RE: Submission #70812-RG-1

Supplementary data (Kim *et al*.)

Supplementary Methods

Mice. To induce Cre-mediated deletion of *Keap1* in tamoxifen-inducible CMVCre-Keap1$^{flox/flox}$, mice were treated with tamoxifen for 5 days (1 mg/mouse/day, intraperitoneal injection). To induce Cre-mediated deletion of keap1 in MxCre-Keap1$^{flox/flox}$, mice were administered with polyriboinosinic acid/polyribocytidylic acid [poly(I:C)] (Sigma, St. Louis, MO) for 7 days (25 mg/kg/day)(1). Deletion of *Keap1* exons 2-3 were confirmed by PCR analysis using primers: forward 5'-GAG TCC ACA GTG TGT GGC C -3', reverse 5'-GAG TCA CCG TAA GCC TGG TC-3'. Generation of Nrf2-deficient C57BL/6 mice was described previously (2).

Isolation of bone marrow (BM) cells and flow cytometry. BM cell isolation and flow cytometric analysis of cell surface markers was performed as previously described (3, 4)**.** Briefly, BM cells were flushed from the long bones (tibias and femurs) of mice with Iscove's Modified Dulbecco's Medium media with 2% FBS. Before staining, BM red blood cells (RBCs) were lysed using ACK Lysing Buffer (Quality Biological Inc, Gaithersburg, MD). Of note, for detection of stromal cells, RBC lysis step was not performed, instead APC-Ter119 was use as RBC markers and PE-CD45 as hematopoietic cells marker. For detection of progenitor cells, whole BM cells were incubated with biotin-conjugated monoclonal antibodies to lineage markers (Miltenyi Biotec, Cambrige, MA), including CD5, CD11b, CD45R, Anti-7-4, Anti-Gr-1 and Anti-

Ter-119. PE-Sca1, APC-c-kit, PerCPcy5.5-CD16/32 and FITC-CD34 (eBioscience, San Diego, CA) were co-stained with lineage markers. Note that to detect biotin-conjugated lineage markers, streptavidin-conjugated APC-Cy7 (eBioscience, San Diego, CA) was used. Stained cells were analyzed using an LSRII flow cytometer (BD Biosciences, La Jolla, CA). For sorting CD34-LSK cells, FITC-CD34 (eBioscience, San Diego, CA) antibody was used in addition to c-kit, sca1 and lineage markers.

Quantitative real-time RT-PCR. Total RNA was extracted from cells or tissues using the RNeasy Mini Kit according to the manufacturer's recommended protocol (Qiagen Inc, Gaithersburg, MD). Quantitative real-time RT PCR analyses were conducted using assay on demand probe sets (Applied Biosystems, Foster City, CA), and reactions were analyzed using the ABI 7000 Taqman system as previously described (5). GAPDH or ActB was used for normalization.

ROS analysis. ROS levels were determined by flow cytometry using H2DCFDA dye (Invitrogen, Frederick, MD) respectively as previously described (6, 7).

Peripheral Blood analysis. Complete blood count was analyzed by a Hemavet® 950FS (Drew Scientific Inc, Dallas, TX).

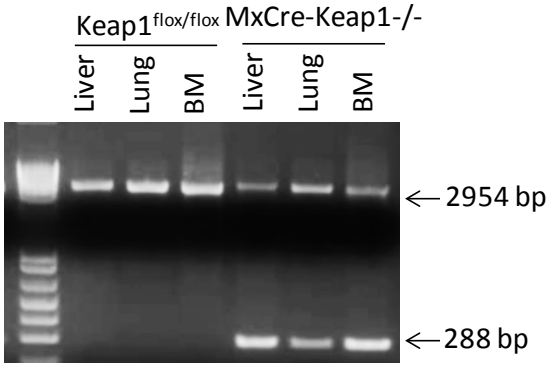
Colony-forming cell (CFC) assays. CFC assays were performed using MethoCult M3434 (Stem Cell Technologies, Vancouver, Canada) as previously described (8).

H and E staining. femurs and tibiaefrom one leg/animal were dissected, fixed for 24 hours in 10% neutral buffered formalin, and processed, including decalcification and hematoxylin and eosin (H&E) staining (Histology Core, Johns Hopkins).

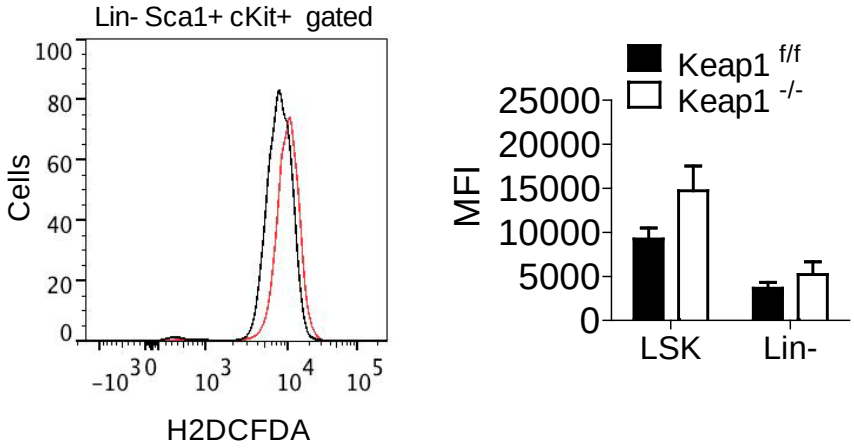
Statistical Analysis. Unless otherwise noted, P values were calculated using a two-tailed Student's t-test with unpaired analysis.

Supplementary Figures

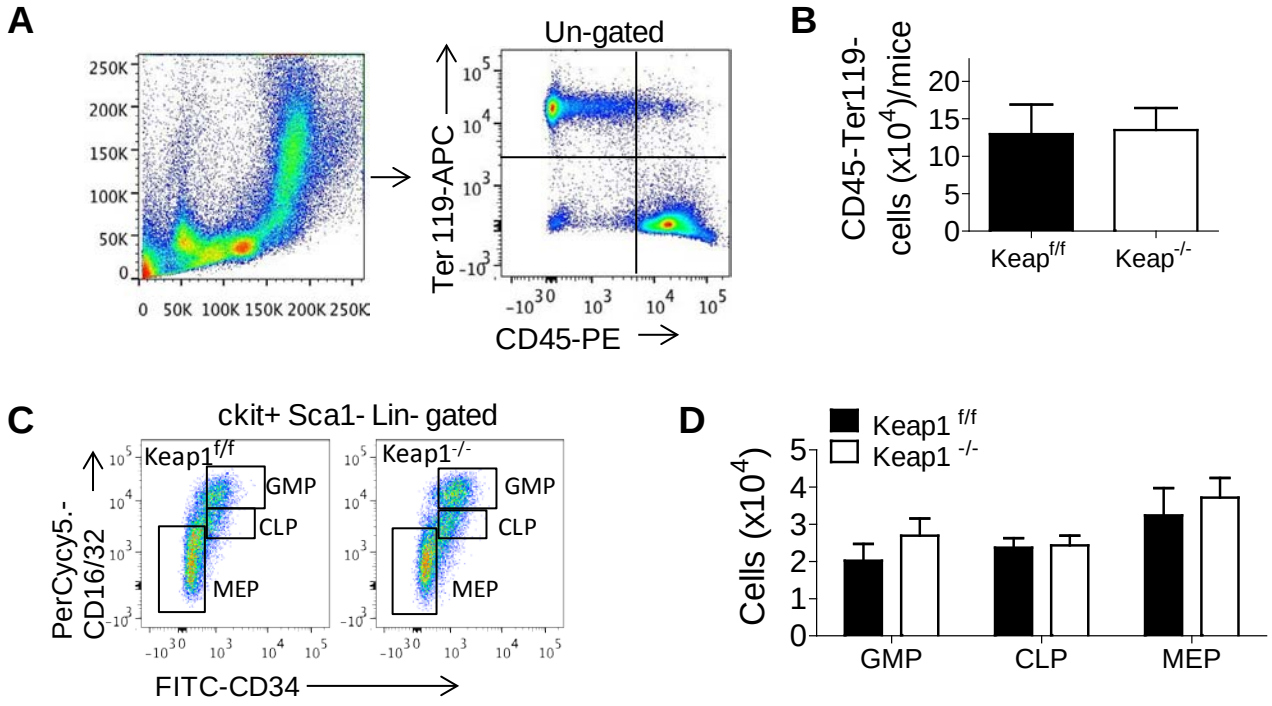
Supplementary Figure 1. PCR analysis showing the genomic deletion of exons 2 and 3 of the Keap1 gene in BM cells, liver and lung isolated from Keap1$^{flox/flox}$ and MxCre-Keap1$^{flox/flox}$ mice following poly(I:C) treatment (25 mg/kg/day for 7 days). The deleted Keap1 gene was noted in BM, lung and liver. The 288-bp band represents the Keap1 allele with exons 2 and 3 deleted, and the 2,954-bp band represents the floxed or the wild-type allele.



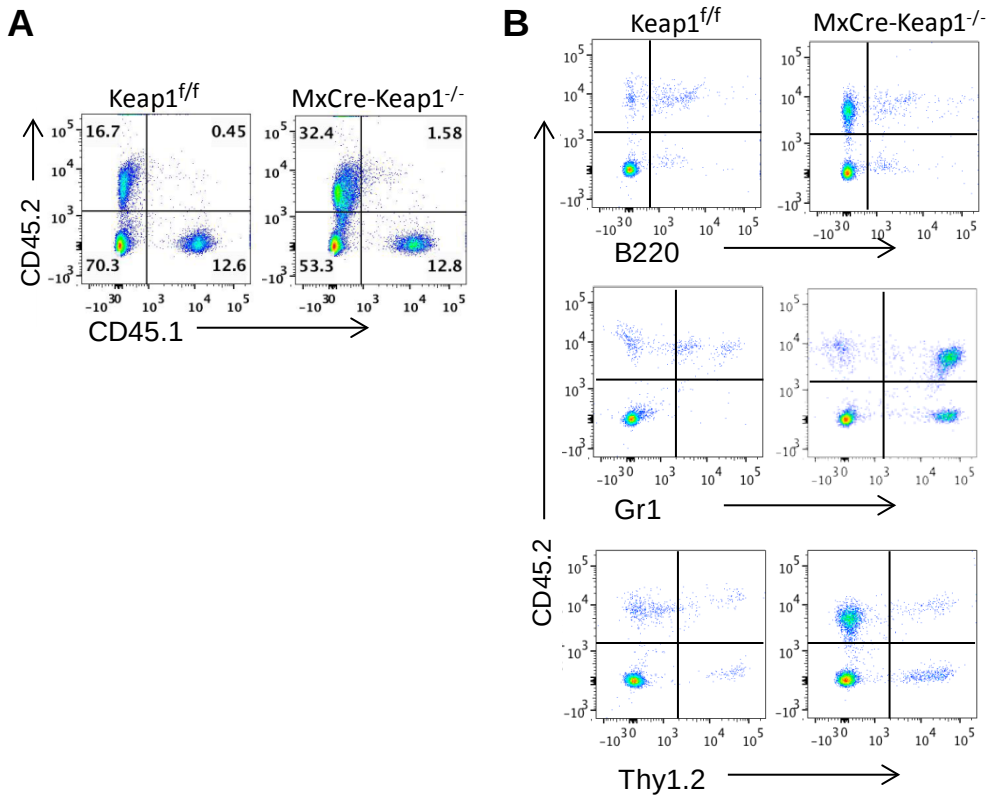
Supplementary Figure 2. **Basal ROS levels are comparable between Keap1$^{flox/flox}$ and MxCre-Keap1$^{-/-}$ LSK cells.** ROS levels was measured by flow cytometry in the LSK population isolated from Keap1$^{flox/flox}$ and MxCre-Keap1$^{flox/flox}$ mice 4 weeks after poly(I:C) treatment (25 mg/kg/day for 7 days). MxCre-Keap1$^{-/-}$ is represented as Keap1$^{-/-}$ in the graph legends.



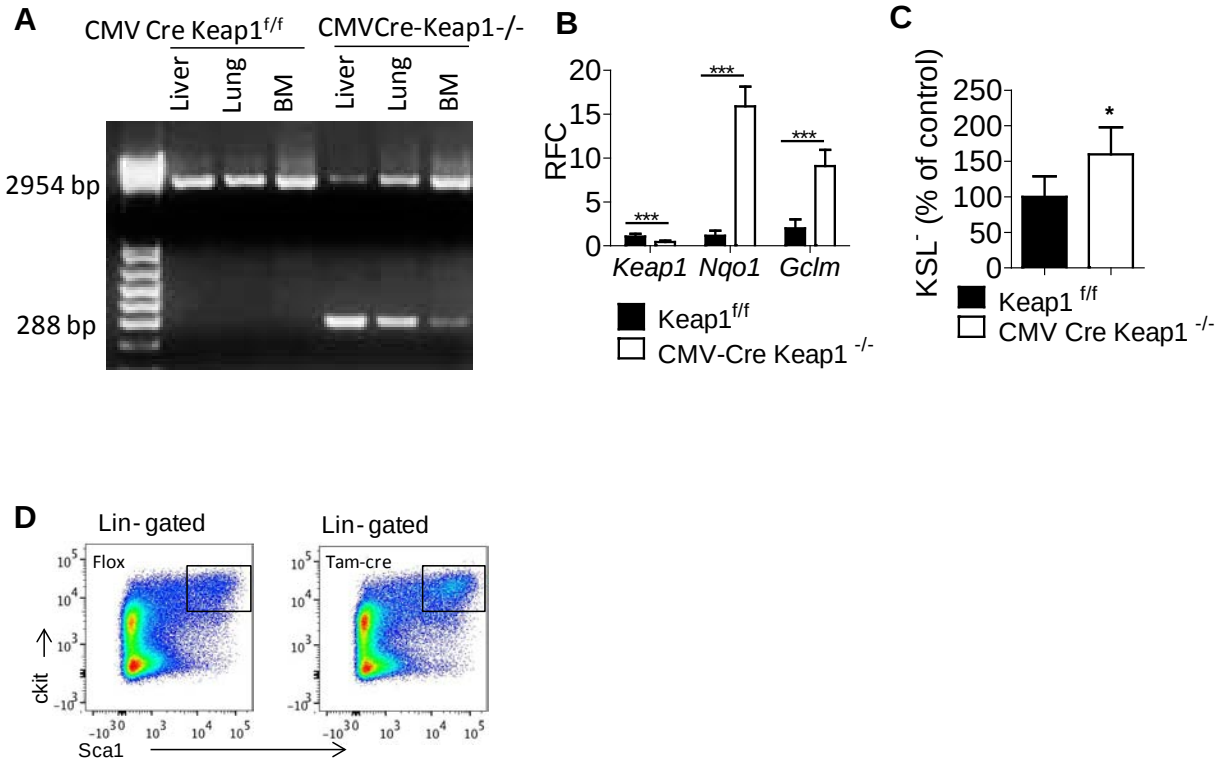
Supplementary Figure 3. **Analysis of stromal cells and multilineage progenitor cells in the bone marrow of MxCre-Keap1$^{-/-}$ and floxed control mice under steady-state condition. (A-B)** Representative flow cytograms depicting BM stromal cells identified as CD45^{-}Ter119^{-} (A) and total number of BM stromal cells (B) in MxCre-Keap1$^{-/-}$ and floxed control mice under steady-state condition. **(C-D)** Representative flow cytograms depicting multilineage progenitor cells (C) and total number of multilineage progenitor cells in BM of MxCre-Keap1$^{-/-}$ and floxed control mice (D) under steady-state condition.



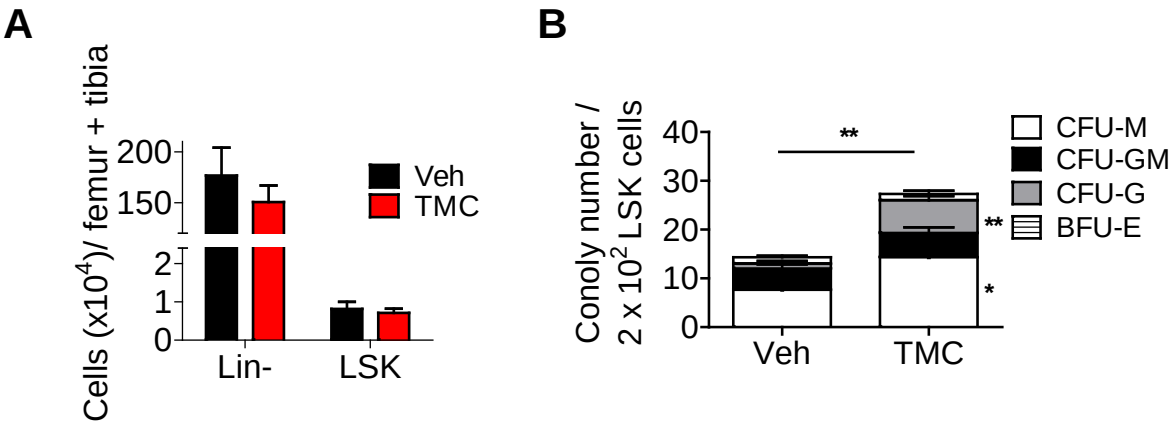
Supplementary Figure 4. Representative flow cytograms depicting donor cell chimerism in the secondary recipient. (A-B) Representative flow cytometric analysis of peripheral blood donor cells (CD45.2) engraftment (A) and B220+, Gr1+ and Th1.2 engraftment (B) at 2 weeks after transplantation in the secondary recipient transplanted with bone marrow from poly(I:C) treated primary recipient transplanted with LSK cells harvested from Mx-CreKeap1$^{flox/flox}$ and Keap1$^{flox/flox}$ mice as depicted in Figure 2D.



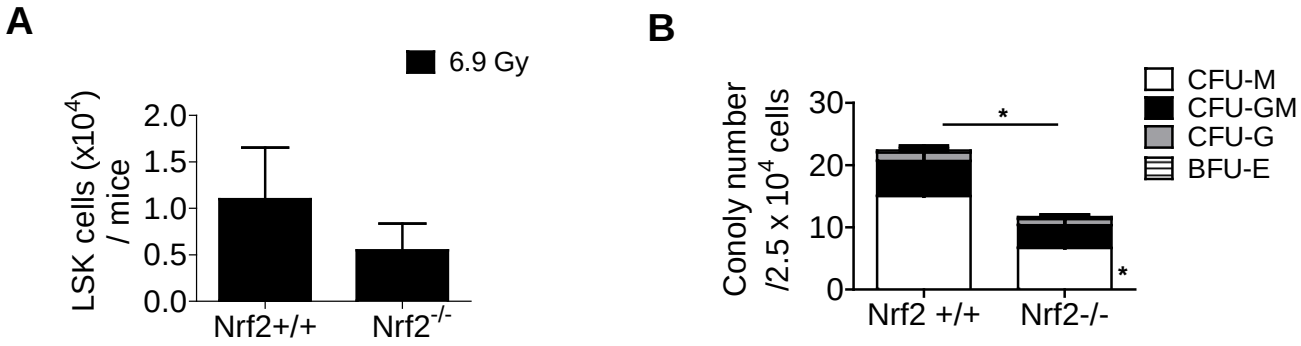
Supplementary Figure 5. Tamoxifen treatment induced Nrf2 activity and higher number of hematopoietic stem cells in the bone marrow of Tamoxifen-inducible CMVCre-Keap1$^{flox/flox}$ mice compared to Keap1$^{flox/flox}$. **(A)** PCR analysis showing the genomic deletion of exons 2 and 3 of the Keap1 gene in BM cells, liver and lung isolated from Keap1$^{flox/flox}$ and CMV-Keap1$^{flox/flox}$ mice following treatment of tamoxifen for 5 days (1mg/mouse/day, i.p). The 288-bp band represents the Keap1 allele with exons 2 and 3 deleted, and the 2,954-bp band represents the floxed or the wild-type allele. **(B)** Relative gene expression of *Keap1, Nqo1 and Gclm* in total BM cells and **(C)** Total number of LSK cells in BM of CMV-CreKeap1$^{-/-}$ mice and floxed control mice following tamoxifen treatment (*P < 0.05, ***P < 0.001). **(D)** Representative flow cytometric analysis of c-kit and Sca-1 expression in LSK progeny cells in the co-cultures on day 7 (experimental design depicted in Figure 2G).



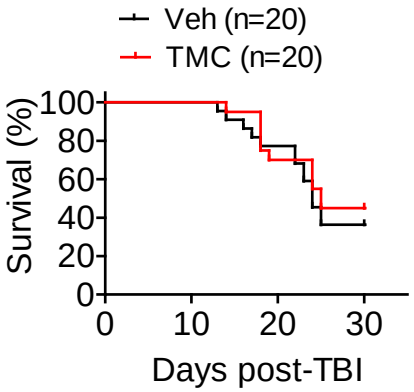
Supplementary Figure 6. TMC administration increased functional hematopoietic progenitor cells in normal mice under steady-state conditions. Normal healthy mice were orally administered TMC or vehicle every 48h (6 doses in total). (**A**) Total number of lineage negative (Lin-) or LSK cells in femur and tibia of un-irradiated mice administered orally with TMC or vehicle. (**B**) Colony-forming units (CFUs) per BM isolated from un-irradiated mice administered orally with TMC or vehicle. Significant compared to vehicle; *P < 0.05.



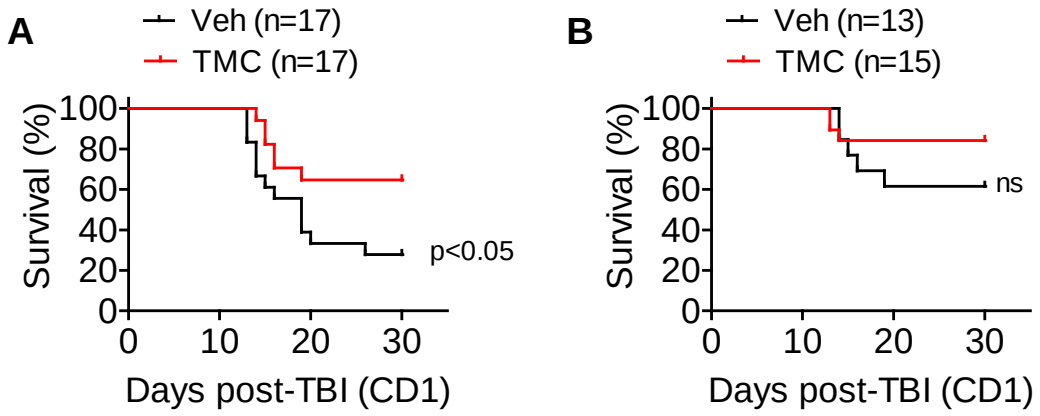
Supplementary Figure 7. Nrf2-deficient mice exhibit greater myelosuppression following TBI. (**A**) Total number of LSK cells analyzed in femur and tibia of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice 48 h after TBI (6 Gy). Significant compared to TBI, *P < 0.05; significant compared to $Nrf2^{-/-}$, ** P < 0.01. (**B**) Colony-forming units (CFUs) per BM isolated from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice 48 h following TBI. Significant compared to TBI; *P < 0.05.



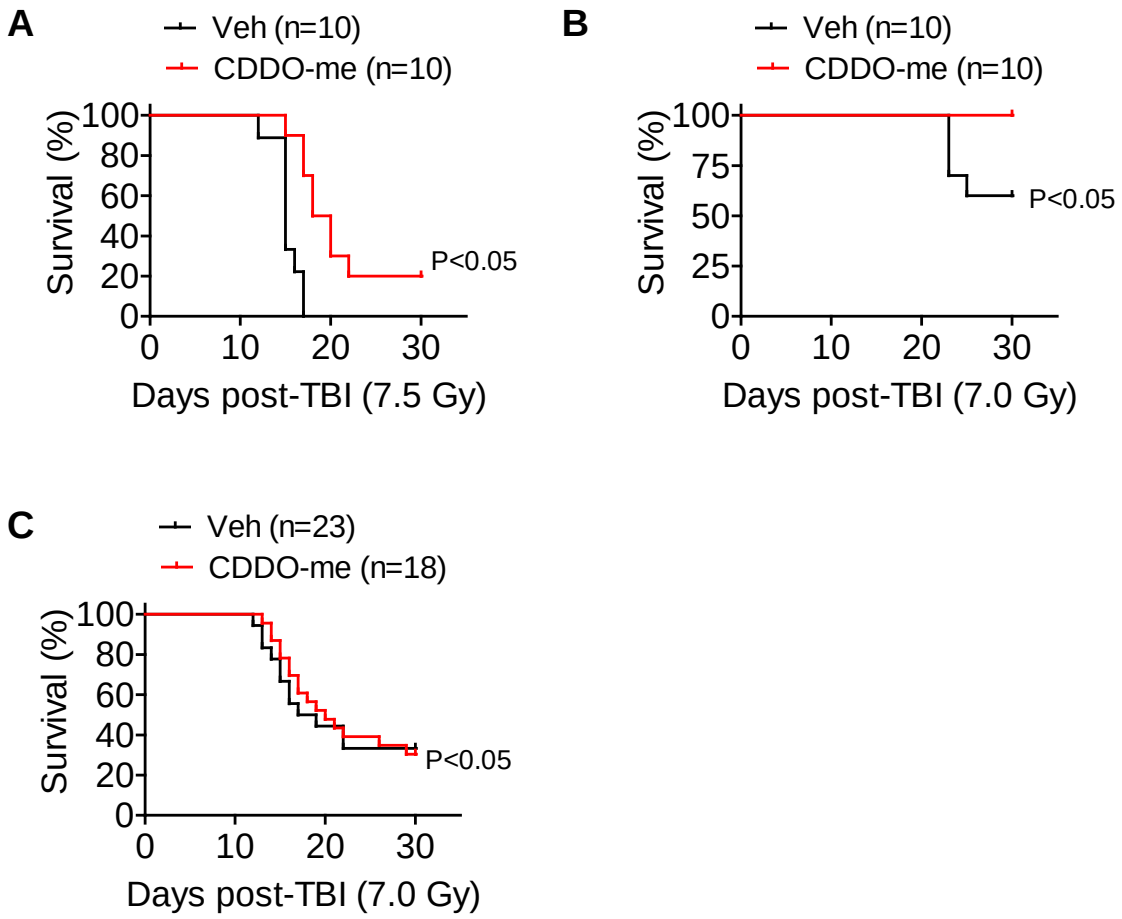
Supplementary Figure 8. TMC showed poor radiomitigative efficacy when administered beginning 48h after TBI. Thirty-day survival curve of C57BL/6 mice treated with TMC or vehicle 5 times, once every other day, beginning 48h after TBI.



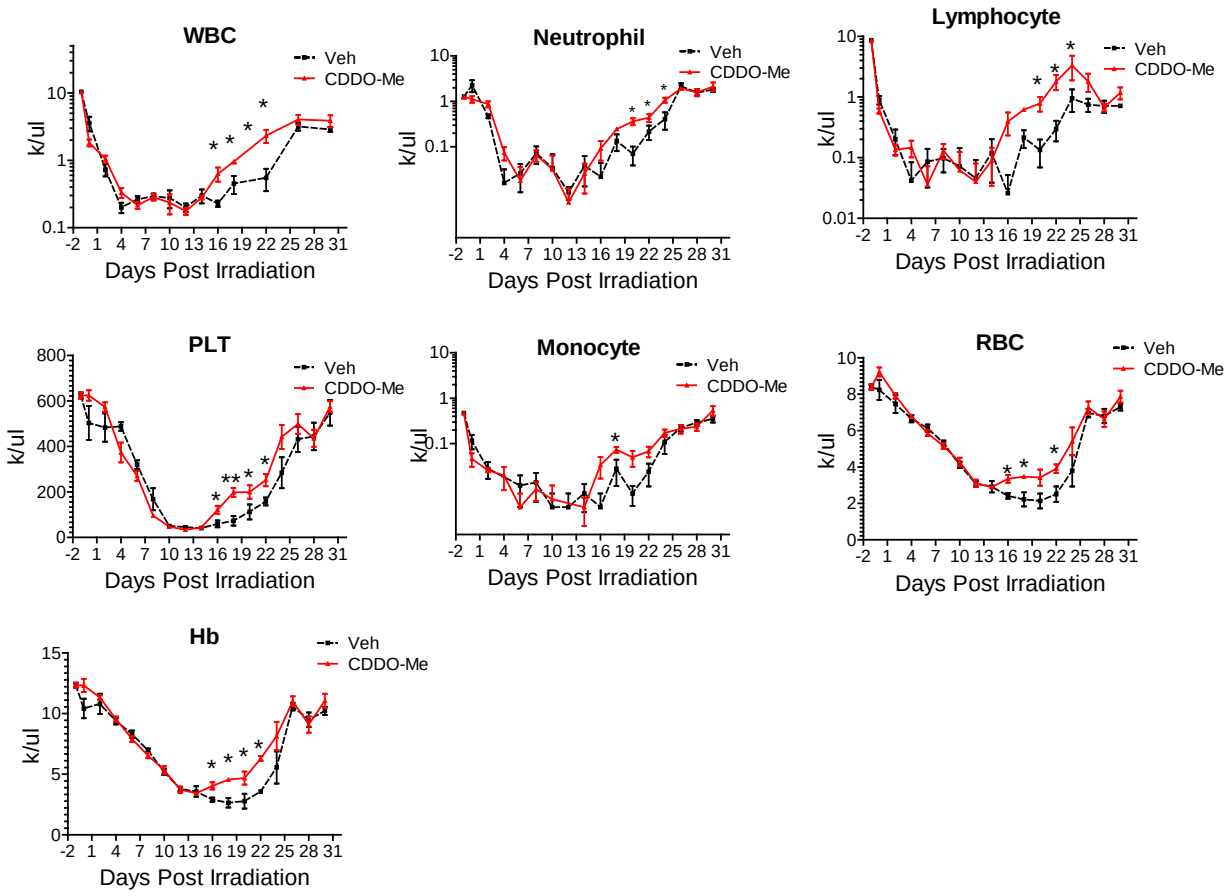
Supplementary Figure 9. Radiomitigative efficacy of TMC to improve 30-day survival of CD-1 strain of mice following TBI. Thirty-day survival rate of CD-1 mice administered TMC or vehicle 6 times (once every 48 h) beginning 24 h after 6.9 Gy (A) and 6.5 Gy (B).



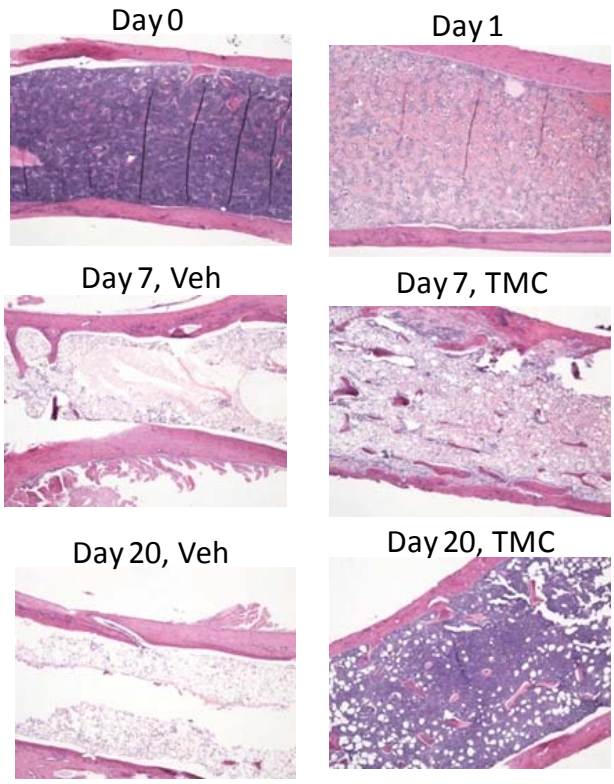
Supplementary Figure 10. Administration of Nrf2 activator CDDO-Me mitigates TBI-induced mortality in mice in an Nrf2-dependent manner. (A-C) Mice were orally administered with vehicle (10% DMSO, 10% cremaphore-EL and 80% PBS) or CDDO-Me (10μmol/kg) 5 times, once every 48h beginning 24h after TBI. **(A, B)** Thirty-day survival rate of C57/BL6 mice after 7.5Gy (A) and 7.0Gy (B). **(C)** Thirty-day survival rate of $Nrf2^{-/-}$ mice (C57BL/6) administered with vehicle or CDDO-Me 5 times (once every 48h) beginning at 24h after 7.0Gy TBI.



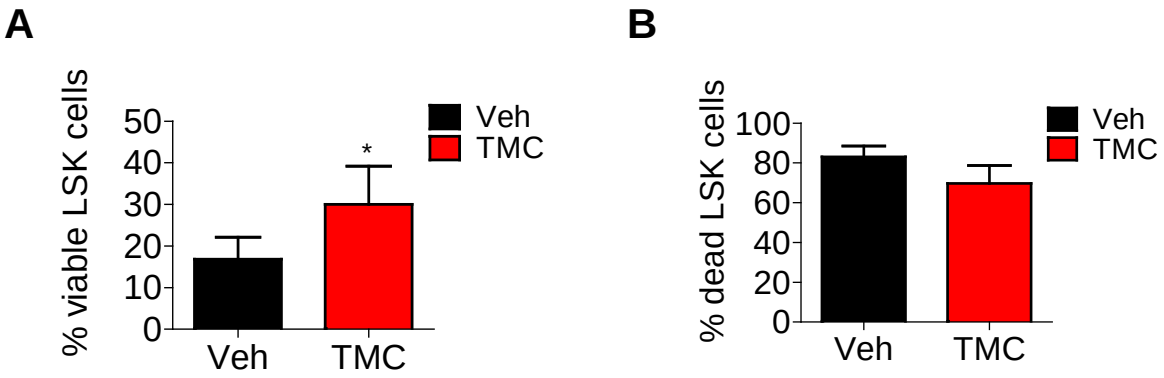
Supplementary Figure 11. Administration of Nrf2 activator CDDO-Me mitigates TBI-induced myelosuppression in mice. C57/BL6 mice were administered with vehicle or CDDO-Me 24h after TBI and four additional treatments every 48 hours after initial treatment. At each time period indicated, a cohort of mice (n=5) were sacrificed and differential blood cell counts (white blood cell (WBC), neutrophil, lymphocyte, platelet (PLT) monocyte, red blood cell (RBC) and hemoglobin (Hb) were analyzed in the peripheral blood. *p<0.05, **p<0.01, ***p<0.001



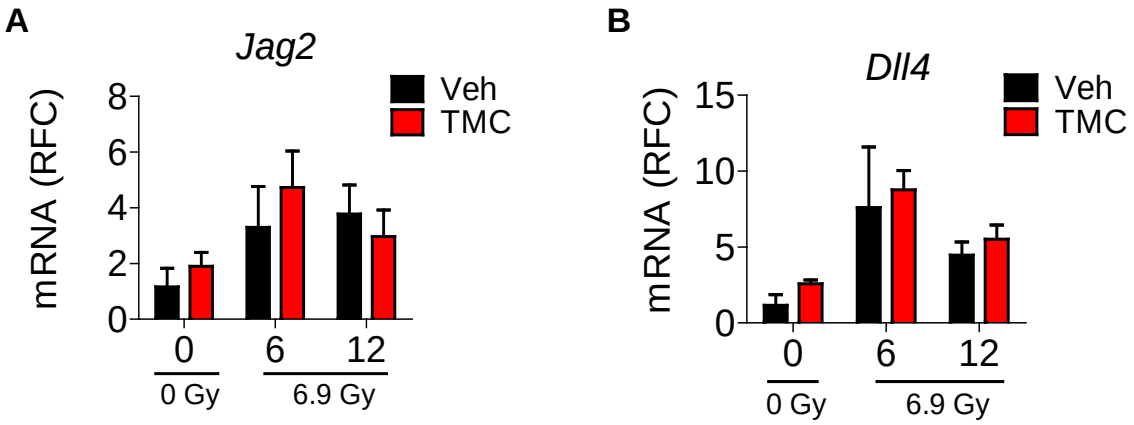
Supplementary Figure 12. TMC administration accelerates BM recovery in mice following TBI exposure. Representative images of H&E staining showing longitudinal sections of the femurs of TMC- or vehicle-treated mice isolated on day 0 (un-irradiated), day 1, day 7 and day 20 post-TBI. Treatment with TMC or vehicle was initiated 24 h post-TBI.



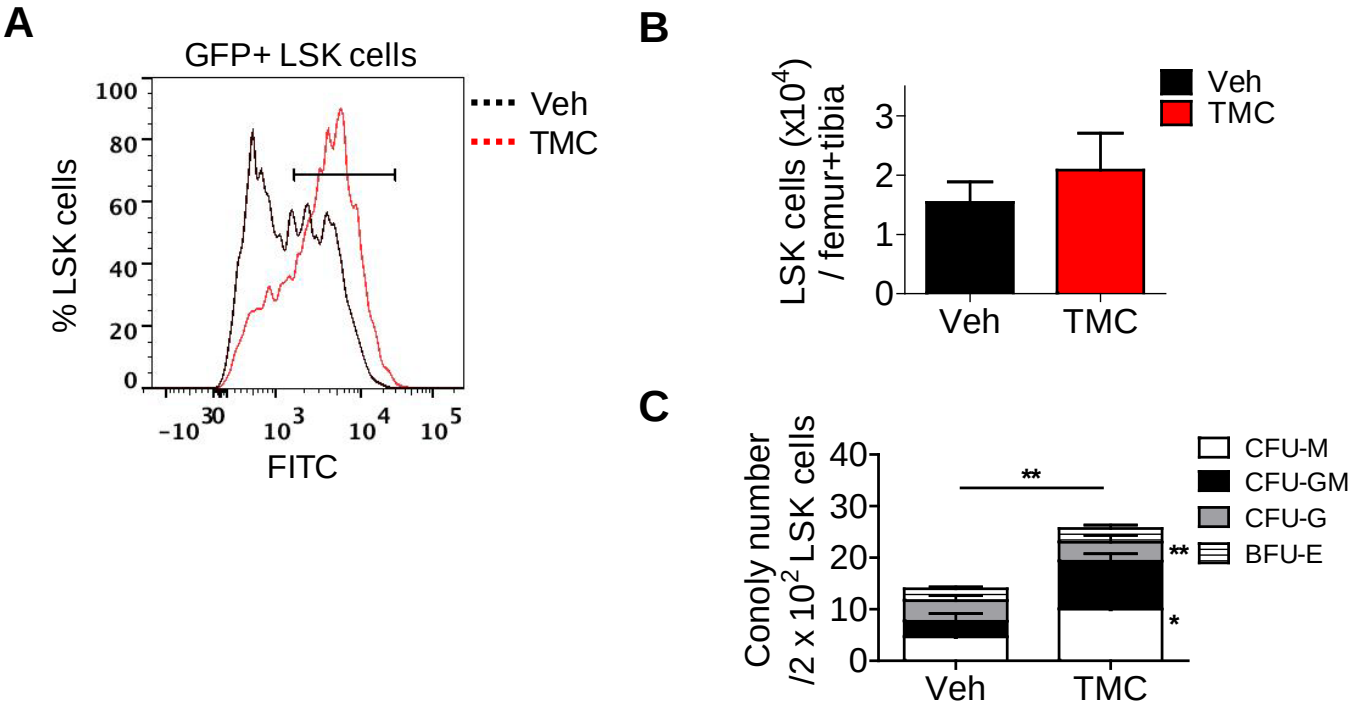
Supplementary Figure 13. **Flow cytometric analysis of live or dead LSK cells in TMC- or vehicle-treated mice on day 3 post-TBI.** LSK cells were stained with a fixable Aqua Live/Dead dye (A-B). Data represented as mean ± SD of percentage live or dead LSK cells.



Supplementary Figure 14. TMC administration does not alter the expression of Jag2 and Dll4 in BM cells of irradiated mice. A-B) Temporal expression of Jag2 (A) and Dll4 (B) mRNA in BM cells isolated from TMC or vehicle-treated mice on days 6 and 12 post-TBI. Data represented as mean of relative fold change (RFC) ± SD, n = 4-5 mice per time period.



Supplementary Figure 15. TMC administration up-regulated notch signaling in un-irradiated TNR mice. TNR mice were administered orally with 6 doses (once every 48 h) of TMC or vehicle. (**A,B**) Percent of GFP-positive LSK cells and total number of LSK cells in BM of TNR mice administered with TMC or vehicle. Significant compared to vehicle. (**C**) BM CFCs in the TNR mice treated with TMC or vehicle. Data represented as mean (± SD) of CFCs; n = 5 mice/group; repeated 3 times.



Supplementary tables

Supplementary Table 1. Complete blood count in Keap1$^{f/f}$ and MxCre-Keap1$^{-/-}$ mice treated with or without poly(I:C) (1 dose/day for 7 day, i.p).

	Keap1$^{f/f}$	MxCre-Keap1$^{f/f}$
WBC (K/µL)	7.5 ± 2.8	8.9 ± 3.7
Neutrophils (K/µL)	2.5 ± 1.1	4.2 ± 2.7
Lymphocytes (K/µL)	4.3 ± 1.6	4.4 ± 1.1
RBC (M/µL)	7.6 ± 0.7	8.8 ± 0.2
HB (g/dL)	11.1 ± 0.9	11.9 ± 0.4
HCT (%)	33.7 ± 2.8	36.3 ± 0.6
PLT (K/µL)	675 ± 216	1053 ± 71*

* $p<0.05$, n=10

Supplementary Table 2. Expression of Nrf2-regulated genes (heme oxygenase-1 (Ho-1); NAD(P)H dehydrogenase quinone 1 (Nqo1); and glutamate-cysteine ligase, modifier subunit (Gclm)) in BM cells and other tissues in mice orally administered 6 doses (once every 48 h) of TMC or vehicle. Data are expressed as mean of relative fold change (RFC) compared to vehicle.

	Ho-1 (RFC)		*Nqo1 (RFC)*		*Gclm (RFC)*	
Organ	Veh	TMC	Veh	TMC	Veh	TMC
Bone marrow cells	1.1 ± 0.61	1.5 ± 0.29*	1.1 ± 0.60	2.5 ± 0.53	1.0 ± 0.28	2.1 ± 0.42
Lung	1.1 ± 0.46	1.4 ± 0.33*	1.0 ± 0.69	2.7 ± 0.47*	1.1 ± 0.38	1.5 ± 0.10
Small intestine	1.0 ± 0.52	310.5 ± 22.7*	1.0 ± 0.57	39.5 ± 12.0*	1.0 ± 0.13	87.4 ± 56.5*
Liver	1.0 ± 0.32	2.7 ± 1.62*	1.0 ± 0.29	11.5 ± 3.32*	1.0 ± 0.10	1.7 ± 0.36*
Brain	1.0 ± 0.27	2.4 ± 0.66*	1.0 ± 0.06	6.2 ± 1.35*	1.1 ± 0.43	3.5 ± 1.52*

* $p<0.05$, n=9-10

Supplementary Table 3. Body weight, relative organ weight, blood clinical chemistry and complete blood count of mice orally administered with vehicle or TMC (400mg/kg/day for 15 days).

	Vehicle	TMC
Body Weight (g)	23.12 ± 1.1	22.21 ± 1.8
Relative organ weights		
Liver (g)	1.477 ± 0.04	1.632 ± 0.17
Spleen (g)	0.069 ± 0.006	0.067 ± 0.008
Heart (g)	0.168 ± 0.01	0.158 ± 0.02
Right Kidney (g)	0.221 ± 0.02	0.208 ± 0.02
Left Kidney (g)	0.188 ± 0.01	0.203 ± 0.02
Clinical Chemistry		
Gamma GT (µ/L)	4.0 ± 1.00	3.0 ± 1.00
Alanine Aminotransferase (µ/L)	52.3 ± 11.02	46.7 ± 3.21
Aspartate Aminotransferase (µ/L)	68.0 ± 15.39	74.0 ± 11.00
Alkanine Phosphatase (µ/L)	122.7 ± 5.51	110.7 ± 6.81
Blood urea Nitrogen (mg/dL)	19.7 ± 1.53	17.3 ± 2.52
Creatinine (mg/dL)	0.37 ± 0.06	0.37 ± 0.06
Complete blood count		
WBC (K/µL)	3.8 ± 0.51	4.46 ± 1.25
Neutrophils (K/µL)	0.6 ± 0.22	1.15 ± 0.83
Lymphocytes (K/µL)	2.9 ± 0.44	2.91 ± 0.66
RBC (M/µL)	9.5 ± 0.54	9.13 ± 0.73
HB (g/dL)	13.1 ± 0.53	12.84 ± 0.60
HCT (%)	47.9 ± 2.36	46.70 ± 2.93
PLT (K/µL)	1166 ± 116	1092 ± 179

Supplementary Table 4. Expression of Nrf2-regulated genes (cysteine ligase catalytic subunit (Gclc); NAD(P)H dehydrogenase quinone 1 (Nqo1); and glutamate-cysteine ligase, modifier subunit (Gclm)) in different tissues harvested from mice orally administered with 5 doses (once every 48 h) of CDDO-me or vehicle from 1hr post TBI . Data are expressed as mean of relative fold change (RFC) compared to vehicle.

Organ	*Gclc (RFC)*		*Nqo1 (RFC)*		*Gclm (RFC)*	
Kidney	1.0 ± 0.31	1.86 ± 0.17*	1.0 ± 0.12	6.2 ± 1.07	1.0 ± 0.08	1.4 ± 0.09
Thymus	1.0 ± 0.06	2.15 ± 0.37*	1.0 ± 0.45	4.41 ± 0.85*	1.1 ± 0.35	1.8 ± 0.28
Lung	1.0 ± 0.19	2.77 ± 0.33*	1.0 ± 0.16	7.32 ± 0.70*	1.0 ± 0.13	1.81 ± 0.32*
Liver	1.0 ± 0.41	2.93 ± 0.63*	1.0 ± 0.11	12.84 ± 3.45*	1.0 ± 0.18	1.81 ± 0.32*
Colon	1.0 ± 0.13	13.46 ± 0.64*	1.0 ± 0.17	12.5 ± 0.49*	1.0 ± 0.22	13.76 ± 0.48*
Intestine	1.0 ± 0.06	24.39 ± 1.66*	1.0 ± 0.14	8.23 ± 0.57*	1.0 ± 0.14	7.42 ± 0.78*
Stomach	1.0 ± 0.19	17.42 ± 4.27*	1.0 ± 0.16	4.94 ± 0.291*	1.0 ± 0.11	13.76 ± 2.58*

* $p<0.05$, n=5 RFC, relative fold change compare to vehicle treatment

Supplementary references

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