Sensitizing Acute Myeloid Leukemia Cells to Induced Differentiation by Inhibiting the RIP1/RIP3 Pathway

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Supplementary Table and Figures

Table S1. List of genes were analyzed in array database.
BCL2L1, BIRC2, BIRC3, CARD11, CASP8, CD40, CD40LG, CYLD, FADD, LTA, MAP3K7, NR2C2, PTGS2, RIPK1, RIPK3, SOD2, TNF, TNFAIP3, TNFRSF10A, TNFRSF10B, TNFRSF11A, TNFRSF1A, TNFRSF1B, TNFSF10, TNFSF11, TRADD, TRAF1, TRAF2, TRAF3, TRAF5, TRAF6, TRAM2, UBE2D3, UBE2N, TAB1, TAB2, TAB3, DDX58, IL1A, IL1B, IL1R1, IRAK1, IRAK2, MYD88, TICAM1, TLR1, TLR2, TLR3, TLR4, TLR5, TLR9, CSF1R, IFNG, IFNGR1, IFNGR2, IRF1, IRF2, IRF3, IRF4, IRF5, IRF7, IRF8, IRF9, SOCS1, SOCS2, SOCS3, CDKN1A, CDKN1B, CDKN2A, CDKN2B, JUN, JUNB, RB1, TP53

Table S2. Nec1 enhances IFN-γ-induced differentiation in primary human AML samples

Patient	Subtype	CFU	Treatment				Mutated Gene
			DMSO	Nec1	IFN-γ	IFN-γ+Nec1	
P1	M1	YES	65 ± 6	18 ± 5*	38 ± 4*	6 ± 2*#	NPM1c+
P2	2nd to MDS	NO					NA
P3	M1	YES	460 ± 60	380 ± 65*	442 ± 25*	229 ± 15*#	NPM1c+
P4	2nd to MDS	NO					NA
P5	M5	YES	218 ± 3	163 ± 4*	164 ± 10*	65 ± 8*#	MLL-AF9
P6	M0	YES	45 ± 3	43 ± 3	34 ± 5*	23 ± 3*#	NPM1c+
P7	M5	YES	54 ± 15	37 ± 4*	39 ± 2*	24 ± 4*#	NPM1c+
P8	2nd to Therapy	YES	504 ± 54	460 ± 74*	522 ± 36	349 ± 32*#	MLL-AF9
P9	2nd to MDS	NO					NA
P10	2nd to MDS	YES	579 ± 33	469 ± 43*	523 ± 56	313 ± 24*#	DNMTR882C
HD							
HD1	Normal $CD34^+$	YES	157 ± 18	207 ± 21	344 ± 25	321 ± 12	
HD2	Normal $CD34^+$	YES	203 ± 12	303 ± 18	329 ± 23	166 ± 35	

*, $p<0.05$, compared to DMSO; #, $p<0.05$, compared to IFN-γ. HD: healthy donor.

Table S3. Cytokine and chemokine profile of *WT*, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ AML cells.

ng/ml	*WT*	*RIP1*$^{-/-}$	*RIP3*$^{-/-}$
IL-1α	760+/-56	689+/-139	598+/-216
IL-1β	1036+/-378	770+/-267*	850+/-256*
IL-2	-	-	-
IL-3	29089+/-3689	31087+/-2564	28800+/-5321
IL-4	-	-	-
IL-5	-	-	-
IL-6	36987+/-5789	45671+/-6321	37611+/-3487
IL-9	-	-	-
IL-10	588+/-112	601+/87	530+/-89
IL-12 (p40)	219+/-23	238+/-53	312+/-44
IL-12 (p70)	318+/-22	416+/-79	356+/-33
IL-13	-	-	-
IL-17A	-	-	-
eotaxin	-	-	-
G-CSF	121+/-12	156+/-21	131+/-39
GM-CSF	36711+/-679	45619+/-389	37700+/-533
IFN-γ	-	-	-
KC	12+/-5	11+/-6	13+/-4
MCP-1 (MCAF)	-	-	-
MIP-1α	323+/-33	378+/-32	430+/-52
MIP-1β	56+/-15	67+/-10	61+/-8
RANTES	-	-	-
TNF-α	15088+/-2376	18110+/-2380	12109+/-2121

WT, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ AML cells were cultured in 4-cytokine medium as described in *Materials and Methods*, and supernatants were collected 24 hours later for cytokine/chemokine profile. Data represent the average of three biologic repeats. High levels of IL3 and IL6 (highlighted in blue) were due to the addition of exogenous IL3 and IL6 in the cultures. *, $p<0.05$, compared to *WT* AML cells

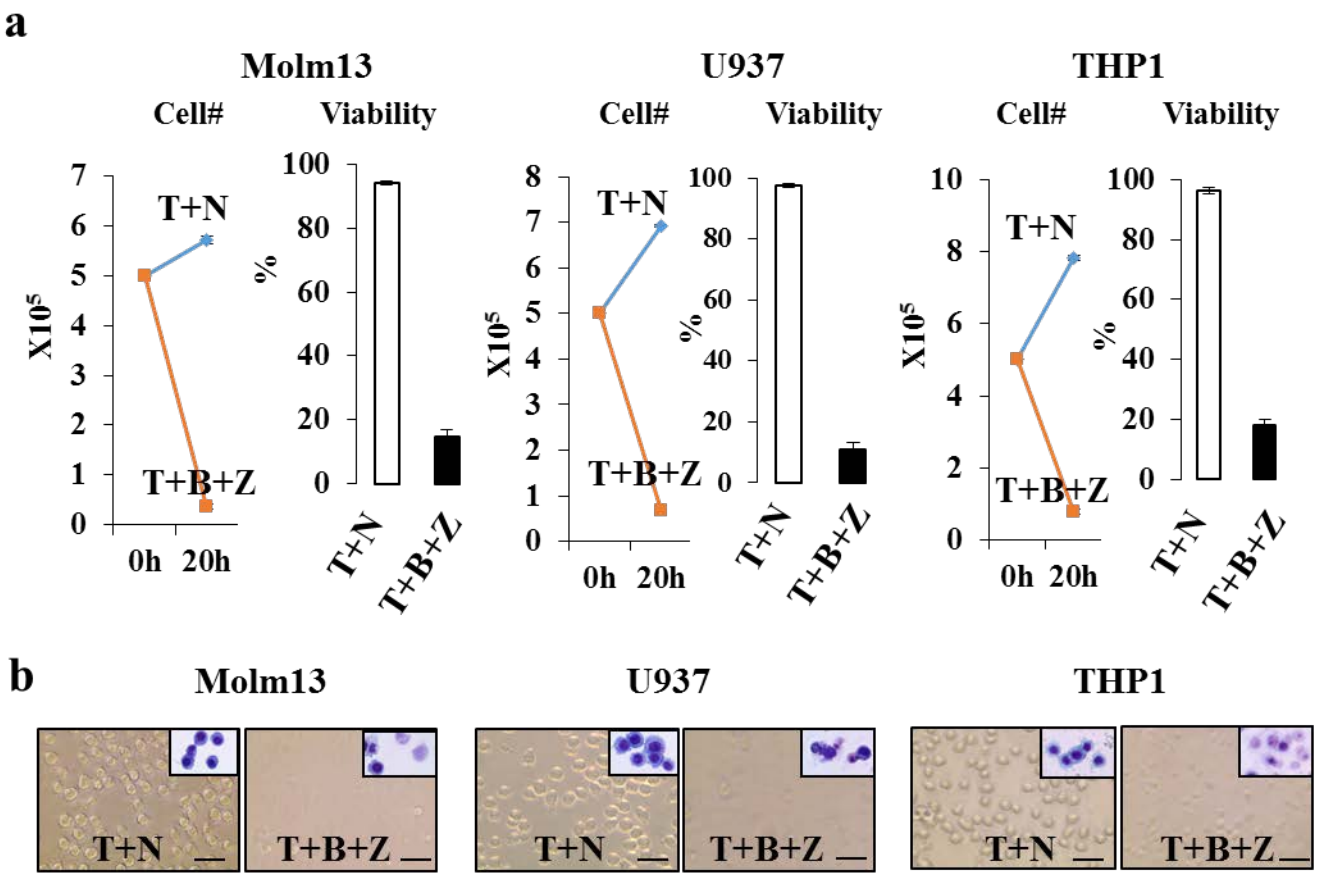


Fig. S1. Induced necroptosis in human AML cell lines. Molm13, U937 and THP1 cells (5×10^5 in 1 mL) were treated with TNF-α (20ng mL^{-1}) plus Nec1 (50 μM) (T+N), or TNF-α (20 ng mL^{-1}) plus Birinapant (1 μM) and Z-VAD (10 μM) for 20 hours and subjected to cell counting and viability analysis by Trypan blue exclusion (**a**). Cells were photographed in the original culture dish and after being pelleted for Giemsa staining on slides (**b**). Scale bar=50 μm.

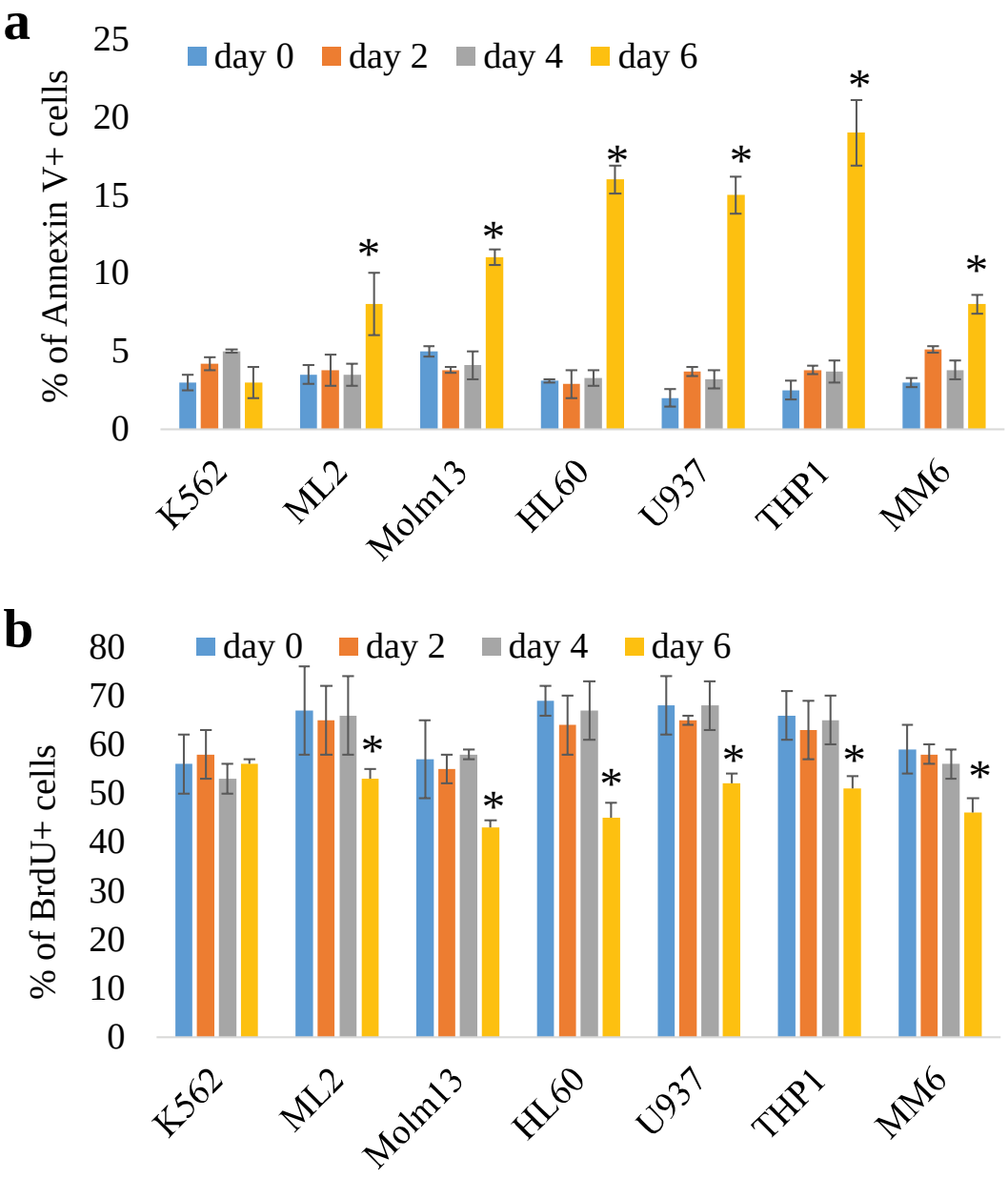


Fig. S2. Inhibition of proliferation and cell death of human AML cell lines can only be detected after 4 days of Nec1 treatment. Human CML cell line K562 and AML cell lines ML-2, Molm13, HL60, U937, THP1, and MM6 were treated with Nec1 (N, 50 μM). Cell death was determined by Annexin-V staining (**a**) and cell proliferation was examined by BrdU pulse-labeling assay (**b**) at the indicated time points. * $p<0.05$, compared to day 0 (untreated group). (**Associated with Fig. 2a**).

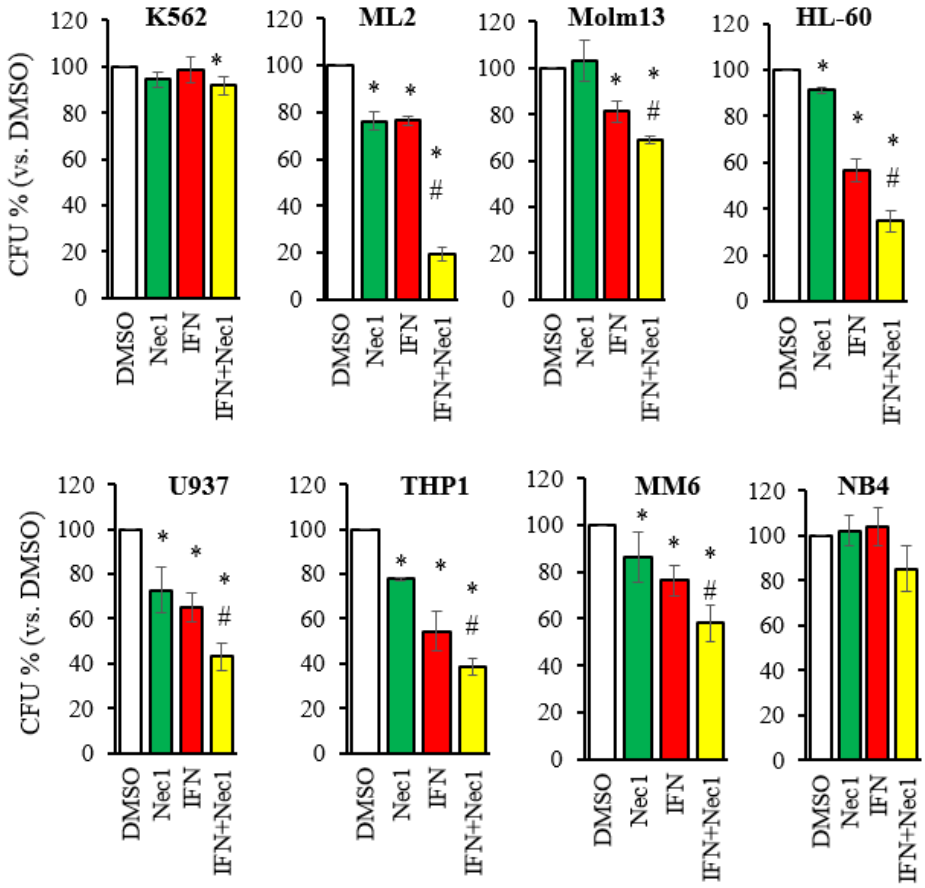
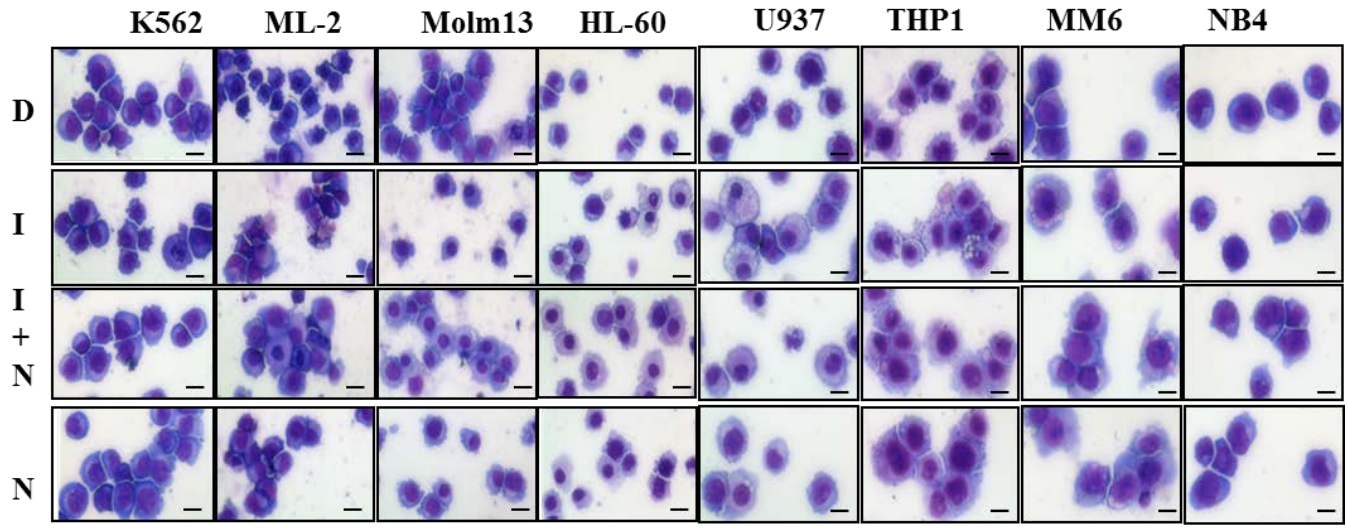


Fig. S3. Reduction of CFU capacity of human AML cell lines after IFN-γ and Nec1 treatment. Human CML cell line K562 and AML cell lines ML-2, Molm13, HL60, U937, THP1, MM6, and NB4 were treated with Nec1 (N, 50 μM), human IFN-γ (I, 5 ng mL^{-1}), IFN-γ +Nec1 and DMSO (D, vehicle control), respectively, for 4 days and 1000 cells were used for CFU assay. CFU were counted 9 days later. Data are presented as the relative results normalized to DMSO group. * $p<0.05$, compared to DMSO, #, $p<0.05$, compared to IFN-γ group. (**Associated with Fig. 2a**).

a.



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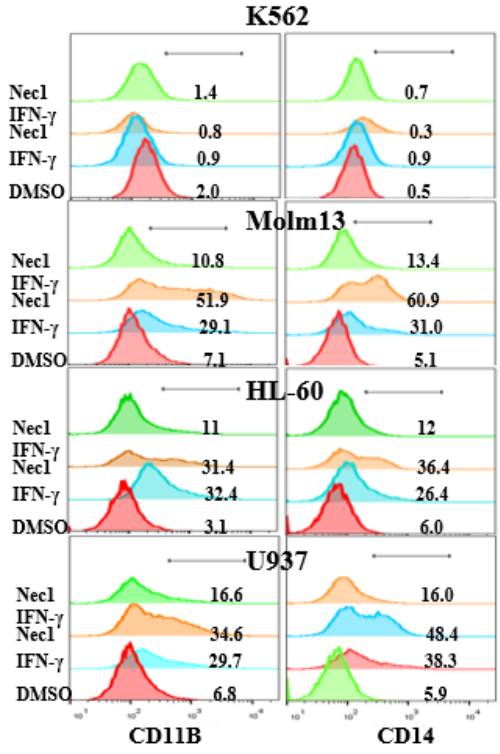

Fig. S4. Nec1 and IFN-γ treatment induced differentiation of human AML cell lines but not cause cell death. Human AML cell lines were treated with Nec1 (50 μM) or IFN-γ (5 ng mL^{-1}) individually or in combination (CML K562 cell line served as a negative control). Cell differentiation was evaluated by cell morphology (**a**) and FACS for CD11b and CD14 (**b**) on day 5. Scale bar=15 μm. (**Associated with Fig. 2a-b**).

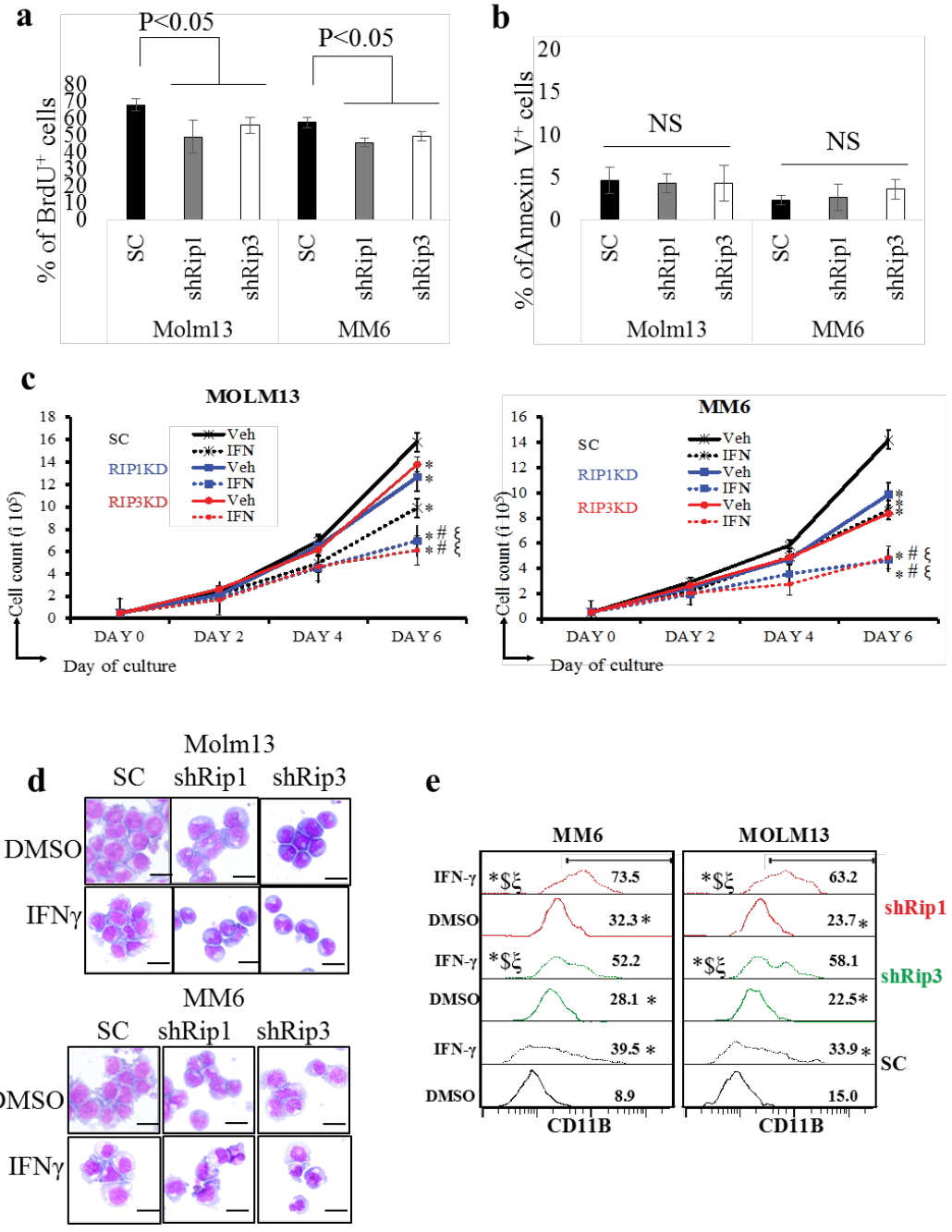

Fig. S5. Knockdown of RIP1 or RIP3 repressed proliferation and sensitized AML cells to IFN-γ – induced differentiation but did not affect survival of AML cells. **a-b**. Molm13 and MM6 cells with knockdown of RIP1 (shRNA#1) and RIP3 (shRNA#1) (as described in **Fig. 2c**) were cultured in regular culture medium. Proliferation was analyzed by BrdU pulse labeling (**a**). Survival was analyzed by Annexin- V/7AAD staining (**b**). **c-e**. Cells were treated with IFN-γ (5 ng mL^{-1}) for 6 days. Cell growth was examined every two days by counting the number of living cells (**c**). Cell differentiation was evaluated by cell morphology (**d**) and FACS for CD11b (**e**). Scale bar=15μm. * p<0.05, compared to DMSO-treated scrambled (SC) group; #, p<0.05, compared to IFN-γ-treated SC group; $^{\xi}$ p<0.05, compared to Veh-treated corresponding shRNA group (**Associated with Fig. 2c**).

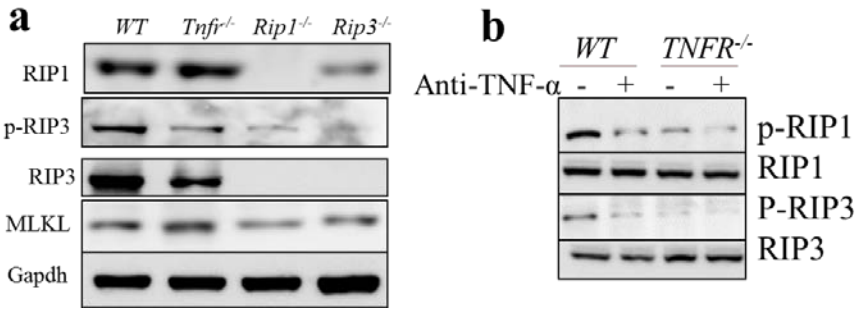

Fig. S6. RIP1-RIP3 signaling is activated AML cells by autocrine TNF-α in *in vitro* culture. a. The expression of RIP1, RIP3 and MLKL in *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ AML cells as shown by Western blotting. **b**. Blocking TNF-α signaling by anti-TNF-α inhibits RIP1-RIP3 signaling in *WT* AML cells but not *TNFR*$^{-/-}$ AML cells. *WT* and *TNFr*$^{-/-}$ AML cells were cultured in the regular culture medium with or without anti-TNFα treatment for 24 hours. Cell lysates were collected to detect p-RIP1 and p-RIP3 levels.

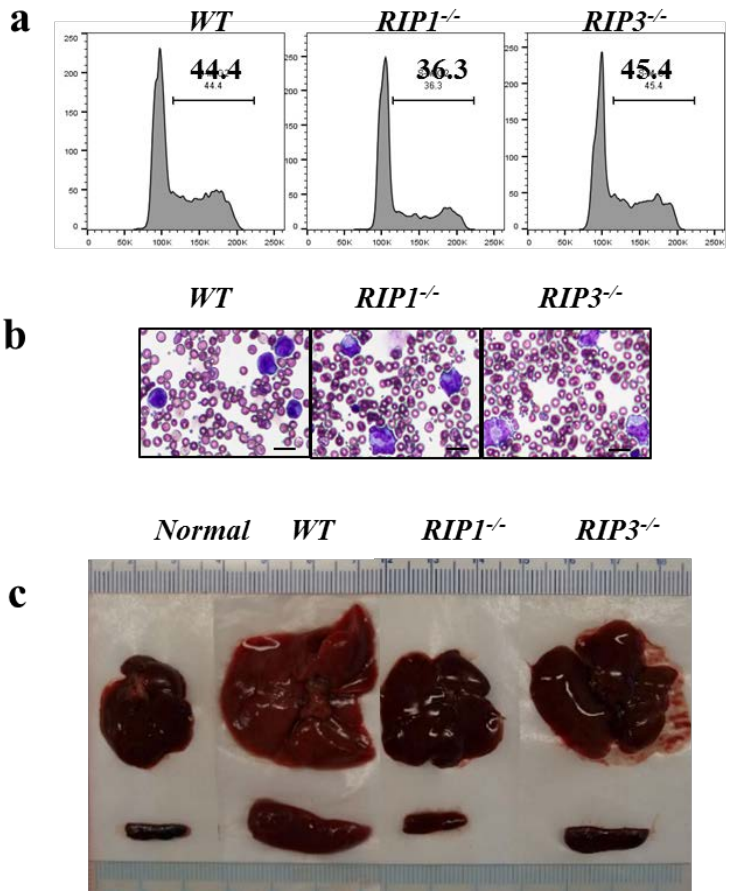

Fig. S7. Cell cycle, differentiation status and leukemogenic capacity of *WT*, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9*-AML cells. **a.** Cell cycle in *WT*, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ MA9 AML cells was analyzed by propidium iodide staining. The numbers show cell percentages in S/M/G2 stage. **b.** Peripheral blood smears stained with Giemsa stain at the time mice became moribund with leukemia. Scale bar= 20 μm. Gene-mutant AML cells are morphologically more differentiated compared to *WT* AML cells. **c.** Livers and spleens were collected from mice which had received *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9*-AML cells (3000/mouse) 25 days post-transplantation. Representative liver and spleen photographs from each genotype are presented. (**Associated with Fig. 3**).

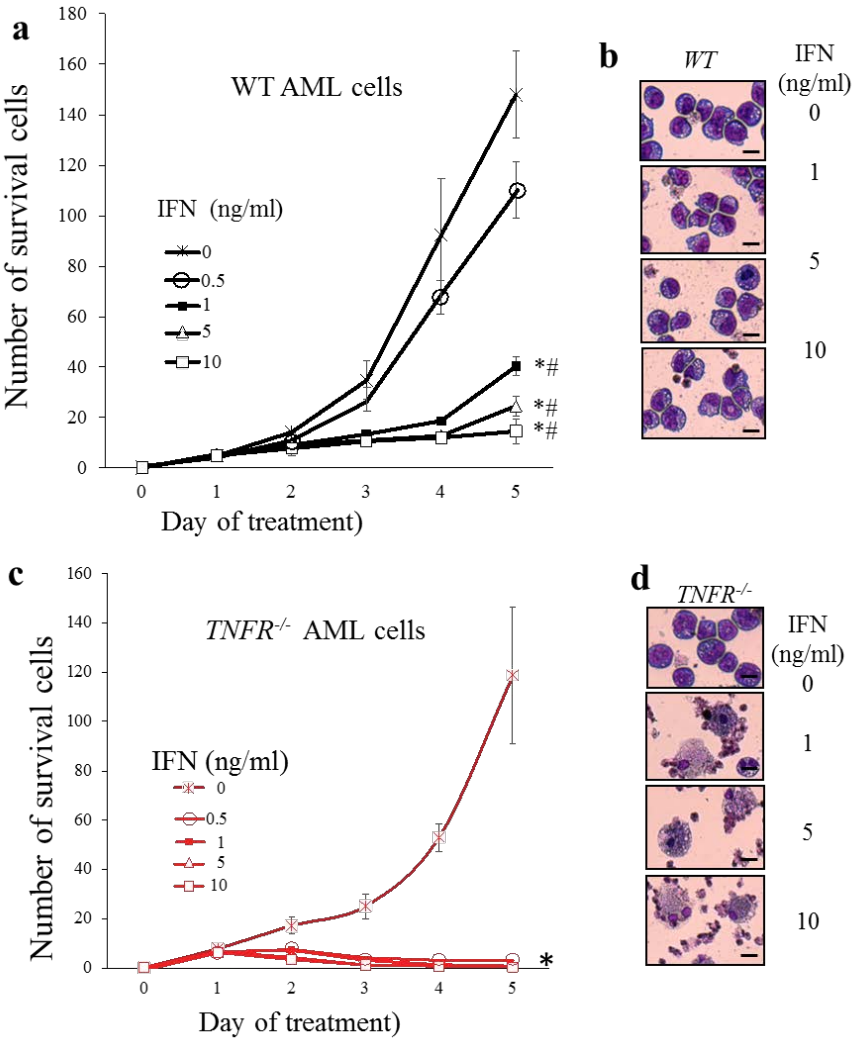

Fig. S8. Response of *WT* AML cells to IFN-γ treatment. *WT* (**a and b**) and *TNFR*$^{-/-}$ (**c and d**)AML cells were treated with IFN-γ at indicated concentrations. Cell growth was examined by counting the number of the live cells daily and plotted on growth curves (**a, c**). On day 5 post-treatment, the remaining cells were collected to assess their morphology by cytospin after Giemsa staining (**b, d**). Scale bar=15 μm. ***,** $p<0.05$, compared to Veh group; # compared to 0.5ng/ml IFN-γ group.

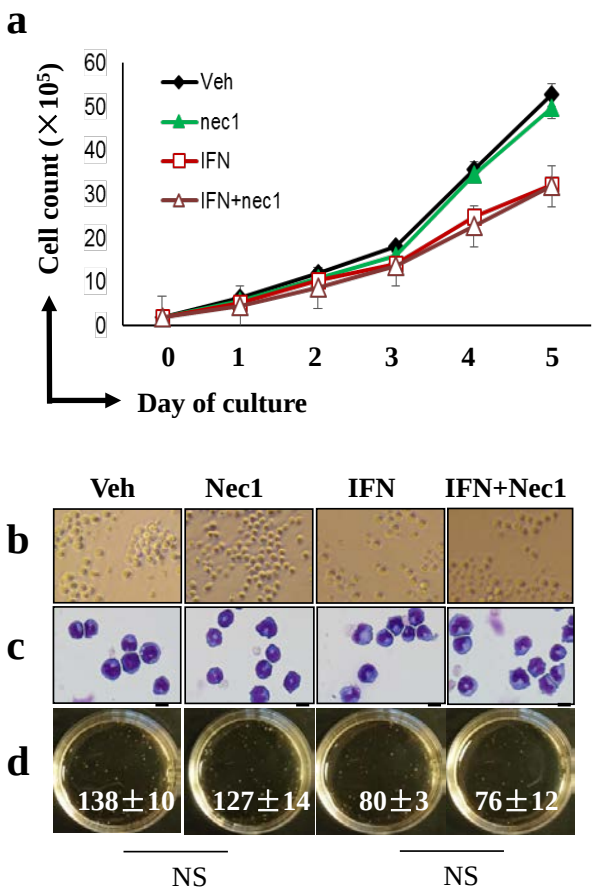

Fig. S9. The specificity of Nec1 in inhibiting RIP1/RIP3 signaling. *RIP3*$^{-/-}$ *MA9*-AML cells were treated with Nec1 (30μM) and IFN-γ (0.1 ng mL^{-1}) individually or in combination, respectively. Cell growth was evaluated by counting the number of the live cells daily; data are plotted on growth curves (**a**). On day 5 following treatment, remaining cells were collected to examine differentiation morphology in the dish (**b**) and after cytospin and following Giemsa staining (**c**). Scale bar=15μm. Treated cells were also subjected to CFU assay (1000/dish). Colonies were photographed and counted after one week of culturing (**d**).

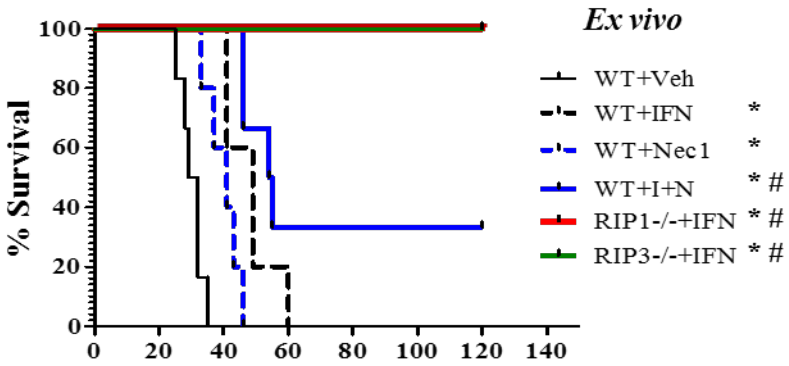

Fig. S10. ***Ex vivo*** **treatment of AML cells and transplantation**. *WT* AML cells were treated with Nec1 (30 μM), mIFN-γ (1 ng mL^{-1}) or Nec1+ mIFN-γ*; RIP1*$^{-/-}$, and *RIP3*$^{-/-}$ AML cells were treated with vehicle or mIFN-γ 1ng mL^{-1} for five days and transplanted into lethally-irradiated mice together with supporting bone marrow (BM) cells. Each mouse received 5000 live cells from the indicated treatment group and 2×10^5 supporting cells (n=5). Animal survival curves were plotted and statistically analyzed based on daily monitoring for mouse mortality. Leukemia was confirmed after the mice had expired * $p<0.05$, compared to WT+Veh; #, $p<0.05$, compared to WT+IFN. (**Associated with Fig. 4**).

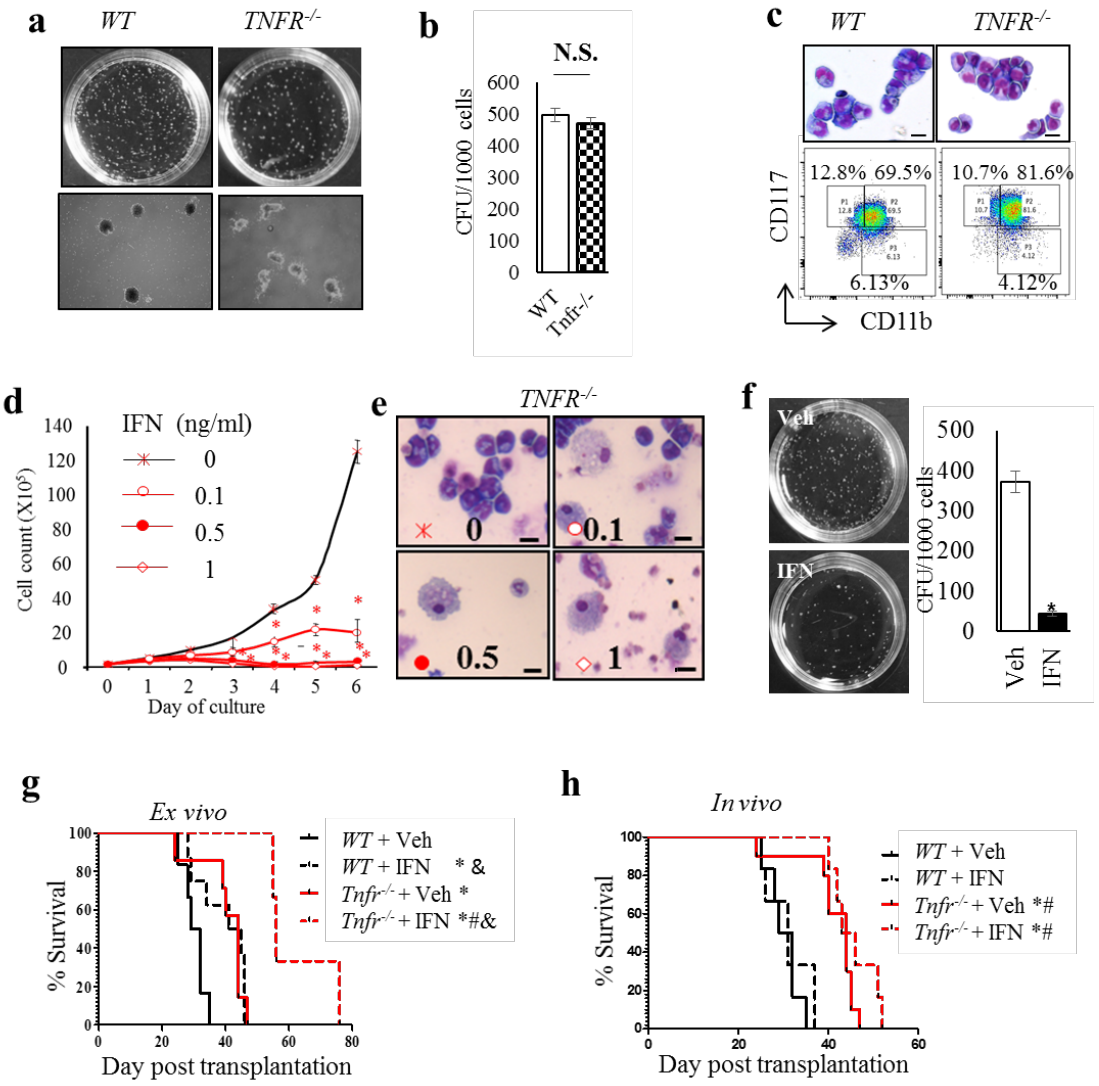

Fig. S11. Genetic inactivation of *TNFR* promotes IFN-γ-induced differentiation *in vitro* but not *in vivo*. **a-c.** CFU assays were performed by seeding 1000 cells/dish. Colonies were photographed (**a**) and counted (**b**) on day 7 of culturing. Cells were collected from colonies, cell morphology was examined after Giemsa staining and CD117/CD11b expression was analyzed by FACS (**c**). **d-f.** *TNFR*$^{-/-}$ AML cells were treated with IFN-γ at the indicated concentrations. Cell growth was plotted (**d**) and cell morphology was examined on day 5 of culturing (**e**). On day 5 of treatment, cells treated with 1ng mL^{-1} mIFN-γ and vehicle control groups were seeded for CFU assay. CFUs were photographed and counted (**f**) after 7 days in culture. **g.** *WT and TNFR*$^{-/-}$ AML cells were treated with mIFN-γ (1ng mL^{-1}) for five days and transplanted into lethally-irradiated mice together with supporting cells. Animal survival curves were plotted and statistically analyzed. * $p<0.05$, compared to WT+Veh; #, $p<0.05$, compared to WT+IFN; &, compared to Veh group of the same cell type. **h.** *WT* and *TNFR*$^{-/-}$ AML cells (5000 /mouse) were transplanted into lethally-irradiated mice together with supporting cells. Starting 15 days post-transplantation, half of the recipient mice from each group were treated with IFN-γ daily for two weeks. Animal survival curves were plotted and statistically analyzed. scale bar=15μm. * $p<0.05$, compared to WT+Veh; #, $p<0.05$, compared to WT+IFN.

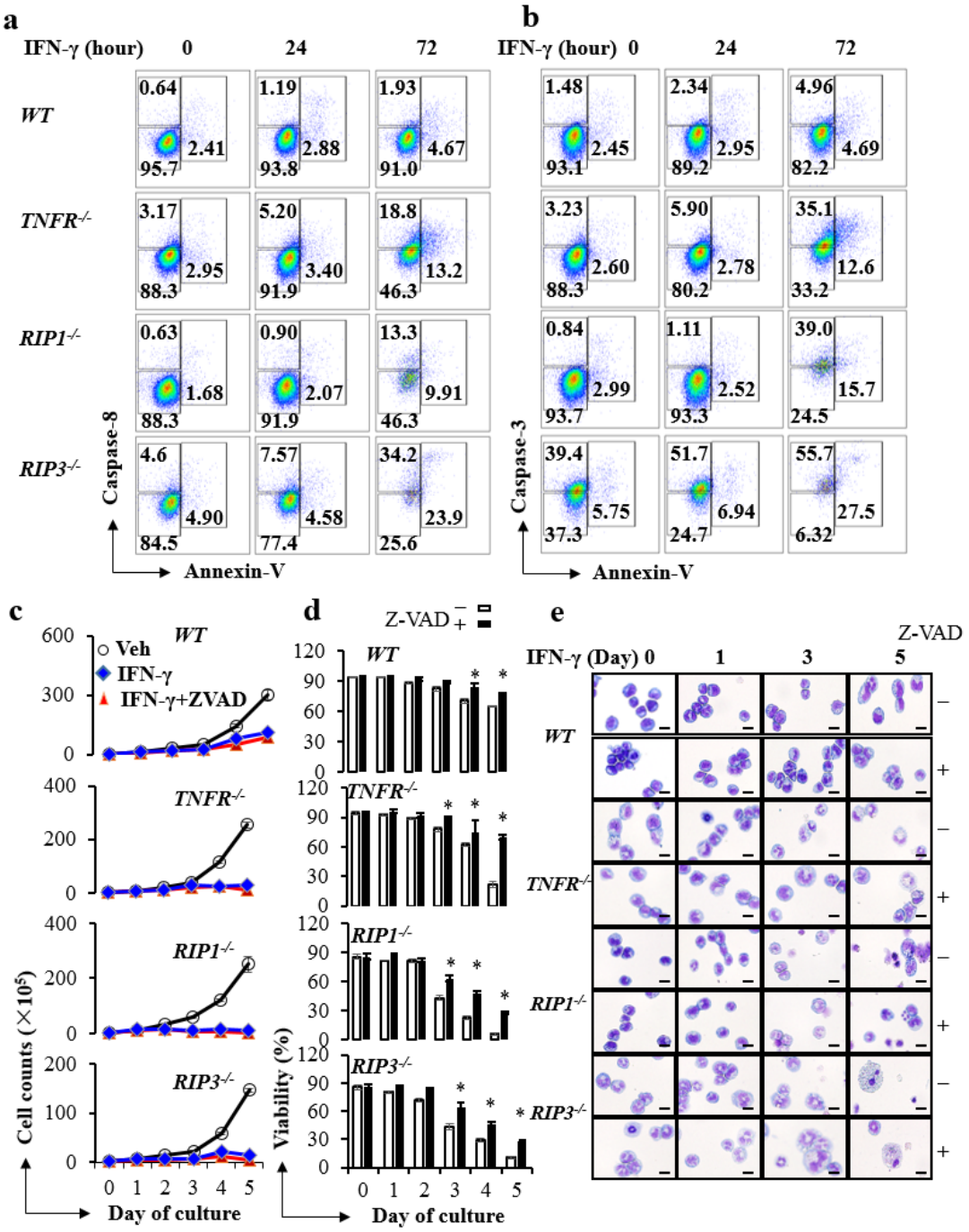

Fig. S12. Activation of caspase-8 and caspase-3 is secondary to induction of differentiation in murine AML cells. *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9*-AML cells were treated with mIFN-γ (1 ng/ml) for 5 days. Apoptosis (Annexin-V^{+}) and activation of caspase-8 (**a**) and caspase-3 (**b**) were measured after 0, 24 and 48 hr. of treatment. *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9*-AML cells were treated with mIFN-γ (1 ng mL^{-1}) in the presence or absence of Z-VAD (10 μM) for 5 days. Cell number (**c**) and viability **(d)** were measured daily and cell morphology (**e**) was examined on alternating days. * p<0.05, comparison of Z-VAD+ and Z-VAD- groups at the same time point; scale bar=15 μm. (**Associated with Fig. 5**).

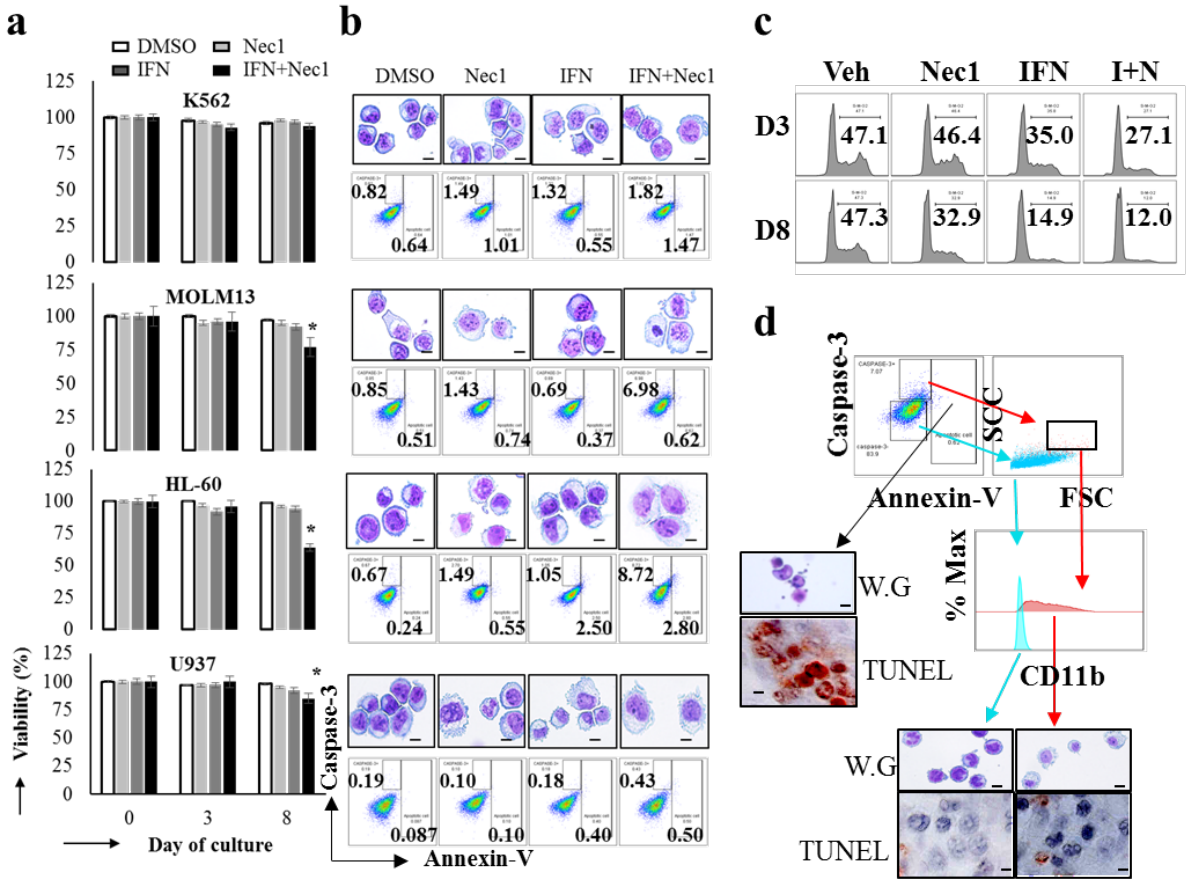

Fig. S13. Cell death, caspase activation, and cell cycle arrest are secondary to differentiation in human AML cells. Human AML cell lines Molm13, HL60 and U937 were treated with Nec1 (50 μM) or IFN-γ (5 ng mL^{-1}) individually or in combination (CML cell line K562 served as negative control). **a.** Cell viability was examined on days 3 and day 8 by Trypan Blue exclusion. **b.** Cell morphology (upper panel) and caspase-3 activation for apoptosis (lower panel) were analyzed on day 8. **c.** Cell cycle was analyzed on day 3 (D3) and day 8 (D8) in HL60 cells by propidium iodide staining. The numbers show percentages of cells in S/M/G2 stages. **d.** On day 8 of treatment with Nec1+hIFN-γ, HL60 cells were stained for activated caspase-3 and Annexin-V. Activated-caspase-3-positive and -negative cells were sorted and stained for CD11b and morphologic analysis (W.G: Wright's Giemsa staining). Annexin V^+ cells were sorted and apoptosis were verified by stained with W.G and TUNEL. Annexin V- cells were used as controls. (**Associated with Fig. 5**).

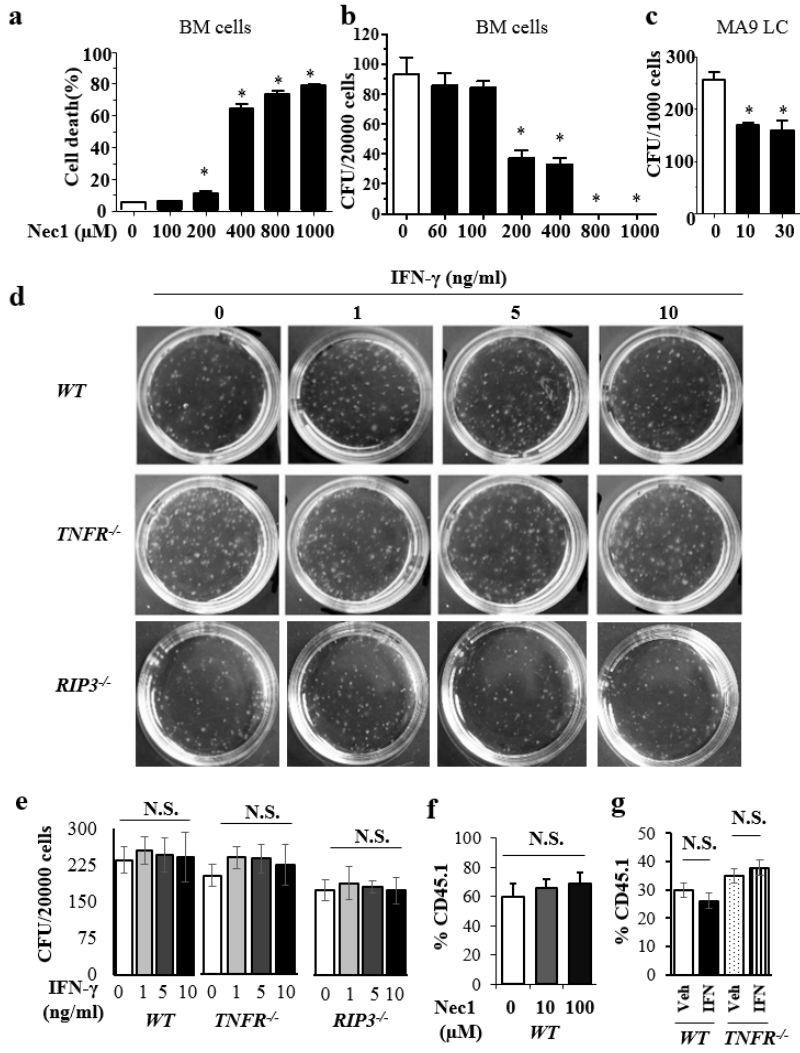

Fig. S14. ***RIP3*** **inactivation did not impact clonogenic ability nor hematopoietic reconstitutive capacity of normal HPSCs.** **a.** $CD117^+$ HSPCs were isolated from BM of *WT* mice. Cells were cultured in liquid medium containing Nec1 at the indicated concentrations (0-1000 μM) for 1 day. Cell death was measured using propidium iodide staining and FACS. **b**. Mononucleated cells (MNCs) isolated from BM of *WT* mice were seeded into methylcellulose (2×10^4/dish) containing Nec1 at the indicated concentrations (0-1000 μM). CFUs were counted on day 7 of culturing (*, $p<0.05$, compared to Veh). **c**. CFU assay was performed for *WT* and *MA9*-AML cells in parallel at a lower concentration range of Nec1 (0-60 μM). **d-e.** BM MNCs prepared from *WT* and $TNFR^{-/-}$ and $RIP3^{-/-}$ mice were seeded into methylcellulose (2×10^4/dish) containing IFN-γ (1-10 ng mL^{-1}). Colonies on the plate were photographed on day 7 of culturing (**d**); numbers of colonies from triplicate dishes for each treatment group were counted and compared (**e**); N.S. = not significant. **f-g.** BM MNCs collected from CD45.1 mice were treated with 10 or 100 μM Nec1 (**f**) or 1 ng mL^{-1} IFN-γ (**g**) for 48 hours. 10×10^5 cells were treated in each group. All live cells in each group were collected and mixed with 10×10^5 CD45.2 support bone marrow cells. Cells from each group were transplanted into 5 lethally-irradiated recipient (CD45.2) mice. The percentages of $CD45.1^+$ WBCs in peripheral blood of recipient mice were examined and compared 3 months later.

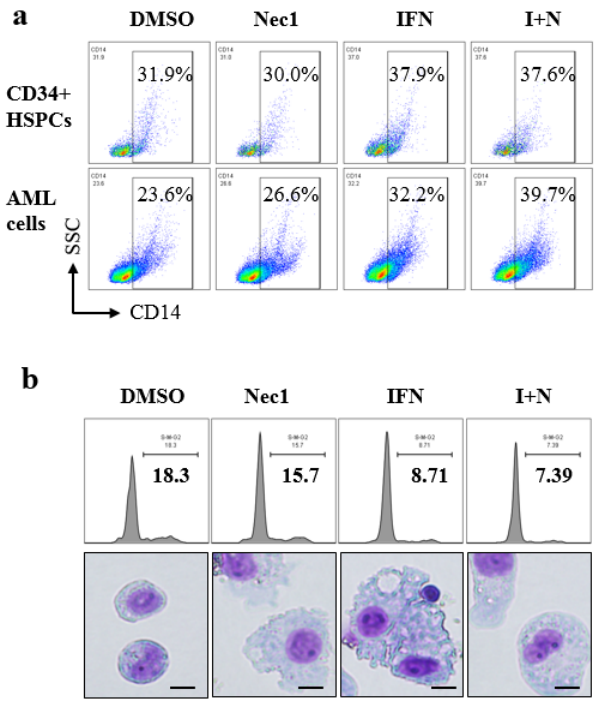

Fig. S15. . Differentiation of primary human AML cells after Nec1 and IFN-γ treatment. Data are representative of one of two healthy donors and one of the 7 colony-forming AML samples. **a.** Human $CD34^+$ HSPCs from healthy donors and primary AML cells from AML patients were seeded for CFU in the presence of DMSO, Nec1 (50 μM), hIFN-γ (5 ng mL^{-1}, IFN), or IFN-γ+ Nec1 (I+N). CD14 expression was analyzed after culturing for 9 days. AML cells were cultured in liquid medium with individual or combined treatment using Nec1 and IFN-γ. Cell cycle and morphology were analyzed after 5 days in culture. In upper panel, numbers show the percentages of cells in S/M/G2 stages. Lower panel: scale bar=15 μm. (**Associated with Fig. 6**).

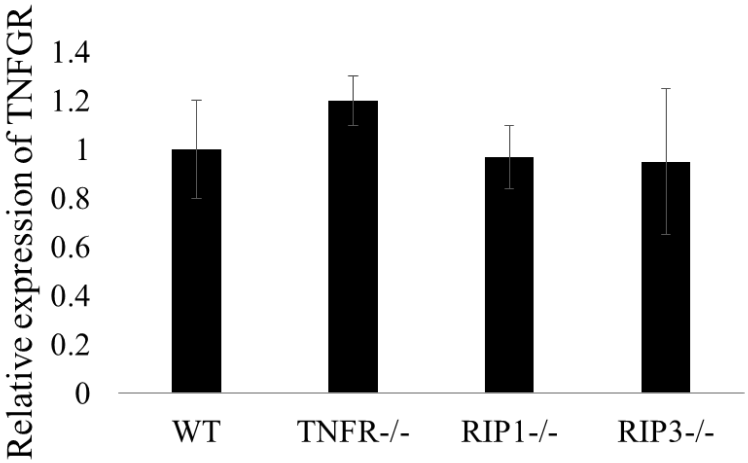

Fig. S16. IFNGR expression among *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9*-AML cells was examined by qRT-PCR assay. (**Associated with Fig. 7**)

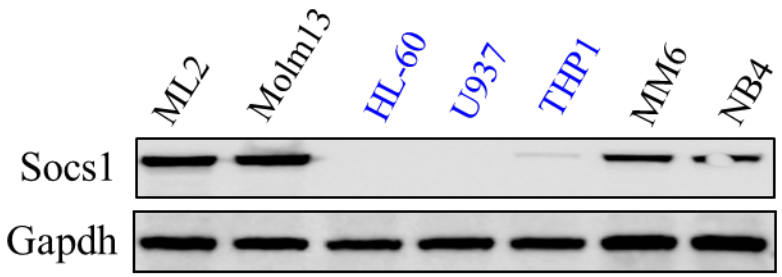

Fig. S17. SOCS1 expression was reduced in HL60, U937 and THP1 AML cells (RIP1/RIP3-low) compared to RIP1/RIP3-high (ML2, Molm13, MM6, and NB4) cells. (**Associated with Fig. 7**).

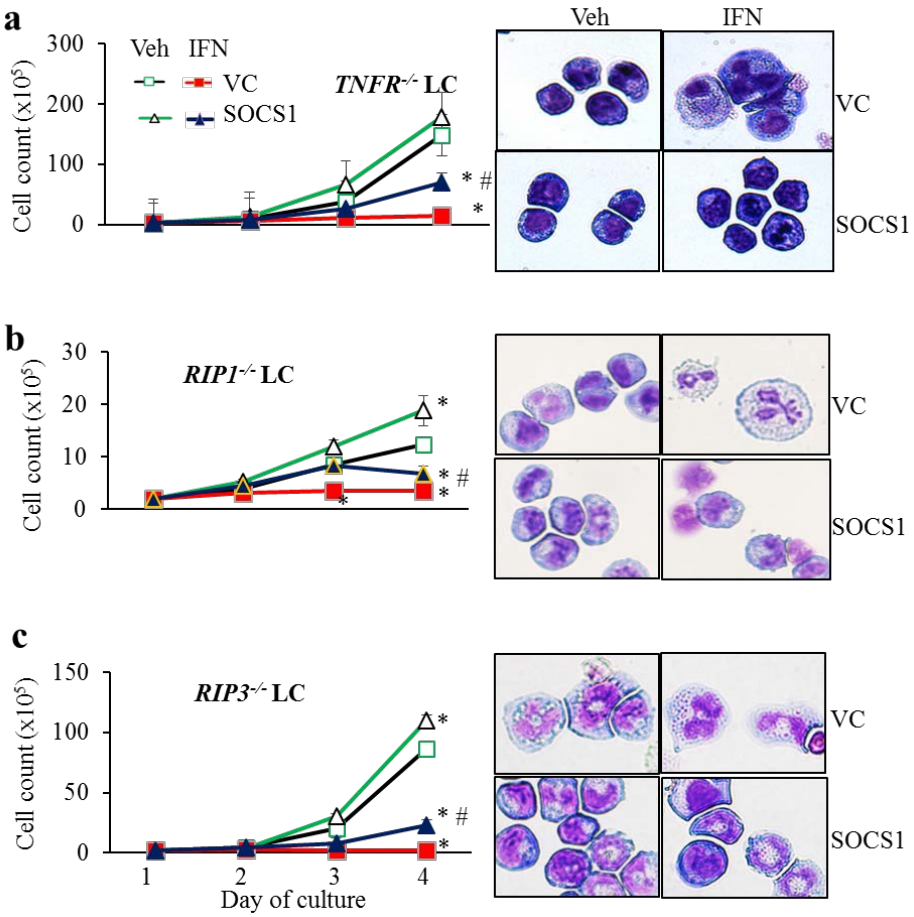

Fig. S18. *TNFR*$^{-/-}$, *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ *MA9* AML cells transduced with vector-only (VC) or SOCS 1 were treated with mIFN-γ (1 ng mL^{-1}), and cell growth was examined daily. Cell morphology was analyzed on day 4. *, $p<0.05$, compared to VC + Veh; #, $p<0.05$, compared to VC + IFN-γ; scale bar=15 μm. (**Associated with Fig. 7**).

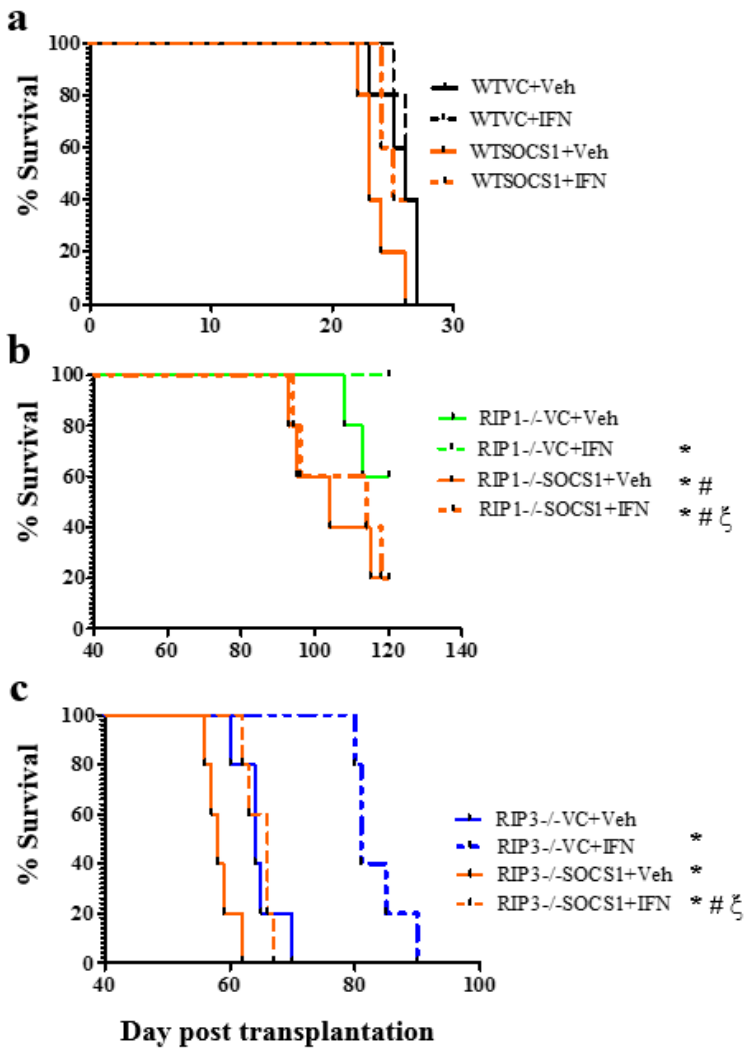

Fig. S19. SOCS1 overexpression partially restored IFN-γ resistance to *RIP1*$^{-/-}$ and *RIP3*$^{-/-}$ AML cells. *WT*, *RIP1*$^{-/-}$, and *RIP3*$^{-/-}$ *MA9* AML cells transduced with vector control (VC) or SOCS1-expressing virus were transplanted into lethally-irradiated mice together with supporting cells. Each recipient mouse received 5000 *WT* AML cells, 20,000 *RIP3*$^{-/-}$ AML cells, or 50,000 *RIP1*$^{-/-}$ AML cells (n=5/group). Beginning 15 days post-transplantation, half of the recipient mice from each group were treated with IFN-γ daily for two weeks. Animal survival curves were plotted and statistically analyzed. * $p<0.05$, compared to WT+Veh; #, $p<0.05$, compared to WT+IFN; ξ, compared to Veh group of the same cell type. (**Associated with Fig. 7**).

Supplementary Materials and Methods

Mice and genotyping. Recipient mice (C57BL6/J) were purchased from the Jackson Laboratory (Sacramento, CA, USA). Mice were permitted 1 week to acclimate to their environment before irradiation and adoptive transplantation. All mice were housed under a 12-hr. light/dark cycle in micro-isolator cages contained within a laminar flow system. All procedures were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals for research purposes and were approved by the Loyola University IACUC (#2013053). The adoptive transfer of leukemic and supporting cells was performed as described in detail previously[1]. *TNFR*$^{-/-}$ mice (B6.129S-TNFRsf1aTNFRsf1b, knockout of both *TNFR 1* and *2*) were purchased from the Jackson Laboratory. Fetal liver and bone marrow (BM) hematopoietic cells were obtained from *RIP1*$^{-/-}$ (*RIP1*-knockout)[23] and *RIP3*$^{-/-}$ (*RIP3*-knockout) mice, respectively[3]. All mutant mice used in this study are of C57BL6/J background. The PCR primer sequences for genotyping of mutant mice are listed in the section on oligonucleotide sequences below.

Generation of genetic-mutant murine AML cells. CD117^{+} HSPCs from *WT*, *TNFR*$^{-/-}$ (knockout of both TNF receptors 1 and 2), *RIP1*$^{-/-}$, and *RIP3*$^{-/-}$ mice were isolated by *EasySep* mouse CD117-Positive Selection Kit (Stem Cell Technologies). Cells were infected with *MA9*-neo-expressing retrovirus. Infected cells were selected with G418 for 1 week in RPMI 1640 medium containing 10% FBS supplemented with rmIL-3 (10 ng mL^{-1}), rmIL-6 (25 ng mL^{-1}), SCF (100 ng mL^{-1}) and GM-SCF (20 ng mL^{-1}) to generate *MA9*-immortalized cells (pre-AML cells in the text) with indicated genetic mutations. Pre-AML cells (2×10^6/mouse) along with supporting BM cells (2×10^5/mouse) were adoptively transplanted into lethally-irradiated mice (8.5 Gy) by tail vein injection. The mice were monitored for leukemia development by examining white blood cell counts and percentages of leukemic blasts in peripheral blood. *WT*, *TNFR*$^{-/-}$, *RIP1*$^{-/-}$, and *RIP3*$^{-/-}$ AML cells isolated from spleens and BM of the corresponding leukemic mice were used in the current study.

Primary human samples. Peripheral blood (PB) samples from AML patients were obtained from the oncology clinic at Loyola University Medical Center in accordance with an approved IRB protocol (IRB # 205151081313). Written consent was obtained from all subjects. The range of leukemic blasts in the PB of all patients was 12-90% when the samples were collected. Only samples consisting of 70-90% blasts were used for the current study. Mono-nucleated cells (MNCs) were enriched by *Ficoll-Paque* gradient centrifugation. MNCs were then cultured in *StemSpan* serum-free medium (StemCell Technologies) supplemented with recombinant human SCF (100 ng mL^{1}), Flt-3L (100 ng mL^{-1}), TPO (20 ng mL^{-1}), IL-6 (20 ng mL^{-1}), and IL-3 (20 ng mL^{-1}). All of these cytokines were obtained from Humanzyme. Following overnight culture, 4×10^4 cells from each sample were harvested and plated onto methylcellulose (StemCell Technologies) containing the indicated doses of Nec1 and IFN. Colonies were counted on day 10 of culturing. In the liquid culture and treatment protocols, cells were cultured in RPMI 1640 medium containing 10% FBS supplemented with the 5 cytokines mentioned above.

AML cell line culture and treatments. AML cells lines ML-2, Molm13 and MM6 were purchased from DSMZ. AML cell lines HL60, U937, THP1, NB4, and K562 were purchased from ATCC**.** All human AML cell lines, including NB4, MM6, ML-2, THP1, Molm13, U937, and HL60 as well as the CML line K562, were cultured in RPMI 1640 containing 10% FBS, whereas all murine AML cells were maintained in the 4-cytokine medium described above. All cells were incubated at 37^{o}C, 100% humidity, and 5% CO_2. For experimental treatments, AML cells were seeded into 24-well plates, 2×10^5 cells mL^{-1} of medium per well, with indicated agents. Cell growth was measured by counting viable cells daily using

an automated cell counter with Trypan Blue staining (TC10, Bio-Rad). Triplicate experiments were performed.

Colony-forming unit (CFU) assay. After indicated treatments, murine AML cells were seeded into *MethoCult GF M3434* medium for CFU assay following the instructions provided by vender (StemCell Technologies). Murine AML cells were seeded at 1000 cells mL^{-1} and BM MNCs were seeded at 20,000 cells mL^{-1} and incubated at 37°C., 100% humidity, and 5% CO_2 for 7 days (AML) or 10 days (BM MNCs). Human AML cells were seeded into *MethoCult* base medium without cytokines at 1000 cells mL^{-1}. CFUs were read on day 10 of culturing. Primary AML patient samples were seeded into *MethoCult* 4035 Optimum without EPO and incubated for 14 days. Numbers of CFUs were counted according to the manufacturer's instructions. Triplicate experiments were conducted.

Retroviral and lentiviral infection. High titer virus was produced by co-transfecting Phoenix cells or 293T cells with retroviral or lentiviral plasmids containing the indicated genes or shRNA together with packaging vectors using *Calphos* Mammalian Transfection Kit (Clontech). Retroviral and lentiviral supernatants were harvested 24 and 48 hours after transfection. AML cells or $CD117^+$ HSPCs were transduced with virally-expressed genes of interest by spinoculation at 32°C., 2000 rpm for 4 hours. The transduced cells were purified by FACS. pMIG-SOCS1-IRES-GFP[4] was provided by Dr. Ji-Long Chen of CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

shRNA knockdown. The lentiviral shRNA plasmids were purchased from Origene (Rockville, MD). The catalog number of RIP1 is TL320591 and of RIP3 is TL320649. Each kit contains 4 shRNAs with GFP reporter. RIP1 and RIP3 knockdown were confirmed in GFP^+ cells by Western blotting. The cells with the highest efficiency of knockdown were used for CFU assay (#1 and 2 for both RIP1 and RIP3).

RNA preparation and qRT-PCR. RNA was isolated using *TRIzol* reagent (Invitrogen). cDNA was generated using *SuperScript® III* Reverse Transcriptase (Life Technologies). mRNA levels of genes of interest were examined by *Taqman* qRT-PCR; primer information is listed in the section on oligonucleotide sequences below. The relative mRNA expression level in each cell type was first normalized to its *GAPDH* control then compared to *WT* samples.

Cell morphologic analysis. Cells collected from liquid culture or CFU assays were subjected to cytospin using a *Thermo Scientific™ Cytospin 4* cytocentrifuge and were stained with Wright's Giemsa. The resultant slides were viewed and photographed using an Olympus BX50 microscope (Olympus America Inc., PA) equipped with a digital camera system (Qimaging Retiga 2000R).

Western blotting. Cell lysates were prepared using Cell Lysis Buffer (Cell Signaling, Cat. # 9803S) followed by a brief sonication. The supernatants were collected after centrifugation at 4°C., 14,000×g for 20 minutes. Samples were separated on a 12% *Ready Gel* Tris-HCl polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) at 150 volts for 45-60 minutes and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) at 100 volts for 60 minutes. Western blot analysis was accomplished by using primary antibodies specific for p-RIP1, RIP1, p-RIP3, RIP3, MLKL, p-MLKL, SOCS1, STAT1, p-STAT1, ERK, and GAPDH, followed by HRP-labeled anti-rabbit or anti-mouse IgG secondary antibody.

Flow cytometric analysis. For intracellular staining, cells were pretreated with PMA (50 ng/ml) for 6 hours in the presence of brefeldin-A (10 μg/ml) in the final 4 hours, followed by a 5-minute fixation with

4% paraformaldehyde and permeabilized with saponin (0.1%). Cells were suspended in FACS buffer (1×PBS supplemented with 2% FBS) at a concentration of 1×10^7 cells mL^{-1} and aliquotted into flow cytometry tubes (100 µl per tube) for antibody staining. Surface staining was performed without fixation or permeabilization. Stained cells were subjected to multi-color analysis using a BD *LSRFortessa*^{TM} flow cytometer. Data were analyzed using *Flowjo* software. During the analysis, cells were first gated on live cells, then further analyzed for specific staining. When two or more gating strategies were used, the gating procedure utilized is specified in the corresponding figures.

Annexin-V staining and caspase activity. Following treatment for a specified time, cells were collected and stained with propidium iodide and alloyphycocyanin (APC)-conjugated Annexin-V in Annexin binding buffer following the manufacturer's instructions (BD Biosciences). For early apoptosis, cells were first gated on PI^- cells. Annexin-V^+ and Annexin-V^- cells were further analyzed for caspase activity and morphology. Caspase activation was determined with caspase-8 and caspase-3 staining kits following the manufacturer's instructions (eBioscience).

Cell cycle analysis. Following treatment for specified times, cells were collected and fixed in 70% cold ethanol and stained with a propidium iodide/RNase solution (Cell Signaling). Cells were gated on the single cell population first, then analyzed for DNA content.

Oligonucleotide sequences.
The PCR primers used for mouse genotyping were:

TNFR1-1 5′-GGATTGTCACGGTGCCGTTGAAG-3′;
TNFR1-2: 5′-TGACAAGGACACGGTGTGTGGC-3′;
TNFR1-3: 5′-TGCTGATGGGGATACATCCATC-3′,
TNFR1-4: 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.

RIP1-1: 5′-TGTGTCAAGTCTCCCTGCAG-3′;
RIP1-2: 5′-CACGGTCCTTTTGCCCTG-3′;
RIP1-3: 5′-CTGCTAAAGCGCATGCTC-3′;
RIP1-4: 5′-CACGGTCCTTTTGCCCTG-3′.

These primers produce a 386bp product in *WT* and a 400bp product in *RIP1*-mutant mice.

RIP3-1: 5′-GGCTTTCATTGTGGAGGTAAGCTGAGA-3′;
RIP3-2 5′-GAACCCGTGGATAAGTGCACTTGAAT-3′.

These primers produce a 280bp product in *WT* and a 320bp product in *RIP3*-mutant mice.

The primers used for Taqman Real-Time PCR for inflammatory mediators were purchased from Life Technologies, including: *IL-1β* (Mm00434228_m1), *TNF-α* (Mm00443260_m1), *TLR-2 (*Mm01213946_g1*)*, *TLR-4* (Mm00445273_m1), MYD-88 (Mm00440338_m1), SOCS1 (Mm00782550_s1, 2nd set: *SOCS1*-1: 5′- CTCCTTGGGGTCTGTTGGC-3′ , *SOCS1*-2: 5′-GGTTGCGTGCTACCATCCTA-3′) and HPRT (Mm01545399_m1).

Sources of reagents used. Rip-1 inhibitor Necrostatin-1 (Nec1) was purchased from Santa Cruz Biotechnology, and birinapant from Selleckchem (Houston, TX). Recombinant murine (rm) IFN, rmIL-3, rmIL-6, rmSCF, rmGM-CSF, rmTNF-α recombinant human (h) IFN-γ and rhIFN-α-2, as well as antibodies for FACS analysis, including PE-conjugated anti-human TNF, APC-anti-mouse CD117, PE-anti-mouse CD11B, APC-anti-human CD14 and PE-anti-human CD11B, APC-Annexin-V, activated caspase-8 and caspase-3 staining kits were purchased from eBioscience (San Diego). Z-VAD-FMK was purchased from R&D system (Minneapolis, MN). Cell Lysis Buffer (10×) for Western blotting was obtained from Cell Signaling (Danvers, MA), and supplemented with proteinase inhibitors and phosphatase inhibitors (Roche Diagnostics). Anti-TNF monoclonal antibody used for blocking TNF signaling in cultures was obtained from Amgen Inc. Western blotting antibodies, GAPDH, STAT1, p-STAT1, SOCS-1, RIP1, RIP3, MLKL, p-MLKL, ERK primary and requisite secondary antibodies were obtained from Cell Signaling. p-RIP (pS227) was purchased from Abcam. p-RIP1 (pS166, rabbit polyclonal) was obtained by GlaxoSmithKline LLC. ATRA, PMA, brefeldin, and saponin were purchased from Sigma (St. Louis, MO).

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