Supplementary Materials

METHODS

Oligonucleotide sequences

The phosphorothioated ODN and antisense strands (AS) of siRNAs were linked using 5 units of C3 carbon chain linker, $(CH_2)_3$ from Glen Research (Sterling, VA). The resulting constructs were hybridized with complementary sense strands (SS) of siRNAs to create chimeric ODN-siRNA constructs (deoxyribonucleotides are underlined). Sequences of single stranded constructs are listed below; the siRNA sequence is specific for mouse *Stat3* gene (NM_213659, bases 1898-1922).

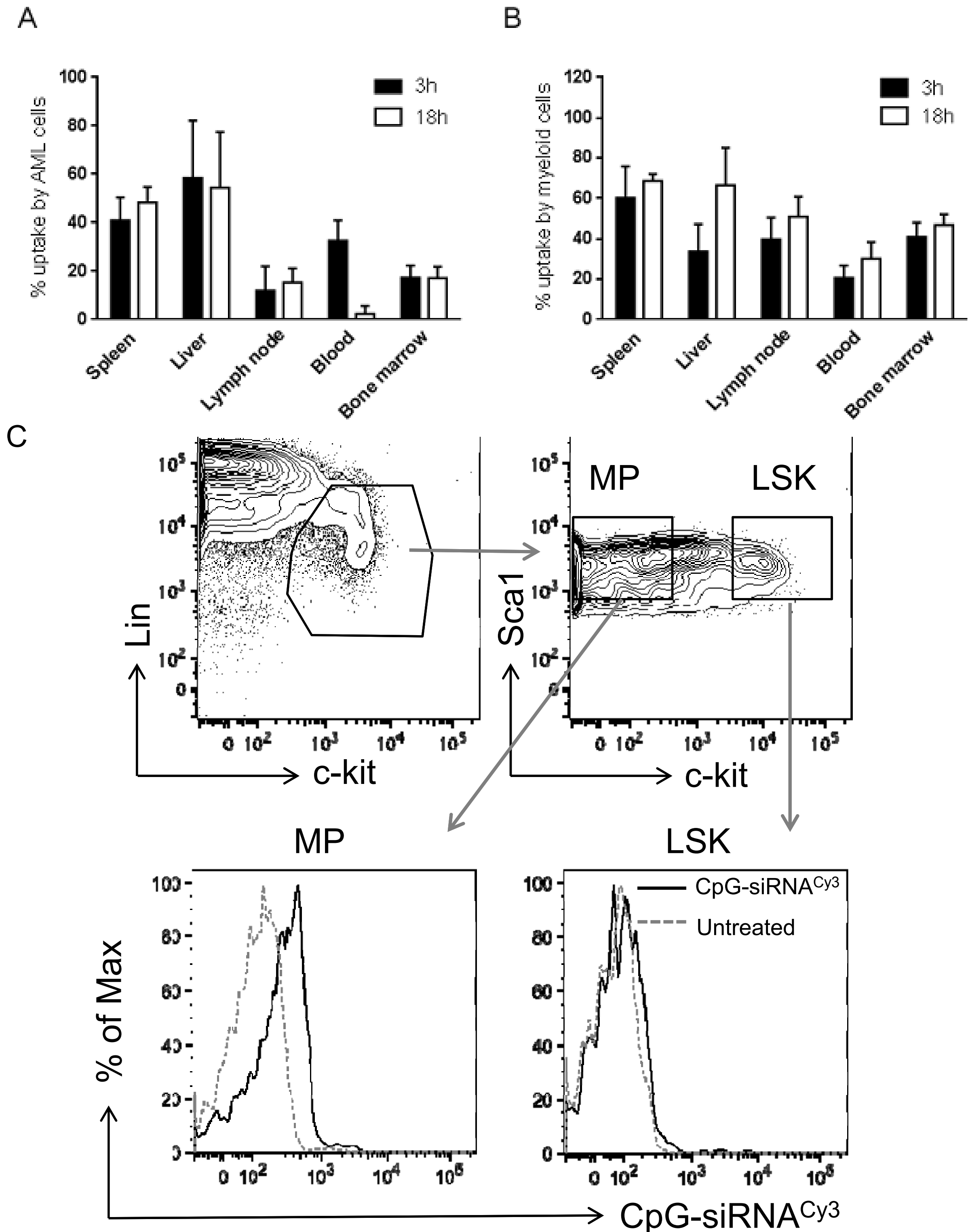
CpG1668-mouse Stat3 siRNA(AS):
5' TCCATGACGTTCCTGATGCT-linker-UUAGCCCAUGUGAUCUGACACCCUGAA 3'

Mouse Stat3 siRNA (SS):
5' CAGGGUGUCAGAUCACAUGGGCUAA 3'

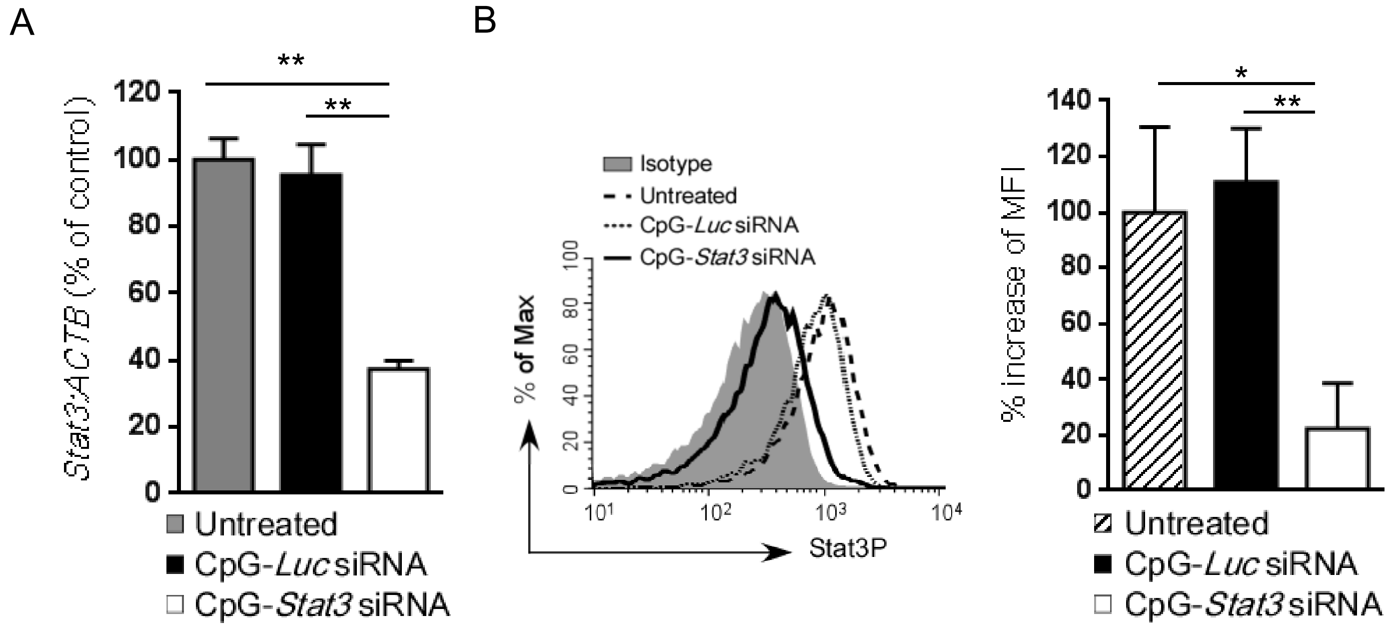
CpG-*Stat3* siRNA and *Stat3* siRNA were fluorescently labeled using Cy3 (Lumiprobe) at the 5' end of siRNA-SS. We have previously verified that modification of CpG-siRNA conjugates with fluorochromes such as Cy3 does not alter their internalization by target cells or gene silencing effect[28]. The sequence of firefly *luciferase*-specific 25/27mer siRNA (Luc1 R 25D/27) used for the CpG(1668)-*Luc* siRNA conjugate molecule was published before[18]. The formation of siRNA duplex was confirmed by electrophoresis in 15% polyacrylamide/7.5M urea gel. To improve serum stability, the second 3' position in the AS of siRNA was 2'OMe-modified (as indicated by double-underline) and then hybridized with complementary SS of *Stat3* siRNA creating CpG-siRNA duplex. All oligonucleotides were manufactured in the DNA/RNA Synthesis Core (COH).

Quantitative real-time PCR and protein assays

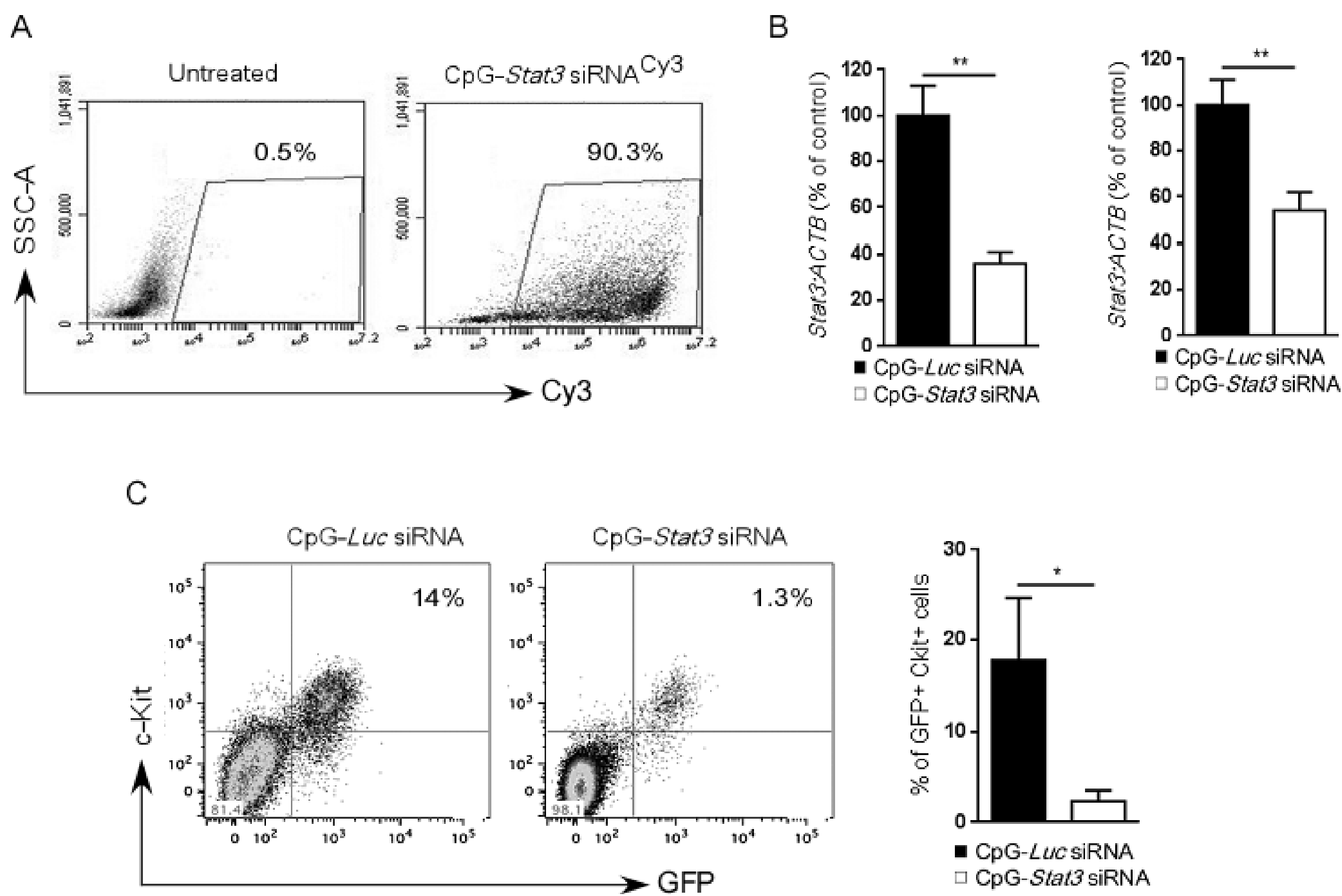
Total RNA isolation and cDNA synthesis were carried out as described previously[20]. Samples were analyzed using specific primers and probe sets (Roche), such as *Stat3*: 5'-GGAATAACGGTGAAGGTGCT-3',5'-CATGTCAAACGTGAGCGACT-3', UPL #25; *Tlr9*: 5'-GAATCCTCCATCTCCCAACAT-3', 5'-CCAGAGTCTCAGCCAGCACT-3', UPL #79; *ACTB* was detected using Reference Gene Assays (Roche) on CFX96 Real-Time PCR (Bio-Rad). Western blot to detect Stat3, Stat3P and β-actin expression was performed as described[24]. Plasma cytokines were analyzed using Bio-Plex arrays (Bio-Rad) at the Clinical Immunobiology Core (COH).



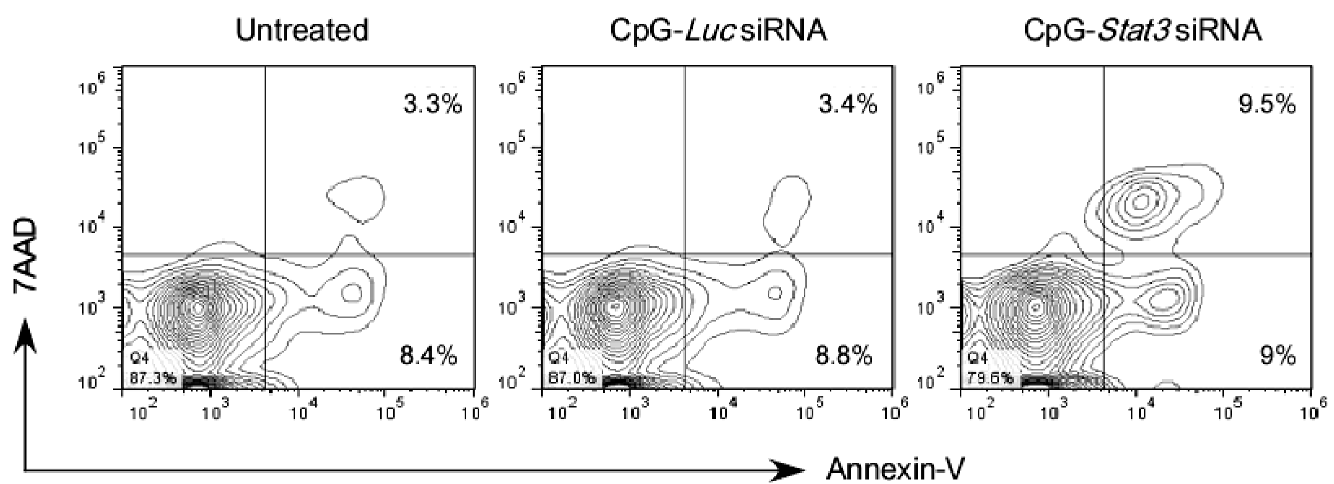
Supplementary Figure 1. CpG-*Stat3* siRNA is efficiently internalized by AML cells, non-malignant myeloid cells but not by hematopoietic stem cells. (A, B) *CMM*+ AML-bearing C57BL/6 mice (*n* = 6) were injected with a single dose of Cy3-labeled CpG-*Stat3* siRNA (5 mg/kg). The biodistribution of fluorescently labeled siRNA was assessed in the indicated organs at 3 or 18 hrs after injection using flow cytometry. The siRNA internalization by c-Kit$^+$/GFP$^+$ AML cells (A) and CD11b$^+$ myeloid immune cells (B) were analyzed and shown separately. Results are representative of two independent experiments. (C) CpG-siRNA^{Cy3} uptake by normal hematopoietic stem cells (LSK: Lin-/Sca1+/cKit+) and myeloid progenitors (MP). Freshly isolated bone marrow cells were incubated in 500 nM CpG-siRNA^{Cy3} for 4 h and analyzed using flow cytometry.



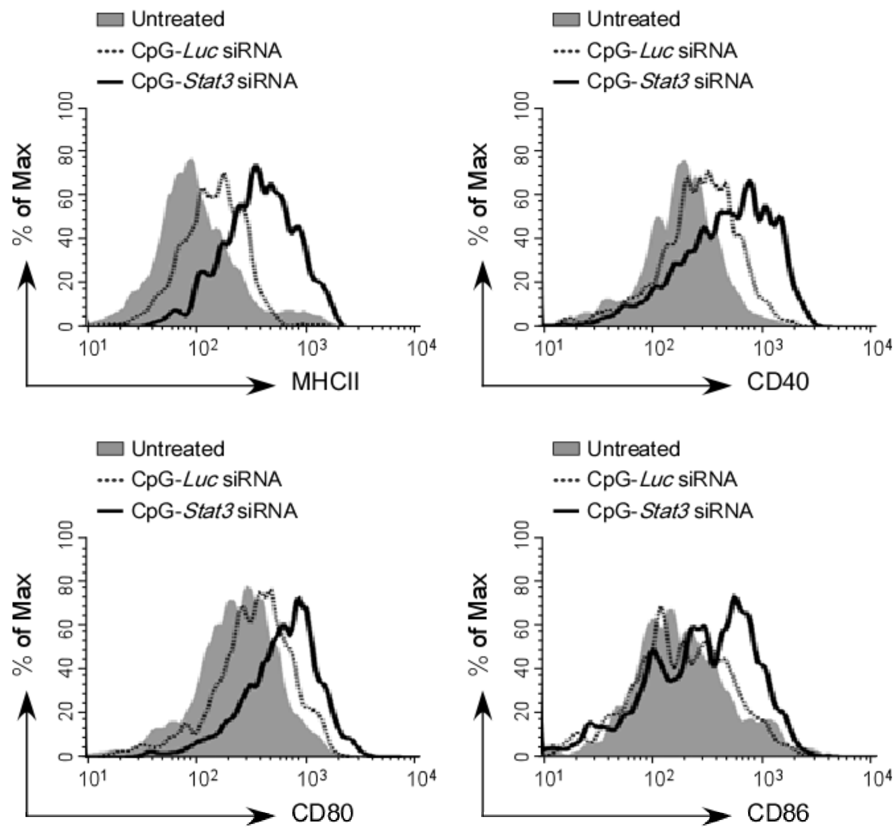
Supplementary Figure 2. CpG-*Stat3* siRNA treatment inhibits Stat3 expression and activity in AML cells *in vivo*. To verify the silencing effect of CpG-*Stat3* siRNA, AML-bearing mice were treated with CpG-*Stat3* siRNA as mentioned in Figure 1.D. (A) Then Stat3 mRNA level in isolated AML cells was measured by qPCR. (B) In the same experimental setup activation status of Stat3 was also measured using flow cytometry after intracellular staining with antibody specific to phospho-Stat3 (Tyr705). Representative histogram overlay data (left panel) and results presenting average mean fluorescent intensities (MFI) for all tested groups (right panel) are shown; means ± SEM (n = 6).

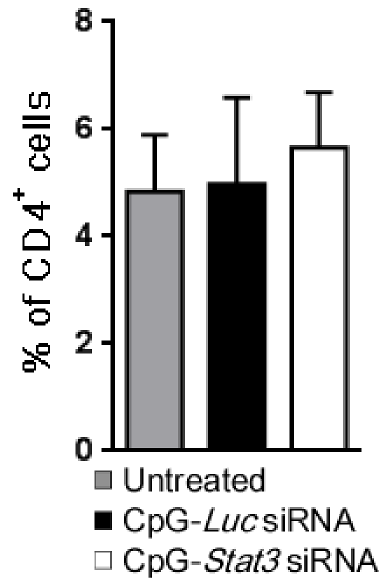


Supplementary Figure 3. CpG-*Stat3* siRNA treatment induces *Stat3* gene silencing and inhibits AML progression in 129S6 model. (A) The internalization of naked CpG-*Stat3* siRNA by *CMM*+ AML cells derived from 129S6 mice. AML cells were incubated with 500 nM of fluorescently labeled CpG-*Stat3* siRNA^{Cy3} conjugate [3] for 30 min without any transfection reagents. Percentages of Cy3+ AML cells were assessed by FACS. (B, C) 129S6 mice were inoculated with syngeneic *CMM*+ AML cells and treated with CpG-*Stat3* siRNA same as C57BL/6 mice, mentioned in Figure 2. (B) *Stat3* mRNA level in isolated AML cells from spleen (left) and bone marrow (right) were measured by qPCR. (C) The reduction in the percentage of circulating c-Kit+/GFP+ AML cells in mice treated using CpG-Stat3 siRNA compared to CpG-Luc siRNA control. Shown are meant ± SEM (*n* = 3).

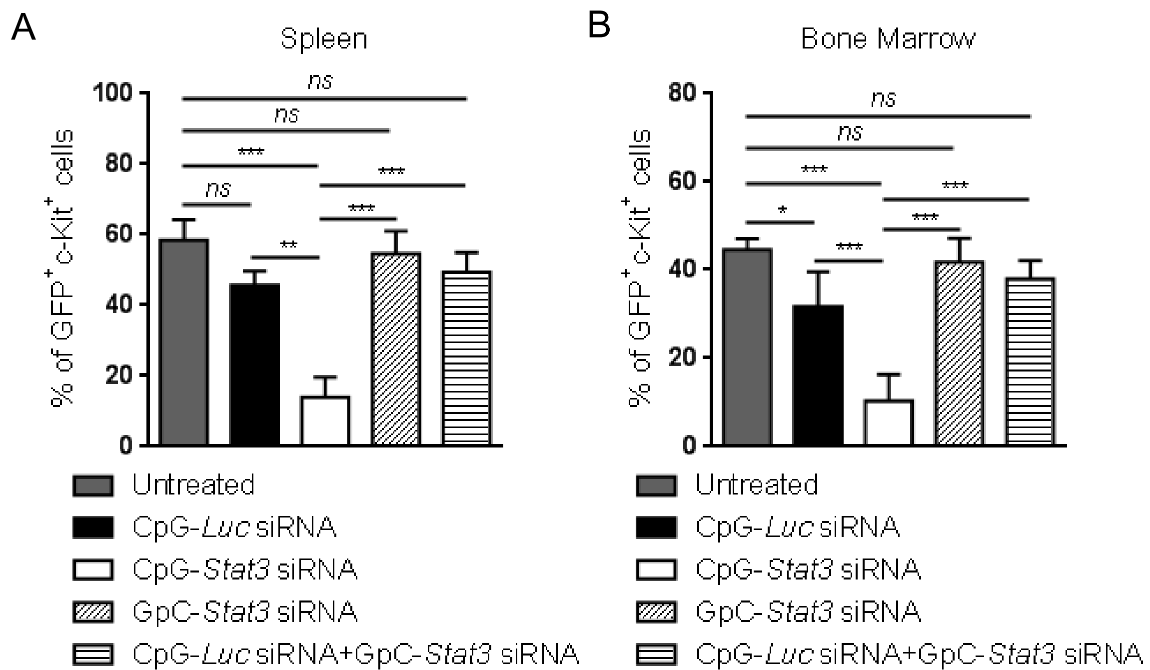


Supplementary Figure 4. Direct cytotoxic effect of CpG-*Stat3* siRNA on cultured *CMM*+ AML cells. Tumor cells were treated *in vitro* using 500 nM CpG-*Stat3* siRNA for 72 hrs. The percentage of cell apoptotic and/or necrotic cells was assessed by flow cytometry after staining for Annexin-V and 7AAD.

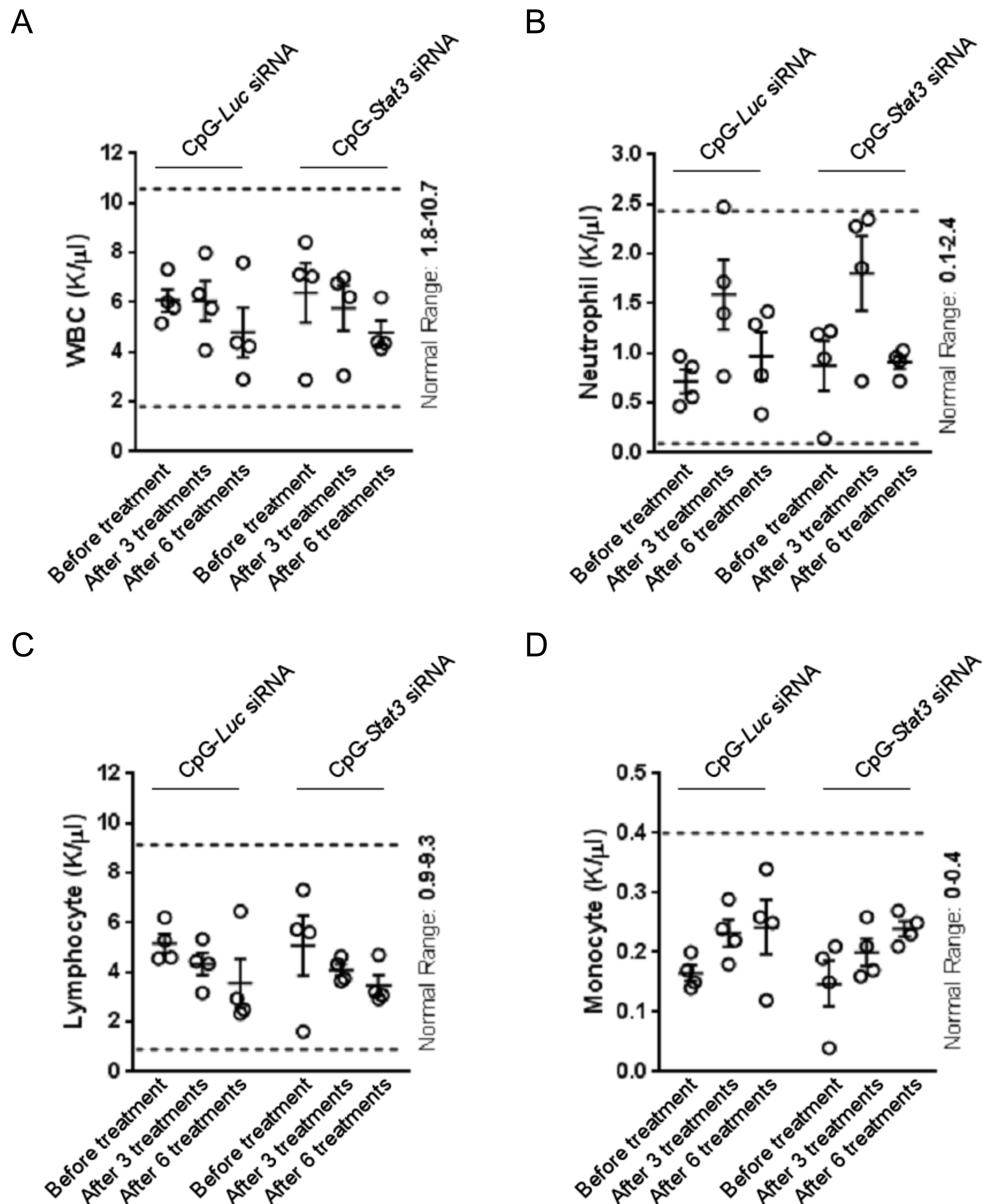




Supplementary Figure 5. CpG-*Stat3* siRNA treatment triggers immune activation of dendritic cells without affecting CD4⁺ T cell numbers in AML-bearing mice. Flow cytometric analysis of (A) MHCII, CD40, CD80 and CD86 expression on the surface of lymph-node CD11c⁺ splenic dendritic cells and (B) the percentage of total splenic CD4⁺ T cells after CpG-*Stat3* siRNA treatment compared to untreated or CpG-*Luc* siRNA treated mice. Mice were treated using CpG-siRNA as described in Figure 2.



Supplementary Figure 6. Antitumor effects of various control CpG- or GpC-siRNA conjugates *in vivo.* C57BL/6 mice were injected i.v. with 1×10^6 *CMM*$^+$ cells. After 2-3 weeks when tumors were engrafted (>1%, ranging 1-5% of AML cells in blood), mice were injected six times with 5 mg/kg of CpG- or GpC-siRNA constructs as indicated every other day and euthanized one day after last treatment. Flow cytometric analysis of GFP$^+$c-Kit$^+$ AML cells in spleen (**A**) and bone marrow (**B**) from various groups of mice. Shown are means ± SEM (5 mice/group).



Supplementary Figure 7. Effect of CpG-*Stat3* siRNA conjugates on leukocyte populations in naïve, tumor-free mice. Naïve C57BL/6 mice were injected six times using CpG-*Stat3* siRNA or control CpG-*Luc* siRNA (5 mg/kg) every other day. The percentages of WBCs (**A**), neutrophils (**B**), lymphocytes (**C**) and monocytes (**D**) were counted using Hemavet Hematology Analyzer before, after 3 and 6 injections of indicated conjugates. Shown are combined results from one experiment on 4 mice/group; means ±. SD; none of the treatment conditions led to statistically significant changes in cell counts.