**TLR9-Targeted STAT3 Silencing Abrogates Immunosuppressive Activity of Myeloid-Derived Suppressor Cells from Prostate Cancer Patients**

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**Abstract**

**Purpose:** Recent advances in immunotherapy of advanced human cancers underscored the need to address and eliminate tumor immune evasion. The myeloid-derived suppressor cells (MDSC) are important inhibitors of T-cell responses in solid tumors, such as prostate cancers. However, targeting MDSCs proved challenging due to their phenotypic heterogeneity.

**Experimental Design:** Myeloid cell populations were evaluated using flow cytometry on blood samples, functional assays, and immunohistochemical/immunofluorescent stainings on specimens from healthy subjects, localized and metastatic castration-resistant prostate cancer patients.

**Results:** Here, we identify a population of Lin⁻CD15⁺CD33⁻ granulocytic MDSCs that accumulate in patients’ circulation during prostate cancer progression from localized to metastatic disease. The prostate cancer-associated MDSCs potently inhibit autologous CD8⁺ T cells’ proliferation and production of IFNγ and granzyme-B. The circulating MDSCs have high levels of activated STAT3, which is a central immune checkpoint regulator. The granulocytic pSTAT3⁺ cells are also detectable in patients’ prostate tissues. We previously generated an original strategy to silence genes specifically in Toll-like Receptor-9 (TLR9) positive myeloid cells using CpG-siRNA conjugates. We demonstrate that human granulocytic MDSCs express TLR9 and rapidly internalize naked CpG-STAT3siRNA, thereby silencing STAT3 expression. STAT3 blocking abrogates immunosuppressive effects of patients-derived MDSCs on effector CD8⁺ T cells. These effects depended on reduced expression and enzymatic activity of Arginase-1, a downstream STAT3 target gene and a potent T-cell inhibitor.

**Conclusions:** Overall, we demonstrate the accumulation of granulocytic MDSCs with prostate cancer progression and the feasibility of using TLR9-targeted STAT3siRNA delivery strategy to alleviate MDSC-mediated immunosuppression. Clin Cancer Res; 21(16); 3771–82. ©2015 AACR.

**Introduction**

Prostate cancer remains the most common malignancy in men in the United States (1). Although localized prostate cancers are curable with surgery or standard therapeutic regimens, the 5-year survival rate of patients with the advanced metastatic tumors is reduced to 20% to 30%. Androgen deprivation therapies control the recurrent disease only for limited time until the development of metastatic castration-resistant prostate cancers (mCRPC). Current chemotherapeutic regimens for mCRPC have limited efficacy and are plagued by the highly toxic effects to normal tissues (2). The first FDA-approved immunotherapeutic approach to mCRPC, using autologous cellular vaccinations, showed promising although modest improvement in patients’ survival (3). Growing evidence suggests that prostate tumor microenvironment can block immune responses using a wide array of immune checkpoint mechanisms likely extending beyond PD-1 blockade (4–7). Human tumors recruit and expand population of potentially immunosuppressive myeloid-derived suppressor cells (MDSC) that were associated with progression and poor patients’ survival (8–10). Depending on the expression of lineage-specific immune markers, MDSCs can be divided into CD14⁺CD15⁻CD33⁺ monocyctic MDSCs (M-MDSC) and CD14⁻CD15⁺CD33⁺ granulocytic MDSCs (G-MDSC), also known as polymorphonuclear neutrophil-MDSCs (PMN-MDSC; ref. 8). The generation of MDSCs is a result of tumor-induced skewing of monocyte differentiation from macrophages/DCs into M-MDSCs and later into G-MDSCs, a dominant myeloid population in cancer patients’ circulation (11). First study that reported circulating prostate cancer–associated MDSCs in patients with the localized disease identified these cells as CD14⁺/HLA-DR⁻ M-MDSCs (12). Whether G-MDSCs are contributing to prostate cancer progression remains unknown. However, there is accumulating evidence linking G-MDSCs with immunosuppression in human...
genitourinary tumors, such as renal and bladder cancers (13–16).

Preclinical studies in mouse tumor models showed that MDSC depletion or targeting their expansion and recruitment increases the effectiveness of cancer therapies, including anti-PD-1 immunotherapies (8, 17). We also recently observed that resistance of renal carcinoma patients to pazopanib, a multi-receptor tyrosine kinase inhibitor and antiangiogenic agent, correlated with accumulation of CD15+ G-MDSCs in circulation compared with patients responding to therapy (18).

The expansion of MDSCs in the tumor microenvironment is induced by cancer-derived mediators, such as VEGF, HGF, G-CSF, IL6, and IL10 (19). Downstream signaling induced by majority of these factors converges on signal transducer and activator of transcription 3 (STAT3), which plays central role in the expansion of MDSCs. The activation of STAT3 promotes immunosuppressive functions, including production of ROS, iNOS, and IDO (19, 21). Targeting STAT3 is therefore an attractive strategy to alleviate MDSC-mediated immunosuppression in the tumor microenvironment without the need for myeloid cell depletion.

We previously generated an original method to silence genes associated with progression of prostate cancers from localized to metastatic disease. We also tested the feasibility of using CpG-STAT3 siRNA to abrogate immunosuppressive functions of prostate cancer–associated G-MDSCs on effector T-cell activity. Overall, these studies provide a rational for application of CpG-STAT3 siRNA strategy to immunotherapy of human prostate cancers.

**Materials and Methods**

**Patients**

Blood specimens were collected prospectively (after informed consent was obtained) from patients under two independent protocols, IRB-11020 and IRB-10058 (COH). In the IRB-11020, selected patients were diagnosed with high-risk localized prostate cancers. Blood specimens were collected at the baseline before patients underwent prostatectomy. Patients in the IRB-10058 were diagnosed with metastatic castration-resistant prostate cancers (mCRPC) and were later treated with docetaxel chemotherapy. Blood specimens were collected at baseline and after 4 months of docetaxel chemotherapy applied in 3 weekly cycles. Prostatectomy specimens were acquired from patients with high-risk, localized prostate cancers under IRB-10151 protocol (COH). Each protocol and the relevant informed consent were approved by the institutional scientific review committee, data safety monitoring board, and the institutional review board at City of Hope. All patients enrolled provided written informed consent, and the study was conducted in accordance with the amended Declaration of Helsinki and the International Conference on Harmonization Guidelines.

**PBMC isolation and flow cytometry**

PBMCs and plasma were separated using Vacutainer CPT tubes (BD Biosciences) within 2 hours after collection by centrifugation at 1,800 × g for 20 minutes at room temperature. Fresh PBMCs were used for phenotypic analysis of myeloid immune cell populations, 1 × 10⁶ of PBMCs were preincubated with FcγRI/IR-specific antibody to block unspecific binding and then stained with fluorescein-labeled antibodies to HLA-DR, CD11b, CD14, CD3, CD19, CD56, CD114, CD33, and CD34 (eBiosciences). For analysis of intracellular markers, we used PBMCs previously frozen in optimized Cryostor CS5 media (Biolyte). Freeze–thaw procedure reduced CD15 staining causing decrease in the percentage of CD15⁺CD33⁺ cells (Supplementary Fig. S1), however, reductions of G-MDSC percentages were consistent between various patients. Thus, it was feasible and acceptable to compare identical handled cryopreserved samples to assess relative changes of G-MDSC population during disease progression. For intracellular staining, PBMCs were first stained for surface markers, then fixed and permeabilized using BD fixation and perm/wash buffer, respectively, following the manufacturer’s recommendations. After blocking in human serum, cells were stained using fluorescein-labeled antibodies specific to TLR9 (eBiosciences), tyrosine 705-phosphorylated STAT3 (pSTAT3; BD Biosciences), or Arginase-1 (R&D Systems). Flow cytometric data were collected on BD-Accuri C6 Flow Cytometer (BD Biosciences) or MACSQuant (Miltenyi Biotec) and analyzed using FlowJo software (TreeStar).

**MDSC isolation and treatment**

For analysis of immunosuppressive functions, myeloid cell populations were isolated from fresh blood samples using
FACS Aria III cell sorter (BD Biosciences) or magnetic enrichment (STEMCELL Technologies). For the latter, CD14+ cells were first removed from total PBMCs using specific antibodies (eBiologics) and then CD14+ CD15− cells were selected using CD15-specific antibodies (eBiologics). Purity of isolated cells was evaluated by flow cytometry, which detected single-cell population (data not shown). For the analysis of STAT3 activation and ARG1 expression, frozen PBMCs were thawed and cultured for at least 2 hours in 20% plasma from the same patient. These conditions were sufficient to restore the maximum levels of STAT3 signaling as determined in preliminary studies (Supplementary Fig S2). Then selected myeloid cell populations were isolated using high-speed cell sorting using FACS-Aria (BD Biosciences) or magnetic separation using specific antibodies (STEMCELL Technologies). For the latter, after removal of CD14+ myeloid cells from total PBMCs using CD14-specific antibodies (eBiologics), the CD14+ CD15− cells were enriched from the remaining PBMCs using CD15-specific antibody (eBiologics). For uptake studies, enriched MDSCs were treated using different concentrations of FITC-labeled CpG-STAT3 siRNA followed by flow cytometry to assess the uptake. The sequences of human cell-specific CpG-siRNAs were reported before (23, 24). All CpG-siRNA conjugates were synthesized at DNA/RNA Synthesis Core (COH) by using 5 units of C3 chemical chain, (CH3), (Glen Research) to link the D19 oligodeoxynucleotide to antisense strands (AS) of siRNAs. The resulting constructs were hybridized to complementary siRNA sense strands (SS) to generate CpG-siRNA conjugates. To test the effect of STAT3 or Arginase-1 inhibition on MDSC function, the isolated MDSCs were treated with 500 nmol/L of STAT3 siRNA or 20 μmol/L nor-NOHA (Cayman Chemical Company) and then cocultured with autologous T cells.

Quantitative real-time PCR and protein assays

For quantitative PCR (qPCR), total RNA was extracted from isolated MDSCs using the RNeasy Plus kit (Qiagen). After cDNA synthesis using iScript kit (Bio-Rad), samples were analyzed using sets of probes from the Universal Probe Library and specific primer pairs for human STAT3: 5′-CTGCCATATGCCTAGAAGAC-3′, 5′-CCCCCTTGTAGAAACATTTTGC-3′, UPL #25; TLR9: 5′-TGTAGAACATCTCCTCGCTGA-3′, 5′-GAGAGACACCCC-GGTGCAG-3′, UPL #56. SsoAdvanced SYBR Green Supermix (Bio-Rad) was used to analyze human ARG-1, iNOS, and IDO expression in patient MDSCs with following sets of primers: ARG-1: 5′-GTCTCTCAACGACAGCC-3′ and 5′-GCTCAAG-TGCAGAAAGAGA-3′; iNOS: 5′-ATCTGTCCTGAGTGCAGT-3′ and 5′-TCCAGACAAATCCACACG-3′; IDO: 5′-CATCTGA-CAAATGATCATAAG-3′ and 5′-CAGTGGCACATTAACCTCC-CTC-3′. Sequence-specific amplification was analyzed on the CFX96 Real-Time PCR Detection System (Bio-Rad). The data were normalized to the TBP expression and the relative expression levels were calculated using the 2−ΔΔCt method. Concentrations of cytokines and growth factors were assessed in plasma specimens using Human Cytokine 30-plex protein assay (Invitrogen) on FLEXMAP 3D System (Luminex) at the Clinical Immunobiology and Correlative Studies Laboratory (City of Hope).

Immunohistochemistry and confocal microscopy

For immunohistochemical analysis, 4-μm sections were deparaffinized and immunostained using antibodies specific for CD15 (Abcam) or pSTAT3 (Cell Signaling Technology). The slides were analyzed first using upright AX70 microscope (Olympus) followed by whole-slide imaging using NanoZoomer scanner and NDP v.2.5 software (Hamamatsu Photonics). To assess the morphology of CD15 HICD33LO and CD15LO cell lysates and blood serum from different stage prostate cancer patients were measured using the QuantiChrom Arginase Assay Kit (BioAssay Systems). To prepare lysates, isolated MDSCs were lysed with 10 mmol/L Tris-HCl (pH 7.4) containing protease inhibitors (Complete Mini, Roche) and 0.4% Triton X-100 for 15 minutes on ice. For blood serum samples, urea was removed using Amicon Ultra-0.5 centrifugal filter devices (Millipore).

Statistical analysis

The Unpaired t test was used to calculate the two-tailed P value to estimate statistical significance of differences between two experimental groups. One- or two-way ANOVA plus Bonferroni posttest were applied to assess the statistical significance of differences between multiple treatment groups or patients from different stages and healthy donors. Statistically significant P values are indicated in the figures with asterisks: *, **, ***, P < 0.001; **, P < 0.01; *, P < 0.05. Data were analyzed using Prism v.6.0 software (GraphPad).
Figure 1.
CD15\textsuperscript{+} myeloid cells accumulate in peripheral blood and tumor tissues in prostate cancer patients with disease progression. A and B, flow cytometric analysis of fresh PBMCs from healthy subjects and prostate cancer patients with localized or metastatic disease. Representative dot plots (A) and graphs combining data from all subjects (B) showing percentages of CD15\textsuperscript{+}CD33\textsuperscript{LO} or CD15\textsuperscript{+}CD33\textsuperscript{HI} cells in prostate cancer patients' circulation at localized prostate cancers (PC; n = 11) or metastatic tumors (mCRPC; n = 10) compared with healthy donors (n = 5). Shown are means ± SD. C, representative histograms showing expression of lineage markers (Lin = CD3/CD19/CD56), CD11b, CD14, HLA-DR, and CD114 (G-CSFR) among CD15\textsuperscript{+}CD33\textsuperscript{LO} (top row) and CD15\textsuperscript{+}CD33\textsuperscript{HI} (bottom row) cells. D, cellular morphology of sorted, cytospinned, and stained CD15\textsuperscript{+}CD33\textsuperscript{LO} or CD15\textsuperscript{+}CD33\textsuperscript{HI} myeloid cells.Representative microphotographs showing monocytic (top) and granulocytic polymorphonuclear (PMN) phenotype (bottom) of CD15\textsuperscript{+}CD33\textsuperscript{LO} and CD15\textsuperscript{+}CD33\textsuperscript{HI} cells, respectively. E, CD15\textsuperscript{+} cells in cancer patients' prostate tissues have granulocytic and PMN phenotype. (Continued on the following page.)
Results

Prostate cancer progression is associated with the accumulation of circulating CD15HI CD33LO granulocytic myeloid cells

We used flow cytometry to phenotype immune cell populations in blood derived from patients with localized or metastatic prostate cancers compared to healthy subjects. As shown in Fig. 1A and B, increased percentages of circulating myeloid cell subsets were more sensitive indicators of tumor presence and progression than changes in lymphoid cell populations, such as regulatory T cells, or in plasmacytoid DCs (Supplementary Fig. S3). Compared with controls, both groups of prostate cancer patients showed significant accumulation of blood CD15HI CD33LO granulocytic myeloid cells, whereas the percentage of CD15LO CD33HI myeloid cells did not change (Fig. 1B). In addition, the percentage of CD15HI CD33LO granulocytic myeloid cells was increased more than twice in mCRPC patients compared with patients with localized tumors (Fig. 1B). The percentage of CD15HI CD33LO myeloid cells in mCRPC patients remained elevated even after standard docetaxel treatment regimen (Supplementary Fig. S4). Further phenotypic characterization indicated that both myeloid cell populations share expression of the common myeloid marker CD11b while being negative for lineage-markers CD3, CD19, and CD56 (Fig. 1C). However, monocytic-lineage marker CD14 and HLA-DR molecules were expressed only by CD15LO CD33HI and not by CD15HI CD33LO (Fig. 1C). Therefore, CD15HI CD33LO cells resemble the granulocytic subtype of MDSCs as also indicated by G-CSFR/CD114 expression, which is associated with this subpopulation (Fig. 1C; ref. 26). To verify this observation, we next analyzed cytomorphology of both populations after cell sorting. As expected, CD15HI CD33LO cells show polymorphonuclear (PMN) morphology typical for granulocytic cells (top) compared with mononuclear CD15LO CD33HI cells (bottom; Fig. 1D).

Figure 2.

CD15HI MDSCs isolated from prostate cancer patients inhibit proliferation and activity of autologous T cells. A to C, CD15HI CD14- granulocytic and CD15- CD14+ monocytic cell populations freshly enriched from metastatic prostate cancer patients’ PBMCs were cultured separately with autologous T cells in presence of CD3-/CD28-specific antibodies for stimulation. A, representative flow cytometry data showing T-cell proliferation assessed by CFSE dilution after 3 days of coculture. B, combined results of T-cell proliferation assays from 5 patients showing percentage of total T-cell proliferation at different T:myeloid cell ratios. C, proliferation of CD4+ and CD8+ T cells when incubated at 1:1 ratio with or without the indicated myeloid cell populations; means ± SD (n = 5). D and E, CD15HI CD14- myeloid cells inhibit production of IFNγ and granzyme B by activated CD8+ T cells. T cells were cocultured with either one of myeloid cell populations at 1:1 ratio as above. The intracellular levels of IFNγ and granzyme B were measured using flow cytometry. Representative dot plots and bar graphs showing percentages of CD8+ IFNγ+ T cells (D) and CD8+ Granzyme-B+ T cells (E) after 3 days of culture; shown are means ± SD (n = 5). Asterisks, statistically significant differences.

(Continued) Representative results of immunohistochemical staining on FFPE sections from two different patients (top and bottom); scale bar, 100 μm. F, mature CD68+ neutrophils are only minor fraction of circulating CD15HI CD33LO cells in prostate cancer patients. The expression of CD68 was assessed on granulocytic cells from healthy subjects or prostate cancer patients. Shown are representative dot plot graphs (two left panels) and the summary of results from 6 different patients (right bar graph); means ± SD (n = 6). G, plasma levels of G-CSF and several other growth factors/chemokines increase with prostate cancer progression in contrast to reduced levels of proinflammatory IFNα. Luminex-based analysis of plasma samples from prostate cancer patients with localized (n = 25) or metastatic tumors (n = 15) compared with healthy individuals (n = 4). Asterisks, statistically significant differences; means ± SD.
PMN features of CD15<sup>+</sup> cells, such as partly segmented nuclei, were also detectable in immunohistochemical stainings of prostatectomy sections derived from mCRPC patients (Fig. 1E). However, flow cytometric results detected only a small fraction of mature CD16<sup>HI</sup> neutrophils within CD15<sup>HI</sup>CD33<sup>LO</sup> cell compartment, which indicates that majority of these cells are immature granulocytes (Fig. 1F). Our further comparative analysis of plasma samples from patients with localized versus metastatic tumors indicated correlation between the elevated numbers of CD15<sup>HI</sup>CD33<sup>LO</sup> cells and the increase in plasma concentrations of G-CSF, HGF, bFGF, and IL8 with the concomitant reduction of proinflammatory IFN<sub>α</sub> (Fig. 1G). Overall, prostate cancer progression from localized to metastatic disease correlates with changes of cytokine/growth factor environment that may promote accumulation of circulating CD15<sup>HI</sup>CD33<sup>LO</sup> granulocytic cells.

G-MDSCs inhibit proliferation and activity of autologous T cells

We next verified whether prostate cancer–associated CD15<sup>+</sup> granulocytic cells show immunosuppressive activity. The effect on the proliferation and activities of autologous T cells was assessed using CD14<sup>+</sup> CD15<sup>+</sup> monocytes as a negative control. As shown in Fig. 2A and B, the CD14<sup>+</sup> CD15<sup>+</sup> myeloid cells reduced CD3/CD28-driven T-cell proliferation to approximately 50% at 2:1 or approximately 80% at 1:1 ratio of T cells to myeloid cells, respectively. In contrast, the presence of CD14<sup>+</sup> CD15<sup>+</sup> monocytes did not significantly alter T-cell proliferation. The CD14<sup>+</sup> CD15<sup>+</sup> myeloid cells had similar effect on both autologous CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Fig. 2C) as well as on allogeneic T cells (Supplementary Fig. S5). The CD14<sup>+</sup> CD15<sup>+</sup> myeloid cells but...
not monocytes from mCRPC patients, also strongly inhibited IFNγ (Fig. 2D) and granzyme B production by effector CD8+ T cells (Fig. 2E). These results confirmed that CD15+ myeloid cells accumulating in the circulation of prostate cancer patients are functionally MDSCs.

**G-MDSCs in circulation show higher level of pSTAT3**

Recent clinical studies suggested that STAT3 contributes not only to MDSC expansion, as suggested by mouse tumor models, but plays a critical role for the expression of several mediators of immunosuppression (21, 27). Thus, we used flow cytometry to assess levels of activated and tyrosine-phosphorylated STAT3 (pSTAT3) in G-MDSCs during prostate cancer progression. As shown in Fig. 3A, CD15+CD33+ cells from patients with both localized and metastatic disease had elevated levels of intracellular pSTAT3 compared to control healthy subjects. These changes reflected increased STAT3 activation rather than upregulated expression of STAT3 total protein, which did not change between healthy subjects and prostate cancer patients (Fig. 3B). Importantly, the percentage of G-MDSCs with high STAT3 activity increased significantly during disease progression, reaching maximum in mCRPC patients (Fig. 3C). Next, we verified whether pSTAT3+ granulocytic cells are present not only in circulation but also in the prostate cancer microenvironment. Sections of prostatectomy-derived tumors (n = 10) were stained using immunohistochemistry for pSTAT3, counterstained, and microscopically analyzed. pSTAT3+ cells with PMN morphology were present in patients’ samples and could be found in prostate tissues, including lumen of benign glands (Fig. 3D). Thus, we conclude that granulocytic CD15+/pSTAT3+ cells are commonly present in mCRPC patients’ circulation and likely reach into the prostate tumor microenvironment.

**Prostate cancer–associated G-MDSCs secrete high levels of Arginase-1**

To assess whether G-MDSCs actively contribute to prostate cancer–associated immune evasion, we compared iNOS, IDO, or ARG1 mRNA levels in CD15+/pSTAT3+ G-MDSCs sorted from cancer patients in comparison with CD15+/pSTAT3− cells in healthy control subjects. The qPCR analysis indicated more than 100-fold increase in ARG1 levels in cancer patients’ samples (Fig. 4A). In contrast, the IDO mRNA analysis indicated only 5-fold while there was no effect on iNOS expression in the same group of samples. These results were further corroborated by measuring ARG1 enzymatic activity directly in cellular lysates prepared from CD15+CD14+ cells derived from metastatic prostate cancer patients versus healthy subjects. CD15+CD14+ cells from mCRPC patients showed about 10-fold increase in ARG1 activity compared with controls (Fig. 4B). In addition, the overall frequency of ARG1 expressing CD15+CD33+ cells from patients with both localized and metastatic tumors, respectively (Fig. 4D). These changes were paralleled by significantly elevated enzymatic activity of ARG1 in patients’ plasma samples during disease progression. Compared with baseline levels in healthy controls, the ARGI1 activity was doubled or tripled in patients with localized or metastatic tumors, respectively (Fig. 4E). Overall, these data implicate the role of ARG1 in

**Figure 4.**

The percentage of arginase-expressing MDSCs increases with prostate cancer progression. A and B, Arginase 1 expression and activity is highly elevated in G-MDSCs from mCRPC patients. Levels of ARG1 mRNA in comparison with iIDO and iNOS transcripts (A) as well as intracellular activity of Arginase 1 (B) were assessed in CD15+CD14+ G-MDSCs using real-time qPCR and QuantiChrom assays, respectively. Shown are means ± SD (n = 4). C, prostate cancer progression correlates with increase in the percentage of arginase-expressing CD15+CD33+ G-MDSCs. Flow cytometric analysis comparing PBMCs from healthy individuals (n = 4) with prostate cancer patients with localized disease (n = 6) or mCRPCs (n = 6); means ± SD. D, high intracellular levels of arginase expression in G-MDSCs from prostate cancer patients compared with granulocytes from healthy subjects as assessed using flow cytometry. Representative histograms (three left panels) and bar graph combining all data (right) from one of three experiments are shown; means ± SD. E, plasma levels of arginase activity increase with disease progression as measured in blood samples from healthy individuals (n = 4), prostate cancer patients with localized (n = 6) and metastatic disease (n = 6); means ± SD. Asterisks, statistically significant differences.
Targeted STAT3 silencing using CpG-STAT3siRNA strategy abrogates immunosuppressive activity of granulocytic MDSCs. A, CD15^HICD33^LO G-MDSCs express TLR9 at mRNA or protein levels as assessed by using real-time qPCR (left) or flow cytometry (right), respectively. CD19^+ B cells and CD3^+ T cells were used as positive and negative controls for qPCR analysis (left), respectively. B, dose- and time-dependent internalization of CpG-STAT3siRNA by CD15^HICD33^LO MDSCs. PBMCs from prostate cancer patients were incubated with fluorescently-labeled CpG-STAT3siRNA or unconjugated STAT3siRNA for the indicated times and doses without any transfection reagents. (Continued on the following page.)
promoting and sustaining the immunosuppressive microenvironment in human prostate cancers.

Prostate cancer-associated G-MDSCs express TLR9 and are effectively targeted by CpG-STAT3siRNA conjugates

We previously demonstrated that ligands for the intracellular TLR receptors can deliver siRNA molecules into mouse and human target cells (23, 24). The unformulated CpG-siRNA conjugates silence specific genes both in vitro and in vivo. As verified using real-time qPCR, CD15+/CD3+ MDSCs isolated from prostate cancer patients’ blood express TLR9 at both mRNA and protein levels similar to B cells used as a positive control (Fig. 5A; ref. 24). Next, we determined whether G-MDSCs can internalize CpG-STAT3siRNA. PBMCs from late-stage prostate cancer patients were incubated for various times and doses with fluorescently labeled CpG-siRNA15-9 or unconjugated siRNA15-9 without any transfection reagents. After 30 minutes of incubation, the uptake of CpG-STAT3siRNA exceeded 80% of G-MDSCs and continued to increase with longer incubation times (Fig. 5B, left two panels). At 4 hours, the CpG-STAT3siRNA was internalized by the majority of cells already at the lowest 100 nmol/L concentration (Fig. 5B, middle). In contrast, the G-MDSCs did not internalize the unconjugated STAT3siRNA even after 4 hours of incubation at 500 nmol/L (Fig. 5B, right). Confocal microscopy studies showed perinuclear/cytoplasmic localization of the siRNA part of the molecule after uptake by target G-MDSCs (Fig. 5C). This is consistent with the TLR9-mediated mechanism of endosomal release of processed CpG-siRNA conjugates (23). Finally, we examined whether CpG-STAT3siRNA treatment will result in target gene silencing in G-MDSCs. Magnetically enriched G-MDSCs were incubated for 48 hours with 500 nmol/L CpG-siRNA conjugates targeting STAT3 or luciferase genes, the latter being used as a negative, nontargeting control. Both STAT3 expression and pSTAT3 levels were assessed using flow cytometry with western blotting. CpG-STAT3 siRNA treatment reduced STAT3 mRNA expression by approximately 70% (Fig. 5D) and as a result also STAT3 phosphorylation and total protein levels (Fig. 5E). Importantly, STAT3 inhibition did not affect G-MDSC viability as verified by flow cytometry (Supplementary Fig. S6). Next, we determined the effect of CpG-STAT3siRNA on immunosuppressive functions of G-MDSCs. The isolated CD15+CD14+ cells were pretreated using CpG-STAT3 siRNA or control CpG-Luc siRNA overnight and then cocultured with autologous CD3+ T cells (1:1 ratio) with CD3/CD28 costimulation for additional 72 hours. As shown in Fig. 5F, CpG-STAT3siRNA alleviated most of the G-MDSC–mediated effect on T-cell proliferation. Under the same experimental conditions, we analyzed IFNγ and granulocyte B production by CD8+ T cells. Both IFNγ (Fig. 5G) and granulocyte B (Fig. 5H) expression levels were restored to approximately 70% and approximately 80% of control levels, respectively, after STAT3 blocking in G-MDSCs using CpG-STAT3siRNA. Overall, these results suggest the feasibility of using CpG-STAT3siRNA strategy for functional depletion of prostate cancer–associated MDSCs.

CpG-STAT3siRNA blocks ARG1 production and impairs G-MDSC activity

To gain insights into the mechanism(s) of G-MDSCs loss of function, we assessed the effect of STAT3 inhibition on ARG1 expression. CD15+CD14+ cells were isolated from late-stage prostate cancer PBMCs and cultured for 48 hours in the presence of 500 nmol/L CpG-STAT3siRNA or CpG-Luci siRNA. As assessed using flow cytometric and colorimetric assays, both ARG1 expression (Fig. 6A) and enzymatic activity (Fig. 6B) were reduced by approximately 60% in G-MDSCs following STAT3 silencing. To verify whether ARG1 actually contributes to STAT3-mediated suppression of T-cell activity by G-MDSCs, we repeated T-cell proliferation assays using specific inhibitor of arginase activity (nor-NOHA; ref. 21). Prostate cancer–derived G-MDSCs (CD15+CD14+) were pretreated with nor-NOHA alone, CpG-STAT3 siRNA alone, or both reagents combined and then cocultured with autologous T cells with CD3/CD28-mediated stimulation (Fig. 6C). As expected, G-MDSCs treated with nor-NOHA used as a single agent reduced by about half the immunosuppressive effect of G-MDSCs on T-cell proliferation (Fig. 6D). However, STAT3 blocking using CpG-siRNA alone eliminated most of the G-MDSC effect while the combination with nor-NOHA didn’t improve this effect any further (Fig. 6D). Overall, these studies provide a proof-of-principle for targeting immunosuppressive STAT3–ARG1 signaling axis in G-MDSCs as a potential therapeutic strategy to disrupt the immunosuppressive microenvironment in prostate cancers.

Discussion

Here, we provide the first evidence of TLR9+ granulocytic MDSCs that accumulate in prostate cancer patients in correlation with disease progression. We also document that these prostate cancer–associated G-MDSCs require high levels of immunosuppressive STAT3 signaling and Arginase-1 activity protein for suppressing effector T-cell activity. While our results are based on limited number of patients, pSTAT3-positive granulocytic cells were consistently found not only in blood but also patients’ prostatectomy specimens. Finally, we suggest therapeutic strategy for the functional elimination of G-MDSCs using CpG oligonucleotide-mediated delivery of STAT3 siRNA into these TLR9+ myeloid cells. Overall, our findings indicate new and potentially safer therapeutic approach to mitigate immunosuppression in...
prostate cancer patients using gene- and cell type-specific inhibitory oligonucleotides.

The relevance of these results for human cancer therapy is underscored by the lack of clinically relevant strategies for eliminating MDSC-dependent immune evasion. In contrast to results of preclinical studies in mice (28), with notable exception of sunitinib effect in renal cancer patients, current cancer therapies usually fail to reduce the elevated levels of MDSCs (Supplementary Fig. S4; refs. 8, 15, 18). The G-MDSCs were found in circulation of patients with other genitourinary malignancies, such as renal and bladder cancers and several different solid tumors (11, 13, 15, 16). However, due to complex pattern of surface molecules, partly overlapping with other immune cell lineages, targeting MDSCs through antibody-mediated depletion is challenging. Successful targeting of MDSCs requires identification of functional markers associated with the tumor microenvironment, as shown by promising preclinical results on targeting CXCR2-mediated MDSC tumor trafficking (17). Our findings suggest that intracellular signaling mediators of immunosuppression may provide alternative and even more precise functional MDSC biomarkers. This is of importance given the difficulty in identifying MDSCs among other immune cell populations using surface markers or nuclear morphology, as in case of discerning G-MDSCs from mature neutrophils (29, 30). Thus, the contribution of mature neutrophils to the overall immunosuppressive effect cannot be excluded (29). The constitutive activation of STAT3, a known master regulator of immunosuppression, was lately reported in MDSCs associated with several types of human cancers, such as melanoma, head and neck, renal, breast, and pancreatic cancers (15, 21, 27, 31–33). Correspondingly, we have found that majority of granulocytic cells that were infiltrating prostate tissues showed activation of STAT3. These observations merit further studies in a larger set of specimens to validate whether the presence of CD15+/pSTAT3+ and/or CD15+/ARG1+ cells can serve as an indicator of the G-MDSC-induced immunosuppressive microenvironment in prostate cancers. It is known that immunosuppressive activity of G-MDSCs depends mainly on Arginase-1 expression rather than iNOS activity for their function (19). This is consistent with our data from patients’ G-MDSCs, in comparison with normal granulocytic cells, that showed highly elevated levels of ARG1, weak induction of IDO and no detectable effect on iNOS expression. These results seem to reflect different level of STAT3 involvement in the regulation of these target genes. In MDSCs, STAT3 was shown to have potent and direct effect on ARG1, while it collaborates with noncanonical NF-kB signaling to promote IDO expression (19, 21, 32). In contrast, STAT3 activity is

![Graphs and images](Figure 6.)
ment (Fig. 1G). Downstream TLR9 signaling through MyD88 and multiple soluble factors released from the tumor microenvironment (24, 37, 38). TLR9 expression in prostate cancer patients’ G-MDSCs is likely associated with the multiple soluble factors released from the tumor microenvironment (Fig. 1G). Downstream TLR9 signaling through MyD88 and NF-κB contributes to myeloid cell-driven inflammatory responses in reaction to tissue stress and injury (39–41). Interestingly, two reports showed that intratumoral injections of high doses of synthetic TLR9 agonists, CpG oligodeoxynucleotides (ODN), can trigger MDSC differentiation and reduce their immunosuppressive potential in mice (34, 35). These effects were suggested to be either direct or mediated through activation of plasmacytoid DCs and production of type I IFNs. However, numerous clinical trials using CpG ODNs proved that generation of systemic antitumor immune responses is challenging due to immunosuppressive effects of the tumor microenvironment (42). Other studies in mouse tumor models showed that activation of TLRs in myeloid cells can in fact promote expression of immunosuppressive molecules critical for MDSC function, such as Arginase-1, S100A8, II.10, and PGE2 (19, 43–45). The functional dichotomy of TLR9 effects results from cross-talk with other signaling pathways operating under normal or tumor-induced inflammatory conditions. The outcome of TLR9 signaling seems to be defined by a multilayered negative feedback regulation through STAT3 (41, 46). Our own studies in mouse solid tumor models revealed that preexisting STAT3 activity in tumor-associated myeloid cells, such as macrophages and MDSCs, can skew TLR9 signaling toward supporting tumor angiogenesis while blocking antitumor immunity (25, 46). Whether TLR9 contributes to immunosuppressive potential of prostate cancer–associated MDSCs remains to be established in more extensive molecular studies.

STAT3 provides an attractive therapeutic target in tumor-associated myeloid cells (20, 41). At the same time, the role of STAT3 signaling in T lymphocytes is complex. While STAT3 reduces antitumor activity of effector CD8 T cells and promotes generation of tumor-promoting Th17 cells, it is also necessary for the generation of memory T cells and prolonged antitumor immune responses (47–49). Thus, targeting STAT3 for cancer immunotherapy requires myeloid cell-specific strategies to avoid unpredictable adverse effects and toxicities. For example, recent study in mouse melanoma models demonstrated that small-molecule drug targeting upstream STAT3 activators, JAK1/2 kinases, reduced numbers of MDSCs while increasing their immunosuppressive potential and blocking T-cell proliferation (50). We previously demonstrated that TLR9 agonists, CpG ODN, can be utilized for delivery of therapeutic siRNA molecules into TLR9+ cells in both mouse and human systems (23, 24). Targeting STAT3 with parallel TLR9 activation using CpG-STAT3siRNA was shown to eliminate tumor-igenic effects of TLR signaling in mouse tumor models (25). Here, we demonstrated that CpG-siRNA strategy allows for targeting of TLR9+ G-MDSCs to eliminate their immunosuppressive functions without myeloid cell depletion. Further studies should determine whether CpG-STAT3siRNA only modulates G-MDSC activity or induces their differentiation into mature granulocytes or DCs/macrophages. Correspondingly, we previously observed that CpG-STAT3siRNA induces activity of neutrophils against blood cancer xenotransplants in NSG mice (24). Independently from this article, we recently identified that prostate cancer propagating cells in human tumors express TLR9 and it is feasible to target this cancer cell population using CpG-siRNA approach (Moreira and Kortylewski; unpublished results). Taken together, our findings provide support for application of CpG-STAT3siRNA strategy to immunotherapy of advanced prostate cancers alone or in combination with immunotherapies, such as Sipuleucel-T treatment or emerging T-cell-based therapies. Disruption of STAT3 signaling in the tumor microenvironment with concurrent TLR9 stimulation has potential to disrupt the central immunosuppressive circuit paving way to effective antitumor immune responses without toxicities associated with pharmacologic agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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