SUPPLEMENTARY APPENDIX

Necroptosis in spontaneously-mutated hematopoietic cells induces autoimmune bone marrow failure in mice

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Necroptosis in spontaneously-mutated hematopoietic cells induces autoimmune bone marrow failure in mice

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Table 1. Characteristic features of transgenic mice

Mouse strain		Growth	Reproductivity (pups/litter)	Life span
Cre^{-}	*Tak1*$^{f/f}$	Normal	Normal (6-10)	>2 years
	Tak1$^{f/f}$*Tnfr*$^{-/-}$	Normal	Normal (6-10)	>2 years
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Ifnγ*$^{-/-}$	Normal	Normal (6-10)	>2 years
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Rip3*$^{-/-}$	Normal	Normal (6-10)	>2 years
Cre^{+}	*Tak1*$^{f/f}$	Generally normal before 8 month	Normal (6-10) before 8 month old	14-22 months
	Tak1$^{f/f}$*Tnfr*$^{-/-}$	Stop gaining BW at 2 mos. of age	No	3-7 months
	Tak1$^{f/+}$*Tnfr*$^{-/-}$	Normal	Normal, used to breed	>2 years
	Tak1$^{f/+}$*Tnfr*$^{-/-}$ +anti-CD4	Regain BW	N/D	Up to 10 months
	Tak1$^{f/+}$*Tnfr*$^{-/-}$ +IgG	Do not regain BW	N/D	3-7 months
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Ifnγ*$^{-/-}$	Normal	Normal	>1.5 years
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Ifnγ*$^{+/-}$	Stop gaining BW at 3-4 mos. of age	No	6-12 months
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Rip3*$^{-/-}$	Normal	Reduced (1-4)	Up to 10 months
	Tak1$^{f/f}$*Tnfr*$^{-/-}$*Rip3*$^{+/-}$	Stop gaining BW at 2 mos. of age	No	3-7 months

Color codes and abbreviations: no fill=normal; green=normal after intervention; brown=severe BMF; light brown=chronic BMF; BW=body weight; N/D= not determined

Supplementary Figures with Legends

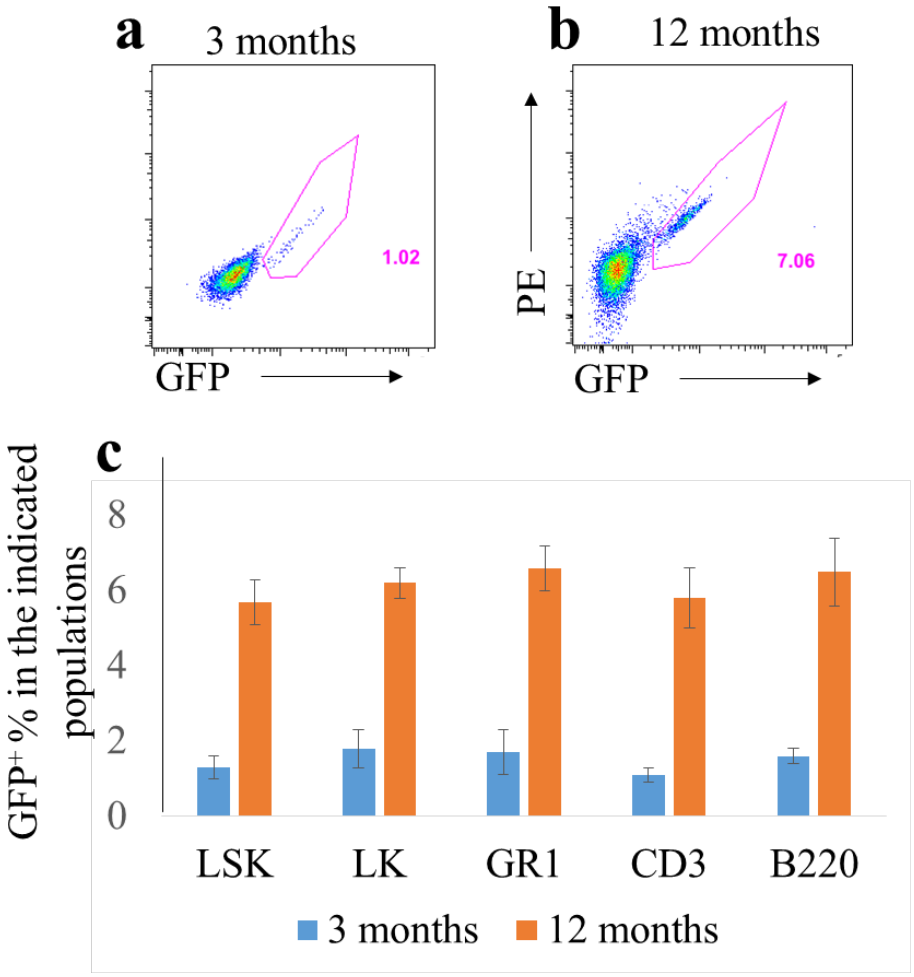

Figure S1. Linkage of Mx1Cre-induced flox-mediated recombination in BM cells as shown by Rosa-stop-GFP reporter assay. *Mx1Cre* mice were crossed with *ROSA26-EGFP*^{f} mice to generate *Mx1Cre*$^{+}$*ROSA26-EGFP*^{f} mice. GFP expression in these mice reliably reflects the Cre-induced flox-mediated recombination in target cells. Consistent with our previously reported findings[1], we found that at 3 months of age, 1-3% of GFP can be detected in all lineages of BM cells including LSK HSPCs, LK progenitors, Gr1^{+} granulocytes, CD3^{+} T cells and B220^{+} B cells. The percentage of GFP^{+} cells was increased during aging and reached 5-7% at 12 months of age in BM cells. **a** and **b.** Representative flow cytometric data for analysis of GFP^{+} cells in BM mononuclear cells at 3 months of age (**a**) and 12 months of age (**b**). **c**. Average GFP^{+}% in indicated populations of BM cells at indicated time points. Three mice for each time point were analyzed.

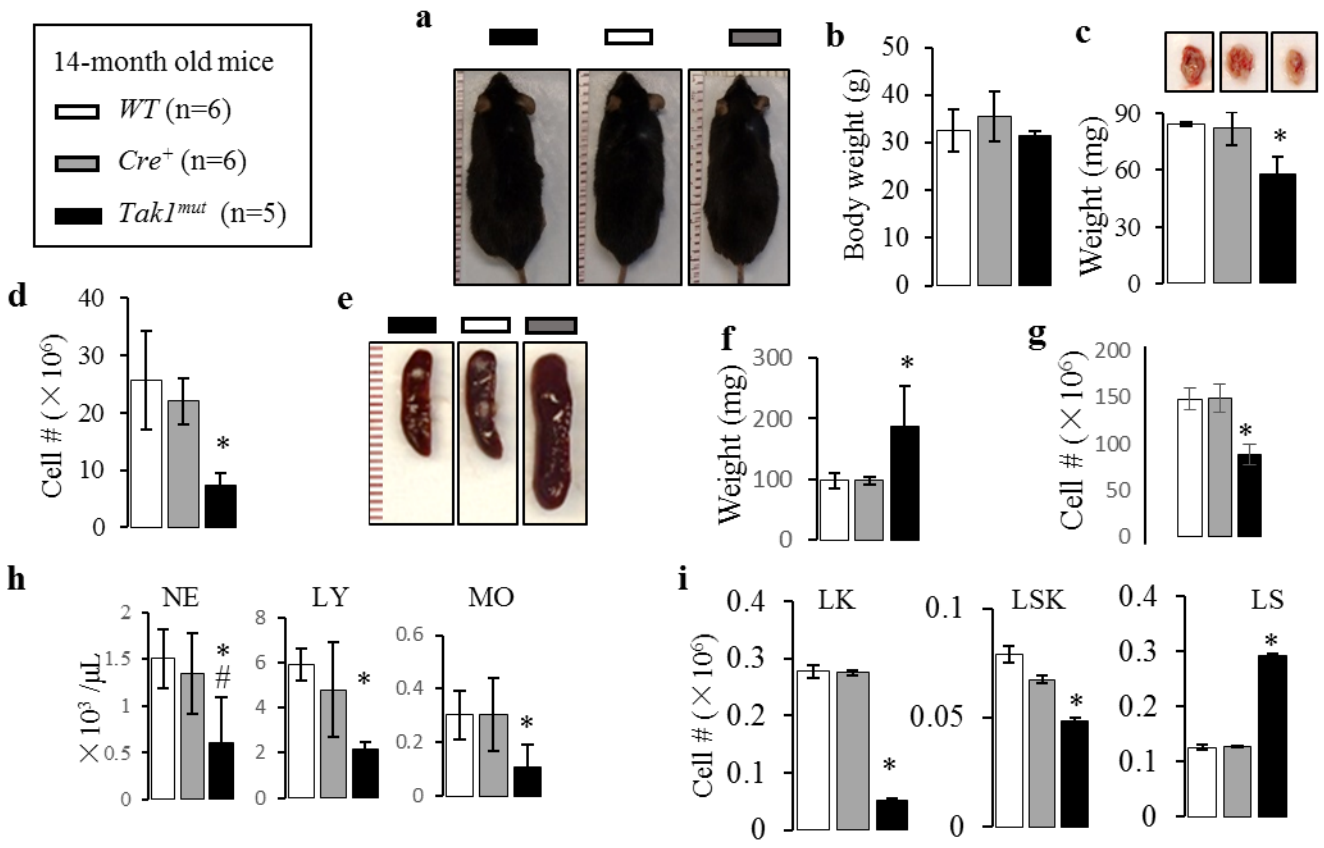

Figure S2. Spontaneous *Tak1* deletion in HSPCs results in chronic BMF (associated with Figure 1). *Tak1*^{mut} mice and their *WT* and *Cre*$^{+}$ littermates were analyzed at age 14 months (n=5~6/group) for overall phenotype (**a.** photographs of body; **b**. body weight) and lymphoid tissues (thymus: **c.** photograph and **d.** weight; spleen: **e**. photograph, **f**. weight and **g.** total nucleated cells). **h.** Numbers of neutrophils (NE), lymphocytes (LY) and monocytes (MO) in peripheral blood. **i.** Absolute numbers of lineage^{-}c-kit^{+}Sca1^{-} (LK), lineage^{-}c-kit^{+}Sca1^{+} (LSK) and lineage^{-}c-kit^{-}Sca1^{+} (LS) populations in bone marrow (BM) from 2 hind legs**.** Data are presented as means ± SD. *, p<0.05 compared to *WT* and Cre^{+} controls.

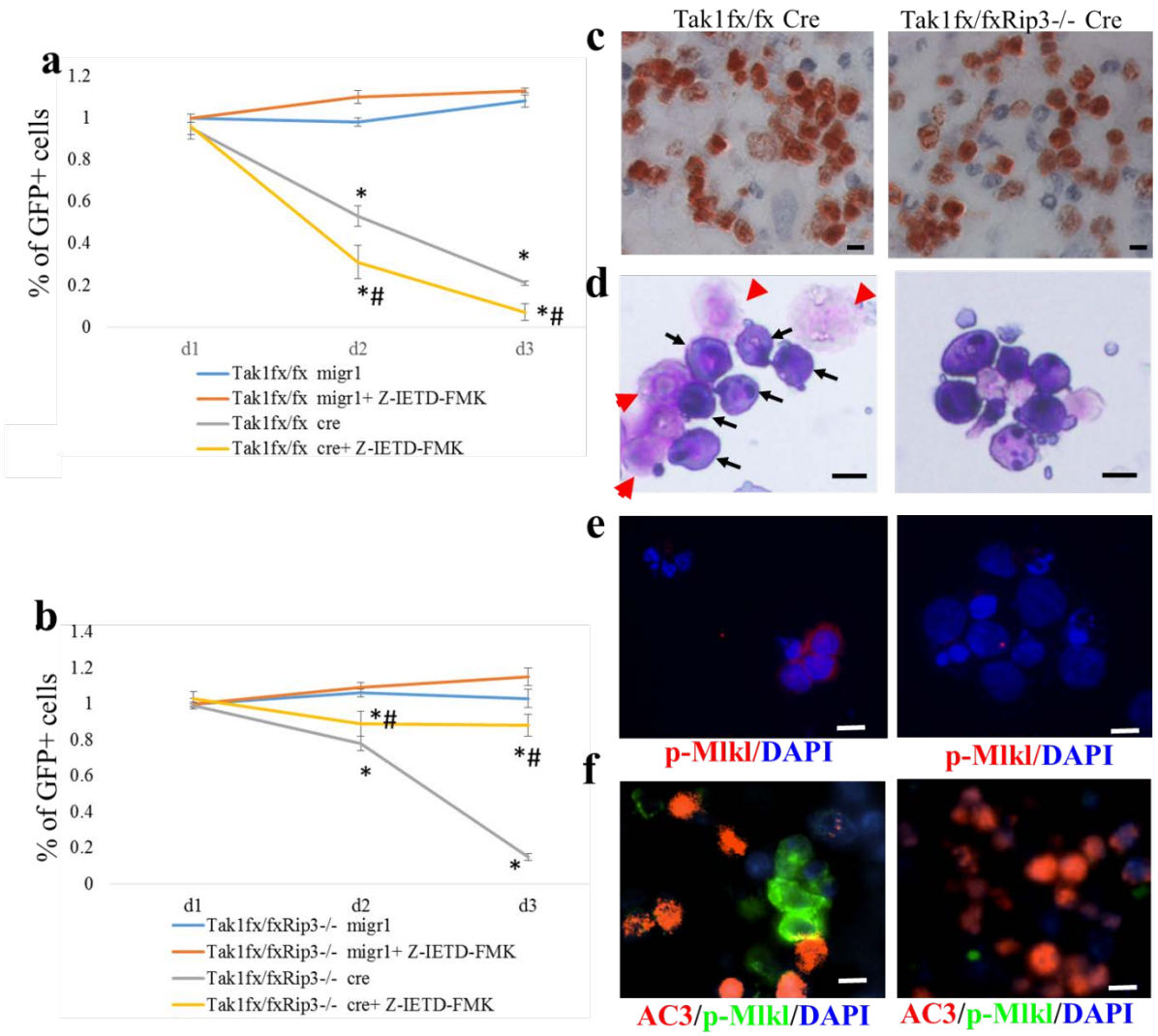

Figure S3. ***Tak1*$^{-/-}$ HSPCs die of caspase –mediated apoptosis and Rip3-mediated necroptosis.** c-Kit^{+} HSPCs were isolated from BM of *Tak1*$^{fx/fx}$ (**a**) and *Tak1*$^{fx/fx}$*Rip3*$^{-/-}$ mice (**b**) and transduced with MSCV-Cre-GFP. GFP percentages with or without caspase inhibitor Z-IETD-FMK treatment were examined on days 1, 2 and 3 post-transduction. Loss of GFP is attributable to death of the transduced HSPCs. *Tak1*$^{-/-}$ *HSPCs* died of necroptosis when caspase 8 was inhibited (**a**); however *Tak1*$^{fx/fx}$*Rip3*$^{-/-}$ HSPCs die of apoptosis, which can be prevented by inhibition of caspase 8 (Z-IETD-FMK is a caspase 8 inhibitor) (**b**). **c-e**. Twenty hours after transduction, the transduced cells were purified by FACS for GFP^{+} and incubated for another 24 hours. Cell death was determined by TUNEL staining (**c**) and Wright's Giemsa staining (**d**, red arrowheads indicate necroptotic cells; black arrows indicate apoptotic cells); necroptosis and apoptosis were further determined by p-Mlkl staining (a reliable marker for necroptosis) (**e**) or p-Mlkl and active caspase 3 (AC3) co-staining (**f**). Apoptotic cells with fragmented nuclei were negative for p-Mlkl (**e**) but positive for AC3. Scale bar=10μm. * P<0.05 compared to Migr1 transduction. # P<0.05 compared to Cre transduction.

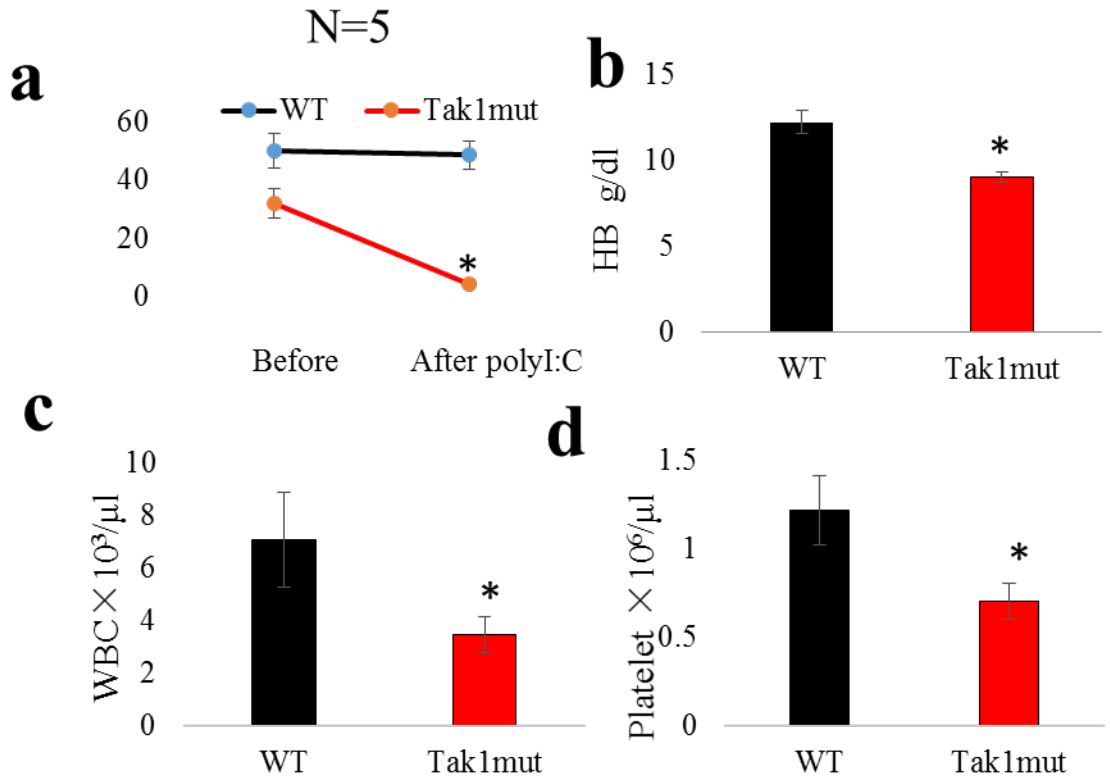

Fig. S4. Pancytopanic phenotype of *WT* recipient mice which had received *Tak1^{mut}* cell transplantation. Sub-lethally-irradiated *WT* recipient mice (CD45.1 background) were transplanted with BM MNCs isolated from *Tak1^{mut}* mice or *WT* littermate controls (CD45.2 background). Five hundred thousand cells were transplanted into each recipient. One month after transplantation, the percentages of donor cells (CD45.2+) in peripheral blood was examined (**a**, before polyI:C). All mice were then injected with polyI:C (1μg/g. body weight) every other day for total three times. Percentages of donor cells (**a**), HB (hemoglobin concentration, **b**), WBC (**c**) and platelet numbers (**d**) were examined 2 months after polyI:C injection. * P<0.05 compared to recipient mice receiving *WT* BM cells.

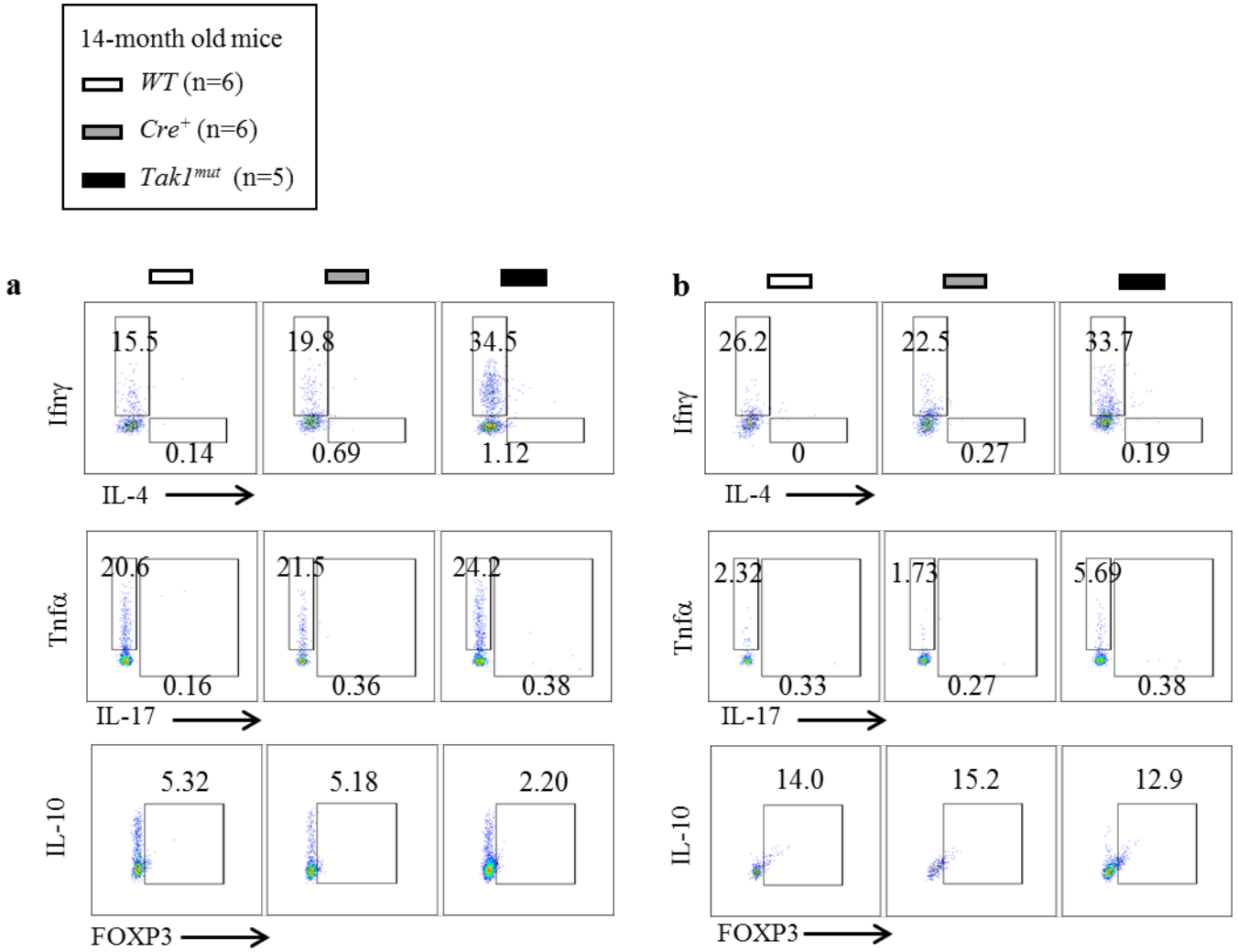

Figure S5. Enhanced Th1 cell responses in *Tak1*^{mut} BMF mice (Associated with Figure 2). a. Representative flow cytometric plots for analysis of CD4^{+} T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD4). **b**. Representative flow cytometric plots for analysis of CD8^{+} T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD8).

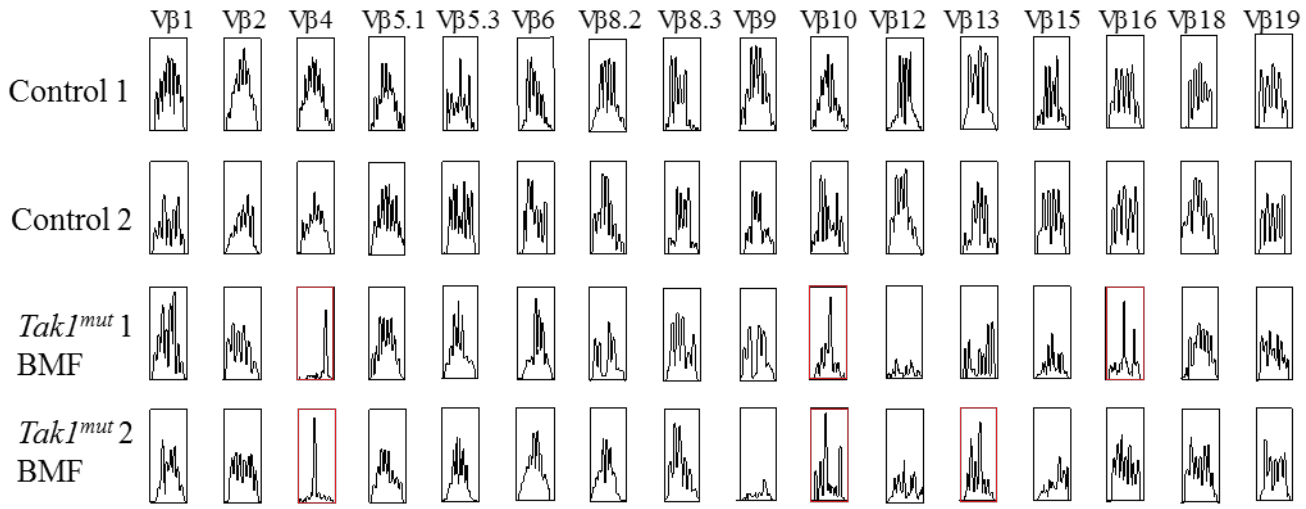

Fig. S6. TCR repertoire of CD4$^+$ T cells in *Tak1^{mut}* BMF mice was analyzed by run-off PCR assay for Vβ subfamily. CD4$^+$ T cells were isolated from *Tak1^{mut}* BMF mice and littermate *WT* control mice by positive selection after direct labelling with monoclonal antibodies to CD4 conjugated with magnetic microbeads. CDR3 fragments were amplified by RT-PCR with a fluorescent anti-sense TCR-constant-β (Cβ) common primer and one of 22 Vβ-family-specific sense primers as reported.[2] The composition of Vβ-CDR3 pools was detected as peaks of fluorescence intensity corresponding to particular CDR3 lengths on an ABI310 DNA sequencer. Spectra lacking the Gaussian-like peak distribution with the predominance of just one or two peaks were classified as abnormal (skewed) and suspicious for oligoclonality/monoclonality (highlighted in red).

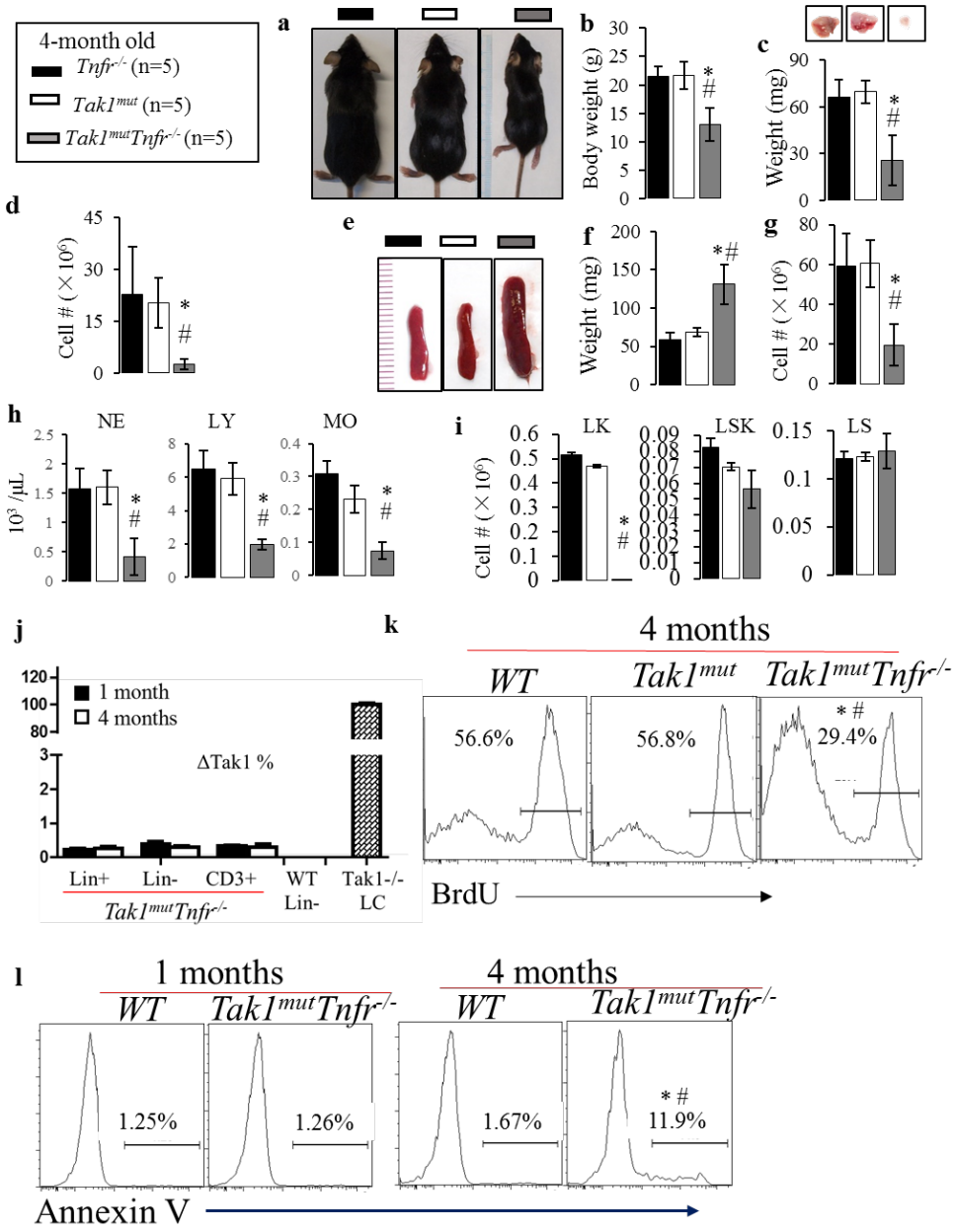

Figure S7. Tnfr deficiency accelerates BMF in *Tak1^{mut}* mice (associated with Fig. 3). *Tak1^{mut}Tnfr$^{-/-}$*, *Tak1^{mut}* and *Tnfr$^{-/-}$* mice were analyzed at age 4 months (n=5/group) for overall phenotype (**a.** photographs of body. **b**.body weights) and lymphoid tissues (thymus: **c.** photograph and **d.** weight; spleen: **e**. photograph, **f**. weight and **g.** total nucleated cells). **h.** Numbers of NE, LY and MO in peripheral blood; **i.** Absolute numbers of LK, LSK and LS populations in BM from 2 hind legs**. j.** Percentages of *Tak1* deletion (*ΔTak1*) in Lin$^-$ HSPCs, Lin$^+$ differentiated BM cells and CD3$^+$ T lymphocytes in *Tak1^{mut}Tnfr$^{-/-}$* mice were examined by quantitative PCR. Lin$^-$ HSPCs from *WT* mice and *Tak1$^{-/-}$* leukemia cells (LCs) were used as negative and positive controls. **k**. Proliferation of c-kit$^+$ HSPCs was examined by BrdU pulse labeling and compared among *WT*, *Tak1^{mut}* and *Tak1^{mut}Tnfr$^{-/-}$* mice at 4 months of age. **l**. Death of c-Kit$^+$ HSPCs was examined by Annexin-V staining and compared between *WT* and *Tak1^{mut}Tnfr$^{-/-}$* mice at the indicated ages. Data are presented as means ± SD. *, $p<0.05$ compared to *Tnfr$^{-/-}$*; #, $p<0.05$ compared to *Tak1^{mut}* mice.

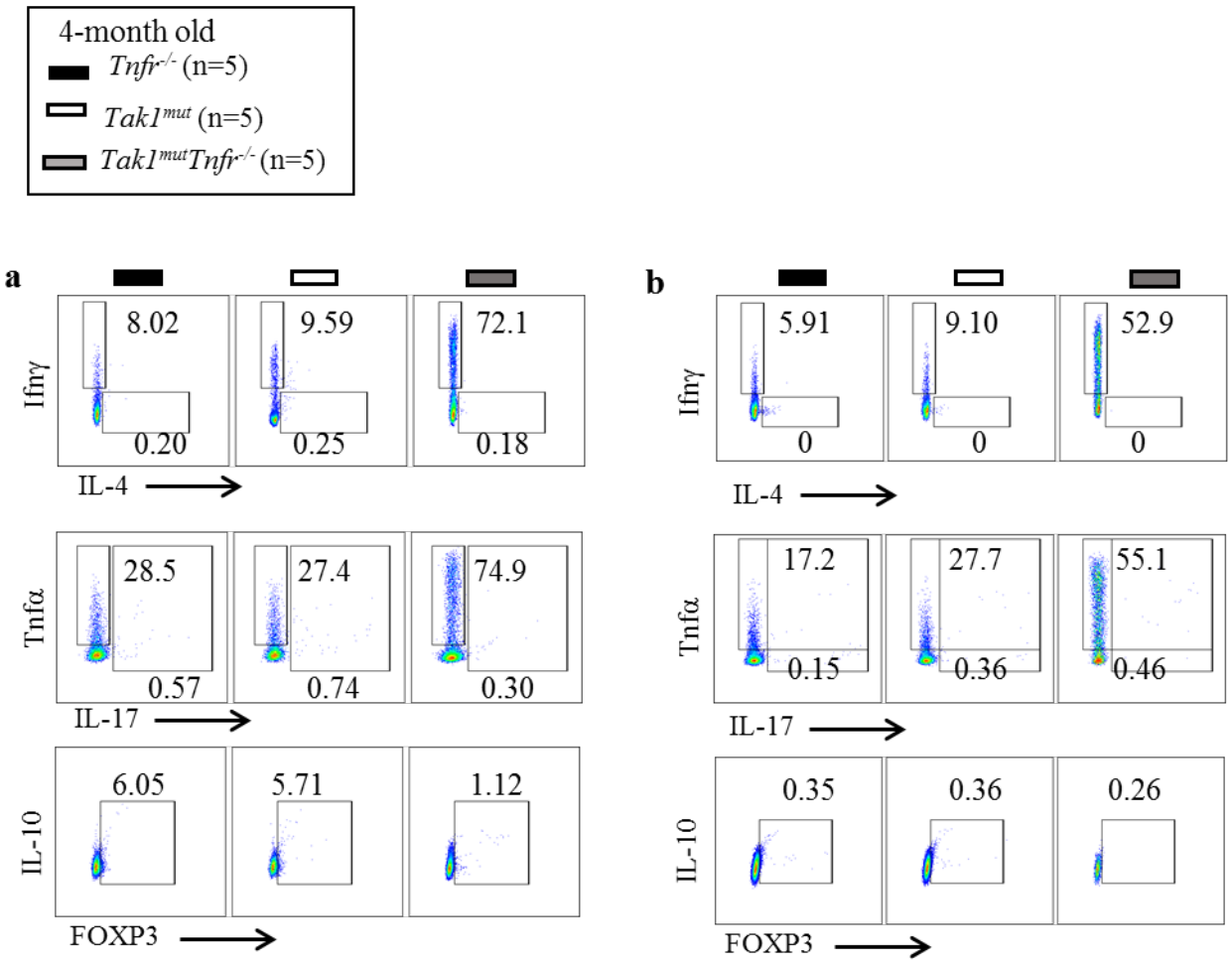

Figure S8. Tnfr deficiency enhances Th1 cell responses in *Tak1*^{mut} mice (associated with Fig. 3). *Tak1*^{mut}*Tnfr*$^{-/-}$ and *Tak1*^{mut} and *Tnfr*$^{-/-}$ mice were analyzed at age 4 months (n=5/group) for T cell analysis. **a**. Representative flow cytometric plots for analysis of CD4^{+} T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD4). **b**. Representative flow cytometric plots for analysis of CD8^{+} T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD8).

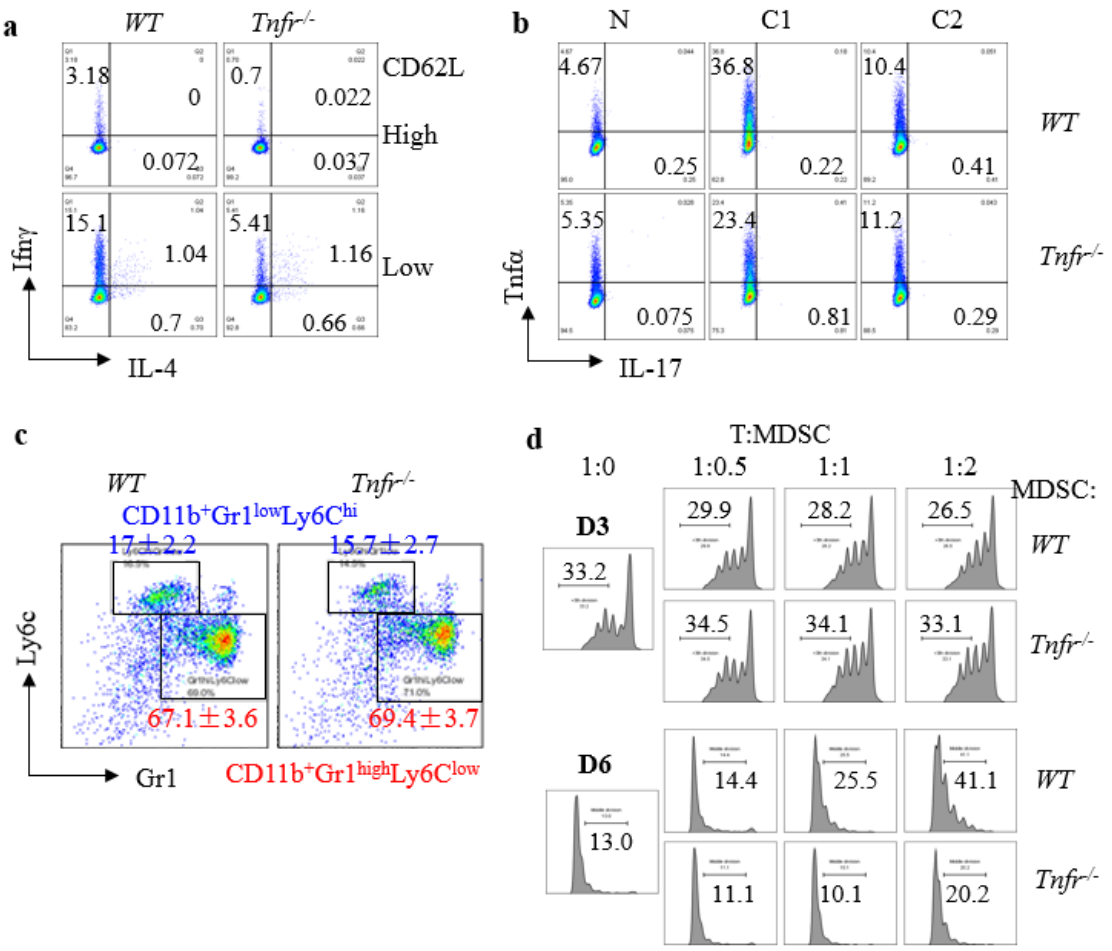

Figure S9. Deficiency in Tnfα signaling enhances the ability of APCs to prime Ifn-γ-expressing T cell development and reduces ability of MDSCs to suppress T cell proliferation (associated with Fig. 4). **a.** T cells from *WT* and *Tnfr*$^{-/-}$ spleens were first isolated with a pan-T cell isolation kit, then separated with anti-CD62L using AutoMACS. Cells were stimulated with PMA + ionomycin for 6 hours and subjected to intracellular staining for cytokines. **b.** *WT* CD62L^{+} T cells were further cultured with *WT* and *Tnfr*$^{-/-}$ APCs for 6 days under three conditions: neutral condition (N), Th1 condition (C1) and Th2 condition (C2). Cells were collected and subjected to intracellular staining for cytokines. **c.** Bone marrow cells from *WT* and *Tnfr*$^{-/-}$ mice were stained for CD11b, Gr1 and Ly6C and analyzed for CD11b^{+}Gr1^{low}Ly6C^{high} and CD11b^{+}Gr1^{high}Ly6C^{low} MDSCs. **d.** CD11b^{+}Gr1^{+} cells were sorted from *WT* and *Tnfr*$^{-/-}$ bone marrow and added to anti-CD3-activated and CFSE-labeled *WT* T cells at indicated ratios and cultured for another 6 days. The CFSE signals were analyzed on days 3 and 6. On day 3, the results were analyzed to show the cells with >3 divisions. On day 6, the results were analyzed to show the cells with a low number of divisions (2-6 divisions). The experiments were repeated independently twice.

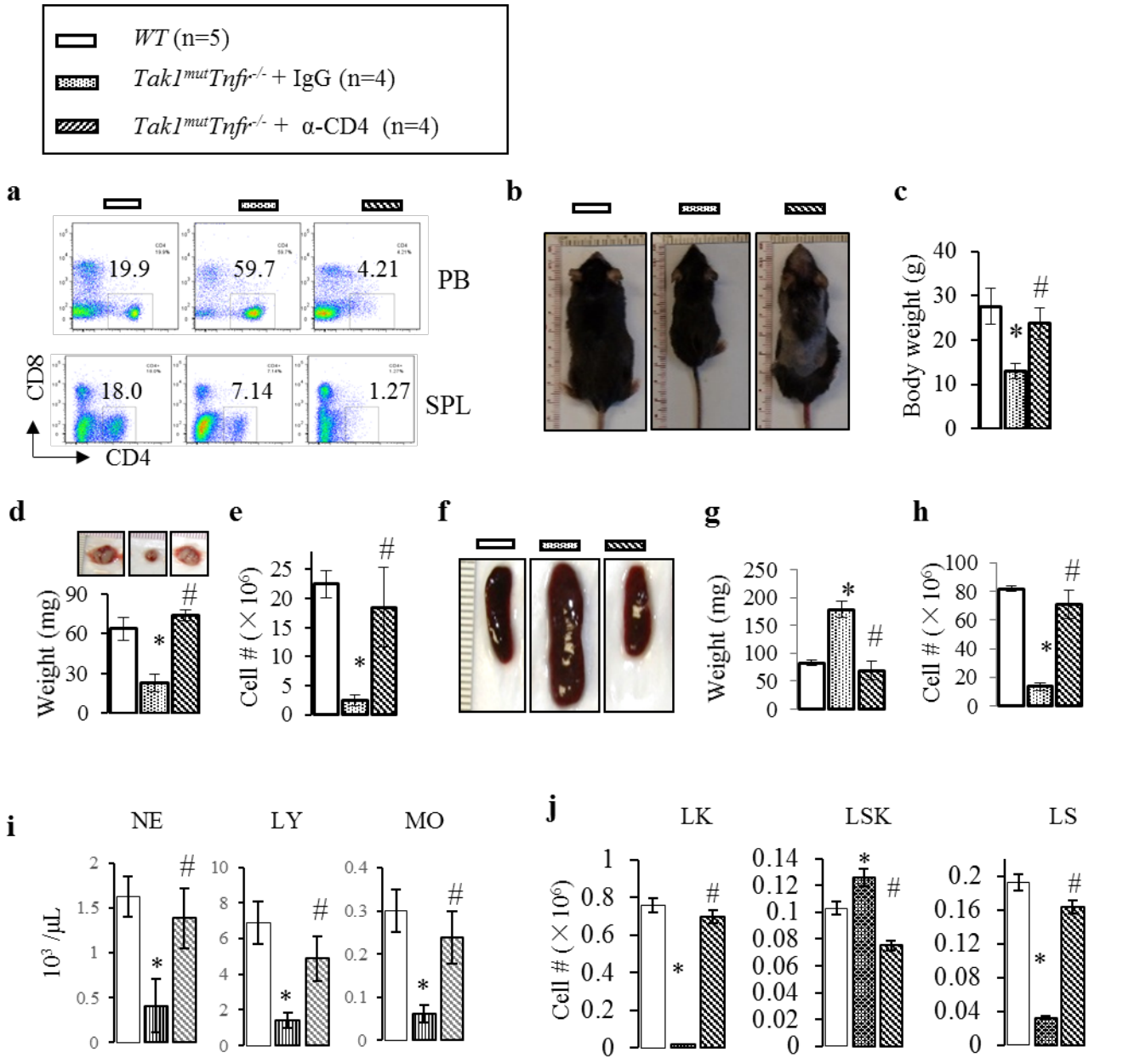

Figure S10. Depletion of CD4$^+$ T cells restores normal hematopoiesis to *Tak1^{mut}Tnfr$^{-/-}$* mice (associated with Fig. 5). *WT* and *Tak1^{mut}Tnfr$^{-/-}$* mice treated with either IgG or anti-CD4 antibody (n=5/group) were analyzed at age 10 months (n=5/group) for CD4 depletion in peripheral blood (**a)** as well as overall phenotype (**a.** photographs of body; **b**. body weight) and lymphoid tissues (thymus: **c.** photograph and **d.** weight; spleen: **e**. photograph, **f**. weight and **g.** total nucleated cells). **i.** Numbers of NE, LY and MO in peripheral blood; **j.** Absolute numbers of LK, LSK and LS populations in BM from 2 hind legs**.** Data are presented as means ± SD. *, $p<0.05$ compared to *WT* controls; #, $p<0.05$ compared to IgG treatment.

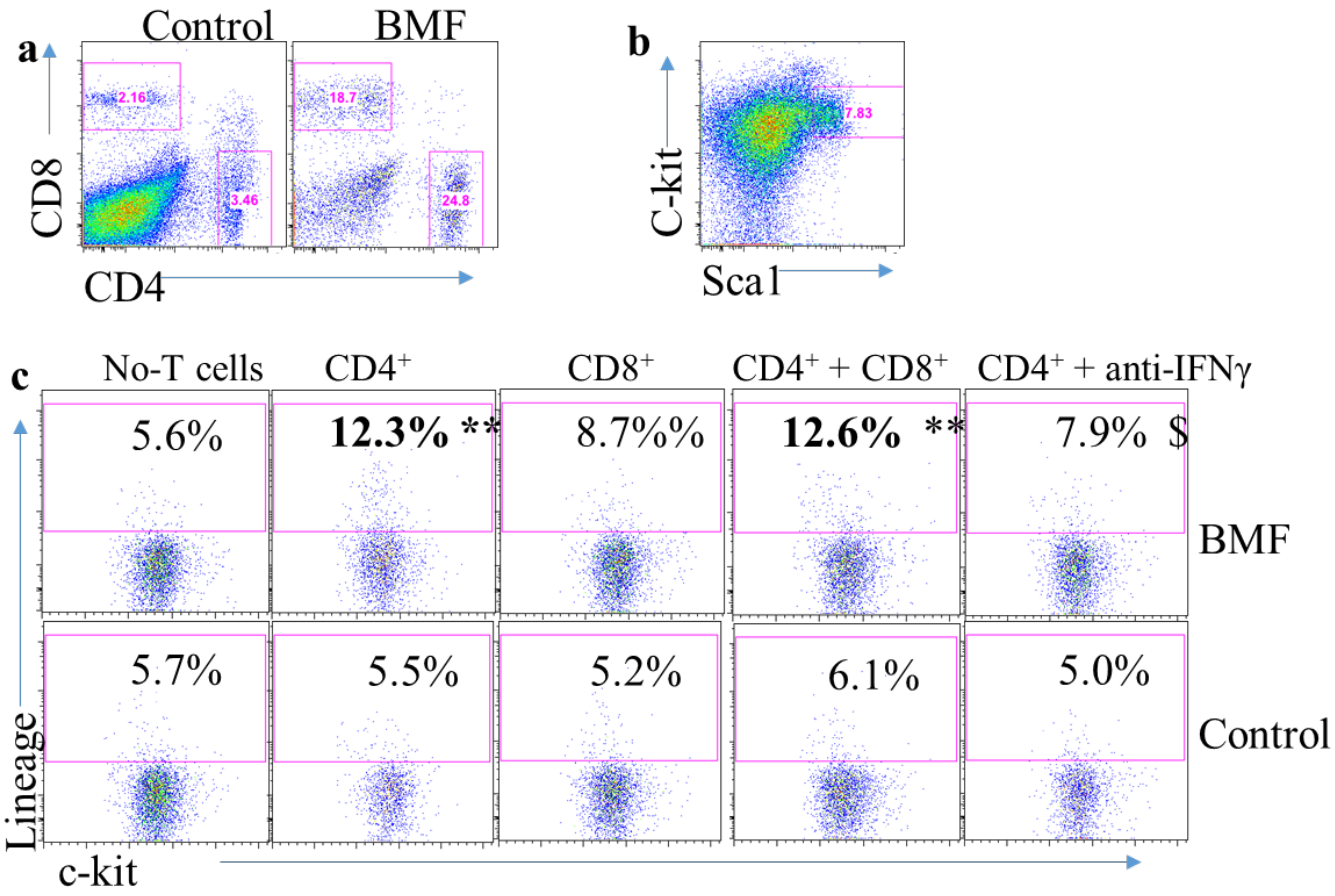

Figure S11. CD4$^+$ T cells isolated from BMF mice promoted differentiation of HSPCs *in vivo* by secreting Ifnγ (associated with Fig. 6). CD4$^+$ and CD8$^+$ T cells were isolated from BMF mice and control mice (**a**). LSK HSPCs were isolated from BM of WT mice (**b**). HSPCs and CD4$^+$ or CD8$^+$ T cells were co-cultured in a stem cell culture medium (*Stemspan*, supplemented with 100ng/ml SCF, 20ng/ml TPO and 20ng/ml FLT3L) containing 25ng/ml of IL2 with or without anti-Tnfγ antibody (20ng/ml) for 5 days. HSPCs in such conditions normally proliferate and maintained an undifferentiated state (c-Kit$^+$) with only a small percentage of cells becoming differentiated (become lineage$^+$). We found that CD4$^+$ and CD8$^+$ cells can only be sustained for 3-4 days without significant expansion. We did not see the direct attack on HSPCs by T cells in any of the experimental groups because there was no significantly increased cell death in the co-culture. After 5 days of co-culture, the number of cells derived from LSK cells were comparable in all experimental groups. However, we observed significantly increased lineage$^+$ cells in the CD4$^+$ cells and CD4$^{+/}$CD8$^+$ cell co-culture groups of BMF mice but not control mice, suggesting CD4$^+$ T cells from BMF mice promote differentiation of HSPCs. Anti-Tnfγ antibody treatment (20ng/ml) can largely prevent such enhanced differentiation in the co-culture, suggesting that CD4$^+$ T cells from BMF mice promote differentiation of HSPCs through secretion of IFNγ.

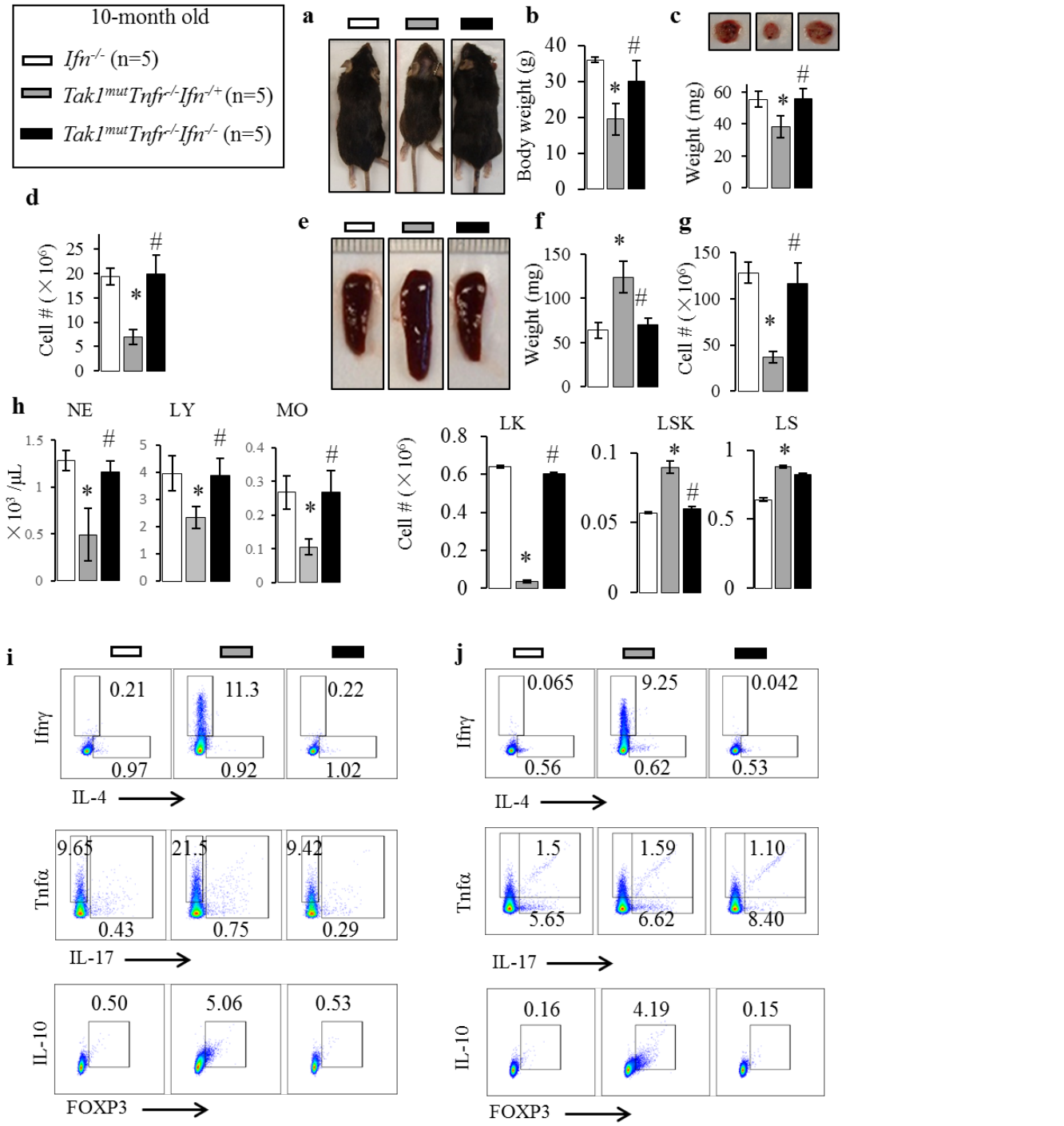

Figure S12. *Ifnγ* knockout prevents BMF in *Tak1*^{mut}*Tnfr*$^{-/-}$ mice. *Ifnγ*$^{-/-}$, *Tak1*^{mut}*Tnfr*$^{-/-}$*Ifnγ*$^{+/-}$ and *Tak1*^{mut}*Tnfr*$^{-/-}$*Ifnγ*$^{-/-}$ mice were analyzed at age 10 months (n=5/group) for overall phenotype (**a.** photographs of body. **b**. body weight) and lymphoid tissues (thymus: **c.** photograph and **d.** weight; spleen: **e**. photograph, **f**. weight and **g.** total nucleated cells). **h.** Numbers of NE, LY and MO in peripheral blood; **i.** Absolute numbers of LK, LSK and LS populations in BM from 2 hind legs. **j**. Representative flow cytometric plots for analysis of CD4$^+$ T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD4). **k**. Representative flow cytometric plots for analysis of CD8$^+$ T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD8). Data are presented as means ± SD. *, p<0.05 compared to *Tnfr*$^{-/-}$ mice; #, p<0.05 compared to *Tak1*^{mut}*Tnfr*$^{-/-}$*Ifnγ*$^{+/-}$ mice.

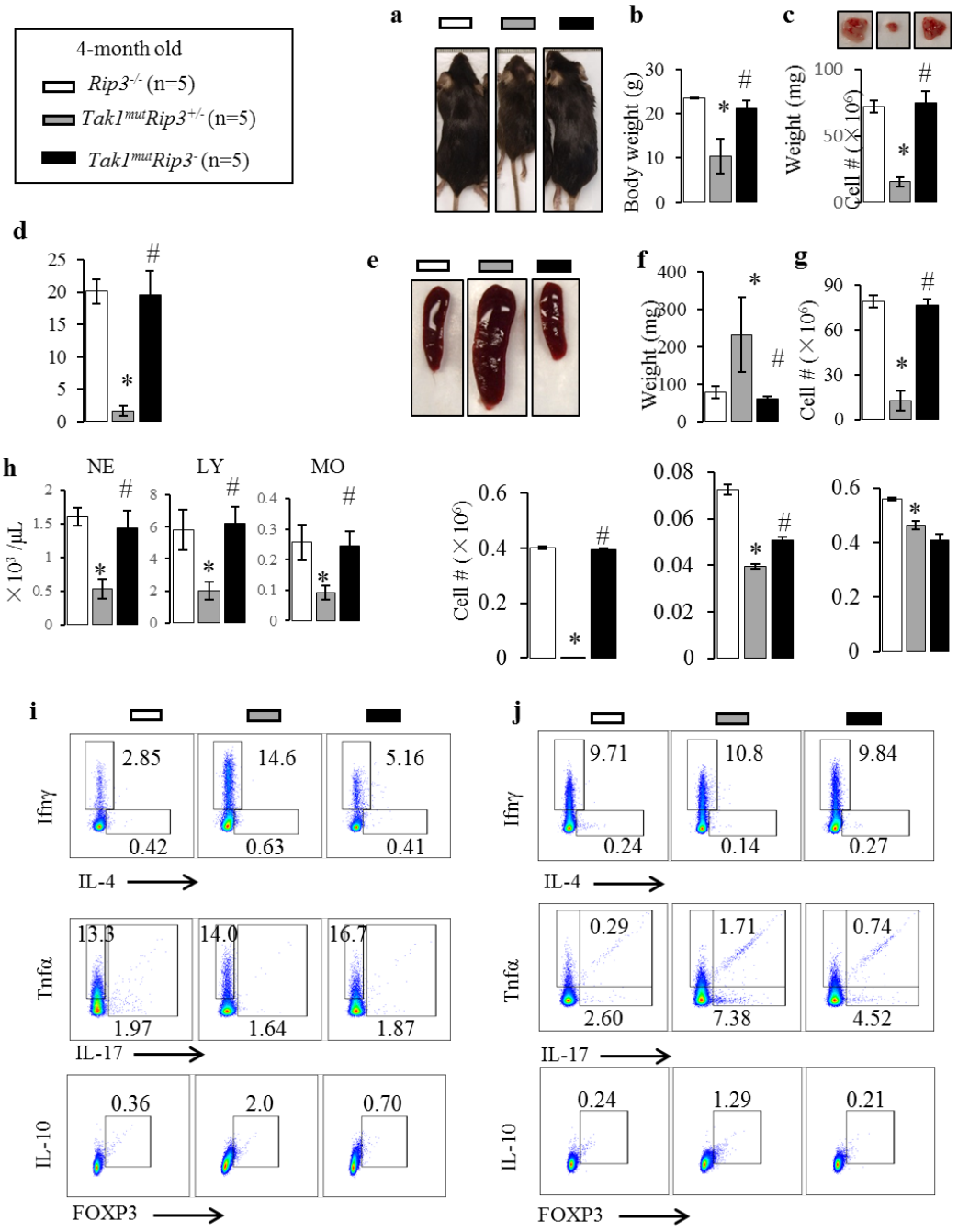

Figure S13. ***Rip3* knockout *prevents BMF in Tak1^{mut}Tnfr$^{-/-}$ mice.*** *Rip3$^{-/-}$*, *Tak1^{mut}Tnfr$^{-/-}$Rip3$^{+/-}$* and *Tak1^{mut}Tnfr$^{-/-}$Rip3$^{-/-}$* mice were analyzed at age 4 months (n=5/group) for overall phenotype (**a.** photographs of body. **b**. body weight) and lymphoid tissues (thymus: **c.** photograph and **d.** weight; spleen: **e**. photograph, **f**. weight and **g.** total nucleated cells). **h.** Numbers of NE, LY and MO in peripheral blood; **i.** Absolute numbers of LK, LSK and LS populations in BM from 2 hind legs**. j**. Representative flow cytometric plots for analysis of CD4$^+$ T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD4). **k**. Representative flow cytometric plots for analysis of CD8$^+$ T cell effector subsets (gates: live nucleated cells, lymphocytes, CD3 and CD8). Data are presented as means ± SD. *, p<0.05 compared to *Rip3$^{-/-}$* mice #, p<0.05 compared to *Tak1^{mut}Tnfr$^{-/-}$Rip3$^{+/-}$* mice.

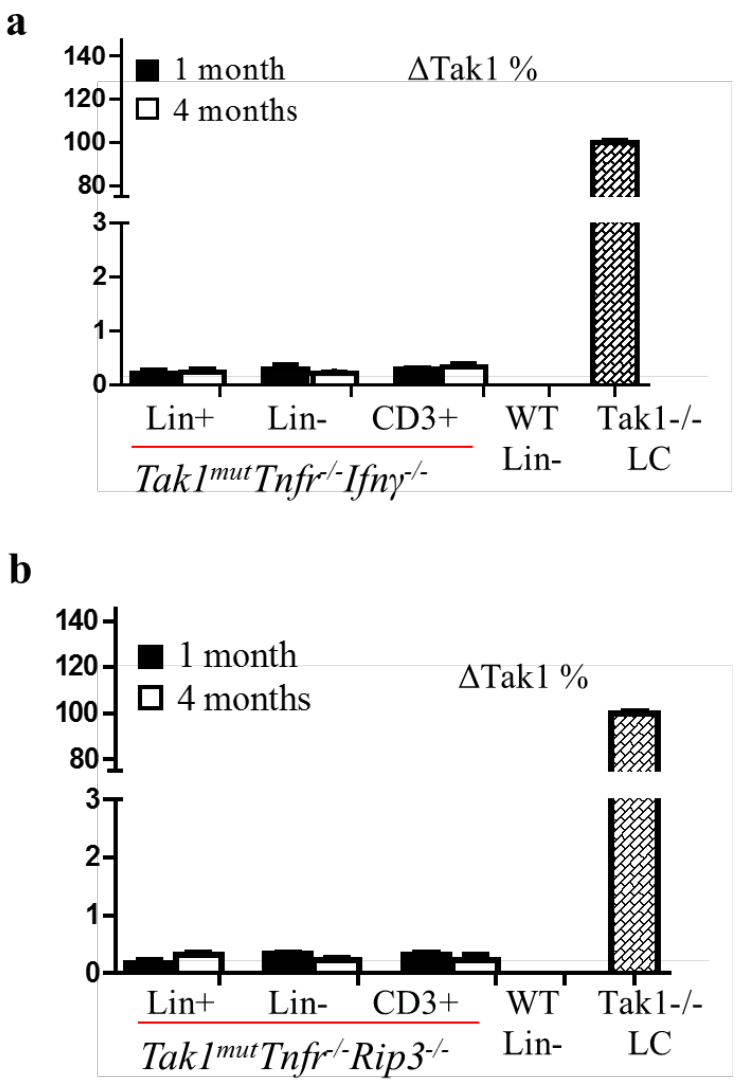

Figure S14. Percentage of *Tak1* deletion *(ΔTak1)* cells in BM of *Tak1^{mut}Tnfr$^{-/-}$Ifnγ$^{-/-}$* and *Tak1^{mut}Tnfr$^{-/-}$Rip3$^{-/-}$* mice at indicated ages. Percentages of *ΔTak1* cells in Lin^{-} HSPCs, Lin^{+} differentiated BM cells and CD3^{+} T lymphocytes in *Tak1^{mut}Tnfr$^{-/-}$Ifnγ$^{-/-}$* mice (**a**) and *Tak1^{mut}Tnfr$^{-/-}$Rip3$^{-/-}$* mice (**b**) at indicated ages were examined by quantitative PCR. Lin^{-} HSPCs from *WT* mice and *Tak1$^{-/-}$* leukemia cells (LCs) were used as negative and positive controls.

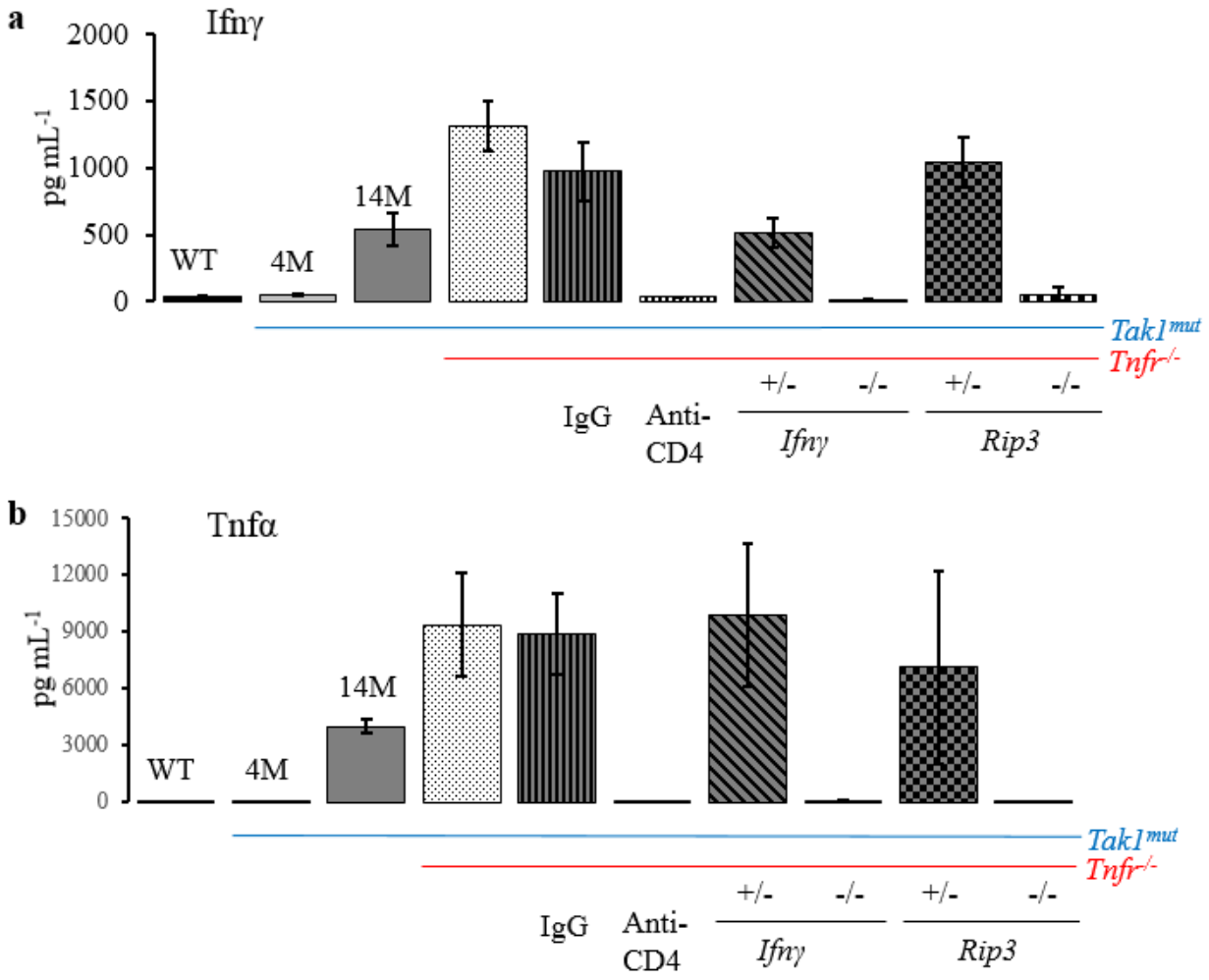

Figure S15. Serum levels of Ifnγ and Tnfα in experimental mice. Serum samples were subjected to ELISA for Ifnγ (**a**) and Tnfα (**b**) in all types of mouse strains used in the current study.

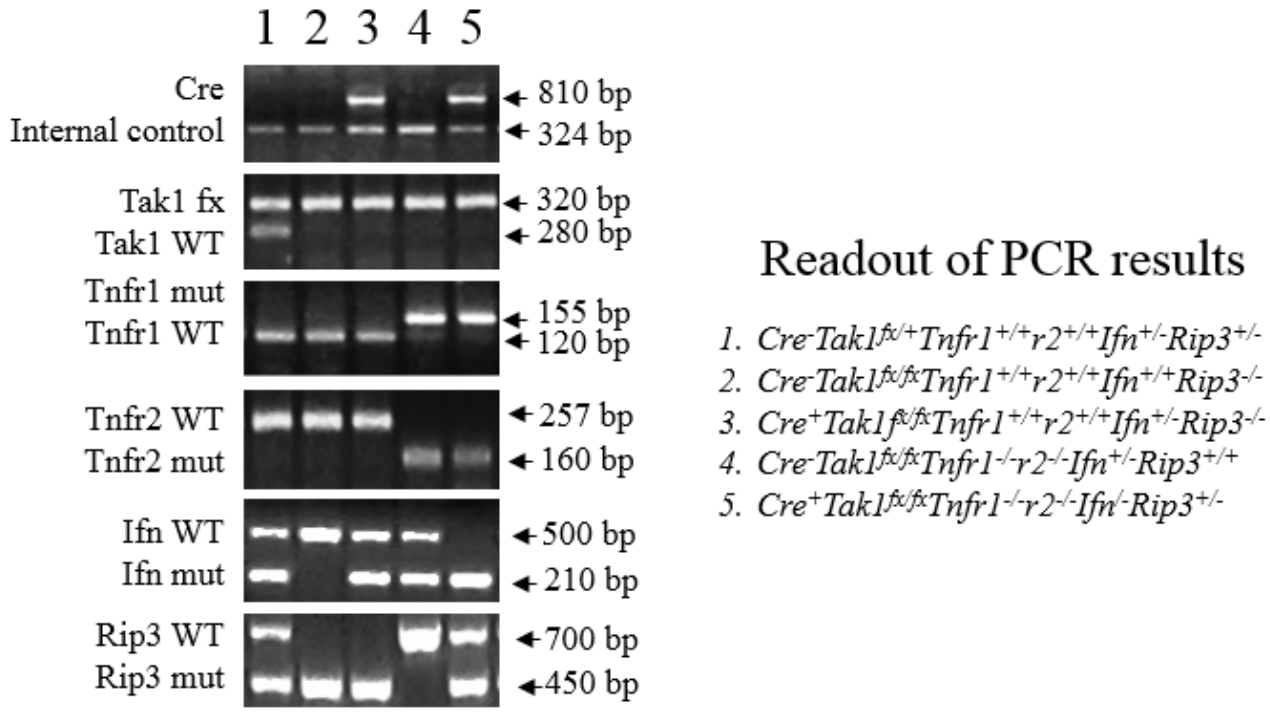

Figure S16. Genotyping of transgenic and knockout mice. DNA was extracted from tail snap tissues and mouse genotypes were analyzed by PCR using the primers listed below.

PCR primers for mouse genotyping:

Cre-1: 5′-CTAGGCCACAGA ATTGAA AGATCT-3′;
Cre-2: 5′-GTAGGTGGAAATTCTAGCATCATCC -3′;
Cre-3: 5′-TACCTGGAAAATGCTTCTGT-3′;
Cre-4: 5′-TGATCTCCGGTATTGAAACT-3′;
Primers for Cre-1 and Cre-2 produce an 810bp product in Cre^+ mice, and primers for Cre-3 and Cre-4 produce a 324bp product in both Cre^+ and Cre^- animals. The latter serve as a PCR amplification control.

Tak1-1: 5′- GGCTTTCATTGTGGAGGTAAGCTGAGA-3′;
Tak1-2: 5′- GGAACCCGTGGATAAGTGCACTTGAAT -3′;
These primers produce a 280bp product in *WT*, and a 320bp product in $Tak1^{fx/fx}$ mice.

Tnfr1-1 5′-GGATTGTCACGGTGCCGTTGAAG-3′;
Tnfr1-2: 5′-TGACAAGGACACGGTGTGTGGC-3′;
Tnfr1-3: 5′-TGCTGATGGGGATACATCCATC-3′,
TnfrR1-4: 5′-CCGGTGGATGTGGAATGTGTG-3′.
These primers produce a 120bp product in *WT* and a 155bp product in *Tnfr1*-mutant mice.

Tnfr2-1: 5′-CCGGTGGATGTGGAATGTGTG-3′;
Tnfr2-2: 5′-AGAGCTCCAGGCACAAGGGC-3′;
Tnfr2-3: 5′-AACGGGCCAGACCTCGGGT-3′.
These primers produce a 257bp product in *WT* and a 160bp product in *Tnfr2*-mutant mice.

Ifnγ-1: 5′- CCT TCT ATC GCC TTC TTG ACG-3′
Ifnγ-2: 5′- AGA AGT AAG TGG AAG GGC CCA GAA G -3′
Ifnγ-3: 5′- AGG GAA ACT GGG AGA GGA GAA ATA T-3′
These primers produce a 500bp product in *WT* and a 210bp product in *Ifnγ*-mutant mice

Rip3-1: 5′- CGCTTTAGAAGCCTTCAGGTTGAC-3′
Rip3-2: 5′- GCAGGCTCTGGTGACAAGATTCATGG-3′
Rip3-3: 5′- CCAGAGGCCACTTGTGTAGCG-3′.
These primers produce a 700bp product in *WT* and a 450bp product in *Rip3*-mutant mice.

Additional Methods

Detection of the percentage of cells with Tak1 deletion (*ΔTak1*) in mouse BM. Lin$^-$ HSPCs, Lin$^+$ differentiated hematopoietic cells and CD3$^+$ T cells were isolated from BM of *Tak1*^{mut}, *Tak1*^{mut}*Tnfr*$^{-/-}$, *Tak1*^{mut}*Tnfr*$^{-/-}$*Ifnγ*$^{-/-}$ and *Tak1*^{mut}*Tnfr*$^{-/-}$*Rip3*$^{-/-}$ mice at the indicated time points. Genomic DNA was extracted from these cells. Quantitative PCR was performed to detect *ΔTak1* in these cells using the primers: 5'-GCAACTTCGACAACTTGCCTTCCTGTG-3' and 5'-GCACTTGAATTAGCGGCCGCAAGCTTAT AACT-3'. PCR using primers for the *Pten* gene (5'−CTCCTCTACTCCATTCTTCCC−3 and 5'−ACTCCCC CAATGAACAAAC−3) were used as endogenous controls for DNA input. DNA isolated from Lin$^-$ cells from wild-type mouse BM and DNA from a *Tak1*$^{-/-}$ leukemic cells (LCs) were used as negative and positive controls, respectively. The relative copy numbers of *ΔTak1* in each type of experimental cells were calculated as: lg2(Δ*Tak1* Ct –*Pten* Ct). The percentage of *ΔTak1* cells in the experimental cells = (relative copies of *ΔTak1* in the experimental cells / relative copies of *ΔTak1* in *Tak1*$^{-/-}$ LCs) × 100.

Antibodies used in these studies. All antibodies were purchased from *eBioscience* (San Diego, CA, clone and catalog# follows for each): anti-CD4-eflour 450 (GK1.5, 48-0041), anti-Ifnγ-Alexa Fluor-488 (XMG1.2, 53-7311), anti-IL-17A-PE-Cy7(eBio17B7, 25-7177), anti-Tnf-α-PE(MP6-XT22, 12-7321), anti-IL-10-PerCp-Cy5.5 (JES5-16E3, 45-7101) and anti-IL-4-APC (17-7041, 17-7041), anti-CD8-FITC (53-6.7, 11-0081). Antibodies used for T cell activation include: anti-CD4-ef450 (GK1.5, 48-0041), anti-

CD69-PerCp-Cy5.5 (H1.2F3, 45-0691), anti-CD44-APC (IM7, 17-0441), and anti-CD62L-FITC (MEL-14, 11-0621); anti-CD3-ef450 (145-2C11, 48-0031), anti-CD8-ef780 (53-6.7, 47-0081). Antibodies used for stem cell analysis include: anti-Lin-FITC (CD3, 145-2C11, 11-0031; B220, RA3-6B2, 11-0452; GR1, RB6-8C5, 11-5931, CD8, 53-6.7, 11-0081, Ter119, TER-119, 11-5921), anti-Sca1-PE (D7, 12-5981), anti-c-kit-APC (2B8, 17-1171), anti-CD8-APC (53-6.7, 17-0081), anti-CD4-FITC (GK1.5, 17-0041), anti-CD41-FITC(MWReg30, 11-0411), and anti-Mac1-PE (M1/70, 12-0112).

ELISA. When mice were subjected to analyses for BM HPSCs and T cells, peripheral blood sera were collected and immediately stored at -80°C. Tnfα and Ifnγ concentrations were determined using mouse ELISA kits (BioSource, Camarillo, CA) according to the manufacturer's protocol.

T cell proliferation analysis. T cells from spleens and lymph nodes were isolated by autoMACS using a pan-T cell isolation kit (Miltenyi Biotec). Cells were labeled with 1 μM CFSE (V12883, Invitrogen) and cultured under specified conditions as indicated. The reductions of CFSE fluorescence were analyzed using FACS as the indicator of T cell division.

T cells and APC co-culture. Purified *WT* T cells were co-cultured with *WT* and *Tnfr*$^{-/-}$ APCs (T cell-depleted splenocytes irradiated at 2500 rad) under three conditions. 1) neutral condition: 3μg/ml anti-CD3, 3μg/ml anti-CD28, and 10 U/ml rmIL-2; 2) Th1 condition: 3 μg/ml anti-CD3, 3 μg/ml anti-CD28, and 1 U/ml rmIL-2, 10 μg/ml anti-IL-4, and 3); or Th2 condition: 3 μg/ml anti-CD3, 3 μg/ml anti-CD28, and 10U/ml rmIL-2, 5 ng/ml rmIL-4, 10μg/ml anti-IL-12. All cytokines and antibodies were purchased from *eBiosciences* except for IL-12 which was obtained from *BioXcell*. After 1 week of culturing, cells were collected and stained for intracellular cytokines as described above.

BrdU pulse-labeling for analyzing the proliferation of HSPCs. As we reported previously (He et al. 2004), mice were injected intraperitoneally with BrdU (10mg./kg. body weight) twice daily for 2 d. Forty-eight hours after the first injection, BM mononuclear cells (MNCs) were collected from mice and stained with FITC-conjugated lineage antibodies and PE-c-Kit. Cells were stained further with APC BrdU Flow Kits (BD Pharmingen) following the protocol provided by the vender. Percentage of $BrdU^+$ cells within $Lin^-c\text{-}kit^+$ HSPCs was analyzed by flow cytometry.

Annexin-V staining for death of HSPCs. As we reported previously [3], BM MNCs were collected from mice at indicated time points and stained with FITC-conjugated lineage antibodies and PE-c-Kit. Cells were further stained with APC-Annexin-V Apoptosis Detection Kit (e-Bioscience) following the instructions provided by the vender. Percentage of $Annexin^+$ cells among $Lin^-c\text{-}kit^+$ HSPCs was analyzed by flow-cytometry.

In vitro *co-culture of CD4$^+$ and CD8$^+$ T cells with HSPCs*. $Lin^-Sca1^+c\text{-}Kit^+$ HSPCs were isolated from *WT* C57Bl6/J mice. $CD4^+$ T cells and $CD8^+$ T cells were isolated from *Tak1^{mut}Tnfr$^{-/-}$* BMF mice and *WT* control mice (all mice were maintained in a C57Bl6/J background). HSPCs and T cells were mixed in a 1:5 ratio and co-cultured in *StemSpan*™ serum-free medium containing 100ng/ml SCF, 20ng/ml TPO and 20ng/ml FLT3L and 25ng/ml mIL2 in the presence or absence of 20ng/ml Ifnγ for 5 days. Proliferation, survival and differentiation of HSPCs were analyzed.

MDSC functional assay. Isolated *WT* T cells were labeled with CFSE and activated with plate-bound anti-CD3 (1μg/ml) and soluble anti-CD28 (0.5μg/ml) for 24 hours. MDSCs ($CD11b^+Gr1^+$ cells) were sorted from BM and mixed with the activated T cells at the indicated ratios. Cells were cultured for 6

days. On days 3 and 6, cells were stained with anti-CD3-eflour 450 and subjected to FACS analysis for the CFSE intensity.

References

1 Zhang, J. *et al.* p27 maintains a subset of leukemia stem cells in the quiescent state in murine MLL-leukemia. *Mol Oncol*, doi:10.1016/j.molonc.2013.07.011 (2013).

2 Currier, J. R. & Robinson, M. A. Spectratype/immunoscope analysis of the expressed TCR repertoire. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 10**, Unit 10 28, doi:10.1002/0471142735.im1028s38 (2001).

3 Tang, M. *et al.* TAK1 is required for the survival of hematopoietic cells and hepatocytes in mice. *J Exp Med* (2008).