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Supplemental information

Bi-functional CpG-STAT3 decoy oligonucleotide triggers multilineage differentiation of acute myeloid leukemia in mice

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SUPPLEMENTAL METHODS

Global transcriptomics

RNA sequencing libraries were prepared with mRNA-HyperPrep kit (Kapa Biosystems). The final libraries were validated with the Agilent Bioanalyzer DNA High Sensitivity Kit and quantified with Qubit. Sequencing was performed on HiSeq 2500 (Illumina) with the single read mode of 51cycle. Real-time analysis (RTA) 2.2.38 software was used to process the image analysis. RNAseq reads were aligned to the mouse reference genome (mm10) using the TopHat (v.1.3.1) or STAR (v2.7.9) software. RNASEQ expression was quantified as reads per kilo base of total exon length per million mapped (RPKM) reads using Partek Genomics Suite v.6.12.0713/7.18.0723. Minimally expressed genes (<0.1 RPKM) were excluded. Gene Expression measurement was log2 transformed with 0.01 offset for differential gene expression analysis using ANOVA with FDR<0.05 and fold change >1.5.

Single cell transcriptomics

The libraries were sequenced with the paired end setting of 28 Read1 and 101 Read2 cycles, 10 index i7 and i5 cycles on NovaSeq 6000 platform (Illumina) using S4 Reagent kit v1.5 (Illumina). Raw sequencing data were processed using the 10x Genomics's Cell Ranger analysis pipeline (v.7.1.0) for sample demultiplexing, barcode processing, alignment with mm10 mouse genome, filtering, and UMI counting. The scRNA-seq data were analyzed using R Seurat Package (v4). The low quality cells are further filtered by three quality control filters: >10% mitochondrial reads, <5% ribosomal reads or <200 detected features. The doublets were identified and removed using DoubletFinder. The normalization and variance stabilization was performed with SCTransform (v2) with regressing out percentage of mitochondrial reads. 2,000 most variable genes were selected to calculate the first 40 principal components for the downstream analysis. The expression profiles were clustered using Leiden algorithm and visualized using two-dimensional UMAP. The cell type of each cell cluster was identified and annotated using known maker genes. The differentially expressed genes of each cell cluster were found with pseudo-bulk method using Deseq2. The pathway enrichment analysis was done using fGSEA with Kegg, Reactome and GO BP databases.

TCGA data analysis

The RNA sequencing data processed with STAR – Counts workflow and raw .idat files for DNA methylation data were downloaded were downloaded from The Cancer Genome Atlas with TCGAbiolinks package [10.1093/nar/gkv1507, 10.1093/nar/gkv1507]. This resulted in 193 and 147 cases for DNA methylation and RNA sequencing respectively. Variance stabilizing transformation from DESeq2 package was utilized for RNA sequencing counts normalization [10.1186/s13059-014-0550-8.]. The Noob background correction method with dye-bias normalization and removal of SNP loci and methylation probes with low detection p-values was performed in minfi package [10.1093/bioinformatics/btu049], which was also utilized to annotate probes. Correlations of mean promoter DNA methylation with gene expression and genes expression correlation with each other were tested with Kendall test. Information about CBFB-MYH11 fusion were downloaded from a study by Vadakekolathu et al. [10.1126/scitranslmed.aaz0463]; U-Mann-Whitney test was utilized to test for gene expression differences with regard to this feature.

Oligonucleotide sequences used for the generation of the inducible Stat3 and Irf8 shRNA cell lines

CMM-tetON-shStat3 and CMM-tetON-shIrf8 cells were generated by transducing parental CMM cells using tetON-shRNA/mCherry-expressing lentiviruses selected from 3-4 shRNA sequences. The shRNA variants selected for further in viivo studies were underlined:

Stat3 shRNA-1: 5' CCGGCCAGACCACTACTGAATATAACTCGAGTTATATTCAGTAGTGGTCTGG 3'
Stat3 shRNA-2: 5' CCGGTCAACAAATTAAGAAACTGGTTTGTGTAGCCAGTTTCTTAATTTGTTGAC 3'
Stat3 shRNA-3: 5' CCAACGACCTGCAGCAATATTCAAGAGATATTGCTGCAGGTCGTTGG 3'
Irf8 shRNA-1: 5' CCGGGAGGAGCTGATCAAGGAACCTCTCGAGAGGTTCCTTGATCAGCTCCTC 3'
Irf8 shRNA-2: 5' CCGGACCACCACCTGCCTTGAAGCTCGAGCTTCAAGGCAGGTGGTGG 3'
Irf8 shRNA-5: 5' CCGGACTCATTCTGGTGCAGGTACTCGAGTACCTGCACCAGAATGAG 3'

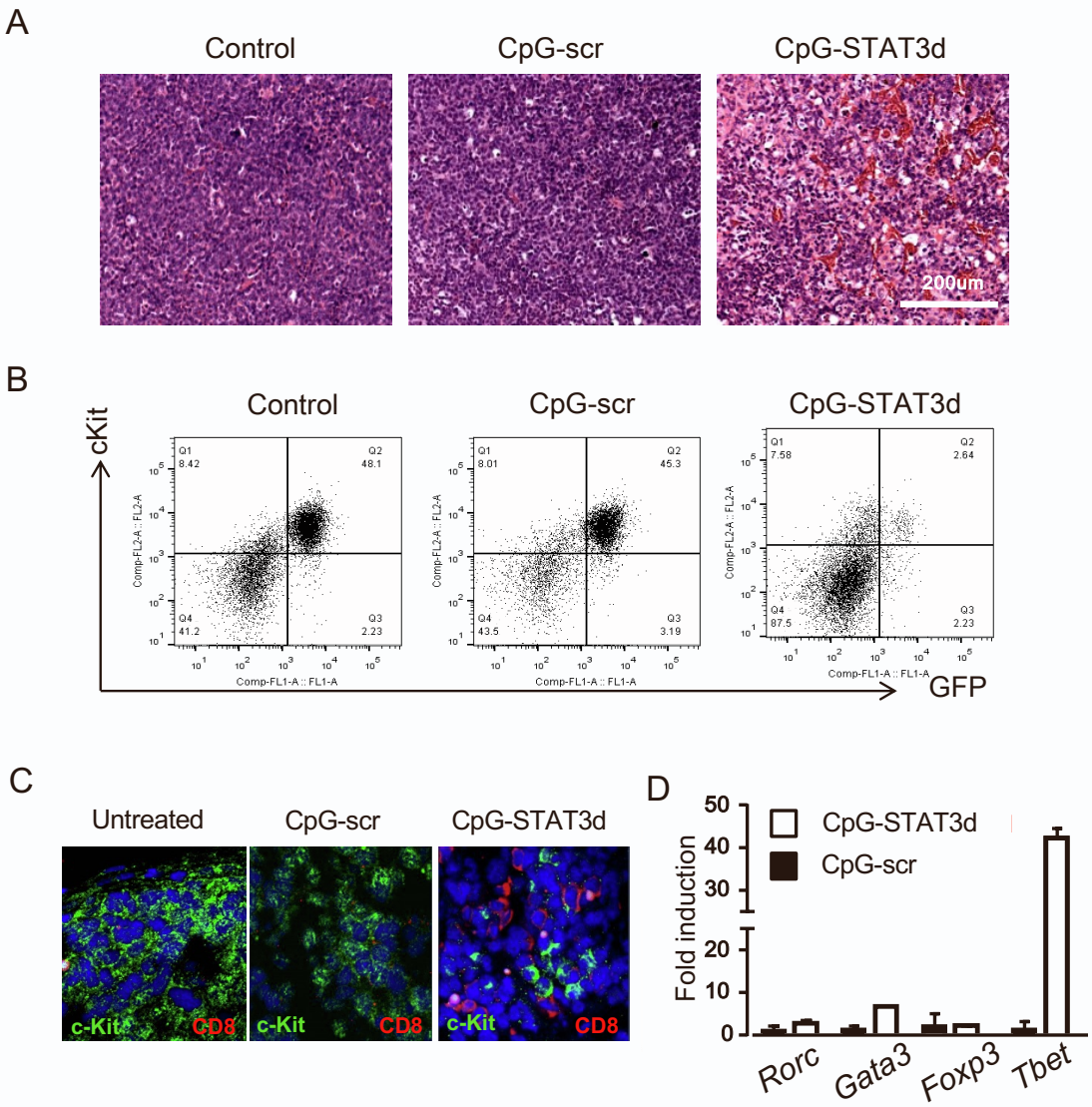

Figure S1. The in vivo activity of CpG-STAT3d oligonucleotide against *Cbfb/Myh11/Mpl* (CMM) leukemia in mice. (**A, B**) CpG-STAT3d oligonucleotide induces regression of bone marrow-localized CMM leukemia in mice. C57BL/6 mice were intravenously injected with $1x10^6$ leukemia cells. After tumors were established (1-2% of AML cells in blood), mice were treated IV using CpG-STAT3d, control CpG-scr oligonucleotides (5mg/kg) or PBS every other day for 6 times. Mice were euthanized one day after the last treatment to assess leukemia burden. (**A**) H&E staining of the fixed and decalcified tibia bone marrow; scale bar=200μm. (**B**) CpG-STAT3d but not CpG-scr oligonucleotides reduce the percentage of GFP+/c-Kit+ CMM leukemic cells. Shown are data representative for two independent experiments. (**C, D**) CpG-STAT3d upregulated expression of Th1-specific immune responses and CD8 T cell infiltration into spleens of leukemia-bearing mice. (**C**) Immunofluorescent staining of spleen sections indicating CD8 T cell infiltration to AML cell clusters in spleens from CpG-STAT3d- but not from control-treated mice. (**D**) The expression of transcription factors representing key CD4 T cell subsets was assessed using qPCR in total RNA samples isolated from spleens of CMM-bearing mice treated as indicated.

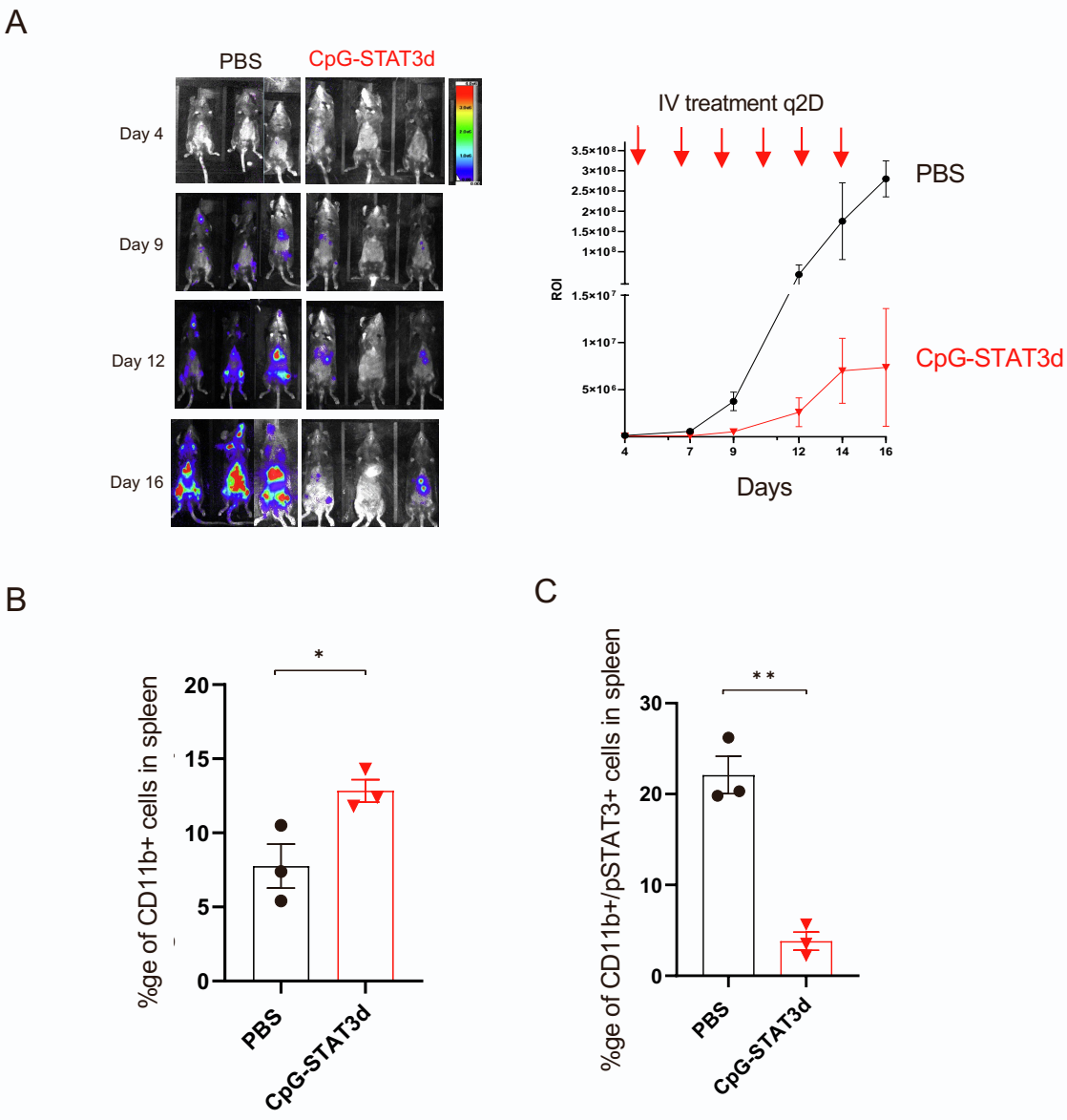

Figure S2. CpG-STAT3dODN inhibits progression of C1498 myelomonocytic leukemia in mice. (A) Whole body bioluminescent imaging of luciferase expressing C1498 AML progression in mice treated using IV injections of 5 mg/kg CpG-STAT3d or PBS control every other day for six times starting from day 4 after leukemia engraftment ($0.5x10^6$ C1498 cells/mouse). Left – representative images; right – bioluminescent signal quantification; means±SEM (*n*=3). (B) The expression of CD11b as a marker of myeloid cell differentiation and (C) the level of activated/tyrosine-phosphorylated STAT3 factor in myeloid cells in spleens from the treated mice. Spleens were harvested from all mice two weeks after treatment initiation (day 17 after tumor challenge) for cytofluorimetric analysis.

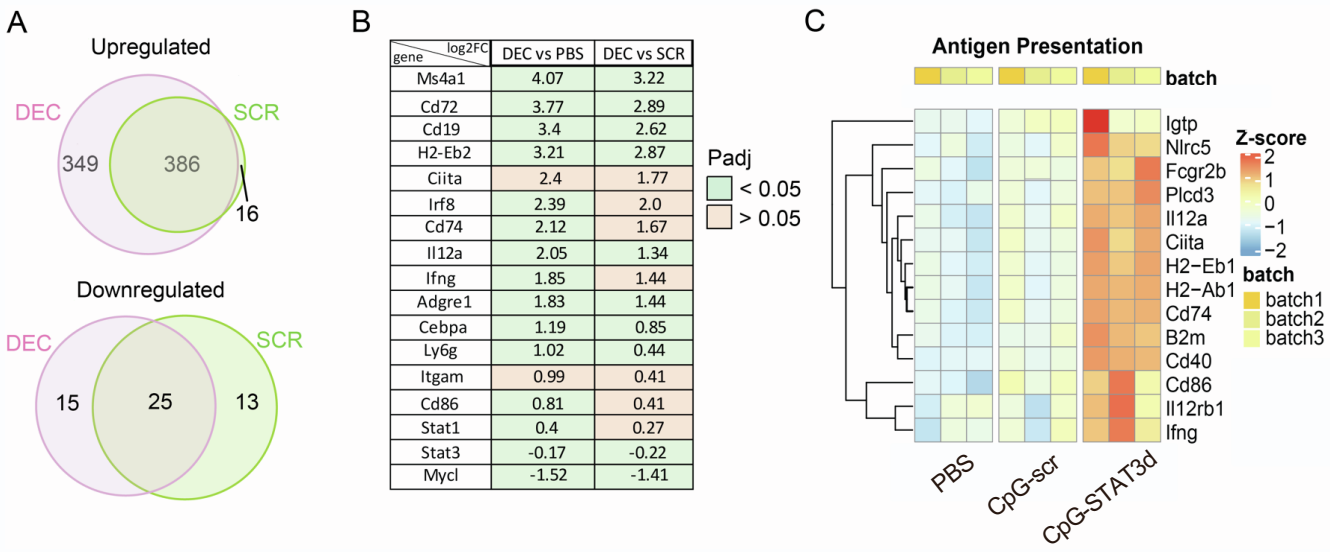

Figure S3. CpG-STAT3d shows unique gene regulation pattern including activation of antigen-presentation related targets compared to control CpG-scr oligonucleotide. AML-bearing mice were treated IV using CpG-STAT3dODN or control CpG-scrODN (5mg/kg) every other day for 3 times as in Figure 1A before RNAseq analysis on leukemic cells. (A) Venn diagrams indicate that nearly all (95%) of the genes upregulated in the CpG-scr were also upregulated in CpG-STAT3d ODN-treated cells. (B) Expression levels of key differentiation-related genes with log2FC values. (C) Heatmap of expression levels for antigen-presentation related genes.

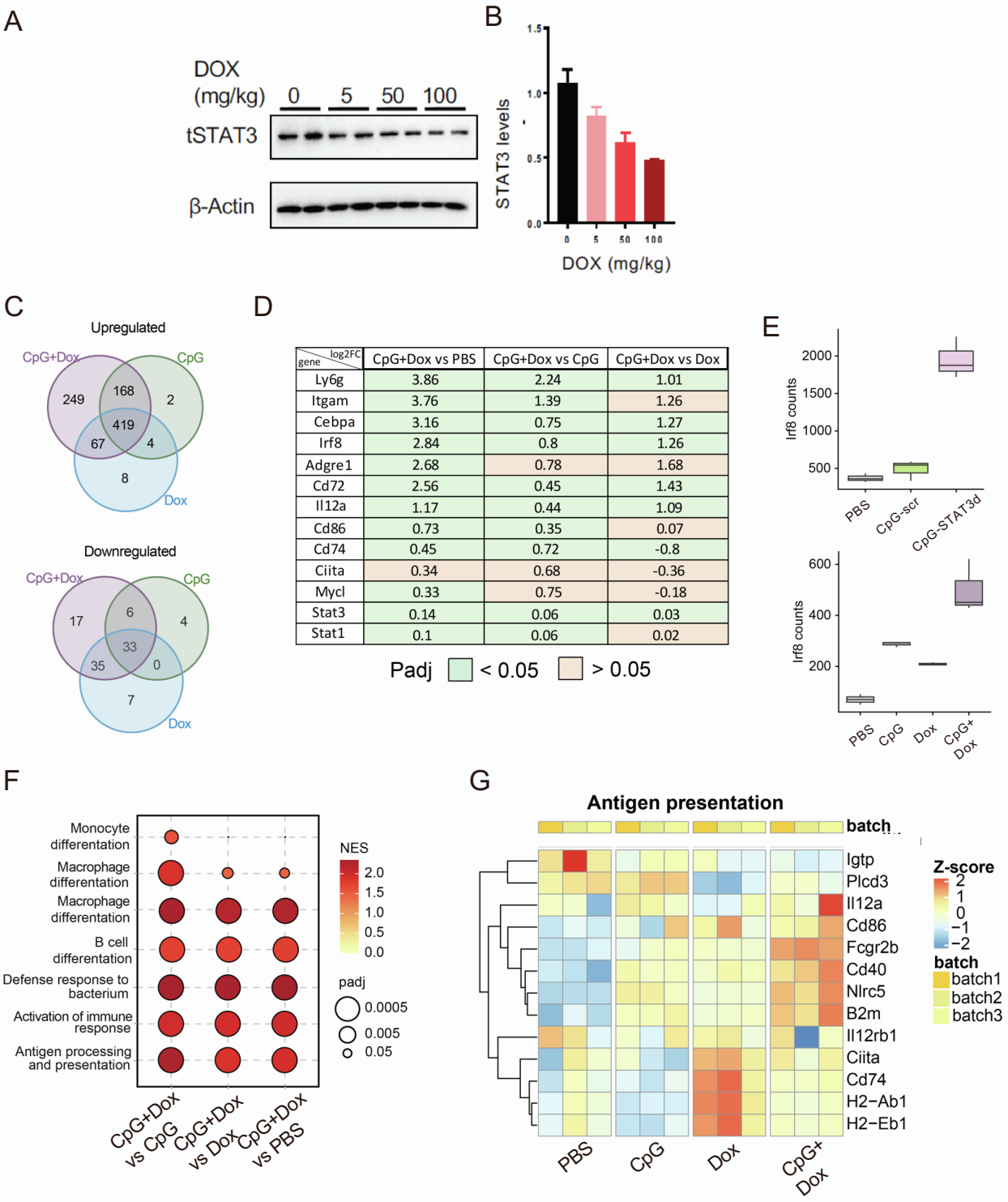

Figure S4. The combination of TLR9-activation and STAT3-inhibition is required to reprogram CMM leukemic cells towards myeloid cell differentiation and antigen presentation. CMM-*tetOn-shStat3* AML-bearing mice were treated IV using PBS, CpG alone, doxycycline (Dox) alone or the combination thereof every other day for 3 times as in Figure 2 before RNAseq analysis on leukemic cells. (A-B) Western blot (A) and quantification of total STAT3 protein levels with or without treatment with different Dox doses in doxycycline-inducible CMM-*tetON-shStat3* cells isolated from spleens. Shown are results from one of three independent experiments. (D) Comparison of *Irf8* expression in two compared RNAseq experiments using decoy oligonucleotide or gene silencing for STAT3 inhibition. (E) Venn diagrams indicate indicating numbers of up- or downregulated genes in the treated groups for the second RNAseq study with inducible STAT3 silencing. (F) The upregulated genes are linked with myeloid cell differentiation and inflammation markers, while the downregulated genes encompass leukemia-associated transcription factors and anti-inflammatory genes. (G-H) GSEA results (G) along with (H) heatmap to depict APC-related gene set expression levels.

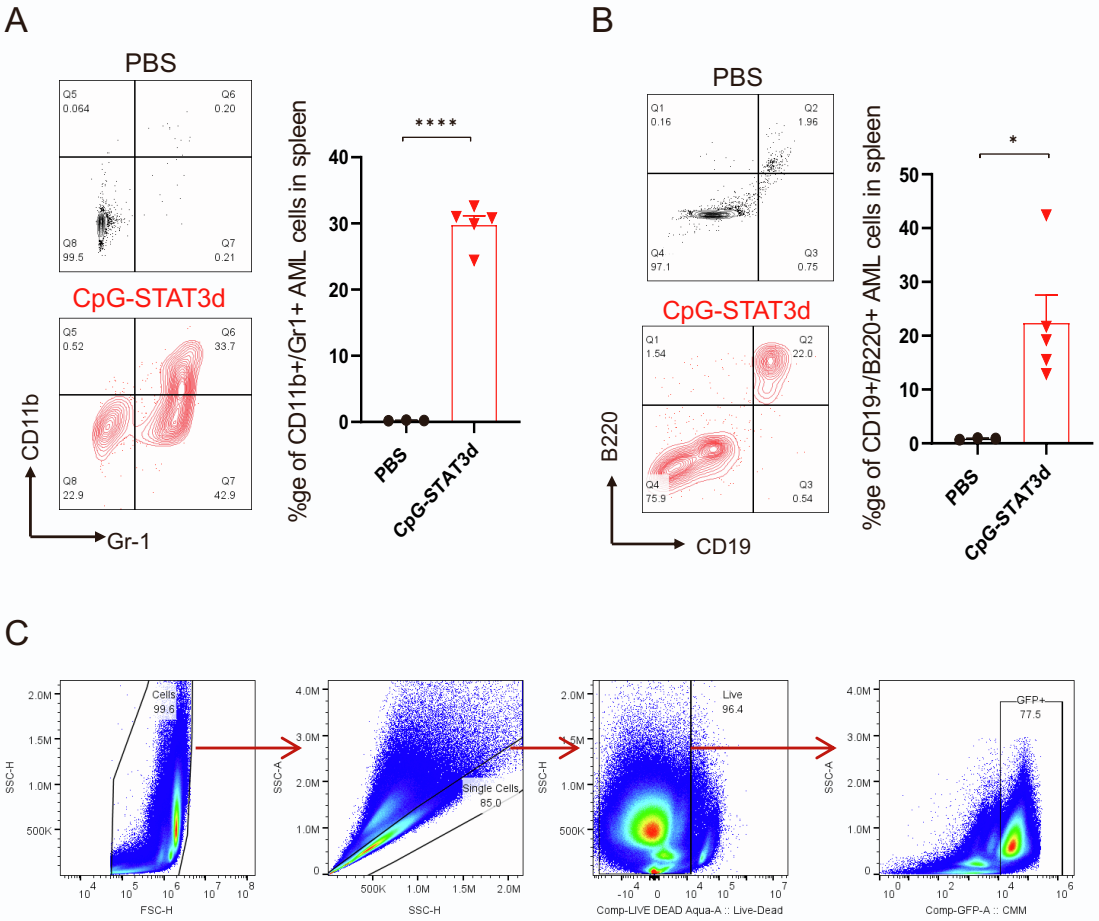

Figure S5. CpG-STAT3d-induced expression of multilineage differentiation markers on CMM leukemic cells. (A) The expression of CD11b and G1 to indicate AML-derived myeloid and granulocytic cell subsets among total population of splenocytes. (B) The expression of CD19 and B220 as B cell lineage markers using flow cytometry on a total population of splenocytes after treatment using CpG-STAT3d ODN for four times over 9 days. (C) Gating strategy used for the flow cytometric analysis of viable, single-cell, GFP-positive AML cells in spleens.

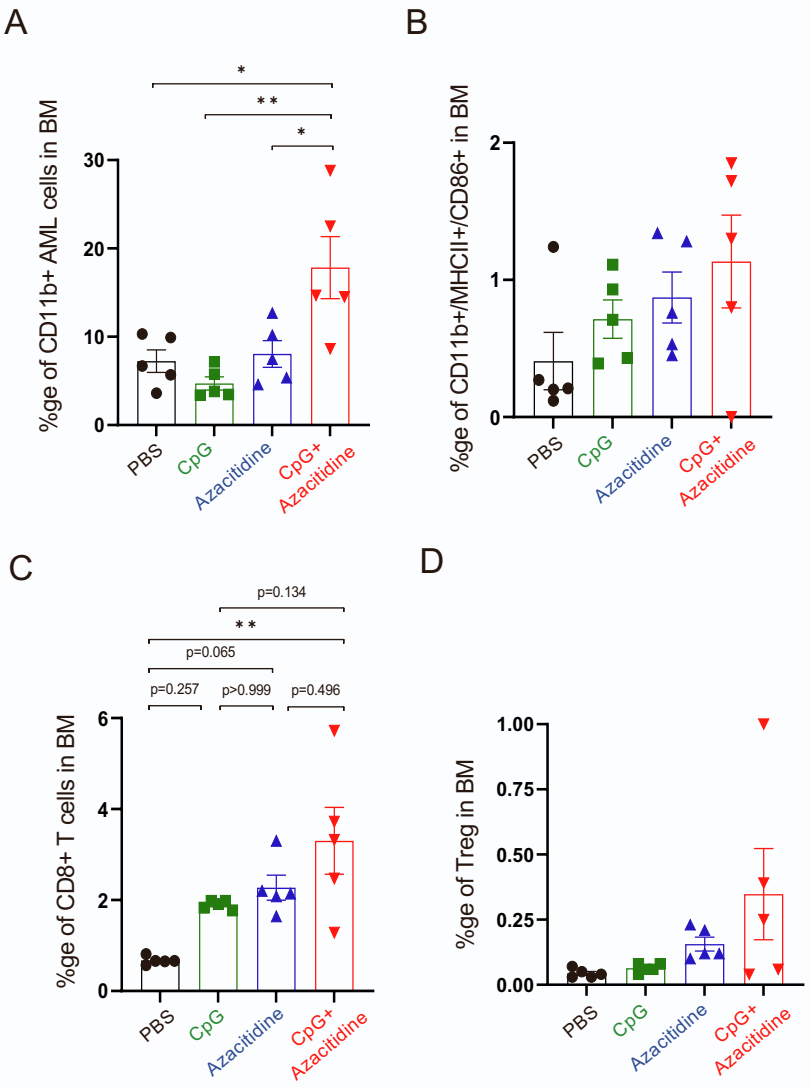

Figure S6. DNMT1 inhibition and CpG/TLR9 stimulation synergize to stimulate CMM cell differentiation and leukemia regression in mouse bone marrow. C57BL/6 mice with established, disseminated CMM leukemia were treated IV using azacitidine (1 mg/kg), CpG oligodeoxynucleotide (1 mg/kg), a combination thereof or PBS every day for 6 times. Two days after the last treatment, mice were euthanized and bone marrow was harvested to analyze AML cell differentiation (A) and maturation (B) as well as CD8 T cell (C) and regulatory CD4 T cell infiltration (D) using flow cytometry; shown are means-±SEM (*n*=5).