Supplemental information for:

Agonistic targeting of TLR1/TLR2 induces p38 MAPK-dependent apoptosis and NFκB-dependent differentiation of AML cells

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SUPPLEMENTAL MATERIALS AND METHODS

Flow cytometric analysis. Staining of TLR1 on human cells was performed using TLR1-PE and isotype IgG1-PE monoclonal antibodies (mAbs), both from eBioscience (San Diego, CA, USA), in combination with a CD34-APC/Cy7 mAb from BioLegend (San Diego, CA, USA), and a CD38-V450 mAb or a CD38-BV711 mAb from BD Biosciences (San Jose, CA, USA). For staining of human TLR2, TLR2-PE/Cy7 (eBiosceince) and isotype IgG1-PE/Cy7 (BioLegend) mAbs were used. $CD34^+CD38^-$ cells from primary human patient and normal CD34-enriched bone marrow (BM) cells were sorted using a FACSAria Fusion (BD Biosciences). Mouse TLR1 expression was assessed using a TLR1-eF660 mAb (eBioscience), and TLR2 expression using a TLR2-AF647 mAb (BioLegend). For lineage distribution analysis of BM and PB, murine cells were stained with CD45.1-APC, CD45.2-BV421, CD11b-BV510, CD3-PE, B220-PE/Cy7, Ter119-PE/Cy5 mAbs (BioLegend), and a Gr1-APC/Cy7 mAb (BD Biosciences). After staining c-Kit-enriched BM cells with Lin-PE/Cy5 (Ter119, B220, CD3, CD11b, Gr1), Sca1-PacBlue, and c-Kit-APC mAbs (BioLegend), $Linage^-Sca1^+c\text{-}Kit^+$ (LSK) BM cells were sorted using a FACSAria IIU (BD Biosciences). To assess TLR1 expression in murine stem and progenitor cells, cells were stained with Linage-cocktail-AF700, Sca1-BV421, c-Kit-BV510, CD16/32-PE/Cy7, Flt3-PE (BioLegend), and CD34-FITC mAbs (BD Biosciences).

Phospho-flow cytometric analysis. For phospho-flow cytometric analysis, murine or human leukemia cells were stimulated, in the absence of cytokine stimulation with Pam3CSK4 for 5 or 20 min. The cells were then fixed using paraformaldehyde (1.6%) for 10 min at room temperature and then washed in phosphate-buffered saline. For permeabilization, cells were resuspended in ice-cold 95% ethanol and immediately vortexed, then washed and stained with antibodies specific for the phosphorylated forms of the intracellular proteins NFκB (PE/Cy7), AKT (PE/CF594) and p38 MAPK (PE/Cy7) (all from BD Biosciences). In antibody-blocking experiments, the human leukemia cells were preincubated for 30 min at 37°C with anti-TLR1 (eBioscience) or anti-TLR2 (BioLegend) mAbs or isotype control mAb (eBioscience) before addition of 100 ng/mL Pam3CSK4.

Cultures of leukemia and normal BM cells. Primary mouse and human cells were cultured in StemSpan™ serum-free expansion medium (SFEM) (StemCell Technologies, Vancouver, Canada) containing 1% penicillin and streptomycin. For mouse *MLL-AF9* leukemia cells, the culture medium was supplemented with 0.5 ng/mL murine interleukin 3 (mIL-3) and 25 ng/mL murine stem cell factor (mSCF) (Peprotech Inc, Rocky Hill, NJ, USA) unless otherwise stated. For mouse *AML1-ETO9*a leukemia cells, the culture medium was supplemented with 25 ng/mL mSCF, 10 ng/mL mIL-3 and 10 ng/mL human interleukin 6 (hIL-6) (Peprotech Inc). For primary human leukemia cells and normal BM cells, the culture medium was supplemented with human thrombopoietin (hTPO), human SCF (hSCF), and human FMS-like tyrosine kinase 3 ligand (hFLT3L), all at 50 ng/mL (Peprotech Inc). Primary $CD34^+CD38^-$ human cells were cultured in S7 medium,[1] comprising Iscove's Modified Dulbecco Medium (IMDM; Sigma-Aldrich, St. Louis, MO, USA), 15% BIT (bovine serum albumin, insulin, transferrin; StemCell Technologies), 0.1 μM 2-mercaptoethanol (Sigma-Aldrich), 1% penicillin and streptomycin, and 1% L-glutamine. The S7-medium was also supplemented with 500 nM SR1, 500 nM UM729 (StemCell Technologies), 100 ng/mL hSCF, 50 ng/mL hFLT3L, 20 ng/mL human granulocyte colony stimulating factor (hG-CSF), and 20 ng/mL human IL-3 (Peprotech Inc.). MA9 cells were previously generated by retroviral expression of the *MLL-AF9* fusion gene in primitive cord blood cells.[2] The MA9 cells were cultured in IMDM containing 15% fetal bovine serum, 1% penicillin and

streptomycin, 1% L-glutamine, and 0.1 μM 2-mercaptoethanol. The MA9 medium was supplemented with hTPO, hFLT3L, hSCF, all at 10 ng/mL.

Differentiation analysis
To assess myeloid differentiation, murine and human leukemia cells were cultured for 2 or 3 days with Pam3CSK4. Murine leukemia cells were stained with a CD11b-BV510 mAb (BioLegend). MA9 cells, primary human leukemia and normal cells were stained with a CD14-APC, CD15-PE and CD11b-PE/Cy7 mAbs (all from BioLegend). In TLR1- and TLR2-antibody blocking experiments, the MA9 cells were stimulated with 10 ng/mL Pam3CSK4. For morphologic analysis, slides with cells were prepared using cytospin (500 rpm, 3 min) followed by standard May-Grünwald-Giemsa (Merck KGaA, Darmstadt, Germany) staining.

Cell cycle analysis
For cell cycle analysis, murine and human leukemia cells were treated with Pam3CSK4, an then fixed and permeabilized as described for the phospho-flow cytometric analysis. The cells were stained with a Ki67-APC mAb (Miltenyi Biotec). Before flow cytometric analysis, cells were washed, and DAPI (BioLegend) was added to the cells.

RNA sequencing analysis. After treating MA9 cells with Pam3CSK4 for 3 or 24 h, cells were harvested, and RNA extracted using a RNeasy Microkit, according to the manufacturer's instructions (QIAgen, Hilden, Germany). RNA libraries were generated using the TruSeq RNA sample prep kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions and sequenced in a NextSeq 500 Desktop Sequencer (Illumina) using the NextSeq 500/550 Mid Output v2 kit, 150 cycles (Illumina). Reads were aligned to the hg19 reference human genome using TopHat 2.0.13.[3] Qlucore Omics Explorer 3.0 (Qlucore, Lund, Sweden) was used to perform statistical analysis, differential gene expression analysis, and visualization of the RNA sequencing data. Gene Set Enrichment Analysis (GSEA[4]) was used to assess enrichment of gene sets in the Pam3CSK4-induced gene expression signatures using an FDR (false discovery rate) of 0.1 as significance cut-off.

SUPPLEMENTAL TABLES

Table S1. Characteristics of AML patients

Patient	TLR1 L/I/H*	Sample type	Age	Sex	Diagnosis	FAB group	WBC, 10^9/L	BM blasts, %	PB blasts, %	Cytogenetic karotype	Molecular aberrations#
AML 1	H	PB	72	F	De Novo	M4	155	91	97	NK	FLT3-TKD
AML 2	I	PB	68	F	De Novo	M4	136	95	72	NK	FLT3-ITD
AML 3	L	PB	74	M	De Novo	M4	15	25	8	ND	NPM1
AML 4	H	PB	65	F	De Novo	M4	94	82	67	47,XX,+8[21] /46XX[4]	NPM1
AML 5	I	BM	62	F	De Novo	M2	18.6	55	62	46,XX,del(5)(q31)[7] /46XX[18]	ND
AML 6	H	BM	67	F	De Novo	M2	4,0	25	25	46XX,t(8;21)(q22;q22)[13] /46,XX[12]	ND
AML 7	L	BM	73	M	De Novo	M2	4.2	70	45	Complex karyotype including del(5q)	ND
AML 8	H	BM	32	M	De Novo	M2	11.5	35	35	45,X,-Y,t(8;21) (q22;q22)[25]	ND
AML 9	L	PB	42	F	De Novo	M4	53.7	44	24	NK	NPM1
AML 10	L	BM	74	M	De Novo	M0	145	90	90	47,XY,+13[20]	ND
AML 11	H	PB	80	M	De Novo	M2	98.9	65	66	NK	ND
AML 12	I	PB	78	M	De Novo	M2	16	57	72	Complex karyotype including del(5q)	ND
AML 13	L	BM	66	F	De Novo	M5	3.3	29	24	ND	ND
AML 14	H	PB	32	M	De Novo	M2	18.3	82	83	46,XY,t(8;21)(q22;q22)[24]	ND
AML 15	H	PB	62	M	De Novo	M2	12.7	76	75	45XY,-7[23] /46XY[2]	ND
AML 16	I	BM	77	F	De Novo	M1	66	90	90	NK	NPM1
AML 17	L	BM	75	M	2º AML	M2 with dysplasia	18	30	8	NK	ND
AML 18	I	BM	74	F	De Novo	M2	1.6	65	-	NK	-
AML 19	H	PB	31	M	De Novo	M2	33.5	62	55	NK	FLT3-ITD
AML 20	I	BM	61	F	De Novo	M1	2.1	31	7	NK	-
AML 21	I	BM	70	F	t-AML	t-AML	8.8	79	46	NK	ND
AML 22	L	BM	80	M	De Novo	M2	2.5	20	-	45,XY,-Y[3] /46,XY[22]	-
AML 23	I	BM	64	M	De Novo	M1	28	95	90	NK	NPM1 /FLT3-TKD
AML 24	H	BM	74	M	De Novo	M5	36.5	34	6	NK	NPM1
AML 25	L	PB	72	M	2º AML	M4	-	51	55	46,XY,inv(9) (p11q12)	-
AML 26	L	BM	51	F	De Novo	M1	53.5	95	95	NK	-
AML 27	L	BM	72	M	De Novo	M4	2.9	70	-	47,XY,+13[18]/46,XY[7]	-
AML 28	I	BM	53	M	De Novo	M4	1.1	70	-	46,XY,t(9;11)(p21:q23)[14] /47,idem,+8[5]/47,idem,+19[4] /46,XY[2]	-

PB: peripheral blood; BM: bone marrow; t-AML: therapy-related AML; NK: normal karotype; ND: not determined; and WBC: white blood cells.
*The patients were divided into groups based on TLR1 expression on $CD34^+CD38^-$ cells; low: TLR1-L (10 patients); intermediate: TLR1-I (9 patients); high: TLR1-H (9 patients). # Molecular aberrations tested in the clinic at diagnosis, including NPM1, FLT3-ITD, FLT3-TKD.

Table S2. TLR1 expression in genetic subgroups of AML

	Number of events	*P*-value	q-value	FC
Fusion genes and partial tandem duplication (PTD)				
RUNX1-RUNX1T1	7	0.000014	0.000041	0.27
CBFB-MYH11	10	0.00084	0.0025	2.37
PML-RARA	16	0.00028	0.00085	0.47
BCR-ABL1	3	0.24	0.37	0.58
MLL-partner	8	0.45	0.68	1.25
MLLT10-partner	6	0.36	0.36	1.36
MLL-PTD	9	0.70	0.76	1.11
GPR128-TFG	3	0.96	0.96	0.98
NUP98-NSD1	3	0.81	0.92	0.89
Other in-frame fusion genes	10	0.064	0.32	0.62
Genes mutated*				
FLT3	49	0.39	0.76	0.89
NPM1	47	0.60	0.60	0.93
DNMT3A	43	0.11	0.28	1.25
IDH2	17	0.075	0.19	1.44
IDH1	16	0.33	0.63	0.81
TET2	15	0.82	0.91	0.95
RUNX1	17	0.11	0.28	1.38
TP53	14	0.60	0.60	1.13
NRAS	12	0.26	0.64	1.31
CEBPA	13	0.95	0.95	1.01
Others, with non of the above genes mutated	43	0.17	0.43	0.83
Others, with non of the above genes mutated and non of the above fusion genes	8	0.52	0.52	1.21
Molecular risk classification				
Good	33	0.0047	0.012	0.65
Intermediate	92	0.089	0.15	1.23
Poor	45	0.40	0.49	1.12
Totalt	173			

FC: fold-change (in specific subgroups relative to all others). *The 10 most frequently mutated genes in AML according to TCGA sequencing data.[5]

Table S3. Gene set enrichment analysis showing gene sets enriched in the Pam3CSK4 signature in MA9 cells (FDR less than 0.1)

Gene set name	Number of genes	FDR (q-value)	Upregulated in	Gene set description	Reference
NFκB_ Glioblastoma	38	<0.001	Pam3CSK4-treated, 3 h	Target genes of NFκB.	Pahl et al., 1999[6]
NFκB_ Glioblastoma	38	<0.001	Pam3CSK4-treated, 24 h	Target genes of NFκB.	Pahl et al., 1999[6]
Biocarta_TOLL_ Pathway	18	0.001	Pam3CSK4-treated, 24 h	Toll-Like Receptor Pathway	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Biocarta_IL6_ Pathway	17	0.080	Pam3CSK4-treated, 24 h	IL 6 signaling pathway	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Myeloid_Cell_ Differentiation	18	<0.001	Pam3CSK4-treated, 24 h	Genes annotated by the GO term GO:0030099. The process whereby a relatively unspecialized myeloid precursor cell acquires the specialized features of any cell of the myeloid leukocyte, megakaryocyte, thrombocyte, or erythrocyte lineages.	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Neut_VS_AML_Up	267	<0.001	Pam3CSK4-treated, 24 h	Differentially expressed between AML and fully differentiated neutrophiles.	Stegmaier et al. 2004[7]
Mono_VS_AML_ Up	300	0.002	Pam3CSK4-treated, 24 h	Differentially expressed between AML and fully differentiated neutrophiles.	Stegmaier et al. 2004[7]
Somervaille_ HighLSC_DN	158	<0.001	Pam3CSK4-treated, 24 h	Probe sets negatively correlated with LSC frequency in both data sets. The expression of 466 probe sets was negatively correlated with LSC frequency across the different molecular subtypes of MLL leukemia and concordantly upregulated from LSC-enriched (c-Kit^{high}) to LSC-depleted (c-Kit^{low}) populations.	Somervaille et al. 2009[8]
Biocarta_Death_ Pathway	17	0.058	Pam3CSK4-treated, 3 h	Induction of apoptosis through DR3 and DR4/5 Death Receptors.	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Biocarta_Death_ Pathway	17	0.001	Pam3CSK4-treated, 24 h	Induction of apoptosis through DR3 and DR4/5 Death Receptors.	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Hallmark_Apoptosis	75	<0.001	Pam3CSK4-treated, 24 h	Genes mediating programmed cell death (apoptosis) by activation of caspases.	Liberzon et al. 2015[9]
KEGG_Apoptosis	43	0.002	Pam3CSK4-treated, 24 h	Apoptosis	MsigDB (http://software.broadinstitute.org/gsea/msigdb)
Hallmark_P53_ Pathway	82	0.018	Pam3CSK4-treated, 24 h	Genes involved in p53 pathways and networks.	Liberzon et al. 2015[9]

FDR: false discovery rate.

SUPPLEMENTAL FIGURES

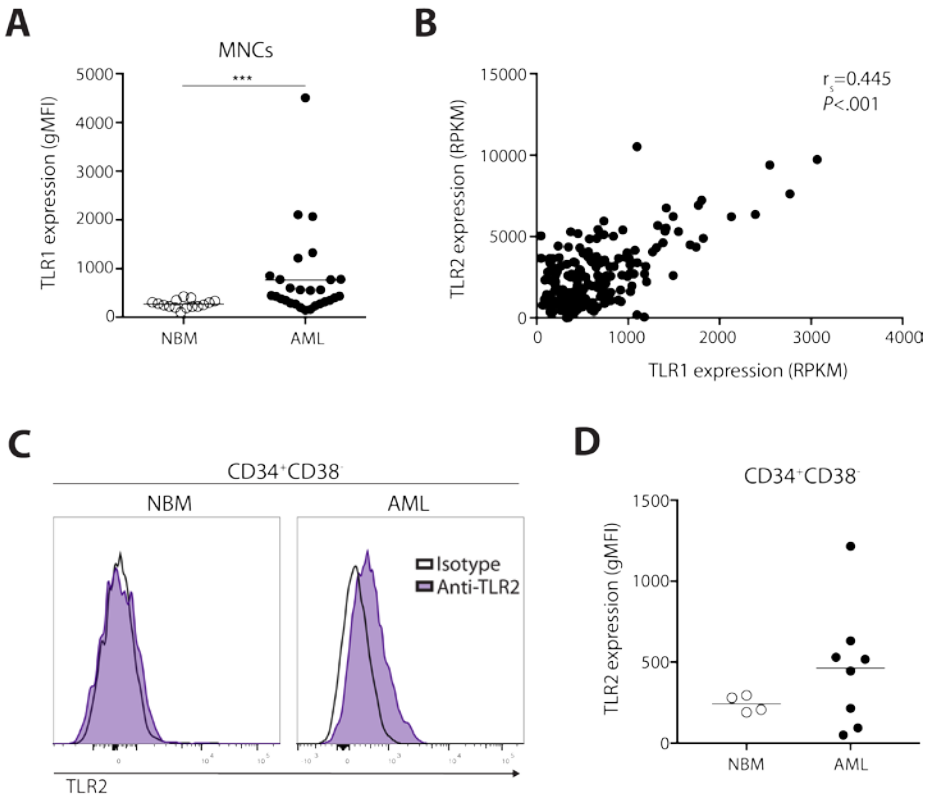

Figure S1. TLR1 and TLR2 are upregulated on primary human AML cells. (A) Geometric mean fluorescence intensity (gMFI) of TLR1 expression in NBM $CD34^+$ cells (n=15) and bulk AML mononuclear cells (MNCs) (n=28) determined by flow cytometry. (B) Correlation between TLR1 and TLR2 RPKM (reads per kilobase million) expression values based on RNA sequencing data from the TCGA database[5] (r_s=0.445; *P*<.001). (C) Histograms depicting TLR2 expression (black line: isotype control antibody, purple: anti-TLR2 antibody) within $CD34^+CD38^-$ cells from representative NBM and AML samples, respectively. (D) TLR2 expression in NBM (n=4) and AML (n=8) $CD34^+CD38^-$ cells. Each dot represents a sample from one patient and horizontal lines indicate mean values for all samples within groups. r_s: Spearman's rank correlation coefficient. *** *P*<.001.

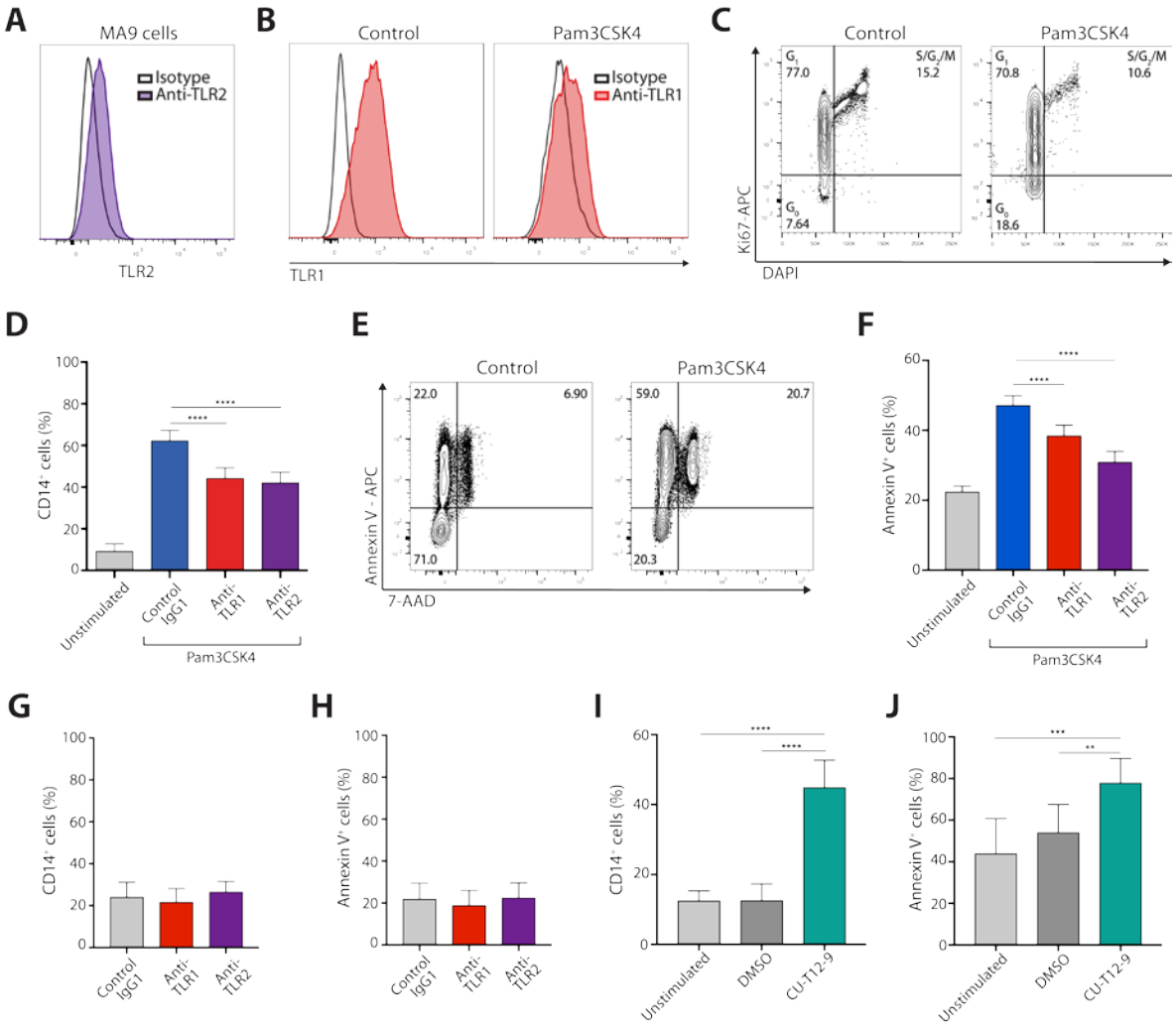

Figure S2. TLR1/TLR2 activation affects cell cycling, myeloid differentiation, and apoptosis of MA9 cells. (A) TLR2 expression on human MA9 leukemia cells. Histogram showing MA9 cells stained for TLR2 (purple) and isotype control (black line) using flow cytometry. (B-F) MA9 cells were treated with or without (control) Pam3CSK4 for 3 days. (B) TLR1 expression on MA9 cells after Pam3CSK4 treatment: TLR1 staining (red) and isotype control (black lines). (C) Contour plots showing cell cycle profiles of representative samples of MA9 cells. (D) Percentage of $CD14^+$ cells assessed by flow cytometry after pre-incubation for 30 min with 50 µg/ml anti-TLR1 or anti-TLR2 blocking antibodies, or an isotype control antibody, before being treated with Pam3CSK4 for 3 days (n=3). (E) Contour plots showing apoptosis profiles of representative samples of MA9 cells analyzed by flow cytometry. (F) Percentage of Annexin V^+ cells after pre-incubation for 30 min with 10 µg/ml anti-TLR1 or anti-TLR2 blocking antibodies, or an isotype control antibody, before being treated with Pam3CSK4 for 2 days (n=3). (G-H) MA9 cells were incubated with anti-TLR1 and anti-TLR2 blocking antibodies or isotype control for (G) 3 days and (H) 2 days. (G) Percentage of $CD14^+$ cells and (H) Annexin V^+ cells (n=3). (I-J) MA9 cells were treated with CU-T12-9 or DMSO control for 3 days. (G) Percentage of $CD14^+$ cells and (H) Annexin V^+ cells (n=3). ** *P*<.01; *** *P*<.001; **** *P*<.0001.

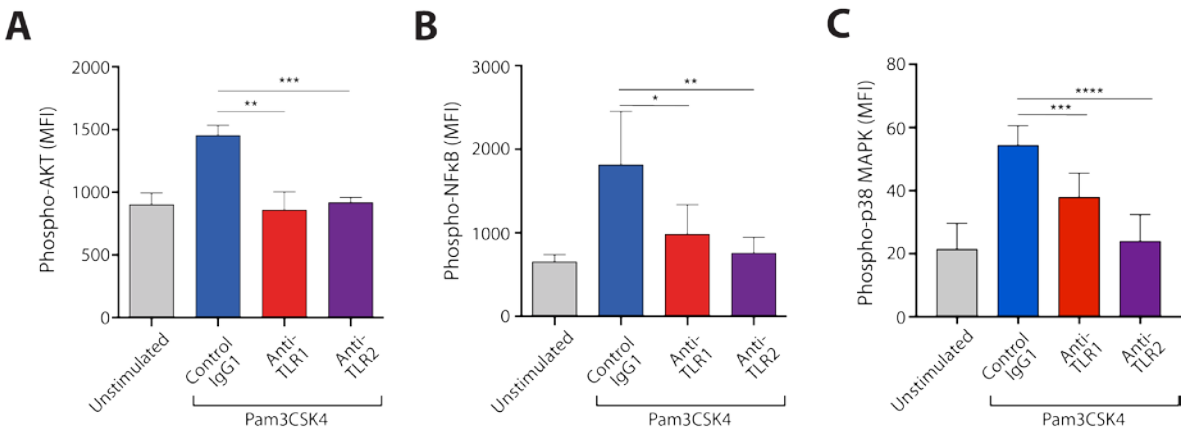

Figure S3. TLR1- and TLR2 blocking antibodies abolish AKT, NFκB and p38 MAPK phosphorylation induced by Pam3CSK4 in MA9 cells. (A) AKT**,** (B) NFκB and (C) p38 MAPK phosphorylation in MA9 cells measured by flow cytometry, following pre-incubation for 30 min with 50 μg/ml anti-TLR1 or anti-TLR2 antibodies or an isotype control antibody, and then 20 min of treatment with 100 ng/ml Pam3CSK4 or no treatment (unstimulated) (n=3). Mean values and SDs are shown. MFI: mean fluorescence intensity. * P<.05; ** P<.01; *** P<.001; **** P<.0001.

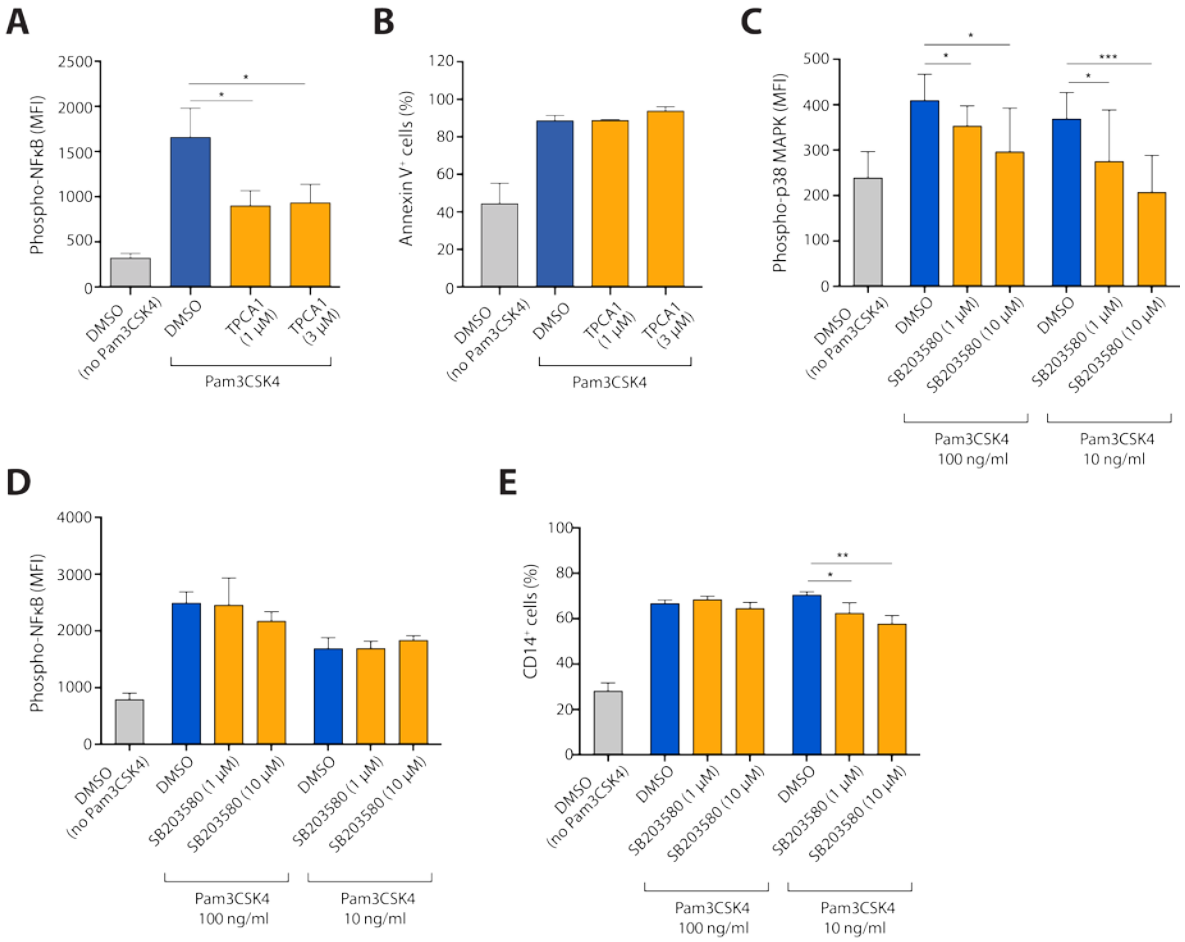

Figure S4. TLR1/TLR2 activation induces apoptosis of MA9 cells in an NFκB-independent manner and p38 MAPK inhibition decreases Pam3CSK4-induced differentiation. (A-B) MA9 cells were pre-incubated with TPCA1 at 1 or 3 μM, or DMSO control for 10 min, before Pam3CSK4 treatment. (A) NFκB phosphorylation after treatment with or without Pam3CSK4 for 20 min (n=3). (B) Percentage of Annexin V^+ cells following treatment with or without Pam3CSK4 for 3 days (n=3). (C-E) MA9 cells were pre-incubated with SB203580 at 1 or 10 μM, or DMSO control for 10 min, before Pam3CSK4 treatment. (C) p38 MAPK and (D) NFκB phosphorylation after treatment with or without Pam3CSK4 for 20 min (n=3). (E) Percentage of $CD14^+$ cells treated with or without Pam3CSK4 for 2 days (n=3). Mean values and SDs are shown. MFI: mean fluorescence intensity. * P<.05; ** P<.01; *** P<.001.

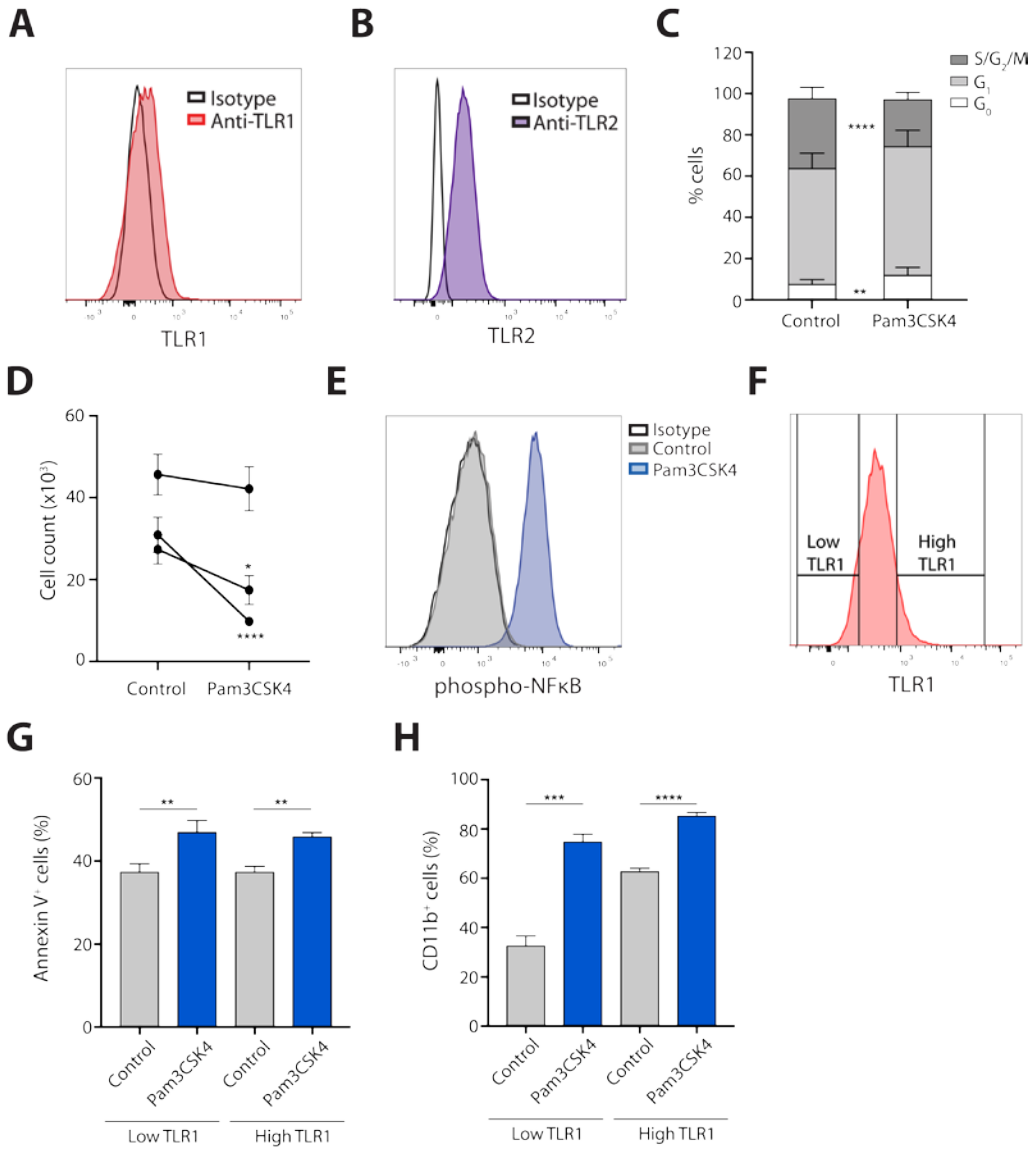

Figure S5. TLR1/TLR2 activation suppresses cell cycling and induces phosphorylation of NFκB in murine *MLL-AF9* AML cells. (A) TLR1 expression on the cell surface of primary murine *MLL-AF9* dsRed^{+} leukemic-granulocyte and macrophage progenitor cells (L-GMPs; Linage^{-}Sca1^{-}cKit^{+}CD34^{+}CD16/32^{+}). (B) TLR2 expression on primary murine c-Kit^{+} *MLL-AF9* AML cells. The histograms show representative samples from leukemic mice stained for TLR1 (red), TLR2 (purple) and isotype control (black line). (C-D) c-Kit^{+} *MLL-AF9* AML cells were cultured for 3 days with or without (control) Pam3CSK4. (C) Cell cycle analysis of AML cells (n=3). (D) Cell count as determined by flow cytometric analysis using CountBright^{TM} beads in three independent experiments. (E) *MLL-AF9* AML cells were stimulated for 5 min with Pam3CSK4 and then NFκB phosphorylation was assessed by phospho-flow cytometric analysis. Depicted in the histogram are unstimulated (control, grey area) and Pam3CSK4-treated cells stained for phospho-NFκB (blue) or isotype (black line) control stained cells. (F) Histogram depicting the gating for the sorting of "Low TLR1" and "High TLR1" expressing c-Kit-enriched *MLL-AF9* AML cells. (G-H) "Low TLR1" or "High TLR1" *MLL-AF9* AML cells were cultured for 3 days with or without (control) Pam3CSK4. (G) Percentage of Annexin V^{+} cells, and (H) CD11b^{+} *MLL-AF9* AML cells assessed by flow cytometry (n=3). * P<.05; ** P<.01; *** P<.001; **** P<.0001.

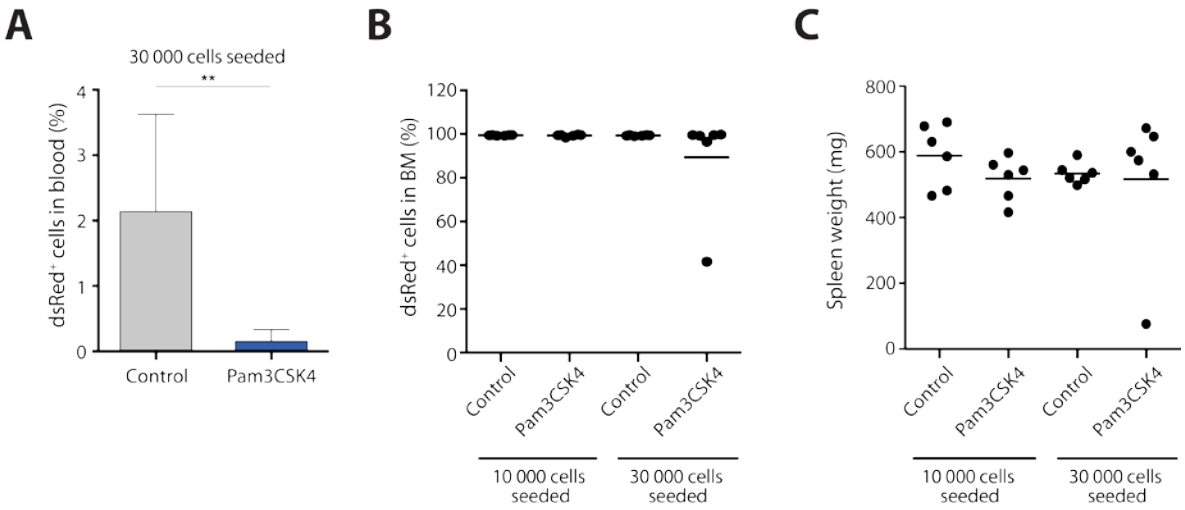

Figure S6. Leukemia burden in mice transplanted with *ex vivo* Pam3CSK4-treated *MLL-AF9* AML cells. Murine c-Kit$^+$ *MLL-AF9* AML cells were seeded at 10 000 or 30 000 cells per well and treated *ex vivo* with or without (control) Pam3CSK4 for three days, then transplanted into sublethally irradiated C57BL/6 mice (6 mice per group). (A) Leukemia burden (dsRed$^+$ cells) in blood at 2 weeks post transplantation was determined by flow cytometry. (B) Frequency of AML cells (dsRed$^+$ cells) in the bone marrow, and (C) spleen weights at sacrifice. Mean values and SDs are shown. ** P<.01.

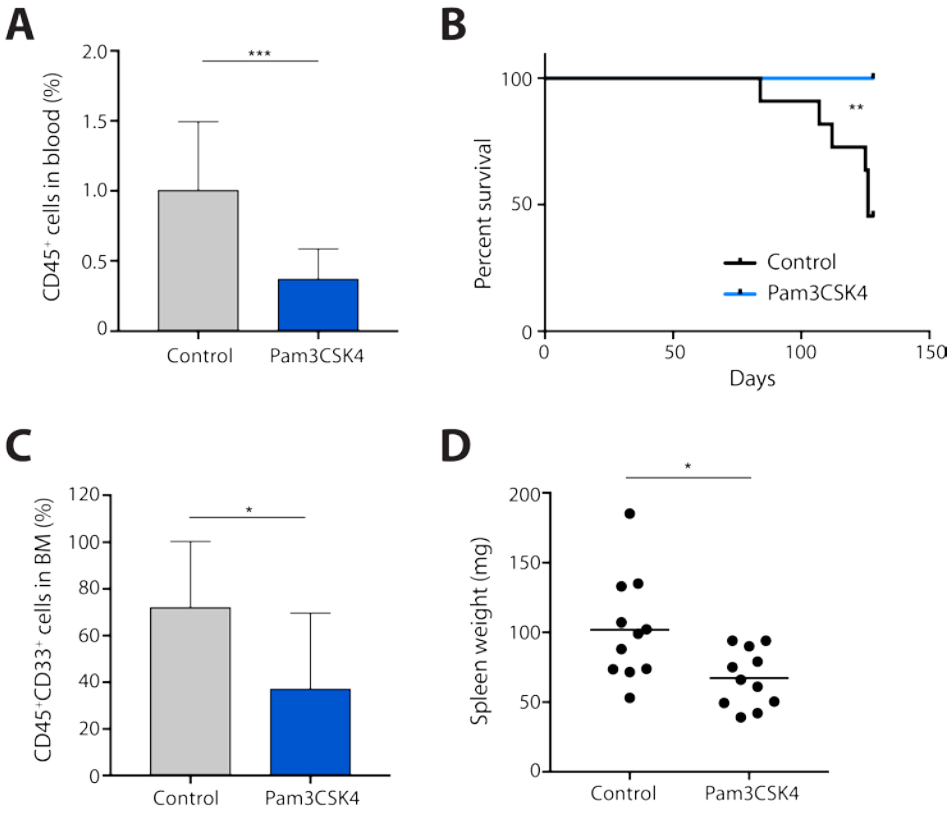

Figure S7. TLR1/TLR2 activation inhibits human leukemia-initiating cells. One million human MA9 cells were seeded in wells and treated with or without (control) Pam3CSK4 for 3 days and then transplanted into sublethally irradiated NSG mice (11 mice per group). (A) Leukemia burden (human $CD45^+$ cells) in blood at 4 weeks post transplantation. (B) Kaplan–Meier curves showing overall survival of the mice. Remaining mice were sacrificed 16 weeks post transplantation. (C) Leukemia burden (human $CD45^+CD33^+$ cells) in bone marrow and (D) spleen weights at sacrifice. Mean values and SDs are shown. * $P<.05$; ** $P<.01$; *** $P<.001$.

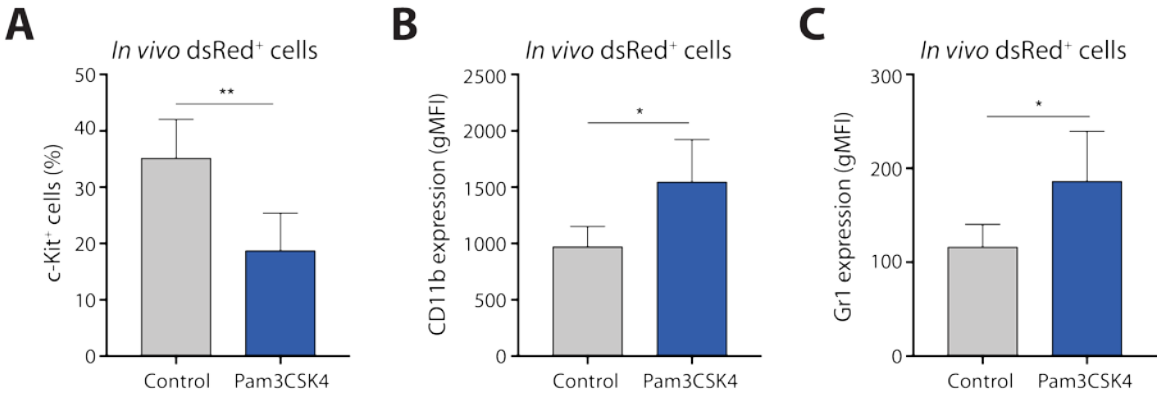

Figure S8. Pam3CSK4 treatment of mice induces differentiation of murine *MLL-AF9* AML cells. 100 000 c-Kit$^+$ *MLL-AF9* AML cells were transplanted into sublethally irradiated recipient mice (5 mice per group) and treated with Pam3CSK4 (100 μg/dose) or PBS (control) by intraperitoneal injections three times per week starting one day post transplantation. (A) Percentage of c-Kit$^+$ cells, (B) CD11b expression, and (C) Gr1 expression within leukemia (dsRed$^+$) cells in BM at day 10 post transplantation. Mean values and SDs are shown. gMFI: geometric mean fluorescence intensity. * P<.05; ** P<.01.

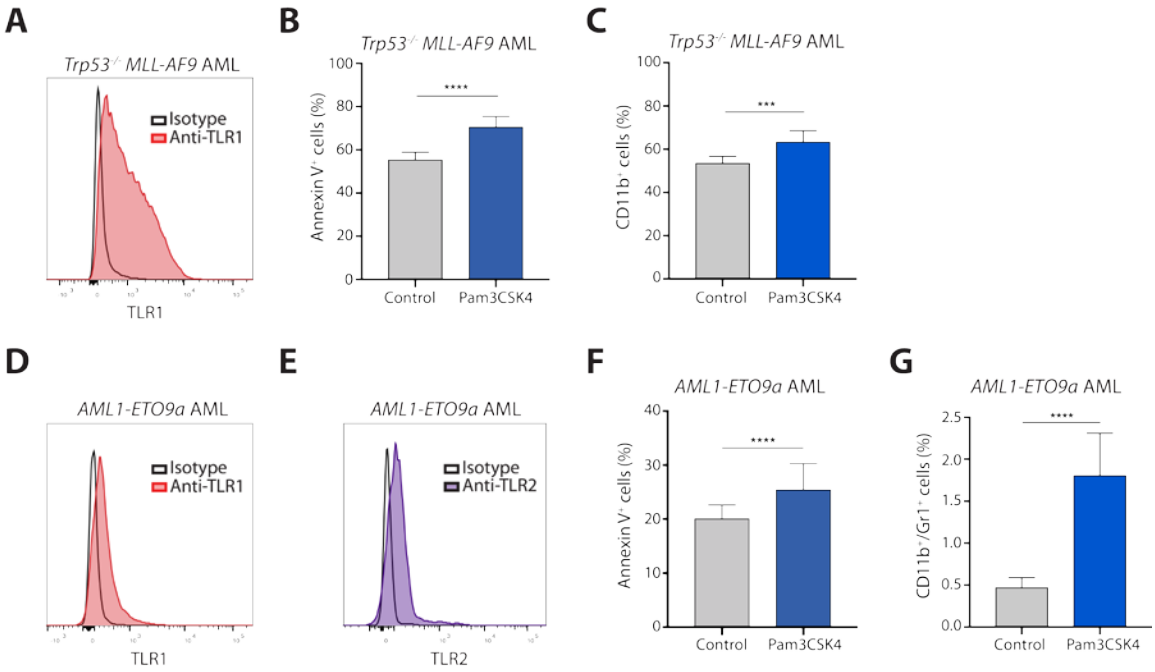

Figure S9. TLR1/TLR2 activation induces apoptosis and differentiation of *Trp53*$^{-/-}$ *MLL-AF9* and *AML1-ETO9a* AML cells. *Trp53*$^{-/-}$ *MLL-AF9* and *AML1-ETO9a* AML cells were treated with or without (control) Pam3CSK4 for 3 days. (A) Histogram depicting TLR1 expression in a representative sample of murine c-Kit^{+} *Trp53*$^{-/-}$ *MLL-AF9* AML cells (black line, isotype control antibody; red, anti-TLR1 antibody). (B) Percentage of Annexin V^{+} and (C) CD11b^{+} *Trp53*$^{-/-}$ *MLL-AF9* AML cells measured by flow cytometry (n=3). Histogram depicting (D) TLR1 and (E) TLR2 expression in a representative sample of murine c-Kit^{+} *AML1-ETO9a* AML cells (black line, isotype control antibody; red, anti-TLR1 antibody; purple, anti-TLR2 antibody). Percentage of (F) Annexin V^{+} and (G) CD11b^{+}/Gr1^{+} *AML1-ETO9a* AML cells (n=3). Mean values and SDs are shown (n=3). *** P<.001; **** P<.0001.

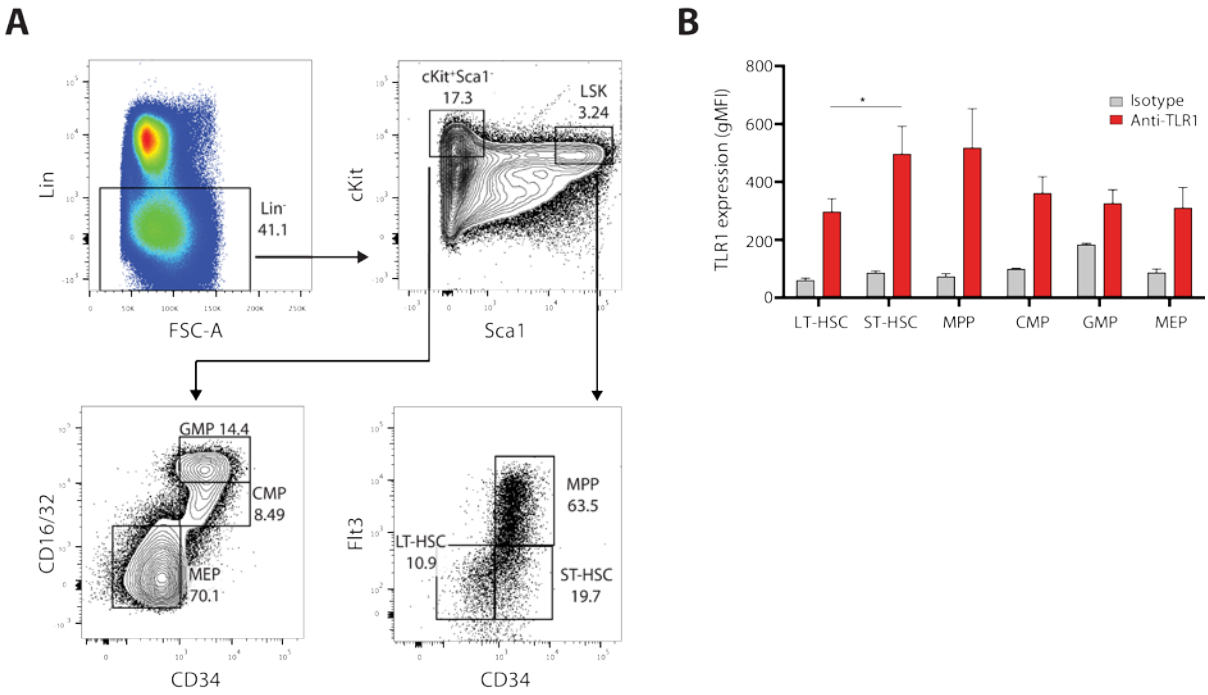

Figure S10. TLR1 expression in murine hematopoietic stem and progenitor cells. (A) Flow cytometric analysis of murine c-Kit-enriched bone marrow cells. The arrows show how the gating was performed. (B) TLR1 (red) and isotype (grey) control stained hematopoietic stem and progenitor cells (n=3; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor; MEP, megakaryocytic and erythroid progenitor). Mean values and SDs are shown. gMFI: geometric mean fluorescence intensity. * $P<.05$.

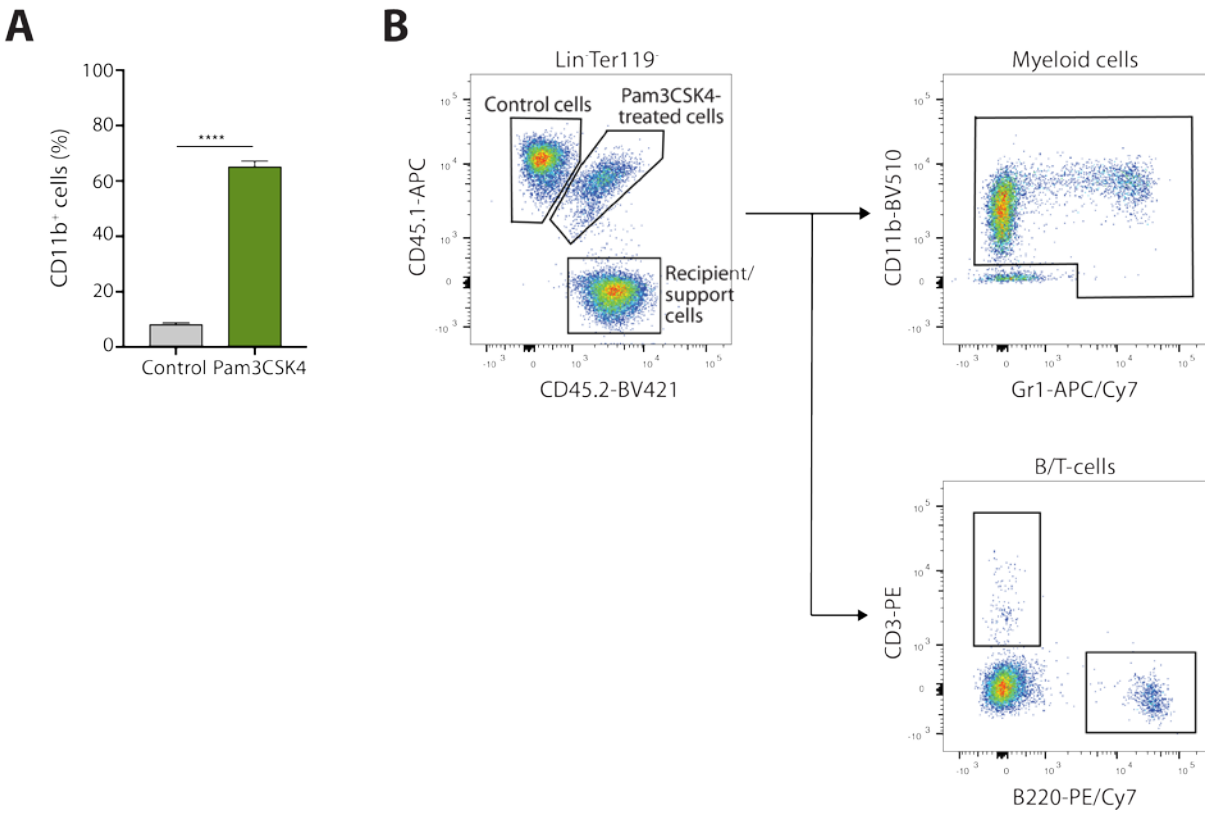

Figure S11. Competitive bone marrow repopulating experiment. (A) Percentage $CD11b^+$ cells from seeded LSK cells following 3 days of treatment with or without (control) Pam3CSK4 (n=5). (B) Sorted LSK cells were cultured for 3 days with ($CD45.1^+CD45.2^+$, Pam3CSK4 group) or without ($CD45.1^+$, control group) Pam3CSK4, then mixed and transplanted into lethally irradiated recipients, along with support cells from $CD45.2^+$ mice (12 mice per group). Shown is flow cytometric analysis of lineage distribution within donor cells in the peripheral blood 16 weeks post transplantation from a representative mouse. The arrows show how the gating was performed. **** $P<.0001$.

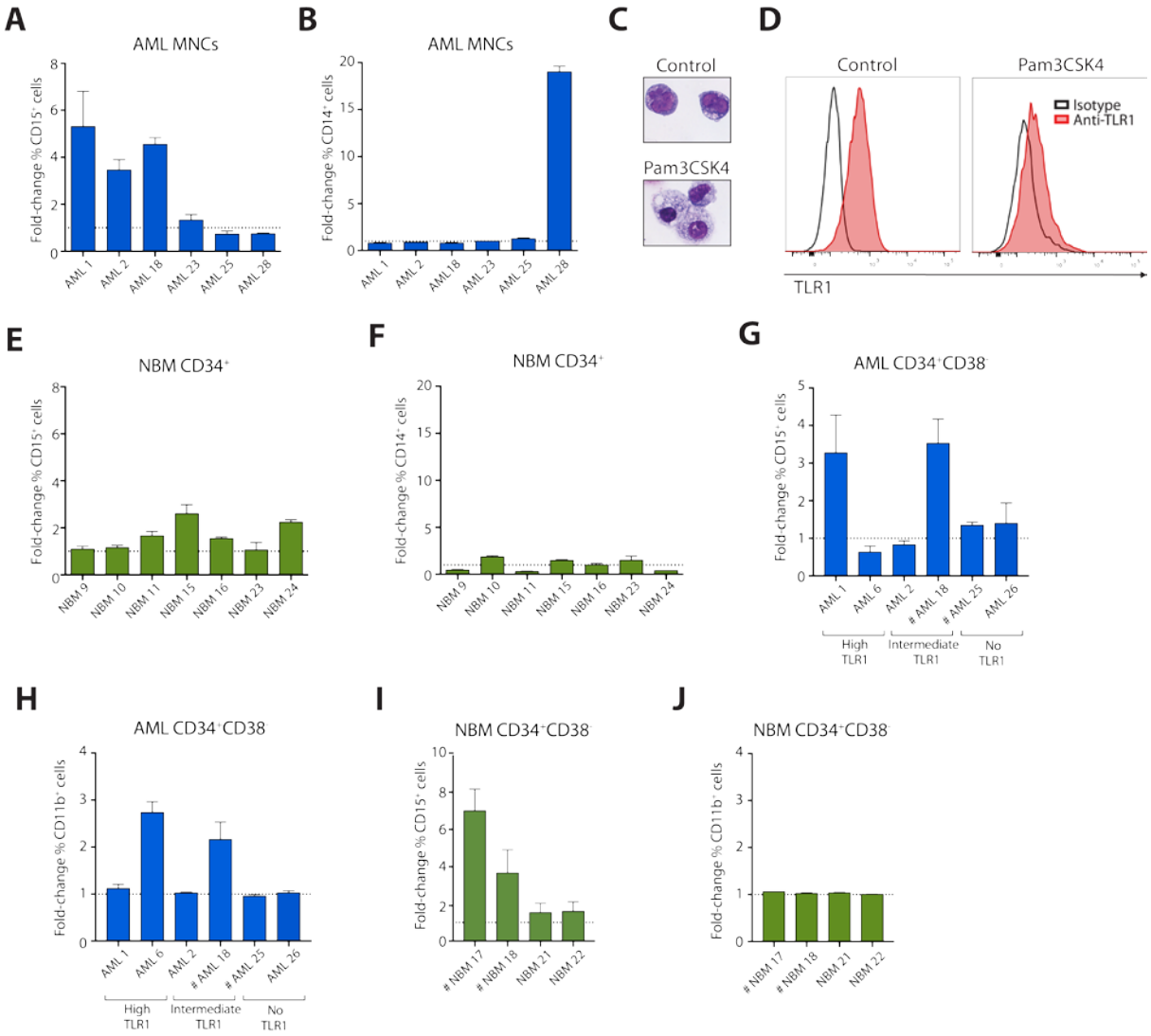

Figure S12. TLR1/TLR2 activation induces differentiation of primary human AML cells. Bulk MNCs or $CD34^+CD38^-$ cells from AML patients, and NBM $CD34^+$ or $CD34^+CD38^-$ cells were cultured for 3 days with or without (control) Pam3CSK4. Fold-change in the percentage of (A) $CD15^+$ and (B) $CD14^+$ Pam3CSK4-treated AML MNCs versus control. (C) May-Grünwald-Giemsa-stained cytospin slides from a representative AML patient sample of MNCs (40x magnification). (D) TLR1 expression on a representative sample of AML MNCs after culture with or without (control) Pam3CSK4. Histograms showing AML MNCs stained for TLR1 (red) and isotype control (black line). Fold-change in the percentage of (E) $CD15^+$ and (F) $CD14^+$ Pam3CSK4-treated NBM $CD34^+$ cells versus the controls. Fold-change in the percentage of (G) $CD15^+$ and (H) $CD11b^+$ Pam3CSK4-treated AML $CD34^+CD38^-$ cells, and fold-change in the percentage of (I) $CD15^+$ and (J) $CD11b^+$ NBM $CD34^+CD38^-$ cells versus the controls. Dotted lines indicate no change in the percentage of cells expressing specific markers. High/Intermediate/No TLR1 was grouped based on TLR1 expression in AML $CD34^+CD38^-$ cells (supplemental Table 1). Data shown is mean of triplicate tests for each sample except for #, indicating duplicate tests. Comparative statistical analysis between treatment and control was performed for the samples with triplicate tests only. Mean values and SDs are shown.

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