue sections were processed with standard immunostaining protocol. In brief, sections were deparaffinized with Xylene (Sigma) and rehydrated with ethanol gradients, followed by antigen retrieval in 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 at 90-100C for 20 minutes. The slides were then cooled to room temperature. Sections were incubated with 0.025% Triton X-100 (Sigma) for permeabilization. Sections were blocked with 1% Bovine serum albumin (Sigma) and then incubated with fluorescence conjugated antibodies over night at 4C in the dark. The following day, sections were washed and mounted with DAPI containing buffer (Thermo Fisher, P36934). Samples were visualized using Olympus Fluoview 1000 with UPlanFL 20X/0.50 objective and analyzed using Fluoview software.

Flow cytometry, Flow imaging, and Cell sorting

Spleen cells and EBIs were harvested from mice. Cells were first stained with Brilliant Violet 510 live/dead dye (BD) to exclude dead cells, and then processed with standard extracellular or intracellular staining. Fortessa LSR (Becton Dickinson) and Accuri (Becton Dickinson) flow cytometers were used for analyses. Flow cytometry data were analyzed with FlowJo software. Imaging data were collected by FlowSight® (Amnis) and analyzed using IDEAS software. Astrios (Becton Dickinson) cell sorter was used for cell sorting. See supplementary Table 1 for detailed antibody information.

Quantitative polymerase chain reaction (qPCR) and gene expression analysis

RNA isolation from cells or tissues was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). qPCR reactions were performed with Taqman probes (Thermo Fisher), and PerfeCTa qPCR SuperMix Master mix (Quanta Biosciences). See Supplementary Table 2 for probe information.

Supplementary Table S1. Flow cytometry antibodies information.

Antibodies	Source	Catalog
Brilliant Violet 421-anti-mouse-Ter119	Biolegend	116233
Alexa647-anti-mouse-Ter119	Biolegend	116218
APC/Cy7-anti-mouse-Ter119	Biolegend	116233
PE-anti-mouse-Ter119	BD	553673
PE-anti-mouse-CD133	eBiosciences	12-1331-82
PEcy7-anti-mouse-CD133	Biolegend	141209
Brilliant Violet 421-anti-mouse-cKit	BD	562609
PE/Cy7- anti-mouse-F4/80	Biolegend	123114
Alexa647- anti-mouse-F4/80	Biolegend	123121
Percp/Cy5.5- anti-mouse-CD11b	BD	550993
FITC- anti-mouse-Vcam-1	BD	553332
Alexa 647- anti-mouse- Vcam-1	Biolegend	105712
PE- anti-mouse-CD169	Biolegend	142404
APC- anti-mouse-CD169	Biolegend	142418
FITC- anti-mouse-Ly6C	BD	553104
APC/Cy7- anti-mouse-Ly6C	BD	560596
Brilliant Violet 421- anti-mouse-CD115	Biolegend	135513
PE- anti-mouse-Ccl2	Biolegend	505903
Alexa647-anti-mouse-MHCII	eBiosciences	11-0081-85
PE-anti-mouse-CD11c	BD Pharmingen	557401
FITC-anti-mouse-CD8α	BD Pharmingen	562367

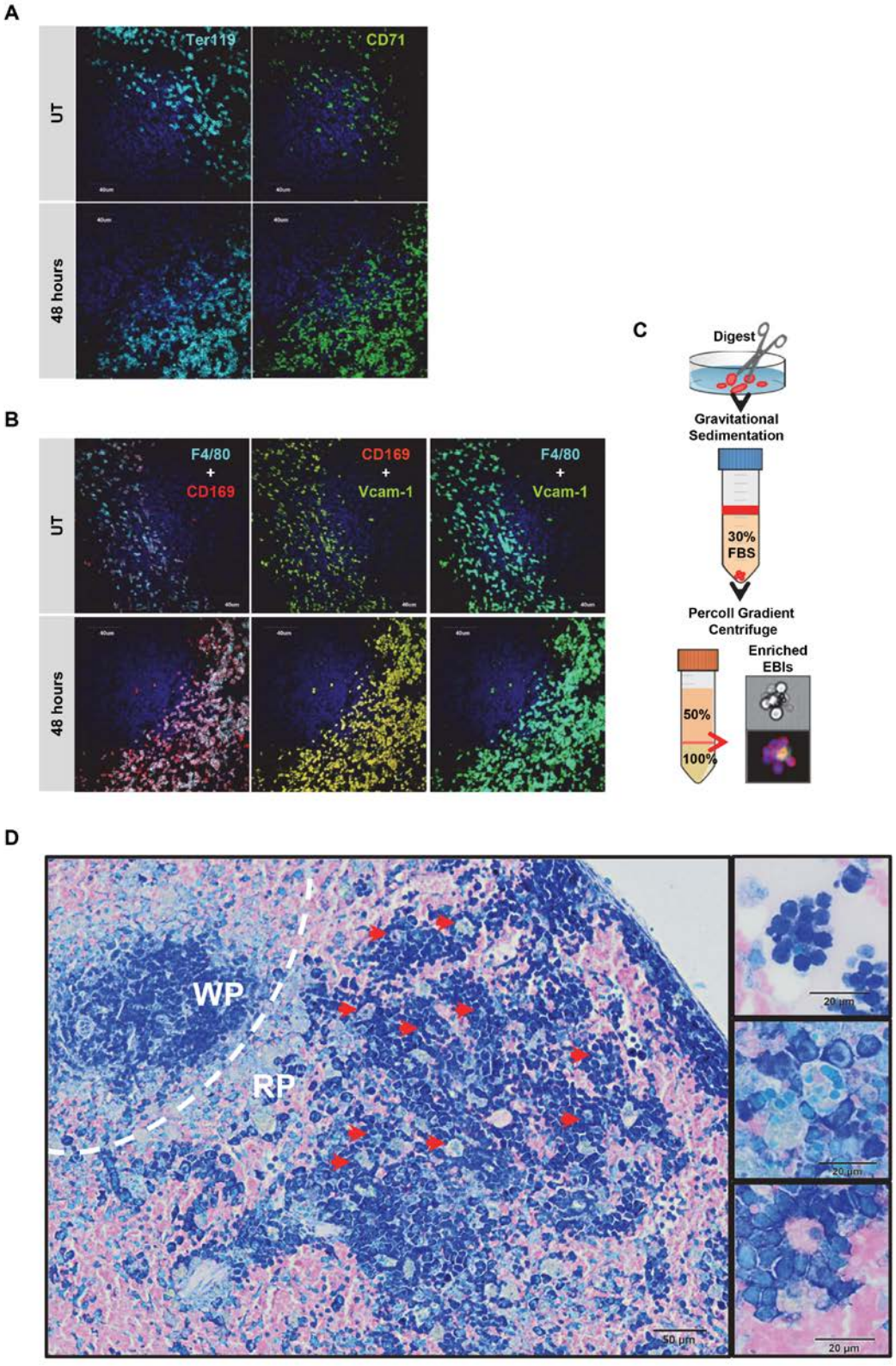
Supplementary Table S2. Taqman probe information.

Gene	Identifier
Bmp4	Mm00432087_m1
Gdf15	Mm00442228_m1
Ccl2	Mm00441242_m1
Spic	Mm00488428_m1

Supplementary References.

1. Bennett LF, Liao C and Paulson RF. Stress Erythropoiesis Model Systems. *Methods in molecular biology.* 2018;1698(91-102).

Figure S1



Supplemental Figure 1

Supplemental Figure 1

(A and B) Immunofluorescence of spleen sections from untreated (UT) mice and mice 48 hours after PHZ treatment. Mice were i.p. injected with a single dose of PHZ at 100 mg/kg body weight. n=3-4 for each group per time point.

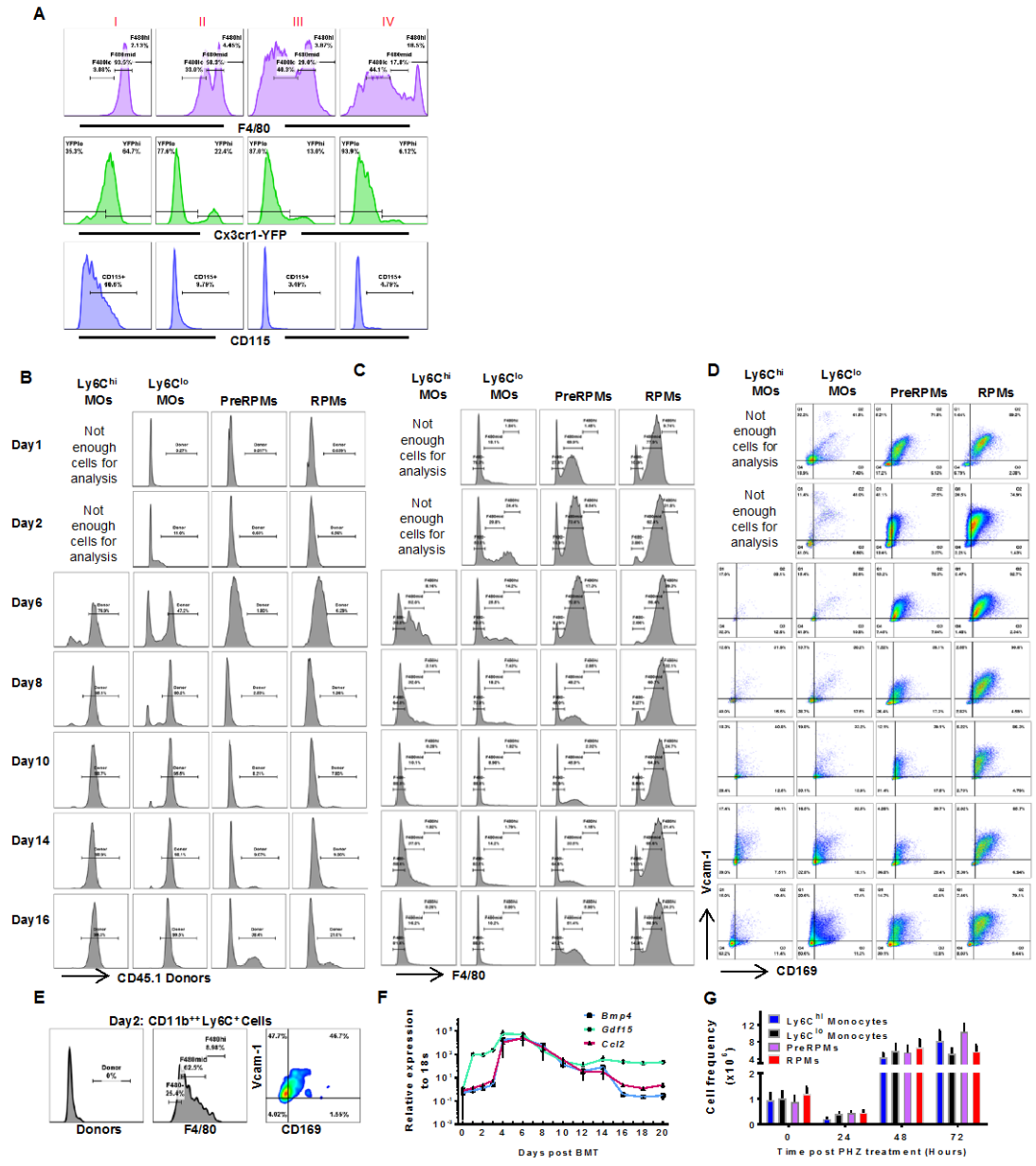
(A) Ter119 and CD71 staining of spleen sections from untreated (UT) mice and mice 48 hours after PHZ treatment.

(B) Pairwise merged immunofluorescence images of Figure. 1A. Spleen sections were stained for F4/80, CD169, and Vcam-1 from untreated (UT) mice and mice 48 hours after PHZ treatment.

(C) Schematic of spleen EBI enrichment procedure. Spleens were minced into small pieces, and incubated in 0.075% (*m/v*) Collagenase IV and 0.004% (*m/v*) DNase I in media for 30min at 37^{o}C with constant shaking. Aggregates were enriched by gravity sedimentation in 30% (*v/v*) FBS for 45min at room temperature, followed by 50%(*v/v*) /100%(*v/v*) Percoll gradient centrifugation. Interface between the 50% (*v/v*) Percoll and 100% (*v/v*) Percoll was collected, washed with PBS, and processed for flow cytometry and FlowSight® imaging.

(D) Wright-Geimsa stain of mouse spleen sections 48 hours after PHZ treatment. Left, EBI location on whole spleen sections. EBIs are pointed by red arrows. White dash line indicates the edge of white pulp and red pulp. WP, white pulp; RP, red pulp; scale bar, 50 μm. Right, three zoomed in pictures of EBIs. Scale bar, 20 μm.

Figure S2



Supplemental Figure S2

Supplemental Figure S2. (Continues from Figure. 2) Characterization of whole spleen cells post BMT.

(A) Continues from Figure. 2A. F4/80, Cx3cr1(YFP derived form Cx3cr1$^{YFP\text{-}CreER}$ mice), and CD115 expression on Ly6C^{hi} monocytes (I), Ly6C^{lo} monocytes (II), Pre-RPMs (III), and RPMs (IV).

(B - E) Continues from Figure. 2B. Characterization of Ly6C^{hi} monocytes, Ly6C^{lo} monocytes, Pre-RPMs, and PRMs from day 1 to day 16 in short-term radioprotective model. Recipient CD45.2 mice were transplanted with 0.5 x 10^6 CD45.1 donor BM cells on day 0. Whole spleen cells were isolated and analyzed using flow cytometry. Gating strategy was applied from Figure. 2A. n= 3 - 4 for each group per time point. RPMs were always F4/80^{hi} CD169^{+} Vcam-1^{+}. Pre-RPMs were phenotypically similar to PRMs from day 1 to day 6, but more resembled MOs post day 6. (B) Percent donor – derived cells, (C) F4/80 expression, (D) CD169 and Vcam-1 expression of Ly6C^{hi} monocytes, Ly6C^{lo} monocytes, Pre-RPMs, and PRMs.

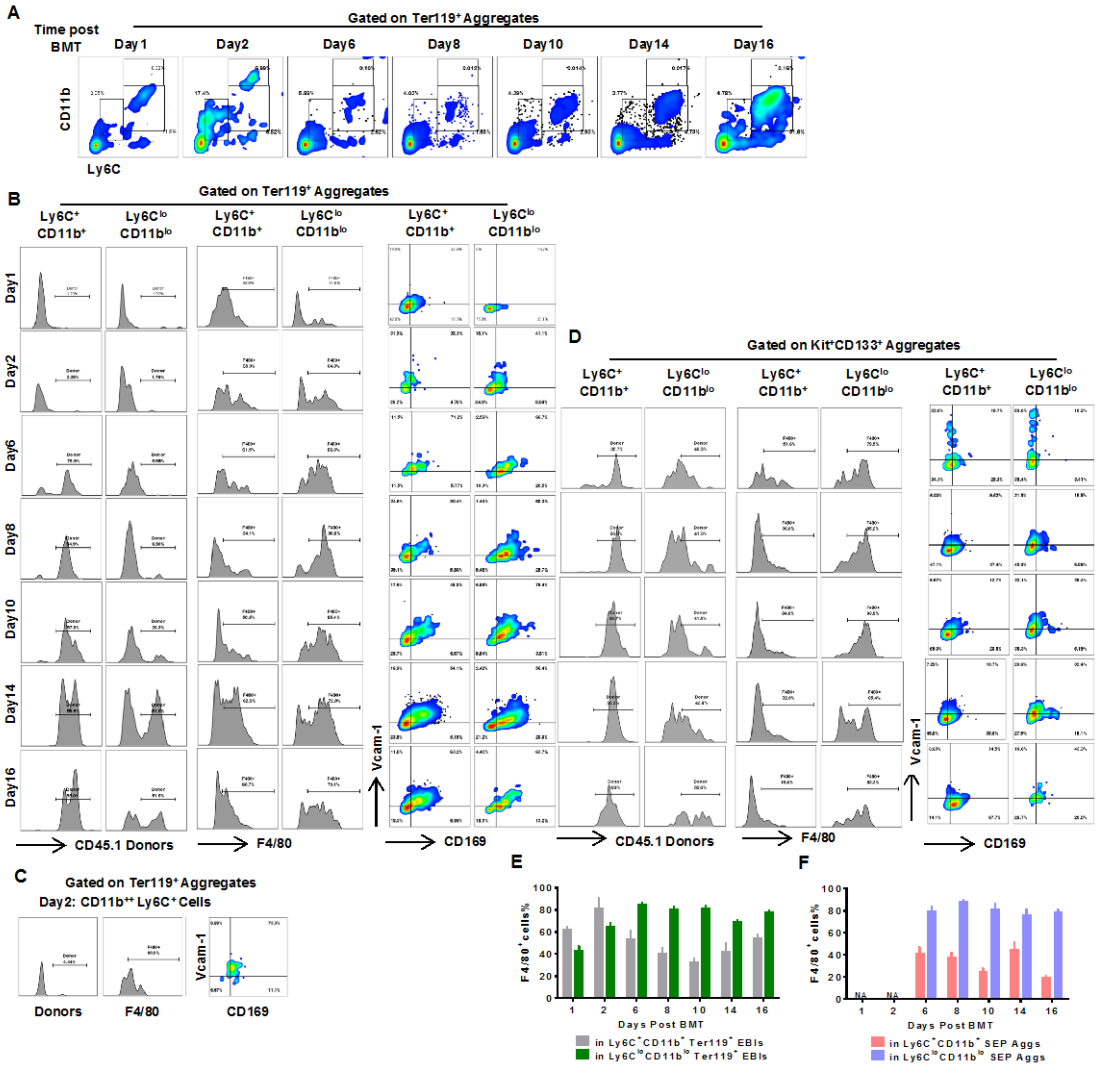
(E) Percent donor, F4/80 expression, and CD169 and Vcam-1 expression of CD11b^{++}Ly6C^{+} cells on day 2 post BMT.

(F) *Bmp4*, *Gdf15*, and *Ccl2* expression relative to 18S rRNA in whole spleen from day 1 to day 20 post BMT.

(G) Quantitation of Ly6C^{hi} monocytes (I), Ly6C^{lo} monocytes (II), Pre-RPMs (III), and RPMs (IV) in PHZ model. n= 3 – 4 each group per timepoint.

Bars are representative of mean ± SEM.

Figure S3



Supplemental Figure S3

Supplemental Figure S3. (Continues from Figure. 2) Characterization of enriched aggregates post BMT.

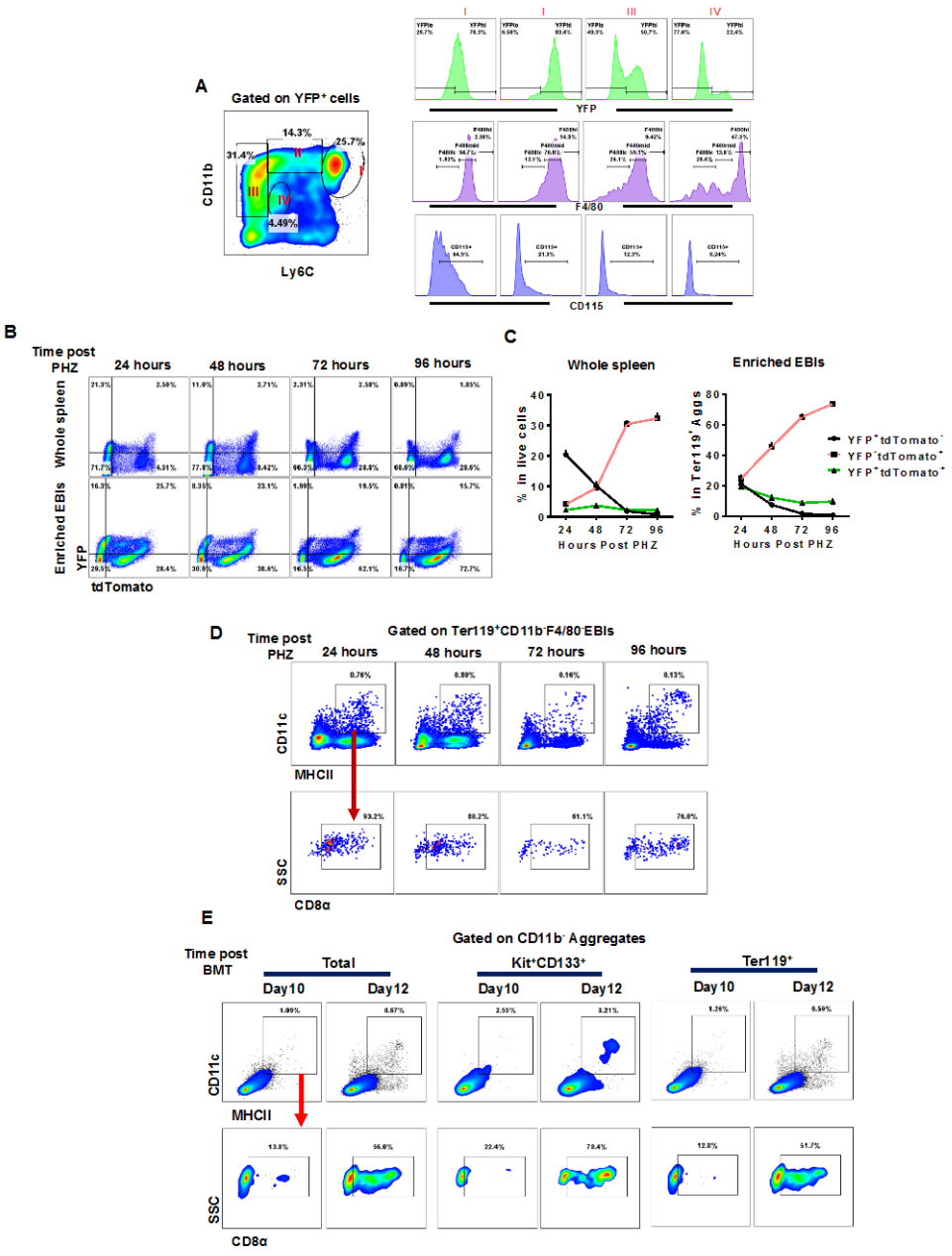
(A - C) Flow cytometry analysis of Ter119^{+}EBIs from day 1 to day 16 post BMT. EBIs were enriched and analyzed by flow cytometry. Ter119 was used to further mark out erythroblast containing aggregates. Gating strategy was simplified to combine Ly6^{hi} monocytes and Ly6^{lo} monocytes into Ly6C^{+}CD11b^{+} monocytes and Pre-RPMs and RPMs into Ly6C^{lo}CD11b^{lo} macrophages, to ensure enough events for analysis. n=3-4 for each group per time point. (A) Representative flow cytometry diagrams. (B) Histograms showing donor composition, F4/80 expression, and CD169 and Vcam-1 expression of Ly6C^{+}CD11b^{+}, and Ly6C^{lo}CD11b^{lo} aggregates in total Ter119^{+}EBIs. (C) Percent donor, F4/80 expression, and CD169 and Vcam-1 expression of CD11b^{++}Ly6C^{+} Ter119^{+}EBIs on day 2 post BMT.

(D) Continues from Figure. 2H. Donor composition, F4/80 expression, and CD169 and Vcam-1 expression of Ly6C^{+}CD11b^{+}, and Ly6C^{lo}CD11b^{lo} aggregates in SEP (Kit^{+}CD133^{+}) aggregates from day 6 to day 16 post BMT.

(E and F) Percentage of F4/80^{+}cells in (E) Ly6C^{+}CD11b^{+} and Ly6C^{lo}CD11b^{lo} Ter119^{+}EBIs, and (F) Ly6C^{+}CD11b^{+}SEP (Kit^{+}CD133^{+}) aggregates and Ly6C^{lo}CD11b^{lo}SEP (Kit^{+}CD133^{+}) aggregates. NA, not applicable (no aggregates present at these time points).

Bars are representative of mean ± SEM.

FigureS4



Supplemental Figure S4

Supplemental Figure S4. (Continuous from Figure. 3)

(A) Gating strategy of *Cx3cr1*^{YFPCreER} mice gated on YFP^{+}cells. YFP expression is driven by Cx3cr1 promotor. Monocytic lineage cells are YFP^{+} cells.

Population I: Ly6C^{hi} monocytes, YFP^{lo}CD11b^{+}Ly6C^{hi}F4/80^{mid}CD115^{+}.

Populatoin II: Ly6C^{lo} monocytes, YFP^{hi} CD11b^{+}Ly6C^{lo}F4/80^{mid}CD115^{lo}.

Population III: Pre-RPMs, YFP^{lo}CD11b^{+}Ly6C$^{lo/-}$F4/80^{+}CD115$^{lo/-}$.

Population IV: RPMs, YFP^{lo}CD11b^{lo}Ly6C^{lo}F4/80^{hi}CD115^{-}.

(B - C) *Cx3cr1*$^{YFPCreER;tdTomato}$ mice were i.p. injected with tamoxifen in corn oil at 75 mg/kg body weight consecutively for 5 days prior single PHZ injection at 100mg/kg. n=4 for each group per time point. At 24, 48, 72 and 96 hours after PHZ treatment, flow cytometry analysis was done to characterize monocytes, pre-RPMs and RPMs in YFP^{+}tdTomato^{neg}cells, YFP^{+}tdTomato^{+}cells and YFP^{neg}tdTomato^{+}cells in whole spleens and Ter119^{+}EBIs.

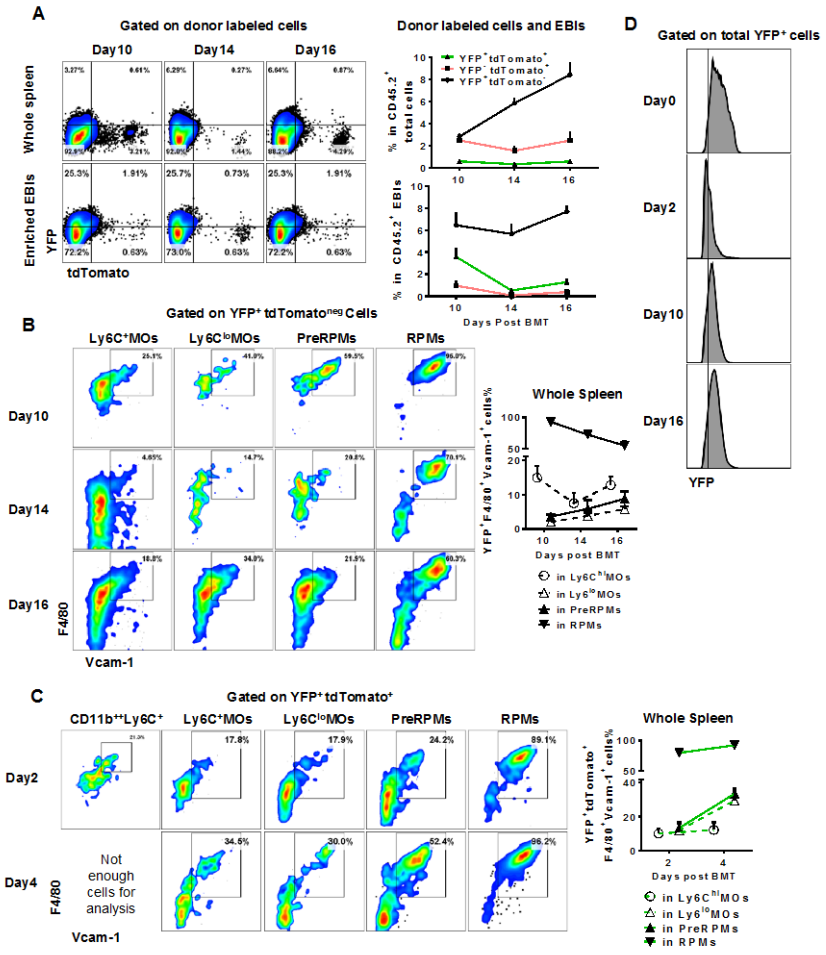
(B) Representative flow cytometry diagrams illustrating total labeled cells YFP versus tdTomato expression.

(C). Percentages of YFP^{+}tdTomato^{neg}cells, YFP^{+}tdTomato^{+}cells and YFP^{neg}tdTomato^{+} cells in whole spleens and Ter119^{+}EBIs.

(D) Flow cytometry analysis of CD11b^{-}F4/80^{-} cells present in Ter119+ EBIs for expression of dendritic cell markers CD11c and MHCII. CD11C^{+}MHCII^{+} cells were analyzed for expression of CD8α, which mark the CD8α^{+} type I conventional dendritic cells.

(E) Flow cytometry analysis of total CD11b^{-} aggregates, CD11b^{-} Kit^{+}CD133^{+}SEP aggregates and CD11b^{-}Ter119^{+} aggregates for dendritic cell markers on day 10 and day 12 post BMT.

Figure S5



Supplemental Figure S5

Supplemental Figure S5. (Continuous from Figure. 4 and Figure. 5)

In early recovery stage, *Cx3cr1*$^{YFPCreER;tdTomato}$ recipient mice were i.p. injected with tamoxifen in corn oil at 75 mg/kg body weight consecutively for 5 days prior irradiation followed by transplantation of CD45.1 wild type donor BM cells. Flow cytometry analysis was applied on gated CD45.2 recipient cells post BMT day 2 and day4.

In middle and late recover stage, where niche monocytes are mostly donor derived. *Cx3cr1*$^{YFPCreER;tdTomato}$ donor mice were i.p. injected with TM in corn oil at 75 mg/kg body weight consecutively for 5 days. BM cells were obtained and transplanted into CD45.1 recipient mice. Flow cytometry analysis was applied on gated CD45.2 donor cells post BMT day 8, day 10, day14 and day 16. Day8 had too few events to analyze and were not included. Gating strategies applied from Figure 1A. n=3 - 4 for each group per time point.

(A) Left, representative flow cytometry charts illustrating YFP and tdTomato expression in whole spleen and enriched Ter119^{+}EBIs on day 10, day 14 and day 16 post BMT. Top right, percent YFP^{+}tdTomato^{neg}, YFP^{+}tdTomato^{+}, and YFP^{neg}tdTomato^{+}cells in CD45.2^{+} recipient cells. Bottom right, percent YFP^{+}tdTomato^{neg}, YFP^{+}tdTomato^{+}, and YFP^{neg}tdTomato^{+}cells in CD45.2^{+} Ter119^{+}EBIs.

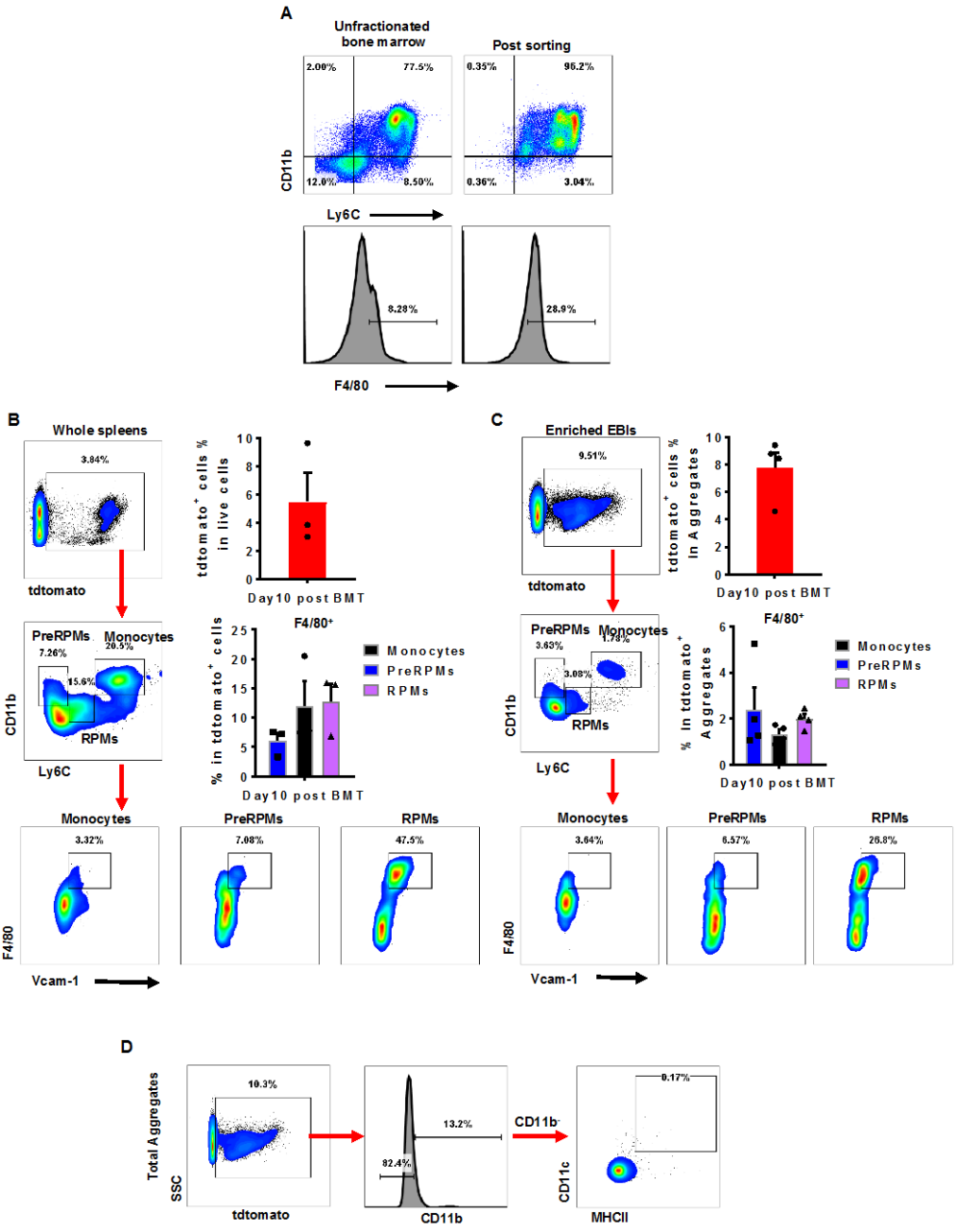
(B) F4/80 and Vcam-1 expression analysis on Ly6C^{hi} monocytes, Ly6C^{lo} monocytes, Pre-RPMs and PRMs, pre-gated on YFP^{+}tdTomato^{neg} cells on day 10, day 14 and day 16 post BMT. (left) Representative flow cytometry diagrams; (right) Percentage F4/80^{+}Vcam-1^{+} cells in each subpopulations within YFP^{+}tdTomato^{neg} cells.

(C) Early stage. F4/80 and Vcam-1 expression analysis on Ly6C^{hi} monocytes, Ly6C^{lo} monocytes, Pre-RPMs and PRMs, pre-gated on YFP^{+} tdTomato^{+}cells. Left, representative flow cytometry charts; Right, percent F4/80^{+}Vcam-1^{+} cells in each subpopulations within YFP^{+} tdTomato^{+}cells.

(D) Flow cytometry histograms of total YFP^{+}cells on days 0, 2, 10 and 14. The mean fluorescence intensity (MFI) increased on days 10 and 14 when compared to day 2.

Bars are representative of mean ± SEM.

FigureS6



Supplemental Figure S6

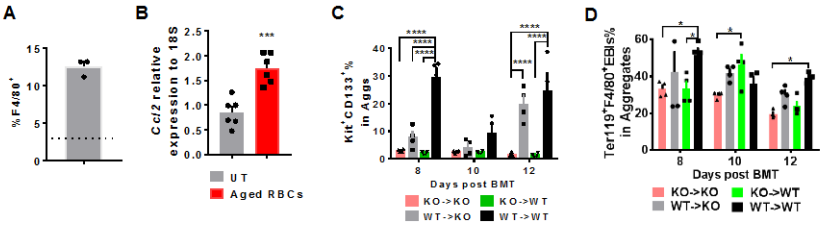
Supplemental Figure S6. Monocytes tracing day 10 post-transplant.

(A) Monocytes purity checking post beads sorting. CD11b, Ly6C and F4/80 were examined by flow cytometry. Purified monocytes were Ly6C+CD11b+F4/80int.

(B) Flow cytometry analysis of spleen and (C) EBI td-tomato+cells by applying the gating strategy described in main Figure 2.

(D) Flow cytometry analysis of total CD11b- tdtomato+ aggregates for dendritic cell markers.

FigureS7



Supplemental Figure S7

Supplemental Figure S7 (Continuous from Figure. 7)

(A - B) Ccl2 transcript analysis of aged RBC treated macrophages. (A) F4/80+ cell percentage in adherent layer generated from mouse spleens. (B) Ccl2 mRNA levels in aged RBC treated layer and untreated layer. n=3 per group with two repeats.

(C) Percentage of Kit+CD133+ aggregates in total enriched aggregates in Ccl2 mutant BMT model.

(D)Ter119+F4/80+EBIs, and there were less Ter119+EBIs in the KO->KO condition throughout the time points compared to WT conditions percentage wise. n=3-4 each group per timepoint. Bars are representative of mean ± SEM.