Leukemia cell-targeted STAT3 silencing and TLR9-triggering generate systemic antitumor immunity

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Running title: CpG-Stat3 siRNA eradicates disseminated leukemia
KEY POINTS:

1. Blocking STAT3 in acute myeloid leukemia cells stimulates their TLR9-induced immunogenicity and antigen-specific activation of CD8+ T cells
2. Systemic delivery of CpG-Stat3 siRNA generates potent adaptive immune responses eradicating disseminated acute myeloid leukemia in vivo

ABSTRACT

Signal Transducer and Activator of Transcription-3 (STAT3) is an oncogene and immune checkpoint commonly activated in cancer cells and in tumor-associated immune cells. We previously developed an immunostimulatory strategy based on targeted Stat3 gene silencing in Toll-like Receptor (TLR9)-positive hematopoietic cells using CpG-siRNA conjugates. Here, we assessed therapeutic effect of systemic STAT3 blocking/TLR9 triggering in disseminated acute myeloid leukemia (AML). We used a model of mouse Cbfb/MYH11/Mpl-induced leukemia, which mimics human inv(16)AML. Our results demonstrate that intravenously delivered CpG-Stat3 siRNA, but not control oligonucleotides, can eradicate established AML and impair leukemia-initiating potential. These antitumor effects require host’s effector T cells but not TLR9-positive antigen-presenting cells. Instead, CpG-Stat3 siRNA has direct immunogenic effect on AML cells in vivo leading to upregulation of MHC class II, costimulatory molecules and proinflammatory mediators, such as IL-12, while downregulating expression of coinhibitory PD-L1 molecule. Systemic injections of CpG-Stat3 siRNA generate potent tumor antigen-specific immune responses, increase the ratio of tumor-infiltrating CD8+ T cells to Tregs in various organs and result in CD8+ T cell-dependent regression of leukemia. Our findings underscore the potential of using targeted STAT3 inhibition/TLR9 triggering to break tumor tolerance and induce potent immunity against AML and potentially other TLR9-positive blood cancers.
INTRODUCTION

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with poor long-term survival of the majority of patients undergoing current chemotherapies. The identification of leukemia-specific antigens and recent clinical advances in cancer immunotherapy underscore the potential for safer and more effective AML treatments. However, adoptive T cell transfer and vaccination strategies were so far hampered by the immunosuppressive tumor microenvironment. The immune tolerance in AML results from the accumulation of immature dendritic cells (DC), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) associated with high expression of Th2 cytokines (IL-4, IL-6, IL-10), TGFβ, or coinhibitory molecules, such as PD-L1. In addition, the myeloid cell-specific antigen-presentation and expression of proinflammatory cytokines/chemokines, such as IL-12, are downregulated in leukemia.

Similar to other blood cancers, AML patients show high frequency of STAT3 activation in leukemic blasts which correlates with decreased disease-free survival. STAT3 is plays a role in promoting AML cell proliferation and survival but whether it contributes to immune evasion was not clearly demonstrated. Earlier studies indicated that STAT3 activation is also common in many tumor-associated myeloid cell populations, which contribute to tumorigenesis. It is an attractive but challenging target for cancer therapy, as pharmacologic inhibition of non-enzymatic protein proved difficult. Targeting tyrosine kinases upstream from STAT3 using small molecule inhibitors of JAK, SRC, c-KIT and FLT3 provided an alternative strategy for AML therapy but therapeutic effects in most clinical trials were short-lived.

Growing evidence suggest that to generate long-lasting effects cancer immunotherapies need to alleviate tumor tolerance before jump-starting antitumor immune responses. We have previously shown that STAT3 activity in tumor-associated myeloid cells hampered the effect of locally administered CpG-oligodeoxyribonucleotide (ODN), a TLR9 ligand and clinically-relevant immunoadjuvant. These results provided a possible explanation for limited clinical efficacy of TLR9 agonists against human cancers, including AML. We later demonstrated that CpG-ODNs can be utilized for cell-specific siRNA delivery, as CpG-siRNA conjugate, to silence genes in mouse and
human TLR9-positive cells\textsuperscript{18-20}. Here, we assessed whether systemically administered CpG-Stat3 siRNA will generate antitumor effects against a genetic mouse model of *Cbfβ/MYH11/Mpl\textsuperscript{*} (CMM\textsuperscript{*})-induced leukemia, which closely mimics human AML with inv(16)(p13;q22) gene fusion in 10\% of patients.
METHODS

Cells and reagents

_Cbfb<sup>+</sup>/56M/Mx-Cre<sup>+</sup> _mice<sup>21</sup> were backcrossed to wild-type C57BL/6 mice for >10 generations to generate the syngeneic AML model. Two weeks after poly(I:C)-induced (Invivogen) expression of CBFβ-SMMHC, bone marrow cells from _Cbfb<sup>+</sup>/56M/Mx-Cre<sup>+</sup> _mice were transduced with retroviral MIG-Mpl vector encoding thrombopoietin receptor and GFP genes to generate transplantable _Cbfb-MYH11/Mpl<sup>+</sup> _mouse AML<sup>22</sup>.

C1498 AML cells expressing SIYRYYGL model peptide antigen (C1498.SIY), recognized by CD8<sup>+</sup> T cells in the context of K<sup>0</sup>, were generated recently<sup>23</sup>. The CpG-siRNAs were synthesized in the DNA/RNA Synthesis Core (COH) by linking CpG-ODN to _Stat3_ or _Luciferase_ siRNAs similarly as previously described (Supplementary Materials)<sup>18,20</sup>.

_in vivo experiments

C57BL/6 and NOD/SCID/IL-2R<sup>γ</sup> KO (NSG) mice (6-8 weeks old) were from the NCI (Frederick, MD) or from the Jackson Laboratory, respectively. TLR9KO mice were originally from S. Akira (Osaka University, Japan). Animal care/procedures were performed in accordance with established institutional guidance and approved protocols from the IACUC (COH). C57BL/6 mice were injected into lateral tail vein with 1×10<sup>6</sup> of _Cbfb-MYH11/Mpl<sup>+</sup> _AML cells in PBS. For CD8<sup>+</sup> T cell depletion, mice were injected i.p. with anti-CD8 antibody (200 μg) on day -6, -3 and 0 before tumor challenge and then twice weekly. Blood was drawn from the retro-orbital venous sinus to monitor the circulating c-Kit<sup>+</sup>/GFP<sup>+</sup> AML cells. After AML levels in blood exceeded 1% which corresponds to 10-20% of bone marrow-residing AML cells (Kuo, unpublished), mice were injected i.v. 6 times with various CpG-siRNAs (5mg/kg) every other day and euthanized a day after the last treatment.

Flow cytometry and immunohistochemistry

Single cell suspensions were prepared by mechanic tissue disruption and collagenase-D/DNase-I treatment as described<sup>24</sup>. The AML cell percentages were determined by GFP and c-Kit expression. For extracellular staining, cells were incubated with fluorochrome-labeled antibodies to MHC class II,
CD40, CD80, CD86, PDL1, CD3, CD4, CD8, CD69 and anti-FcγIII/IIR blocking (eBioscience). For intracellular staining, cells were fixed/permeabilized and stained with TLR9-specific antibodies (eBioscience), Stat3P or FoxP3 (BD) as described\textsuperscript{18}. Fluorescence data were analyzed on BD-AccuriC6 Flow Cytometer (BD) using FlowJo software (TreeStar). Immunohistochemical staining was performed on formalin-fixed/paraffin-embedded bone sections (5 μm) at the Pathology Core (COH).

**Quantitative real-time PCR and protein assays**

Total RNA isolation and cDNA synthesis were carried out as described previously\textsuperscript{20}. The sequences of the specific primers/probe sets are listed in the Supplementary Materials. Western blot to detect Stat3, Stat3P and β-actin expression was performed as described\textsuperscript{24}. Plasma cytokines were analyzed using Bio-Plex arrays (Bio-Rad) at the Clinical Immunobiology Core (COH).

**IFNγ ELISPOT assay**

C57BL/6 mice were injected i.v. with 10\textsuperscript{6} Cbfb/MYH11/Mpl\textsuperscript{+} or C1498.SIY cells. Mice with established tumors were treated with CpG-siRNAs every other day (6×) and euthanized next day. The IFNγ ELISPOT was performed following the manufacturer’s protocol (CellSciences) using 5×10\textsuperscript{5} splenocytes and irradiated (100 Gy) CMM\textsuperscript{+} AML (5×10\textsuperscript{4} cells/well) or SIY peptide (10 μg/ml).

**Statistical analysis**

One- or two-way ANOVA plus Bonferroni post-test were applied to assess statistical significance of differences between multiple treatment groups or in tumor growth kinetics between treatment groups, respectively. Data were analyzed using GraphPad Prism vs4.0 software (GraphPad).
RESULTS

Cbfb-MYH11/Mpl+ acute myeloid leukemia as a target for CpG-Stat3 siRNA

We previously demonstrated that blocking STAT3 signaling augments the efficacy of local TLR9-based immunotherapies against solid tumors\textsuperscript{15,18}. STAT3 is also commonly activated in the majority of human AML\textsuperscript{25}. To test the feasibility of using immunostimulatory CpG-Stat3 siRNA for AML therapy, we selected a transplantable mouse model derived from spontaneous leukemia induced by the co-expression of Cbfb-MYH11 fusion gene and the thrombopoietin receptor Mpl\textsuperscript{21,22}. Cbfb-MYH11/Mpl\textsuperscript{+} (CMM\textsuperscript{+}) leukemic cells are c-Kit-positive and were engineered to express GFP. CMM\textsuperscript{+} cells freshly isolated from C57BL/6 mice showed elevated levels of constitutively active STAT3 (Fig. 1A). As tumor cells of myeloid origin, CMM\textsuperscript{+} cells expressed TLR9 mRNA and protein at levels comparable to macrophages (Fig. 1B). Next, we determined whether CMM\textsuperscript{+} cells internalize CpG-Stat3 siRNA. Freshly isolated AML cells were incubated for various times and doses with CpG-Stat3 siRNA or unconjugated Stat3 siRNA without any transfection reagents. Both molecules were fluorescently-labeled to follow the intracellular siRNA uptake. Already at 30 min, over 80% of CMM\textsuperscript{+} AML cells internalized CpG-Stat3 siRNA (Fig. 1C, left). The conjugate uptake increased with longer incubation and peaked at 4 h. The maximum cell internalization required 250-500 nM concentrations (Fig. 1C, middle). In contrast, the unconjugated Stat3 siRNA was not significantly internalized even after 4 h incubation at 500 nM concentration (Fig. 1C, right). The rapid uptake limits exposure of the naked oligonucleotide to serum nucleases in the environment. Thus, we assessed the potential of using systemic delivery of CpG-Stat3 siRNA for leukemia immunotherapy. To assess \textit{in vivo} biodistribution of CpG-Stat3 siRNA, we used mice with CMM\textsuperscript{+} AML disseminated into various organs. Mice were injected \textit{i.v.} using a single dose of CpG-Stat3 siRNA\textsubscript{Cy3} (5mg/kg) and euthanized 3 or 18 h later. We assessed cellular distribution of Stat3 siRNA\textsubscript{Cy3} in c-Kit\textsuperscript{+}/GFP\textsuperscript{+} leukemic cells and in myeloid immune cells from various organs using flow cytometry. Our results confirmed that \textit{i.v.} injected CpG-Stat3 siRNA\textsubscript{Cy3} targeted both AML cells and normal myeloid immune cells in different organs (Supplementary Fig. 1). As expected for naked oligonucleotides, we observed the highest Stat3 siRNA\textsubscript{Cy3} uptake by AML (~40-50%) and non-malignant myeloid cells (~30-70%) localized in spleen
and liver. In addition, Stat3 siRNA<sup>Cy3</sup> was internalized by ~15-20% of AML cells and ~40-45% of macrophages in both bone marrow and lymph nodes. We also assessed uptake of CpG-siRNA<sup>Cy3</sup> by bone marrow myeloid progenitor and hematopoietic stem cells in naïve mice. Although, myeloid progenitor cells internalized low levels of siRNA<sup>Cy3</sup>, no siRNA uptake was detected in hematopoietic stem cells (Supplementary Fig. 1C). We also did not detect any effect of the CpG-Stat3 siRNA on non-malignant bone marrow cell viability. These results suggested that immunostimulatory CpG-siRNA are suitable for targeting of AML cells together with the tumor-associated myeloid cells with minimal effect on normal hematopoiesis.

**Systemic administration of immunostimulatory CpG-Stat3 siRNA reduces AML burden and improves mice survival**

We evaluated antitumor effects of systemic CpG-Stat3 siRNA injections into *Cbfb-MYH11/Mpl<sup>+</sup>* AML-bearing mice. Syngeneic recipients engrafted with *CMM<sup>+</sup>* AML were injected 6 times every other day using CpG-siRNA specific for Stat3 or Luciferase genes starting when c-Kit<sup>+/GFP<sup>+</sup></sup> AML cells became detectable in peripheral blood (day 7-10). The CpG-Luciferase siRNA was used as a non-targeting control to assess immunostimulatory effects of the conjugate in the absence of STAT3 inhibition. We confirmed that STAT3 expression was reduced at mRNA (Supplementary Fig. 2A) and protein levels in c-Kit<sup>+</sup> AML cells from spleens and/or bone marrows of CpG-Stat3 siRNA-treated mice but not in controls (Fig. 2A). Finally, the *in vivo* CpG-Stat3 siRNA treatment reduced STAT3 activity in splenic AML cells by ~80% as measured by flow cytometry (Supplementary Fig. 2B).

Next, we examined the antitumor effect of targeted Stat3 silencing in TLR9-positive cells in mice. Repeated *i.v.* administration of CpG-Stat3 siRNA reduced percentage of AML cells by ~70-80% in bone marrow, spleen, lymph nodes and in peripheral blood while the CpG-Luciferase siRNA led to ~30% AML reduction compared to untreated mice (Fig. 2B and Fig. 2C). No effect was observed using non-silencing/non-immunostimulatory control conjugate (Supplementary Fig. 6)<sup>18</sup>. The injections of CpG-Stat3 siRNA but not CpG-Luciferase siRNA also normalized splenic (Fig. 2D) and bone marrow cellularities (Fig. 2E). We further confirmed that CpG-Stat3 siRNA administration induces
comparable therapeutic effects against CMM+ AML tumors in mice on different genetic background (129S6) (Supplementary Fig. 3).

Long-term therapeutic effects in AML critically depend on the elimination of leukemia-initiating cells26. To assess the leukemia-initiating potential of CMM+ AML after CpG-Stat3 siRNA treatment, we performed serial transplantation. Briefly, bone marrow-resident c-Kit+GFP+ AML cells were enriched from mice treated for two weeks with various CpG-siRNAs (as in Figure 2), counted and injected into naïve secondary recipients. AML progression in secondary recipients was monitored detecting percentages of c-Kit+GFP+ cells in peripheral blood. Mice that received AML cells from CpG-Stat3 siRNA-treated donors showed significant delay in AML engraftment (Fig. 3A) and extended survival (Fig. 3B) compared to both control groups. Even though control CpG-Luciferase siRNA partially reduced AML burden in donor mice (Fig. 2), it did not reduce leukemia-initiating potential in secondary recipients (Fig. 3A). These results prompted us to evaluate effect of prolonged STAT3-inhibition/TLR9-activation. The repeated CpG-Stat3 siRNA administration twice weekly for over 7 weeks resulted in survival of 60% of mice while no mice survived in CpG-Luciferase siRNA-treated and untreated groups (Fig. 3C). Long term administration of CpG-Stat3 siRNA did not cause obvious adverse effects and/or immunotoxicity. Together, these data demonstrate that targeted STAT3-inhibition/TLR9-activation effectively impairs leukemia-initiating potential of CMM+ AML, thereby preventing tumor recurrence.

Anti-leukemic effects of CpG-Stat3 siRNA depend on intact immune system

We previously demonstrated that targeting STAT3 using CpG-siRNA can induce direct cytotoxic effects against human TLR9-positive hematologic malignancies in immunodeficient mice20. To verify whether intact immune system is required for antitumor effect of CpG-Stat3 siRNA against CMM+ AML, we engrafted leukemic cells into immunodeficient NSG mice. Mice with established tumors (day 7), were treated using 6 repeated i.v. injections of CpG-siRNAs as in Figure 2. Although, CpG-Stat3 siRNA significantly downregulated Stat3 expression in AML cells (Fig. 4A), it failed to restrain leukemia progression in NSG mice (Fig. 4B). These results indicate that direct cytotoxic effect of
STAT3-inhibition/TLR9-activation on CMM+ AML cells in vivo is negligible, corresponding to weak CpG-Stat3 siRNA cytotoxicity to leukemic cell in vitro (Supplementary Fig. 4). We previously observed that local administration of CpG-Stat3 siRNA generates immune responses against solid tumors through direct activation of host’s antigen-presenting cells which in turn stimulate effector T cells27. CD8+ T lymphocytes are not directly activated by CpG-Stat3 siRNA as they poorly internalize conjugates and do not express TLR918,27. Therefore, CpG-Stat3 siRNA administration should not generate anti-leukemic effects in Tlr9-deficient mice due to impaired siRNA processing/release in myeloid and B cell populations18,28. To verify this assumption, we transplanted CMM+ AML tumors in Tlr9−/− mice, treated them using CpG-Stat3 siRNA or CpG-Luciferase siRNA, as in Fig. 4a, to induce target gene silencing (Fig. 4C). Unexpectedly, Tlr9 ablation in target immune cells did not prevent potent anti-leukemic effects of STAT3 targeting (Fig. 4D). Repeated CpG-Stat3 siRNA treatments reduced tumor burden by ~75-80% in bone marrow, spleen and peripheral blood similarly as observed before in wild-type mice (Fig. 2C). Thus, therapeutic effect of targeted STAT3-inhibition/TLR9-activation relies on effector immune cells but it does not require host’s antigen-presenting cells.

**Targeted STAT3-inhibition/TLR9-activation augments immunogenicity of AML cells in vivo**

Earlier studies demonstrated that proinflammatory cytokines can induce in vitro differentiation and antigen-presenting functions of AML cells29,30. Therefore, we analyzed surface expression of antigen-presenting molecules on in vivo-treated leukemic cells. Wild-type mice with established CMM+ AML were injected using CpG-Stat3 siRNA, CpG-Luciferase siRNA or left untreated (as described in Fig. 4A). CpG-Stat3 siRNA treatment induced upregulation of MHC class II, CD40, CD80 and CD86 molecules on splenic GFP+ AML cells, while the immunostimulatory effect of CpG-Luciferase siRNA was insignificant (Fig. 5A). The levels of immunostimulatory molecules induced by STAT3 targeting were comparable between AML cells (GFP+) and normal DCs (GFP−) isolated from lymph nodes of the same mice (Supplementary Fig. 5A). In contrast, levels of a coinhibitory PD-L1 molecule, implicated in tumor immune tolerance, were significantly reduced in AML cells after Stat3 silencing.
We also found that mice from CpG-Stat3 siRNA-treated group but not control animals had highly elevated plasma levels of proinflammatory mediators, such as IFNγ, IL-12 and CXCL9/MIG (Fig. 5C) with concomitant reduction of Th2 cytokines, such as IL-4 and IL-6 (Fig. 5D). Finally, we evaluated whether the CpG-Stat3 siRNA-treated AML cells are capable of T cell activation. In vitro coculture of AML cells freshly isolated from CpG-siRNA-treated mice, with CD3+ T cells isolated from naïve mice, demonstrated the superior potential of CpG-Stat3 siRNA to induce AML-dependent T cell proliferation compared to CpG-Luciferase siRNA-treated or untreated mice (Fig. 5E).

**CpG-Stat3 siRNA increases the ratio of CD8+ effector to regulatory T cells *in vivo***

Next, we investigated whether increased AML immunogenicity in CpG-Stat3 siRNA-treated mice will induce CD8+ T cell infiltration into major leukemia reservoirs such as bone marrow and spleen. As shown in Figure 6A, the average percentage of CD8+ T cells in spleen and bone marrow increased ~5-fold in AML-bearing mice after two-weeks of CpG-Stat3 siRNA treatment (as in Fig. 2) compared to untreated control. The immunostimulatory but non-silencing CpG-Luciferase siRNA increased effector T cell numbers only slightly. Correspondingly, CpG-Stat3 siRNA and not CpG-Luciferase siRNA injections triggered CD8+ T cell activation as indicated by the CD69 upregulation (Fig. 6B). The generation of adaptive anti-cancer immune responses is known to correlate with the increased ratio of effector to regulatory T cells within the tumor. Thus, we evaluated the numbers of CD4+FoxP3+ Treg cells in mice treated for two weeks using CpG-siRNA as described above. Even though percentage of total CD4+ T cells did not significantly change (Supplementary Fig. 5B), we observed an average ~50% reduction in splenic Tregs (CD4+/FoxP3+) in AML-bearing mice after treatment using Stat3 targeting CpG-siRNA and not control conjugate compared to negative control (Fig. 6C). Altogether, these findings indicate that systemic CpG-Stat3 siRNA treatment improves the ratio of effector CD8+ T cells to Tregs thereby generating potent adaptive antitumor immunity in mice with disseminated leukemia.
Anti-leukemic effects of CpG-Stat3 siRNA depend on CD8+ T cell-mediated adaptive immunity

Based on the above results CD8+ T cells are likely implicated in anti-leukemic effects of targeted STAT3-inhibition/TLR9-activation. To corroborate these findings, we depleted CD8+ cells using specific antibodies in AML-bearing mice and then followed with 6 repeated injections of CpG-Stat3 siRNA. The anti-leukemic potential of CpG-Stat3 siRNA was reduced by ~80% in CD8+ T cell-depleted mice compared to controls, proving that effector T cells play key role in AML regression (Fig. 7A). We further assessed whether CD8+ T cell activation following in vivo CpG-Stat3 siRNA treatment is tumor antigen-specific. First, we used the irradiated CMM+ AML cells as a source of polyvalent tumor antigens for activation of splenic T cells isolated from CpG-siRNA-treated mice. ELISPOT assay detected significantly increased production of IFNγ in splenocytes derived from CpG-Stat3 siRNA-treated mice compared to untreated or control treated mice (Fig. 7B). Next, we assessed response to defined tumor antigen using an established AML model of C1498 cells expressing SIYRYYGL peptide31. Preliminary analysis confirmed that C1498.SIY cells are TLR9+ and STAT3P+ similar to CMM+ AML cells (Hossain-Kortylewski, unpublished). Mice with established C1498.SIY tumors were treated in vivo with CpG-Stat3 siRNA, control oligonucleotides or left untreated. Again, ELISPOT assays after the recall stimulation with SIYRYYGL peptide detected increased IFNγ production by T cells from CpG-Stat3 siRNA-treated mice compared to controls (Fig. 7C). Overall, these findings provide further evidence that myeloid cell-specific STAT3-inhibition/TLR9-activation generates tumor-antigen specific adaptive immune responses in AML-bearing mice.
DISCUSSION

We used CpG-Stat3 siRNA conjugate to test the effect of concurrent STAT3 inhibition and TLR9-dependent immunostimulation in mouse AML model\textsuperscript{16}. Our results demonstrate that STAT3 impairs antigen-presenting functions of both non-malignant and leukemic myeloid cells \textit{in vivo}. Moreover, STAT3 inhibition/TLR9 activation in AML cells alone is sufficient for generation of anti-tumor immunity. Dual-function CpG-Stat3 siRNA generated anti-tumor immune responses in two orthotopic AML models, \textit{CMM}\textsuperscript{+} and C1498, independently from mice genetic background. The repeated systemic administration of unformulated CpG-Stat3 siRNA generated effective CD8\textsuperscript{+} T cell-dependent immune responses, thereby leading to remission of disseminated AML in majority of mice. We reported previously that inhibition of STAT3 in non-malignant, tumor-associated myeloid cells triggers immune responses against solid tumors but rarely induced complete tumor regression\textsuperscript{15,18}. The elimination of leukemia after systemic CpG-Stat3 siRNA treatment underscores therapeutic potential of inducing AML cell immunogenicity and breaking tumor immune tolerance.

STAT3 gained recognition as a promising therapeutic target in AML for its oncogenic role in tumor cell proliferation and survival\textsuperscript{8,9}. It was also demonstrated that while driving AML cell proliferation STAT3 suppresses differentiation of leukemic cells\textsuperscript{33}. This is similar to the function of STAT3 in expansion of undifferentiated myeloid cells and inhibition of DC maturation\textsuperscript{34-37}. Our results demonstrate that CpG-Stat3 siRNA-induced leukemia regression primarily depends on AML cell differentiation to APC phenotype rather than on direct tumor cell killing. The potent antitumor immunity leads to systemic AML elimination even though penetration of leukemic cells by intravenously delivered CpG-Stat3 siRNA is incomplete. The resistance of \textit{CMM}\textsuperscript{+} cells to cytotoxic effects of STAT3 inhibition is likely a result of thrombopoietin receptor-induced survival signaling, which involves Jak2/STAT5, Akt and MAPK\textsuperscript{22}. Effective induction of direct tumoricidal effect may also require higher concentration and more frequent administration of CpG-siRNA to rapidly dividing tumor cells. We have previously demonstrated that daily intratumoral administration of CpG(A)-STAT3 siRNA into subcutaneously growing human AML tumors induced cancer cell apoptosis and inhibited tumor growth in immunodeficient NSG mice\textsuperscript{20}. Preliminary results from our \textit{in vitro} studies on primary
AML specimens indicate feasibility of using CpG(A)-STAT3 siRNA strategy to trigger immunogenicity of human leukemia (Hossain-Kortylewski, unpublished). Further studies in humanized mouse AML models will assess whether both enhanced immunogenicity of human AML cells and their decreased survival contribute to the potent therapeutic effect of CpG(A)-STAT3 siRNA.

Majority of AML cells are arrested at the early stage of myeloid cell differentiation, creating an opportunity to initiate and re-direct their maturation towards dendritic cells. Significant effort was dedicated to AML immunotherapies based on ex vivo generation of leukemic-DCs with ability to present leukemic antigens to T cells. However, vaccination strategies using leukemic-DCs did not induce sufficient clinical responses likely due to strongly immunosuppressive AML microenvironment. Our studies support the notion that STAT3 activation in leukemic cells plays a critical role in orchestrating immunosuppression in the AML, particularly through preventing leukemic cell differentiation to antigen-presenting cells. Blocking STAT3 together and TLR9 stimulation augmented expression of MHC class II and costimulatory molecules similarly as previously described in non-malignant myeloid cells, such as DCs. These effects were consistent with increase in plasma levels of proinflammatory mediators including IFNγ, IL-12 or CXCL9/MIG, which are induced by TLR9 activation in mature immune cells. The AML cells are also the likely source of IFNγ and IL-12 detectable at high levels in plasma of CpG-Stat3 siRNA-treated mice. Our earlier studies targeting STAT3 only in tumor-associated myeloid cells resulted in mostly local upregulation of proinflammatory cytokine expression. We observed moderate but significant reduction in surface expression of the coinhibitory PD-L1 molecule. PD-L1 is intensively studied molecule which inhibits antitumor immunity by direct binding to a receptor (PD-1) on activated T cells. Studies in mouse models of acute myeloid leukemia provided compelling that blocking PD-L1 can increase sensitivity of AML cells to antitumor T cell responses. Disrupting PD-1/PD-L1 interaction generated promising results in initial clinical trials in hematologic malignancies, including AML. STAT3 was previously shown to directly control PD-L1 expression in T cell lymphoma and later also in IL-6-induced tolerogenic APCs. In our study, the increased STAT3 activity in AML cells correlated with elevated plasma levels of IL-6, which is known as a hallmark of cancer-induced inflammations and a major
STAT3 activator\(^\text{47}\). High plasma levels of IL-6 are an unfavorable prognostic factor for AML patients’ survival\(^\text{48}\). Therefore, targeting STAT3 by CpG-siRNA approach likely interrupts the tolerogenic feed-forward loop, reducing both STAT3 activation and IL-6 production.

In addition to tolerogenic AML cells and dysfunctional DCs, regulatory T cell have important role in promoting immune tolerance in leukemia\(^\text{4}\). We demonstrate that systemic CpG-Stat3 siRNA treatment reduces Treg numbers, while increasing recruitment of activated CD8 T cells into major leukemia reservoirs, such as spleen and bone marrow. Increased ratio of tumor-infiltrating effector CD8 T cells to Tregs is an indicator of successful cancer immunotherapy correlated to tumor rejection\(^\text{32,49}\). This is likely an indirect effect of CpG-Stat3 siRNA-mediated induction of tumor-antigen specific responses, as confirmed in two independent AML models \textit{in vivo} using peptide antigen and whole irradiated tumor cells. Thus, our approach supports generation of polyvalent and multifaceted immune responses that are more likely to eliminate AML cells together with LSCs, which show selective expression of many leukemia-specific antigens\(^\text{1}\).

Our findings imply that systemic administration of naked CpG-siRNA conjugates avoided major pitfalls in application of TLR agonists as immunoadjuvants, namely desensitization/tolerization of immune cells after repeated TLR stimulation\(^\text{17}\). This is likely an effect of TLR9 triggering liberated from the negative effect of potentially tolerogenic STAT3 signaling\(^\text{15}\). At the same time, we did not observe autoimmune effects of long-term, over two-month CpG-Stat3 siRNA treatment. Due to its role for stem cell renewal, the potential risk of systemic STAT3 inhibition could be deregulation of hematopoiesis\(^\text{12}\). However, as verified in our control experiments CpG-Stat3 siRNA conjugates were not internalized by mouse hematopoietic stem cells and did not significantly affect leukocytes in tumor-free mice (Supplementary Fig. 7). Therefore, the myeloid cell-specific CpG-Stat3 siRNA strategy seems to overcome major challenges faced by pharmacologic inhibitors aimed at this signaling pathway. Further studies using AML models in humanized mice are necessary to evaluate the bi-functional, tumoricidal/immunostimulatory effect of CpG(A)-STAT3 siRNA on both leukemic and immune cells. The optimization of the CpG-siRNA serum stability by chemical modifications, conjugation to high molecular weight polymers or encapsulation should further enhance their
therapeutic efficacy. Altogether, these proof-of-principle studies indicate translational potential of CpG-STAT3 siRNA strategy for treatment of commonly TLR9-positive hematologic malignancies such as AML and potentially also multiple myeloma, B cell lymphoma or certain solid cancers\textsuperscript{20,50,51}. 
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AUTHORSHIP

Contribution: D.M.S.H., C.D-S., Q.Z., A.K., H.L., Y.H.K., M.K. performed research; P.S., A.J. were involved in conjugate design/testing; M.K., Y.H.K. designed experiments; J.K. contributed vital reagents; M.K., Y.H.K., S.F., R.B. analyzed the data; M.K. and D.M.S.H. wrote the manuscript.

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FIGURES

Figure 1. CpG-siRNA strategy allows for targeted delivery of Stat3 siRNA into TLR9-positive Cbfβ-MYH11/MPL+ leukemic cells. (A) Western blot analysis showing constitutive activation of Stat3 in CMM+ AML cells compared to untreated or IL-6-treated RAW264.7 macrophages; β-actin was used as internal loading control. (B) TLR9 expression in CMM+ AML cells. Tlr9 mRNA and protein were assessed using qPCR (left panel) and flow cytometry (right panel). RAW264.7 macrophages and CD3+ T cells were used as positive and negative controls, respectively. (C) Dose- and time-dependent internalization of naked CpG-Stat3 siRNA by CMM+ cells. Both molecules were labeled with Cy3 fluorochrome on the 5’ end of the siRNA (SS) to follow their intracellular uptake. AML cells were incubated with various concentrations of fluorescently labeled CpG-Stat3 siRNA Cy3 conjugate or unconjugated Stat3 siRNA Cy3 for indicated times without any transfection reagents. Percentages of Cy3+ AML cells were assessed by FACS.

Figure 2. Systemic CpG-Stat3 siRNA treatment induces regression of disseminated AML in mice. 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 CpG-Stat3 siRNA or control CpG-Luciferase siRNA (5 mg/kg) every other day and euthanized one day after last treatment. (A) Stat3 silencing in AML cells isolated from spleen (left panel) or bone marrow (right panel) was confirmed using western blotting. (B, C) CpG-Stat3 siRNA treatment reduces the percentage of AML cells in various organs. Flow cytometric analysis of GFP+c-Kit+ AML cells in bone marrows (B, top) or spleens (B, bottom) from various groups of mice. Shown are combined results from 6 mice/group; means ± SEM. Statistically significant differences between groups are indicated with asterisks as follows: ***, P<0.001 and *, P<0.05. (D, E) The effect of CpG-siRNA treatments on spleen (D) and bone marrow (E) cellularities. Shown are representative spleen sizes (D) or histochemical analysis of bone marrow structure (E) in indicated groups of mice. Similar results were derived from 3 independent experiments.
Figure 3. Targeting Stat3 reduces leukemia-initiating potential of *Cbfb-MYH11/Mpl* cells. (A, B) CMM⁺ AML cells were isolated from bone marrows of primary recipient mice treated using CpG-siRNAs (5 mg/kg) injected i.v. 6 times every other day or untreated as described in Figure 2. Magnetically enriched c-kit⁺ AML cells pooled from CpG-Stat3 siRNA, CpG-Luciferase siRNA or untreated mice were pooled, counted and identical cell numbers were injected into secondary recipient mice. (A) AML progression in all experimental groups was monitored weekly using flow cytometry to detect percentages of blood GFP⁺c-Kit⁺ AML cells in the peripheral blood. (B) Survival of mice injected with CpG-Stat3 siRNA-treated AML cells is significantly improved compared with mice that received untreated or CpG-Luciferase siRNA-treated AML cells. (C) Survival curve showing long term anti-tumor effect of systemic administration of CpG-Stat3 siRNA compared to untreated CpG-Luciferase siRNA treated group. Statistically significant differences between CpG-Stat3 siRNA- and CpG-Luciferase siRNA-treated or untreated groups are indicated by asterisks; *P* = 0.0002 and *P* < 0.0001, respectively. Shown are means ± SEM with *n* = 8 per group.

Figure 4. The anti-tumor effect of CpG-Stat3 siRNA is immune-mediated but does not depend on activation of host’s antigen-presenting cells. (A, B) NOD/SCID/IL-2RγKO or (C, D) Tlr9⁻/⁻ mice (*n* = 6) were challenged intravenously with 1×10⁶ CMM⁺ AML cells and treated using CpG-Stat3 siRNA, CpG-Luciferase siRNA or left untreated as in Figure 2. Stat3 gene silencing was confirmed by qPCR (A, C) and percentages of GFP⁺c-Kit⁺ AML cells in different organs were determined by flow cytometry (B, D). Shown are means ± SEM from two independent experiments. Statistically significant differences were indicated by asterisks as follows: ***, *P*<0.001; **, *P*<0.01 and *, *P*<0.05.

Figure 5. Enhanced expression of immune-stimulatory molecules on CpG-Stat3 siRNA treated AML cells in vivo. (A, B) AML bearing C57BL/6 mice were treated with CpG-Luciferase siRNA or CpG-Stat3 siRNA as in Figure 2. The surface expression of MHC class II and costimulatory molecules CD40, CD80, CD86 (A) or coinhibitory PD-L1 molecule (B) on splenic AML cells was assessed by flow cytometry. Shown are representative histogram overlays and bar graphs summarizing results.
from each group of mice (n = 6); mean ± SD. (C, D) Levels of different cytokines in blood plasma of mice treated as in A were quantified using Luminex assays. Shown are means ± SEM (n = 6). Statistically significant P values were indicated as follows: ***, P<0.001; **, P<0.01 and *, P<0.05. (E) In vivo pre-treated splenic AML cells were co-cultured in vitro with autologous T cell at 1:1 for 3 days. T cell proliferation was assessed using CFSE dilution assay. Shown are results representative for 3 independent experiments.

Figure 6. CpG-Stat3 siRNA treatment modifies balance between CD8 and Treg cells in AML mice. (A) Percentages of CD3+CD8+ T cells in the spleen (upper panel) and in the bone marrow (lower panel) of mice treated using CpG-Stat3 siRNA compared to untreated and CpG-Luciferase siRNA-treated groups assessed by flow cytometry. Mice were treated as described in Fig. 2, spleens and bone marrows were collected and analyzed using flow cytometry. (B, C) Representative contour plots and bar graphs showing percentages of activated CD8+CD69+ T cells (B) or CD4+FoxP3+ Treg cells (C) in spleens of AML bearing mice under the same experimental conditions. Flow cytometric analysis after extracellular (B) or intracellular (C) staining for the indicated markers using specific antibodies. Shown are means ± SD (n = 6). Statistically significant differences were indicated by asterisks as follows: ***, P<0.001; **, P<0.01 and *, P<0.05.

Figure 7. Antigen specific CD8+ T cells contribute to anti-leukemic effects of CpG-Stat3 siRNA. (A) CD8+ T cell depletion prevents the antitumor effect of CpG-Stat3 siRNA in vivo. C57BL/6 mice were injected i.p. with CD8-specific, control IgG antibodies or left untreated and then injected i.v. with 1×10^6 CMM+ AML cells. After tumors were established, both antibody-treated groups of mice were injected with CpG-Stat3 siRNA every other day for 6 times. Percentages of AML cells in blood, spleen and bone marrow were determined by flow cytometry. Shown are combined results from one experiment on 6 mice/group; means ± SEM (n = 6). (B) STAT3 targeting using CpG-siRNA results in tumor antigen-specific IFNγ production. Splenocytes from untreated, CpG-Luciferase siRNA- or CpG-Stat3 siRNA-treated mice were incubated overnight with irradiated (100 Gy) CMM+ AML cells.
Production of IFNγ was assessed by ELISPOT assay. Shown are representative images (top) and results combined from the group of 4-6 individual mice. (C) CpG-Stat3 siRNA augments recall response to SIY model tumor antigen. C57BL/6 mice were injected with 1×10^6 C1498.SIY cells i.v. and treated as described above. IFNγ ELISPOT assay was performed using splenocytes from 4-6 individual mice per each group after overnight restimulation with SIY-peptide. Shown are the representative results from two independent experiments; means ± SEM. Statistically significant differences were indicated by asterisks as follows: ***, P<0.001; **, P<0.01 and *, P<0.05.
Figure 1
Figure 2

Panel A: Immunoblot analysis showing the expression of Stat3 and β-actin in cells treated with Cpg-Luc siRNA and Cpg-Stat3 siRNA compared to untreated cells.

Panel B: Flow cytometry analysis showing the percentage of c-Kit+ cells in GFP+ populations for untreated and treated cells with Cpg-Luc siRNA and Cpg-Stat3 siRNA.

Panel C: Bar graphs showing the percentage of GFP+c-Kit+ cells in bone marrow, spleen, peripheral blood, and lymph node for untreated and treated cells with Cpg-Luc siRNA and Cpg-Stat3 siRNA.

Panel D: Diagram comparing the morphology of normal and treated cells.

Panel E: Micrographs depicting the histological appearance of untreated and treated cells with Cpg-Luc siRNA and Cpg-Stat3 siRNA.
Figure 3
Figure 4

A

B

Bone Marrow

Spleen

Peripheral Blood

C

D

Bone Marrow

Spleen

Peripheral Blood

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Figure 5
Figure 6
Leukemia cell-targeted STAT3 silencing and TLR9-triggering generate systemic antitumor immunity

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