TLR9-mediated siRNA delivery for targeting of normal and malignant human hematopoietic cells \textit{in vivo}

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\textbf{Running title}: STAT3 targeting in human TLR9+ blood cancers
KEY POINTS

• New CpG(A)-siRNA oligonucleotides allow for targeting genes specifically in human TLR9-positive immune cells and blood cancer cells.

• Tumoricidal and immunostimulatory properties of CpG(A)-STAT3 siRNA provide a novel therapeutic opportunity for hematologic malignancies.

ABSTRACT

STAT3 operates in both cancer cells and tumor-associated immune cells to promote cancer progression. As a transcription factor, it is a highly desirable but difficult target for pharmacological inhibition. We have recently shown that Toll-like receptor 9 (TLR9) agonists, CpG oligonucleotides, can be used for targeted siRNA delivery to mouse immune cells. Here, we demonstrate that similar strategy allows for target gene silencing in both normal and malignant human TLR9-positive hematopoietic cells in vivo. We have developed new human cell-specific CpG(A)-STAT3 siRNA conjugates capable of inducing TLR9-dependent gene silencing and activation of primary immune cells, such as myeloid DCs, plasmacytoid DCs and B cells in vitro. TLR9 is also expressed by several human hematologic malignancies, including B cell lymphoma (BCL), multiple myeloma (MM) and acute myeloid leukemia (AML). We further demonstrate that oncogenic proteins, such as STAT3 or BCL-XL, are effectively knocked-down by specific CpG(A)-siRNAs in TLR9-positive hematologic tumor cells in vivo. Targeting survival signaling using CpG(A)-siRNAs inhibits growth of several xenotransplanted AML and MM tumors. Importantly, CpG(A)-STAT3 siRNA is immunostimulatory and non-toxic for normal human leukocytes in vitro. Our findings indicate the potential of utilizing tumoricidal/immunostimulatory CpG-siRNA oligonucleotides as a novel two-pronged therapeutic strategy for hematologic malignancies.
INTRODUCTION

The proliferation and survival of the majority of hematologic cancers depends on constitutive activity of STAT transcription factors\(^1,2\). The first evidence linking STAT activity with human blood cancer was derived from studies on multiple myeloma (MM). Permanent activity of STAT3 observed in myeloma cells was critical for their survival due to upregulation of antiapoptotic BCL-X\(_i\) protein\(^3\). Later reports identified constitutive activation of STAT3 not only in myeloma but also in other hematologic malignancies, with the highest frequency in B cell lymphomas (BCL) and in acute myeloid leukemia (AML) patients' blasts\(^1,4,5\). The presence of activated STAT3 in leukemic blasts was associated with decreased disease-free survival of AML patients\(^4\). As a point of convergence for downstream signaling from cytokine and growth factor receptors, STAT3 plays a critical role in mediating cross talk within the tumor microenvironment, which promotes tumor immune tolerance, vascularization and metastasis\(^6\). Because STAT3 operates in both cancer cells and non-malignant tumor-associated cells, it represents a highly desirable target for cancer therapy\(^6\). These important findings instigated numerous attempts to develop STAT3 inhibitors, however pharmacological inhibition of a protein lacking enzymatic activity is challenging\(^4,7\). An additional complication is the close structural similarity between oncogenic STAT3 and functionally distinct STAT1, a transcriptional factor critical for generation of antitumor immunity by interferons\(^8,9\). The inhibitors of tyrosine kinases upstream from STAT3, such as JAK, SRC, c-KIT and FLT3 in leukemia, gained attention as promising blood cancer therapeutics\(^4\). However, the effect of small molecule drugs, including FLT3 inhibitors, in most clinical trials was short-lived\(^10,11\). Other conventional treatment regimens for hematologic malignancies are limited by the high toxicity to normal tissues, development of drug resistance and low disease-free survival rates\(^12\).

The emergence of therapeutic strategies based on RNA interference (RNAi) created a unique opportunity to silence any disease-related target gene\(^13,14\). The major obstacle in the clinical application of RNAi is targeted siRNA delivery into cells of interest\(^15,16\) and sensitivity of the immune system to stimulation by nucleic acids\(^17\). However, immune cells may themselves be essential therapeutic targets in cancer therapy\(^6,18,19\). We have recently demonstrated that ligands for
intracellular receptors, such as TLR9, can be used as targeting moieties for cell-specific siRNA delivery\textsuperscript{20}. Chemically synthesized CpG-siRNA molecules, generated by linking siRNA to a CpG oligodeoxyribonucleotide (ODN), targeted and silenced genes specifically in mouse TLR9-positive immune cells including dendritic cells (DCs), macrophages and B cells \textit{in vitro} and \textit{in vivo}\textsuperscript{20,21}. We demonstrated that CpG-Stat3 siRNA treatment disrupted immunosuppressive signaling network in several solid tumor models resulting in a potent antitumor immunity in mice\textsuperscript{20,22}.

In contrast to the mouse system, expression of human TLR9 in steady state is mostly limited to DCs although it can become upregulated under inflammatory conditions\textsuperscript{23,24}. TLR9 is commonly expressed in many hematologic malignancies, including AML, MM and BCL\textsuperscript{25-28}. Activation of TLR9 was shown either to enhance antigen-presenting functions or to induce apoptosis of primary malignant B cells\textsuperscript{27,29}. TLR9 agonists have been tested in numerous clinical trials as anticancer reagents for treatment of hematologic malignancies including BCL, MM and AML\textsuperscript{29,30}. They were proven safe and well-tolerated by patients and did not seem to induce adverse effects such as tumor cell proliferation and survival reported in some \textit{in vitro} studies\textsuperscript{25,27,29,31}. However, the TLR9 agonists used as single agents or even combined with vaccinations failed to overcome strongly immunosuppressive tumor environment in cancer patients\textsuperscript{29,32}. We have previously shown that STAT3 is an important negative feedback regulator which restricts immunostimulatory effects of several TLRs, including TLR9\textsuperscript{33}. The elimination of STAT3 combined with TLR9 triggering was shown to induce potent immunostimulatory effects in mice\textsuperscript{20}. These results underscore the need for combining TLR9 agonists with treatment strategies disrupting immunosuppressive effects of the tumor microenvironment. As recently demonstrated by Brody \textit{et al}. in a phase I/II study, local radiotherapy combined with intratumoral injections of CpG ODN succeeded in activation of tumor-specific memory T cells in lymphoma patients\textsuperscript{30}.

Tumor cells of hematopoietic origin pose special problems for non-viral siRNA delivery due to their low transfection efficiency\textsuperscript{34}. Here, we demonstrate that TLR9-mediated siRNA delivery using type-A CpG ODN linked to siRNA molecules induces target gene silencing in normal human immune cells and in malignant hematopoietic cells. CpG(A)-siRNA conjugates can overcome the limitations of
small molecule drugs by expanding the list of therapeutic targets in TLR9-positive hematologic malignancies to crucial yet currently non-druggable molecules. Our TLR9 cell-specific siRNA delivery approach is particularly suited for targeting STAT3, which functions both in cancer cells and in tumor-associated immune cells.
MATERIALS AND METHODS

Cell culture

Human RPMI8226, KMS-11, U266 myeloma and mouse A20 lymphoma cells were purchased from ATCC. Human KG1a, MonoMac1, MonoMac6, MOLM13, MV4-11, THP1 and TF1 leukemic cell lines were obtained from Dr. D. Tenen laboratory (Beth Israel Deaconess Medical Center, MA). The primary leukemic samples were received from Dr. Ravi Bhatia (City of Hope) with written informed consent of all patients. Sample acquisition was approved by the CoH Institutional Review Board in accordance with the Declaration of Helsinki. The normal PBMCs were derived from anonymous donors by the Clinical Immunobiology Correlative Studies Laboratory (CICSL) at City of Hope or purchased from CTL (Shaker Height, OH). PBMC-derived monocytes were cultured for 5 days in 12-well cell-culture plates using RPMI 1640 medium containing 10% FBS together with GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) or Flt3-L (100 ng/ml) to generate myeloid (CD1c+) or plasmacytoid (CD303+) DCs, respectively.

Oligonucleotide design and synthesis

The sequences of mouse cell-specific CpG1668(B)-siRNAs were reported before\textsuperscript{20}. To generate human cell-specific CpG-siRNAs, the D19 ODN were linked to antisense strands (AS) of siRNAs using 5 units of C3 carbon chain, (CH\textsubscript{2})\textsubscript{3} (Glen Research, VA). The resulting constructs were hybridized to complementary siRNA sense strands (SS) to generate CpG-siRNA conjugates (deoxyribonucleotides are underlined and asterisks indicate phosphothioation sites):

*Human STAT3 siRNA (SS)*

\texttt{5' GGAAGCUGCAGAAAGAUACGACUGA 3'}

*CpG(A)-STAT3 siRNA(AS)*

\texttt{5' G*G*TGCATCGATGCAGG*G*G*G*-linker-UCAGUCGUAUCUUUCUGCAGCUUCCGU 3'}

*Human BCL-X\textsubscript{L} siRNA (SS)*

\texttt{5' GAGCUAUCAGGAACAGCUAUGGGAG 3'}
CpG(A)-BCL-XL siRNA(AS)
5' G*G*TGCATCGATGCAGG*G*G*G-linker-CUCCCAUAGCUGUUCCUGAUAGCUCCC 3'

Luc siRNA (SS)
5' GGUUCCUGGAACAAUUGCUUUUACA 3'

CpG(A)-Luc siRNA(AS)
5' G*G*TGCATCGATGCAGG*G*G*G-linker- UGUAAAAGCAAUUGUUCCAGGAACCAG 3'

Control RNA (SS)
5' UCCAAGUAGAUUCGAGGCGAGAATG 3'

CpG(A)-control RNA(AS)
5' G*G*TGCATCGATGCAGG*G*G*G-linker- CACUUCGCCGUCGAAUCUACUUGGAUU 3'

For uptake studies, sense strand of STAT3 siRNA was labeled on 5’end using fluorescein or Cy3. Conjugates were added to culture media with reduced 5% FBS to limit degradation by nucleases.

Quantitative real-time PCR and protein assays

Total RNA was extracted from cultured or primary cells using RNeasy Plus kit (Qiagen, CA). After cDNA synthesis using iScript kit (Bio-Rad, CA), samples were analyzed using probes from the Universal Probe Library (Roche, CA) and specific primer pairs for human STAT3: 5’-CTGCCTAGATCGGCTAGAAAAC-3’, 5’-CCCTTTGTAGGAAACTTTTTGC-3’, UPL #25; TLR9: 5’-TGTGAAGCATCCTTCCCTGTA-3’, 5’-GAGAGACAGCGGGTGCAG-3’, UPL #56; BCL-XL: 5’-GCTGAGTTACCGGCATCC-3’, 5’-TTCTGAAGGGAGAGAAAGAGATTC-3’, UPL #10; IL-12/p35: 5’-CACTCCCAAACCTGCTGAG-3’, 5’-CAATCTCTTCAGAAGTGCAAGG-3’, UPL #50; IL-12/p40: 5’-CCCTGACATTCTGCGTTCA-3’, 5’-AGGTCTTGTCCGTGAAGACTCTA-3’, UPL #37; TBP, GAPDH or 18S were detected using Roche’s Reference Gene Assays. The ProbeFinder software (Roche) was used to design probe-primer sets. Sequence-specific amplification was analyzed on the CFX96 Real-Time PCR Detection System (Bio-Rad). The data were normalized to the GAPDH, TBP or 18S expression and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Western blot
analysis to detect TLR9, STAT3, BCL-XL and β-actin expression was performed as described previously19. Concentrations of cytokines in cell culture supernatants from cultured primary PBMCs or DCs were performed at the CICSL core (CoH) using Bio-Plex arrays and Luminex system (Bio-Rad).

**Flow cytometry**

Single cell suspensions of cultured cells or tumor tissues freshly prepared by mechanic tissue disruption and collagenase D/DNase I treatment as described19, were stained with 7AAD and/or fluorochrome-coupled Annexin V (BD Biosciences, CA). For extracellular staining of immune markers, cultured mDC or pDC cells were stained with different combinations of fluorochrome-labeled antibodies to CD11c, CD303, HLA-DR or CD86 (eBioscience, CA). Fluorescence data were collected on a BD-Accuri C6 Flow Cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, OR).

**Confocal microscopy**

For confocal microscopy, cells cultured in 24-well plates on #1.5 coverslips were fixed using 2% paraformadehyde, washed, stained with anti-EEA1 (Santa Cruz, CA) primary, and Alexa 555-coupled secondary antibodies (Life Technologies, CA), mounted in Vectashield Hard-set mounting medium with DAPI (Vector Labs, CA), and analyzed by confocal microscopy using a 63x oil immersion objective on LSM-510 Meta inverted confocal microscope (Zeiss, CA).

**Rapid amplification of cDNA ends (RACE) assay**

5’RACE assay was performed using GeneRacer™ Kit (Life Technologies) using a modified manufacturer’s protocol. Briefly, 150 ng of total RNA isolated from cultured pDCs or MV4-11 tumors was ligated to 250 ng of RNA adapter (Life Technologies), reverse transcribed using iScript kit (Bio-Rad) and amplified by touchdown PCR with adapter- and gene-specific primers. PCR products were used as template in nested PCR with forward adapter-specific (Life Technologies) and reverse sequence-specific (5’-CAGGCACCAGGAGGCACTTGTCT-3’) primers. Nested PCR products were separated on 1% agarose gel and the major products of predicted length were purified using QIAquick Gel Extraction kit (Qiagen). Purified products were sequenced using Hitachi AB Model 3730
DNA Analyzer in DNA Sequencing Core (CoH). Derived sequences were aligned with genomic sequences of STAT3 using Vector NTI software (Life Technologies).

**In vivo experiments**

Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from Institutional Animal Care and Use Committees of the CoH. For s.c. tumor challenge, we injected $1-5 \times 10^6$ of KMS-11, MonoMac6, MV4-11 or A20 tumor cells into immunodeficient 7-8 weeks old NOD/SCID/IL-2RγKO (NSG) mice or into Balb/C mice, respectively. After tumors reached average size of ca. 5-8 mm, mice received daily intratumoral injections of 100 µg (5 mg/kg) of indicated CpG-siRNAs or PBS. In case of A20 cells, established tumors were irradiated locally with single collimated 20 Gy dose from Cs-137 source using MARK I irradiator (J.L.Shepherd, CA). Tumor growth was monitored every other day by caliper measurements.

**Immunofluorescent staining**

Flash-frozen tumor specimens were fixed in paraformaldehyde, permeabilized with methanol and stained with antibodies specific to neutrophils (7/4, Cedarlane, NC) and active-caspase 3 (Cell Signaling, MA), then detected with fluorochrome-coupled secondary antibodies (Invitrogen). After staining the nuclei using Hoechst 33342 (Life Technologies), slides were mounted in Vectashield (Vector Labs) and analyzed by fluorescence microscopy. Shown are representative results from two independent experiments using samples isolated from 4 mice.

**Statistical analysis**

Unpaired t-test was used to calculate two-tailed $P$ value to estimate statistical significance of differences between two treatment groups in the whole study. One- or two-way ANOVA plus Bonferroni posttest were applied to assess statistical significance of differences between multiple treatment groups or in tumor growth kinetics between treatment groups, respectively. Statistically significant $P$ values were indicated in figures and/or legends and labeled as follows: $***$, $P<0.001$; $**$, $P<0.01$ and $*$, $P<0.05$. Data were analyzed using Prism 4.0 software (GraphPad, CA).
RESULTS

CpG-Stat3 siRNA amplifies effects of local radiation therapy against B-cell lymphoma

We have previously shown that TLR9 triggering with simultaneous elimination of the Stat3 immune checkpoint control generates tumor antigen-specific immune responses in mice\textsuperscript{20}. Pioneering studies by several other groups demonstrated that combining TLR9 agonists with local tumor irradiation led to synergistic immunotherapeutic effects in solid and blood cancers, such as B cell lymphoma (BCL)\textsuperscript{29,30,35}. Therefore, we assessed if CpG(B)-Stat3 siRNA molecules can further enhance the effect of local radiotherapy by reducing survival/radioresistance of TLR9-positive tumors and by augmenting the immunostimulatory effects of TLR9 triggering on both tumor and immune cells. For our proof-of-principle experiments in Balb/C mice, we selected TLR9\textsuperscript{+} A20 BCL cells, which showed high levels of constitutively active Stat3 (Fig. 1A). As shown in Fig. 1B, intratumoral injections of CpG(B)-Stat3 siRNA combined with local irradiation of A20 lymphomas resulted in complete tumor rejection in all treated mice. While the treatment with control CpG(B)-Luc siRNA delayed A20 growth, it did not prevent later tumor relapse. Moreover, radiotherapy combined with CpG(B)-Stat3 siRNA generated long term protective immunity against the primary tumor. None of these mice (0/5) developed tumors when re-challenged with the same number of A20 tumor cells injected in the opposite flank. These results prompted us to develop CpG-siRNA strategy for clinical translation as a novel approach to immunotherapy of human TLR9-positive blood malignancies.

CpG(A)-siRNA-mediated target gene silencing in human immune cells

We adapted CpG-siRNA conjugates for targeted gene silencing in normal and malignant human hematopoietic cells which are a challenge for nucleic acid delivery. For the design of the human cell-specific CpG-siRNA (Fig. 2A), we utilized a 25/27mer Dicer-substrate STAT3 siRNA selected from over 20 sequences based on the high silencing efficacy (>95\%)\textsuperscript{36}. The 25/27mer siRNA sequence was selected to enable intracellular uncoupling of both parts of the molecule by Dicer endonuclease\textsuperscript{20}. We conjugated STAT3 siRNA to both B- (CpG7909) and A- (D19) class CpG ODNs. CpG(B)/CpG7909 was previously shown to activate B cell populations and plasmacytoid DCs (pDCs),
whereas CpG(A)/D19 is known for a broad target spectrum including human pDCs, myeloid DCs (mDCs), normal B cells and B cell-derived malignant cells. Our initial studies suggested that CpG(A)-STAT3 siRNA may avoid induction of IL-6 and IL-10 cytokines, which are known to activate STAT3 as a negative feedback regulator of TLR9 signaling in immune cells. As expected both IL-6 and IL-10 were secreted by human PBMCs treated using class B but not class A CpG oligonucleotides and conjugates thereof (Supplementary Fig. 1AB). CpG(A)-STAT3 siRNA had also only minimal effect on IFNα production, which is known as a potential trigger of immunotoxicity in vivo. In contrast, IFNα was highly elevated in PBMCs treated with unconjugated class-A ODN (Supplementary Fig. 1C). Based on these results, CpG(A)-siRNA design was selected for further studies. First, we compared uptake of CpG(A)-STAT3 siRNA and unconjugated STAT3 siRNA by primary human immune cells by flow cytometry. Both molecules were labeled with Cy3 fluorochrome on 5' end of the siRNA passenger (SS) strand to follow the localization of the siRNA part of the molecule. As shown in Fig. 2B, CpG(A)-STAT3 siRNA Cy3 was rapidly internalized by several populations of primary human immune cells in the absence of any transfection reagents. Already within 30-60 min of incubation, CpG(A)-STAT3 siRNA Cy3 was internalized by the majority of CD14+ monocytes, cultured CD1c+ mDCs, CD303+ pDCs and to a lesser extent by CD19+ B cells (Fig. 2B, left column). The maximum conjugate uptake was observed in concentrations ranging from 100 to 250 nM (Fig. 2B, middle column). In contrast, neither naïve T cells (CD3+) nor NK cells (CD56+) internalized the CpG(A)-STAT3 siRNA Cy3. As expected, the internalization of unconjugated siRNA Cy3 was negligible even after 18 h of incubation at the highest concentration (Fig. 2B, right column). We also confirmed that CpG(A)-STAT3 siRNAs in doses of up to 500 nM did not affect viability or levels of mitochondrial enzymes in primary human PBMCs (Supplementary Fig. 2). Taken together, these data suggest that CpG part of the conjugate facilitates the delivery of siRNA into several human immune cell populations without detectable signs of toxicity.

Next, we examined whether CpG(A)-STAT3 siRNA uptake resulted in target gene silencing in primary and cultured immune cell populations. Fresh magnetically separated monocytes and B cells or cultured myeloid and plasmacytoid DCs were incubated for 18 h with 500 nM of CpG(A)-siRNA
conjugates targeting STAT3 or luciferase genes, the latter used as a negative control. As shown in Fig. 2C, STAT3 expression was significantly reduced by about 60% in both cultured mDCs and pDCs. The STAT3 knockdown was less pronounced in primary B cells, likely due to weaker conjugate uptake, and it was undetectable in monocytes. CpG-siRNA-mediated gene silencing in mouse immune cells requires TLR9 expression. Since TLR9 is less ubiquitously expressed in humans than in rodents, we examined if lack of TLR9 limited the silencing effect of CpG(A)-STAT3 siRNA. We assessed the expression of TLR9 mRNA in studied immune cell populations using quantitative PCR (qPCR). As expected, TLR9 was expressed at high levels in quiescent human pDCs and B cells and moderately in mDCs, while it was almost undetectable in monocytes and T cells (Fig. 2D). TLR9 expression is known to be sensitive to changing environmental conditions and was shown to be augmented by certain proinflammatory cytokines. Similarly, monocyte differentiation into pDC or mDC in the presence of Flt3 ligand or GM-CSF/IL-4 (Supplementary Fig. 3), respectively, upregulated TLR9 (Fig. 2D). To assess role of TLR9 triggering for induction of RNAi, we conjugated STAT3 siRNA to GpC ODN with sequence identical to D19 ODN except for the inversion of a CpG motif. The GpC ODN lacks the ability to trigger TLR9 activation although it is still internalized by target cells. The GpC(A)-STAT3 siRNA failed to silence STAT3 in human pDCs as measured by qPCR (Fig. 2E). Thus, TLR9 activation is necessary for CpG(A)-siRNA-induced gene silencing in target immune cells.

**Immunostimulatory properties of CpG(A)-STAT3 siRNA**

CpG(A)-STAT3 siRNA, which triggers TLR9 signaling while suppressing inhibitory effect of STAT3, should have improved immunoadjuvant properties on human immune cells as observed before in mice. We evaluated immunostimulatory effects of CpG(A)-STAT3 siRNA in monocyte-derived, cultured mDCs and pDCs. Immature mDCs (Fig. 3A) or pDCs (Fig. 3B) were incubated for 48h with 500 nM of CpG(A)-STAT3 siRNA or control CpG(A)-Luciferase siRNA and analyzed for immune activation marker expression by flow cytometry. Although both CpG(A)-siRNAs upregulated surface expression of HLA-DR complex and costimulatory molecule CD86, the effect of TLR9 triggering was strongly enhanced by STAT3 gene silencing in tested DC populations. As shown by flow cytometric
analysis, siRNA part of the conjugate did not contribute to the immunostimulatory effect of CpG ODN (Supplementary Fig. 4). Both CpG(A) alone and non-silencing control conjugate (CpG(A)-conRNA) upregulated HLA-DR and CD86 in pDCs to similar levels, while conjugate that does not activate TLR9 (GpC-STAT3 siRNA) failed to show any effect. The pDCs activation correlated with increased expression of mRNAs for both p35 and p40 subunits of IL-12, a critical activator of NK and T cell-mediated immunity (Fig. 3C). We also detected significantly increased secretion of IL-12 in supernatants from pDCs cultured in the presence of CpG(A)-STAT3 siRNA but not in CpG(A)-Luc siRNA-treated controls (Fig. 3D). Finally, we verified that CpG(A)-STAT3 siRNA-induced pDC activation augmented their ability to induce T cell proliferation in comparison to controls (Fig. 3E). Together with lack of conjugate immunotoxicity (Supplementary Fig. 2), these results suggest that CpG(A)-STAT3 siRNA is a potent non-toxic immunoadjuvant in human system.

**TLR9-mediated siRNA delivery into human myeloma and leukemia cells**

Previous studies demonstrated frequent expression of TLR9 mRNA in various malignancies of hematopoietic origin such as myeloma and B cell lymphoma. We analyzed protein expression of TLR9 in 3 MM and 7 AML cell lines using western blotting. TLR9 protein was present at moderate to high levels in 2 out of 3 tested MM and 5 out of 7 AML cells confirming earlier reports (Fig. 4A). Next, we examined the ability of TLR9-positive tumor cells to internalize CpG(A)-STAT3 siRNA using flow cytometry and confocal microscopy. After 1 h incubation with 500 nM CpG(A)-STAT3 siRNAFITC the majority of TLR9-positive MM and AML cells, including primary leukemic blasts derived from AML patients, were FITC-positive as detected by flow cytometry (Fig. 4B). We used confocal microscopy to verify the intracellular localization of CpG(A)-STAT3 siRNAFITC in KMS-11 myeloma and MV4-11 leukemia cells (Fig. 4C). Following internalization, CpG(A)-STAT3 siRNAFITC was accumulated within early endosomes as indicated by near complete colocalization with an early endosomal marker, EEA1 (Fig. 4D). Finally, we determined gene silencing effect of CpG(A)-STAT3 siRNA in KMS-11 cells. As measured by qPCR, STAT3 mRNA expression was reduced by about half within 18 h incubation of KMS-11 cells with 500 nM CpG(A)-STAT3 siRNA in comparison to a negative control.
STAT3 silencing effect of CpG(A)-siRNA in rapidly dividing KMS-11 cancer cells was transient and decreased after prolonged cell culture (data not shown), which is likely an effect of limited stability of naked CpG-siRNA oligonucleotides in serum-containing media. Based on these results, we concluded that human TLR9-positive hematopoietic tumor cells are appropriate targets for CpG-siRNA conjugates.

**In vivo gene silencing using CpG(A)-siRNA conjugates**

We evaluated the feasibility of using CpG(A)-siRNA for targeting survival signaling in TLR9-positive tumors in vivo. First, we assessed the percentage of tumor cells penetrated by intratumorally injected conjugate. MV4-11 leukemia cells were injected s.c. into the immunodeficient NOD/SCID/IL-2Rγnull (NSG) mice. The established MV4-11 tumors, which reached the size of ca. 100 mm³, were injected intratumorally with a single 20 or 100 µg dose of fluorescently labeled CpG(A)-STAT3 siRNA-Cy3. The percentages of viable Cy3⁺ cells were analyzed using flow cytometry in tumors harvested 3 h after injection. The intratumoral uptake of the CpG(A)-siRNA conjugate was dose-dependent and reached 76% of viable cells within MV4-11 tumors at 100 µg dose (Fig. 5A). To verify whether local CpG(A)-siRNA delivery could reduce target protein expression in the whole TLR9-positive tumor, we tested silencing of STAT3 and BCL-X₇ proteins which are known for their survival promoting role in blood cancers. Four daily injections of CpG(A)-siRNAs targeting BCL-X₇ (Fig. 5B) or STAT3 (Fig. 5C) consistently reduced protein levels of both targets by average 65% or 61%, respectively. As confirmed by qPCR, STAT3 mRNA expression was similarly reduced in CpG(A)-STAT3 siRNA-treated MV4-11 tumors (Fig. 5D). To verify whether the observed effect of STAT3 knockdown in MV4-11 tumors resulted from RNAi, we employed the 5'-Rapid amplification of cDNA ends (5'RACE) PCR assay. The presence of the 5'RACE product, which indicates STAT3 mRNA cleavage, was detectable only in tumors treated with CpG(A)-STAT3 siRNA but not CpG(A)-Luc siRNA (Fig. 5E). We further confirmed by DNA sequencing that STAT3 mRNA cleavage occurred within the region targeted by STAT3 siRNA.
Antitumor effects of CpG(A)-siRNA conjugates

Both STAT3 and BCL-XL are known survival promoting factors in multiple myeloma and leukemia. Therefore, we assessed the potential of CpG(A)-STAT3 or -BCL-XL siRNAs to induce direct cytotoxic effects in tumor cells. NSG mice with established (>5 mm in diameter) MV4-11 s.c. tumors were injected daily with 100 µg of CpG(A)-siRNA directed against STAT3, BCL-XL or luciferase (negative control). Local treatment using CpG(A)-siRNA conjugates targeting STAT3 or BCL-XL inhibited growth of subcutaneously growing AML tumors (Fig. 6A, right). The antitumor effects correlated with the induction of tumor cell death, as measured by Annexin V-staining and flow cytometry (Fig. 6A, left). We further verified if CpG-STAT3 siRNA can inhibit growth of other TLR9-positive blood cancers. As shown in Fig. 6B and 6C, intratumoral injections of CpG(A)-STAT3 siRNA effectively silenced STAT3 expression (left panels) and reduced growth of both KMS-11 myeloma and MonoMac6 leukemia (right panels). In contrast, treatment with control CpG(A)-Luc siRNA had no significant growth inhibitory effect (Fig. 6B and C). We further confirmed that antitumor activity of CpG(A)-STAT3 siRNA depends on concomitant TLR9 triggering and STAT3 silencing. Control conjugates which did not silence STAT3 (CpG(A)-conRNA) or did not target TLR9 (GpC(A)-STAT3 siRNA) as well as CpG(A) alone were unable to induce antitumor effects against MV4-11 leukemia (Supplementary Fig. 5).

The immunodeficient NSG mice lack most of the immune cell populations, such as T, B and NK cells, but retain granulocyte activity. Innate immunity contributes to antitumor responses, thus we assessed whether treatment using CpG-STAT3 siRNA led to neutrophil recruitment into the tumor tissue. As shown in the Fig. 7, CpG-siRNA targeting STAT3 but not Luciferase resulted in increased tumor infiltration by neutrophils. In addition, neutrophil partly co-localized with areas of MV4-11 tumors actively undergoing apoptosis as indicated by caspase-3 activity (Fig. 7). Taken together, these results suggest that even in mostly immunodeficient NSG mice, CpG(A)-STAT3 siRNA therapy mobilized residual innate immune cells that potentially contributed to the overall antitumor effect. Our studies validated the feasibility of using this strategy against TLR9-positive hematologic malignancies in vivo and emphasized the need for the development of CpG-siRNA conjugates for systemic delivery.
DISCUSSION

We have developed a siRNA delivery strategy to normal and malignant hematopoietic cells \textit{in vivo}, which is suitable for targeting both tumor cells and the tumor microenvironment. The CpG(A)-siRNA approach allows for efficient cell-specific target gene silencing without complex formulations or delivery vehicles. Our studies confirmed feasibility of using CpG(A)-siRNAs for targeting human TLR9-expressing cells such as plasmacytoid DCs, myeloid DCs, B cells and several myeloid and B cell malignancies\textsuperscript{25-27,37-39}. We demonstrated that while TLR9-negative cells such as monocytes are still able to quickly internalize CpG(A)-siRNA, in the absence of TLR9 expression conjugates do not induce RNAi. Our independent studies delineating the molecular mechanism of intracellular CpG-siRNA processing suggest that TLR9 may in fact participate in the uncoupling and release of siRNA from endosomes (Nechaev and Kortylewski, unpublished). We showed previously that class-B CpG ODNs, which have been tested in numerous clinical studies, control TLR9 activity using negative feedback regulation through STAT3\textsuperscript{33}. The mechanism of STAT3 activation by class-B CpG ODNs in human hematopoietic cells is likely to depend on upregulation of cytokines such as IL-6 and IL-10, as shown in this and other studies\textsuperscript{23,26,32}. The signaling cross-talk within the tumor microenvironment further enhances STAT3 signaling, dampening the immunostimulatory activity of TLR agonists\textsuperscript{33}. In contrast, we did not detect increased levels of STAT3-inducing cytokines in human PBMCs cultured with unconjugated CpG(A) ODNs or CpG(A)-siRNAs. The typical feature of class-A CpG ODNs is induction of type I IFNs, which restricted their clinical application to viral and allergic diseases\textsuperscript{29}. Although unconjugated CpG(A) potently activated IFN\alpha, the CpG(A)-STAT3 siRNA conjugates did not exert such activity on cultured human PBMCs. Type I IFN responses were shown to depend on TLR9 triggering by CpG(A) ODNs tetramers formed through the interaction of guanine-rich 3' ends\textsuperscript{38,41}. Conjugation of the siRNA molecule to CpG(A) ODN is likely to reduce spontaneous multimerization of the conjugate, thereby preventing TLR9-mediated induction of IFN\alpha expression. However, the CpG(A)-siRNA conjugates retained the potent immunostimulatory activity on human DCs. The combined effect of TLR9 triggering and targeting of STAT3 immune checkpoint gene strongly augmented maturation of human APCs similar to previously observed effect in \textit{Stat3}-deficient mice\textsuperscript{33}. 
Importantly, we did not detect mitochondrial toxicity or general cytotoxicity of CpG(A)-STAT3 siRNA on primary human PBMCs in vitro.

Our findings validated that TLR9 expressed by human tumor cells provides a delivery route for therapeutic siRNAs similar as in normal mouse\textsuperscript{20} and human immune cells. Previously developed siRNA delivery strategies employed antibodies, oligonucleotides or RNA aptamers specific to surface receptors on tumor cells\textsuperscript{16,36,42}. In contrast, our approach utilized a ligand to intracellular TLR9 receptor which is ubiquitously expressed in B cell malignancies and acute myeloid leukemia\textsuperscript{25-27}. Dicer RNase, which is critical for uncoupling of CpG-siRNA conjugates, is also commonly expressed in normal and transformed B cells and in the majority of clinical AML samples\textsuperscript{43,44}. Our results demonstrate that repeated administration of CpG(A)-siRNAs specific for oncogenic and/or pro-survival genes, such as STAT3 or BCL-X\textsubscript{L}, can induce tumor cell apoptosis and inhibit growth of several xenotransplanted human MM and AML tumors. STAT3 transcription factor plays a dual tumorigenic role in both cancer cells and non-malignant tumor-associated cells, which do not depend on STAT3 for their survival. It is a highly desirable target for cancer therapy that could induce cancer cell death while activating antitumor immunity with minimal adverse effects\textsuperscript{7}. The NSG mice used in our xenotransplant studies are known to be mostly deprived of functional immune cells except for granulocytes\textsuperscript{40}. Thus, our experiments did not reflect the immunostimulatory potential of CpG(A)-STAT3 siRNAs, which can greatly contribute to the overall antitumor effect\textsuperscript{20}. Further studies using tumor models in humanized mice are required to assess the two-pronged, tumoricidal/immunostimulatory effect of CpG(A)-STAT3 siRNA. In spite of these limitations, our experiments suggest that treatment of human hematologic malignancies using CpG(A)-STAT3 siRNA also resulted in immune cell activation. Targeting STAT3 in AML tumors resulted in the pronounced infiltration of neutrophils, which are known to contribute to the antitumor immunity after elimination of STAT3-dependent cross-talk in the tumor microenvironment\textsuperscript{7,33}. The progress in understanding complex interactions within the tumor microenvironment underscores the need to focus on tumor-associated but non-malignant immune cells for successful anti-cancer strategies. The immunomodulatory drugs are making headway in clinical testing for BCL, MM and AML therapy\textsuperscript{30,45,46}. 
It is also becoming apparent that clinical outcome of cancer immunotherapy can be improved by targeted strategies to alleviate tumor-induced immunosuppression without induction of adverse immune effects\textsuperscript{47}. Recent studies revealed that antigen-presenting cells, such as pDCs, play a tumor-promoting role in MM\textsuperscript{48} and potentially also in AML patients\textsuperscript{49}. CpG(A)-\textit{STAT3} siRNA provides a new method for targeting abnormal human DC populations in the tumor microenvironment to restore antitumor immunity while reducing survival of TLR9-positive tumor cells. Our proof-of-principle studies, combining CpG-Stat3 siRNA with local radiotherapy, demonstrated that targeting both tumor and the tumor microenvironment is effective against radiation-resistant mouse BCL. These results support the clinical translation of CpG(A)-\textit{STAT3} siRNA strategy for \textit{in situ} immunotherapeutic strategies against BCL and also against certain advanced solid tumors, which are TLR9-positive\textsuperscript{26,50}. However, for therapy of systemic diseases such as majority of blood cancers, it is highly desirable to further optimize serum stability and circulatory half-life of CpG(A)-\textit{STAT3} siRNA conjugate. Such optimization includes various chemical modifications of CpG(A)-siRNA sugar backbone, conjugation to high molecular weight polymers or encapsulation of the whole molecule. Our results provide rational basis for further development of CpG(A)-siRNA approach to target oncogenic and immunosuppressive targets, such as STAT3, for clinical application.
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AUTHORSHIP CONTRIBUTIONS

Contribution: M.K., Q.Z., D.M.S.H., S.N., A.K., W.Z., Y.L., performed research; C.M.K., P.S. were involved in conjugate design and testing; R.B. provided clinical samples; M.K., H.Y., S.F., J.J.R., A.R., S.P. designed experiments and/or analyzed the data and M.K. wrote the manuscript.

DISCLOSURE OF CONFLICT OF INTEREST

The authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. Therapeutic effect of local radiation therapy combined with CpG-Stat3 siRNA treatment on TLR9-positive B-cell lymphoma in mice. (A) Stat3 is constitutively activated in TLR9+ A20 BCL cells. Shown are results from the western blot analysis of TLR9, activated and total Stat3 in comparison to β-actin used for loading control; TLR9+ RAW264.7 macrophages and TLR9- CD4 T cells were used for reference. (B) Mice with established s.c. growing A20 B-cell lymphoma were injected intratumorally using CpG(B)-Stat3 siRNA, control CpG(B)-Luc siRNA or PBS only, twice daily (day 16, 17) before radiotherapy (RT), then 6 hours post-RT and two more times every other day (days 20, 22). Local tumor irradiation at single 20 Gy dose on day 18 was indicated by an arrow. Shown is the A20 tumor growth kinetics in the experiment using 5 mice per each treatment group; means ± SEM. Statistically significant differences between groups treated with CpG(B)-Stat3 siRNA and controls treated with CpG(B)-Luc siRNA are indicated with asterisks.

Figure 2. Design of the CpG(A)-STAT3 siRNA conjugate for targeting STAT3 in human TLR9-positive cells. (A) Structure and sequence of the CpG(A)-STAT3 siRNA: CpG(A) ODN (D19 sequence) was conjugated to the STAT3 siRNA guide (AS) strand through a flexible carbon chain linker; deoxyribonucleotides are underlined; asterisks indicate phosphothioation sites; shown is a position of the fluorochrome (Cy3 or FITC) at the 5’ end of the STAT3 siRNA passenger strand. (B) Targeted delivery of STAT3 siRNA into various populations of primary human immune cells in vitro. Human PBMCs were incubated in the presence of fluorescently-labeled CpG(A)-STAT3 siRNA<sup>Cy3</sup> or unconjugated STAT3 siRNA<sup>Cy3</sup> in various concentrations for times as indicated without any transfection reagents. Percentages of Cy3-positive CD14<sup>+</sup> monocytes, CD303<sup>+</sup> (BDCA2<sup>+</sup>) plasmacytoid DCs, CD1c<sup>+</sup> (BDCA1<sup>+</sup>) myeloid DCs, CD19<sup>+</sup> B cells, CD56<sup>+</sup> NK cells and CD3<sup>+</sup> T cells were assessed by flow cytometry. Similar results were obtained from three independent experiments. (C) CpG(A)-STAT3 siRNA treatment leads to STAT3 gene silencing in various immune cell populations. Monocytes, mDCs, pDCs and B cells were incubated with 500 nM of CpG-siRNAs targeting STAT3 or Luciferase (as negative control) for 18 h. STAT3 expression was measured using
qPCR. Shown are results normalized to *TBP* gene expression levels from one of three independent experiments; *STAT3* expression level in control CpG-*Luc* siRNA-treated samples was set as 100%; means ± SEM (n = 3). Statistically significant differences are indicated with asterisks; ***, *P*=0.0005; **, *P*=0.0034; *, *P*=0.03. (D) *TLR9* is expressed in target immune cell populations sensitive to CpG(A)-siRNA-mediated gene silencing. *TLR9* expression was measured by qPCR in enriched populations of monocytes, T cells and B cells or in cultured mDCs and pDCs. The results are representative of two independent experiments performed in triplicates; means ± SEM. (E) *STAT3* silencing by CpG(A)-siRNA conjugates depends on *TLR9* targeting and activation. *STAT3* expression was assessed by qPCR in cultured pDCs treated for 18 h using CpG(A) alone, CpG(A) linked to non-silencing control RNA, non-targeting GpC(A)-*STAT3* siRNA or CpG(A)-*STAT3* siRNA. Shown are results representative for one of two independent experiments in triplicates; means ± SEM.

**Figure 3. CpG-STAT3 siRNA stimulates the immune activity of human dendritic cells in vitro.** Cultured myeloid DCs (A) or plasmacytoid DCs (B) were incubated for 48h in the presence of 500 nM of CpG-siRNAs targeting *STAT3* or *Luciferase* (as negative control). The surface expression of HLA-DR and CD86 immune activation markers on both DC populations was assessed by flow cytometry. (C, D) IL-12 expression in pDCs treated as described above was evaluated in total RNA samples (C) or in cell culture supernatants (D) using qPCR or Luminex assays, respectively. Presented are results averaged from 3 independent experiments and analyzed for statistical significance; means ± SEM (n = 3); ns, not significant. (E) *STAT3* blocking in cultured human pDCs augments their immunostimulatory effect on T cells. Allogeneic CD3+ T cells were labeled using CFSE and incubated for 5 days at 1:2 ratio with pDCs pre-treated as indicated. T cell expansion was assessed by CFSE dilution using flow cytometry.

**Figure 4. CpG-siRNA uptake and gene silencing in TLR9-positive myeloma and leukemia cells.** (A) Majority of tested MM (left) and AML (right) cells express high levels of TLR9 protein. Representative results from at least two western blotting analyses were shown; positions of TLR9 and
β-actin for loading control are indicated. (B) CpG-siRNA is quickly internalized by established MM and AML cells as well as by patients’ AML blasts in vitro. Cells were incubated with 500 nM of FITC-labeled CpG-STAT3 siRNA for 1 h. The percentage of FITC-positive cells was analyzed by flow cytometry. (C, D) CpG-STAT3 siRNA is accumulated in early endosomes shortly after intracellular uptake. KMS-11 myeloma cells (C) and MV4-11 leukemia cells (C, D) were treated with 500 nM of CpG-STAT3 siRNAFITC for 1 h. The intracellular localization of the conjugate was assessed by confocal microscopy; green, CpG-STAT3 siRNAFITC; red, EEA1 (early endosome marker); blue, nuclear staining with DAPI. (E) Target gene silencing in cultured KMS-11 cells incubated with 500 nM of CpG-STAT3 siRNA for 18 h. Shown are results averaged from 4 independent experiments analyzed by qPCR and normalized to GAPDH.

Figure 5. In vivo delivery of CpG-siRNAs induces RNAi and abrogates target gene expression in TLR9-positive leukemia cells. (A) Dose-dependent uptake of Intratumorally injected CpG-STAT3 siRNA by TLR9-positive MV4-11 cells. Tumor cells were injected subcutaneously into NSG mice. After tumors were established, mice were injected i.t. using 20 or 100 µg of Cy3-labeled CpG-STAT3 siRNA. The percentage of Cy3-positive cells was analyzed by flow cytometry in cell suspensions prepared from tumors harvested 3 h after injection. (B, C) Intratumoral injections of CpG(A)-siRNA can effectively silence expression of BCL-XL or STAT3 proteins in xenotransplanted MV4-11 leukemia. NSG mice were injected with MV4-11 AML cells. After tumors were established (d 7), mice were treated four times with daily i.t. injections of CpG(A)-siRNAs targeting BCL-YL (B) or STAT3 (C). Protein levels of both BCL-XL and STAT3 were evaluated using western blotting with β-actin as loading control in samples derived from single tumors for both experimental groups. Band intensities were quantified by densitometry using ImageJ software based on identically exposed images. Shown are results from one of two independent experiments analyzed for statistical significance; **, $P=0.003$ and ***, $P<0.0001$. (D, E) RNAi-mediated STAT3 gene knock down in MV4-11 tumors treated using i.t. injections of CpG(A)-siRNAs as above. (D) STAT3 gene silencing in samples from 4 individual tumors was verified by qPCR and normalized to TBP expression; shown are means ± SEM. (E)
CpG(A)-STAT3 siRNA-induced cleavage of STAT3 mRNA in MV4-11 tumors in vivo. Tumors injected i.t. using CpG(A)-siRNAs as indicated were harvested 18 h later. Total RNA samples were analyzed by 5'RACE-PCR assay followed by DNA sequencing detecting mRNA cleavage within the targeted region (partial sequences are shown); arrow indicates the major RACE product of predicted length (300 nts).

**Figure 6. Therapeutic antitumor effects of CpG(A)-siRNAs targeting STAT3 or BCL-X₁ in xenotransplanted leukemia and myeloma models.** (A) Intratumoral delivery of CpG(A)-siRNAs targeting STAT3 or BCL-X₁ inhibits s.c. growth of MV4-11 leukemia and results in augmented tumor cell death. NSG mice were challenged using 5x10⁶ MV4-11 cells injected s.c. After tumors were established (at the average diameter 8 mm), mice were injected i.t. daily using 100 μg of CpG-siRNAs as indicated. Tumor growth was measured using caliper (right). At day 16 tumors were harvested and the percentage of apoptotic Annexin V-positive tumor cells was assessed by flow cytometry (left). Statistically significant differences between CpG(A)-STAT3 or BCL-X₁ siRNAs and CpG(A)-Luc RNA-treated groups (from two-way ANOVA test) are indicated by asterisks; means ± SEM (n = 5). (B, C) Local treatment using CpG(A)-STAT3 siRNA (as above) leads to STAT3 gene silencing (B and C, left graphs; by qPCR), tumor cell death (B, middle; by flow cytometric analysis of Annexin V-positive tumor cells) and reduced growth rate of human KMS-11 myeloma (B) and MonoMac6 leukemia (C) in NSG mice. Shown are representative results from two independent experiments (A, B) or from a single experiment (C) using 6 mice per each treatment group.

**Figure 7. Local delivery of CpG(A)-STAT3 siRNA into xenotransplanted leukemia induces tumor cell death and stimulated neutrophil infiltration.** (A, B) Effects of intratumoral delivery of CpG(A)-STAT3 siRNA on tumor cell apoptosis and neutrophil infiltration. Mice xenotransplanted with MV4-11 leukemia were treated by four i.t. injections of CpG(A)-siRNA targeting STAT3 or Luc for negative control. Tumors were harvested one day after the last treatment, frozen and sectioned. (A) Tissue cryosections were immunofluorescently stained using antibodies to neutrophils (7/4, green)
and activated caspase-3 (red). Slides were counterstained using Hoechst33342 to visualize nuclei (blue) and analyzed by fluorescent microscopy. Shown are representative results from two independent experiments using tumor sections from 4 mice per each group; arrows indicate contact sites between neutrophils and cells undergoing apoptosis. (B) Quantification of neutrophil infiltration using at least 20 randomly chosen fields. Statistically significant differences between treatment groups (from two-way ANOVA test) are indicated by asterisks; ***, $P<0.001$; means ± SEM ($n = 4$).
Fig. 1AB

A

Biochemical analysis by Western blotting: A20, T cells, and RAW264.7 cells were analyzed for TLR9, Stat3P, Stat3, and β-actin expression.

B

Graph showing tumor volume over time for different treatments:
- PBS + IR, n = 5
- CpG(B)-Luc siRNA + IR, n = 5
- CpG(B)-Stat3 siRNA + IR, n = 5

RT indicates the time of treatment initiation.

Tumor volume (mm³)

Days

0 20 40 60 80 100
Monocytes

B cells

NK cells

T cells

pDCs

mDCs

CpG (A) 5’ G*G*TGATCGATGCGG*G*G GAGUAGUCGUAAUCUCUCUGACGUCCCUCCGU 3’

STAT3 siRNA

linker AGUCAGCAUAGAAAAGACGGAAGG

CpG (A)-STAT3 siRNA

CpG (A)-STAT3 siRNA

STAT3 siRNA

% of Max

Cy3

Fig. 2AB
A

Fig. 5

Untreated 20 μg 100 μg CpG-siRNA

B

CpG(A)-Luc siRNA  CpG(A)-BCL-XL siRNA

Bcl-XL  β-actin

C

CpG(A)-Luc siRNA  CpG(A)-STAT3 siRNA

STAT3  β-actin

D

E

5’RACE product (300 nt)

5’RACE product:

STAT3 mRNA target:

PBS     CpG(A)-LUC siRNA     CpG(A)-STAT3 siRNA

5’GGAGTAGAAA GATACGACTG 3’
adaptor

5’GCUGCAGAAA GAUACGACUG 3’
A

![Bar chart showing Annexin V+ cells (% of total) for CpG-LUC siRNA, CpG-STAT3 siRNA, and CpG-BCL-XL siRNA.](image)

**Annexin V+ cells (% of total)**

- CpG-LUC siRNA
- CpG-STAT3 siRNA
- CpG-BCL-XL siRNA

B

![Bar chart showing STAT3:GAPDH (% of control) for CpG-LUC siRNA and CpG-STAT3 siRNA.](image)

**STAT3:GAPDH (% of control)**

- CpG-LUC siRNA
- CpG-STAT3 siRNA

C

![Bar chart showing STAT3:GAPDH (% of control) for CpG-LUC siRNA and CpG-STAT3 siRNA.](image)

**STAT3:GAPDH (% of control)**

- CpG-LUC siRNA
- CpG-STAT3 siRNA

Fig. 6ABC
Fig. 7

A

Hoechst  Neutrophils  Active caspase 3  Merged

PBS

CpG(A)-Luc siRNA

CpG(A)-STAT3 siRNA

B

Neutrophils (cells/section)

PBS  CpG(A)-LUC siRNA  CpG(A)-STAT3 siRNA

***
TLR9-mediated siRNA delivery for targeting of normal and malignant human hematopoietic cells in vivo

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