Tumor and Stem Cell Biology

JAK-STAT Blockade Inhibits Tumor Initiation and Clonogenic Recovery of Prostate Cancer Stem-like Cells

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Abstract

Interleukin (IL)-6 overexpression and constitutive STAT3 activation occur in many cancers, including prostate cancer. However, their contribution to prostate stem and progenitor cells has not been explored. In this study, we show that stem-like cells from patients with prostate cancer secrete higher levels of IL-6 than their counterparts in non-neoplastic prostate. Tumor grade did not influence the levels of expression or secretion. Stem-like and progenitor cells expressed the IL-6 receptor gp80 with concomitant expression of pSTAT3. Blockade of activated STAT3, by either anti-IL-6 antibody siltuximab (CNTO 328) or LLL12, a specific pSTAT3 inhibitor, suppressed the clonogenicity of the stem-like cells in patients with high-grade disease. In a murine xenograft model used to determine the in vivo effects of pSTAT3 suppression, LLL12 treatment effectively abolished outgrowth of a patient-derived castrate-resistant tumor. Our results indicate that the most primitive cells in prostate cancer require pSTAT3 for survival, rationalizing STAT3 as a therapeutic target to treat advanced prostate cancer. Cancer Res; 73(16); 5288-98. ©2013 AACR.

Introduction

Prostate cancer is the most common cancer in men (1). Although the disease often responds initially to androgen-ablation therapy, the cancer recurs in the majority of patients. For these patients with castration-resistant disease, few therapeutic options exist, thus novel therapies are urgently required. There is accumulating evidence that the inflammatory cytokine interleukin (IL)-6 is involved in the etiology of prostate cancer. IL-6 serum levels are elevated in patients with advanced prostate cancer (2), and levels correlate with tumor burden and poor prognosis (3, 4). The ligand and receptor are expressed in both premalignant and malignant prostatic tissue (5, 6) suggesting that the pathway is constitutively active. Accordingly, inhibition of receptor binding using an antibody specific for the ligand-specific subunit gp80 causes regression of prostate tumors in vivo (7, 8), showing the potential of IL-6/IL-6 receptor interactions as a cancer therapy. IL-6 receptors (IL-6R) are composed of an IL-6-specific receptor subunit (gp80) and a signal transducer, gp130 (9). IL-6 uses Janus-activated kinase (JAK)-STAT as mediators of signal transduction (10), primarily STAT3 in prostate cancer (11). Constitutively activated STAT3 has been found in many types of cancer, including leukemia, (12) squamous cell carcinoma of the head and neck (HNSCC; ref. 13), multiple myeloma (14), breast (15, 16), and prostate cancer (11). Introduction of an activating mutant of STAT3 is sufficient to induce transformation of immortalized cells (17), suggesting that the JAK-STAT signaling pathway, through STAT3 activation, is sufficient in mediating tumorigenesis. More recent studies have suggested that STAT3 activation is important for the tumorigenic ability of cancer stem cells in glioblastoma (18), lung (19), and colon cancer (20).

Cancer stem cells have been isolated from a wide range of cancers including leukemia (21), breast (22), brain (23, 24), lung (25), colon (26), pancreas (27), and prostate (28). In prostate cancer, cells with a basal phenotype, CD44+/α6β1hi/CD133+/CD166hi (28), CD49fed/CD24d/CD49fhi/CD133−/CD166hi (29, 30), and CD44+/α6β1hi/CD133− (31) have many of the properties of cancer stem cells. Gene expression profiling of CD44+/α6β1hi/CD133− cancer cells, from primary cultures, revealed significant over-representation of several components of the JAK-STAT signaling pathway (32) providing further evidence of the role of this signaling pathway in prostate cancer.

Current studies on the prostate have failed to account for the heterogeneity at the cellular level as well as the differences between patients. The aim of this study was to examine the role of IL-6 on the fate of stem-like cells derived from patients with prostate cancer. Here, we show that the most undifferentiated cells in cancers express elevated levels of IL-6 and the IL-6-specific receptor. We also show that the JAK-STAT signaling pathway...
pathway is constitutively active in the most primitive cells, and lastly that inhibition of the pathway significantly decreases colony-forming ability in vitro and tumor-initiation in immuno- compromised mice.

**Materials and Methods**

**JAK-STAT inhibitors and CNTO 328**

The pan-JAK inhibitor 2-tetra-butyl-9-fluoro-3,6-dihydro-7H-benzo[h]-imidazo[4,5-f]isoquinoine-7-one, pyridine 6 (P6) was obtained from Calbiochem. The monoclonal antibody CNTO 328 (siltuximab) was obtained from Johnson & Johnson. CNTO 328 is a chimeric (murine-human) IgG1 monoclonal antibody against IL-6, which blocks ligand binding. LLI12 was developed as previously described (33). LLI12 binds directly to the phosphoryl tyrosine 705 (pTyr705)–binding site of the STAT3 monomer.

**Tissue collection, isolation, and culture of tumor cells**

Human prostate tissue was obtained with patients' consent and full ethical approval from patients undergoing radical prostatectomy and from patients undergoing TURP for prostate cancer and from patients undergoing TURP of the prostate for benign prostatic hyperplasia (BPH; Table 1). Grade and stage of tumor were confirmed by histologic examination, by an uropathologist, of representative fragments. Epithelial cultures were prepared and characterized as described previously (34). Cell cultures were maintained in stem cell media (SCM) consisting of keratinocyte growth medium supplemented with (EGF, bovine pituitary extract; Invitrogen Ltd.) 2 ng/mL stem cell factor [First Link (UK) Ltd.], 100 ng/mL cholera toxin (Sigma-Aldrich Company Ltd.), and 1 mg/mL granulocyte macrophage colony-stimulating factor [GM-CSF; First Link (UK) Ltd.]. Cells were cultured in the presence of irradiated (60 Gy) STO (mouse embryonic fibroblasts) cells. After expansion, $\alpha_3\beta_1^{\text{hi}}/\text{CD}133^{+}$ (stem-like), $\alpha_3\beta_1^{\text{low}}/\text{CD}133^{+}$ (transit-amplifying), and $\alpha_3\beta_1^{\text{low}}$ (committed basal) cells were isolated by magnetic activated cell sorting (MACS) (Miltenyi Biotec), as described previously (35).

**Generation of xenografts and isolation of tumor cells**

All animal work was approved by the University of York (York, United Kingdom) Animal Procedures and Ethics Committee and conducted under a United Kingdom Home Office License. Human prostatic tissue was obtained, with informed consent, from an 83-year-old patient undergoing palliative channel TURP for advanced prostate cancer. At diagnosis, the tumor was graded Gleason 4+5 and the patient was placed on androgen- ablation therapy. At xenotransplantation, the patient was castrate-resistant.

The fresh sample was cut into 5-mm sections, mixed with 10% Matrigel at 4°C (BD Biosciences), and immediately implanted into the subcutaneous tissues of 2 BALB/c/e Rag2$^{-/-}$;γC$^{-/-}$ mice. This strain was chosen as human cells can be engrafted with high efficiency, as the mice are deficient in T, B, and natural killer cells (36). Mice were bred in our facility and were housed in individually ventilated cages. Once tumors reached 1.5 cm$^3$, which was considered a humane endpoint, the mice were sacrificed and the tumors were either reimplemented into further mice or the tissue was digested for further experiments. Tumor latency was initially 93 days, but reduced to 45 days with serial transplantation. To maintain the tumor xenograft as “near-patient,” tumors were reestablished from frozen cells after five passages in mice.

To generate single cells from xenografts, tumors were minced into small cubes (3 mm$^3$) and incubated in RPMI-1640 (Invitrogen) containing 5% fetal calf serum (FCS; Invitrogen) and collagenase type I at 200 IU/mL (Lorne Laboratories) for 20 hours at 37°C. Cells were washed in Dulbecco "A" PBS (Oxoid Ltd.) and further disrupted by trituration through a 21G blunt needle (Scientific Laboratory Supplies Ltd.). The cell suspension was then incubated in 0.05% trypsin/EDTA for

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30 minutes at 37°C, passed through a 70-μm cell strainer and a Ficoll gradient (Ficoll-Paque Plus; GE Healthcare Life Sciences) to further enrich for viable cells. Mouse lineage+ and endothelial cells (CD31+) were depleted by indirect labeling with biotinylated anti-lineage+ antibody cocktail (Miltenyi Biotec) and CD31 (ER-MP12; 1:20 dilution; AbD Serotec) for 10 minutes at 4°C. Anti-biotin antibodies conjugated to Microbeads were used as secondary labeling reagent and Lin-/CD31+ cells were collected according to the manufacturer’s instructions (Miltenyi Biotec).

Quantitative real-time PCR
Total RNA was extracted from sorted αβ1hi/CD133+, αβ1hi/CD133−, αβ1loi/CD133+, and αβ1loi cells, derived from malignant and nonmalignant primary cultures, using Qiagen RNasea mini-columns, according to the manufacturer’s protocol. RNA was reverse transcribed, using random hexamers (Invitrogen) and reverse transcriptase (SuperScript II; Invitrogen). Real-time PCR (RT-PCR) was conducted using TaqMan gene expression presynthesized reagents and master mix (Applied Biosystems). Reactions were prepared following the manufacturer’s protocol. All reactions were carried out in triplicate on 96-well PCR plates (ABI PRISM; Applied Biosystems) in an ABI PRISM 7000 sequence detection system (Applied Biosystems). Standard thermal cycling conditions included a hot start of 5 minutes at 50°C and 10 minutes at 95°C, followed by up to 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data analysis was conducted using Microsoft Excel software. Expression values are presented relative to the endogenous control gene, RPLP0.

IL-6 ELISA
Sorted αβ1hi/CD133+, αβ1hi/CD133−, and αβ1loi cells, from malignant and nonmalignant prostate primary cultures, were plated (in triplicate) onto 96-well collagen-coated plates (BD-Biocat; BD Biosciences) at a density of 1 × 104 cells, together with 4 × 105 irradiated STO cells. Conditioned medium was collected over 48 hours and was immediately stored at −20°C. The concentrations of secreted IL-6 in the conditioned media were measured by using the R&D Systems’ Quantikine ELISA Kit, according to the manufacturer’s instructions. Briefly, conditioned media or IL-6 standards were incubated for 2 hours at room temperature in 96-well polystyrene microplates, coated with a mouse monoclonal antibody against IL-6. After washing, an antibody against IL-6 conjugated to alkaline phosphatase was added and incubated for 2 hours at room temperature. A further wash step was conducted, substrate and amplifier was added, and the plate was read at 485 nm.

Cell-based ELISA for detection of STAT3
A cell-based ELISA was used to measure the levels of phosphorylated STAT3 (Tyr705) and total STAT3 simultaneously in primary prostatic cell cultures (R&D Systems), according to the manufacturer’s protocol. Briefly, primary prostate cells (1 × 104) were plated overnight, in triplicate, onto a black collagen-I–coated 96-well plate, washed, and fixed with 4% paraformaldehyde (PFA; w/v). Cells were subsequently incubated with primary antibodies followed by horseradish peroxidase (HRP)- and alkaline phosphatase (AP)-conjugated secondary antibodies. Relative fluorescence units (RFU) of phosphorylated STAT3 and total STAT3 were then measured.

Flow cytometry
Lin−/CD31− tumor cells (isolated from xenografts) were incubated with antibodies to CD44 (clone C26; BD Pharmingen) and CD24 (clone 32D12; Miltenyi Biotec), or an isotype control (Miltenyi Biotec), for 10 minutes at 4°C. Dead cells were excluded using SYTOX Blue (Invitrogen).

For the detection of pSTAT3 and androgen receptor, Lin−/CD31− cells were incubated with Live/Dead stain (Invitrogen) before labeling with cell surface CD44 and CD24 (as above). Cells were then fixed with 1.5% formaldehyde (v/v) for 5 minutes at room temperature, permeabilized with ice-cold methanol for 10 minutes at 4°C, and finally incubated with anti-phospho-STAT3 antibody [Tyr705 (1:10); Cell Signaling Technology] and androgen receptor (Clone 441; Santa Cruz Biotechnology, Inc.), for 30 minutes at 4°C. All cells were analyzed on a CyAn ADP flow cytometer (DakoCytomation) and data processed using Summit v4.3 software (Beckman Coulter Ltd.).

Histochemistry, immunohistochemistry, and immunocytochemistry
Histochemistry was conducted on formalin-fixed, paraffin-embedded mouse xenografts. Sections were stained with hematoxylin and eosin and classified according to Gleason grading (37). Immunohistochemistry was conducted on formalin-fixed, paraffin-embedded normal adult prostate, antigen retrieval with citric acid (pH 6.0), at 97°C for 30 minutes, was followed by incubation with antibodies to IL-6R (rabbit polyclonal C-20; 1:100; Santa Cruz Biotechnology, Inc.), CK5 (mouse monoclonal XM26; 1:100; Vector Laboratories Ltd.), or an isotype control. For the detection of IL-6R, anti-rabbit conjugated to Alexa Fluor 488 was used and anti-mouse Alexa Fluor 586 was used for the detection of CK5 ( Molecular Probes; Invitrogen). Sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector Laboratories Ltd.) and visualized using a Zeiss LSM510 meta confocal microscope.

Immunocytochemistry was conducted on sorted αβ1hi/CD133+, αβ1hi/CD133−, and αβ1loi primary cells. The cell isolates were allowed to adhere for 2 to 3 hours, onto collagen-coated slides, blocked with 10% serum and incubated with primary antibody against IL-6R (rabbit polyclonal C-20; 1:100) or an isotype control for 1 hour. Cells were then fixed with 4% PFA, washed twice with 50 mmol/L NH4Cl, and visualized using anti-rabbit Alexa Fluor 488 and DAPI.

Western blot analysis for detection of phosphorylated STAT3
Cell lysates were prepared, from primary prostate cultures, by adding lysis buffer (CytoBuster Protein Extraction Reagent; Novagen; Merck Chemicals Ltd.) to culture plates for 5 minutes at room temperature. Complete protease inhibitor (EDTA-free Protease Inhibitor Cocktail Tablet; Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Sigma) were then added. Equal amounts of protein were resolved on a 10% to 12%
SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore). Membranes were blocked with 5% semidried milk for 1 hour and then incubated with primary antibodies against phospho-STAT3 (Tyr705; L-500), STAT3 (1:1,000; Cell Signaling Technology), and β-actin (1:10,000; Sigma). Primary antibodies were incubated overnight at 4°C or 1 hour at room temperature followed by incubation with goat anti-mouse immunoglobulin G (IgG) immunoglobulins-HRP or goat anti-rabbit IgG immunoglobulins-HRP secondary antibodies. Membranes were visualized using BM Chemiluminescence Western Blotting Substrate (POD; Roche) and enhanced chemiluminescence (ECL) Hyperfilm (GE Healthcare Life Sciences).

Clonogenic recovery
Primary prostate cultures were treated with either 5 μM/L P6 for 16 hours, 10 μg/mL CNTO 328 for 6 days, or 1 μM/L LLL12 for 24 hours. CD133+ (stem-like) and CD133+ (progenitor) cells were then isolated, counted, and plated on to 35-mm collagen-coated plates (BD Biocoat; BD biosciences) at a cell density of 100 cells per well in the presence of irradiated STO feeders. Colonies were subsequently scored if they contained more than 32 cells (5 population doublings) usually between 14 to 28 days after treatment.

Tumor initiation
Mouse depleted tumor cells (Lin−/CD31−) were treated overnight with 0.5 to 10 μM/L LLL12 or vehicle control. The cells were counted and injected subcutaneously into 6- to 8-week-old Rag2−/−γC−/− mice at limiting dilutions, together with 2 × 10^5 irradiated STO feeder cells in Matrigel. The mice were monitored until the tumors reached 1.5 cm^3 or up to 120 days.

Statistical analysis
Student t test and Kaplan–Meier survival analyses were calculated using Sigma Plot. The log-rank test was used to compare Kaplan–Meier curves. The χ² tests (to determine tumor initiation frequencies and pairwise tests) were calculated using ELDA software (38).

Results
IL-6 expression is highest in undifferentiated, hormone-naive, cancer cells
Primary cultures were generated from tumor tissues of 11 patients with prostate cancer at Gleason grade 6 and above (who had not undergone treatment before biopsy; samples 1–11) and 7 patients who had undergone androgen-ablation therapy (samples 12–18; Table 1). Primary cultures were also generated from 11 patients with the benign condition, BPH.

Quantitative RT-PCR (qRT-PCR) analysis revealed that the more undifferentiated, stem-like (CD44hi/CD133hi) and transit-amplifying cells (CD44+/CD133−) from tumor biopsies expressed 7- and 6-fold higher levels of IL-6 than the more differentiated (committed basal; CD44+, CD133−) cells (Fig. 1A). Because of patient variability, this difference in IL-6 expression did not reach statistical significance. The increase of IL-6 expression in the stem-like and transit-amplifying population was not observed in patients with either benign disease or those who had undergone androgen-ablation therapy. Within the group of patient who had undergone androgen...
ablation, the levels of IL-6 increased with the differentiation of the epithelial cells.

We then assessed the levels of IL-6 secreted from sorted cell populations over 48 hours in culture (Fig. 1B). Similarly, significantly higher levels of IL-6 ($P < 0.05$) were secreted from the stem-like population (from patients with cancer) compared with those with benign disease and the more differentiated cells. In contrast, cultures from patients with benign disease secreted more IL-6 from the transit-amplifying population, compared with either stem-like cells or cell populations committed to differentiation ($P < 0.05$).

These data indicate that IL-6 expression is significantly higher in more stem-like cells from patients with prostate cancer, but only in those patients who had not undergone androgen-ablation therapy, whereas secreted levels of IL-6 were significantly higher in patients with cancer, irrespective of treatment. These data also show that tumor grade did not influence the levels of expression or secretion.

**Stem-like cells express the IL-6R**

We next looked at the expression of IL-6R (gp80) using a combination of immunohistochemistry and immunofluorescence of sorted cell populations (Fig. 2). Analysis of prostate tissue sections showed strong expression within the luminal cells, with weak to absent expression within the basal layer (Fig. 2). However, closer examination, at higher magnification, revealed that rare cells within the basal layer, marked with CK5 (a basal cell marker), coexpressed the receptor (Fig. 2A, inset, arrows). To confirm whether those rare cells were the stem-like population, basal cells (derived from 2 patients with BPH and 1 patient with cancer) were sorted into three populations (stem-like cells, transit-amplifying, and committed basal), and subsequently stained with antibodies to gp80 (Fig. 2B). We observed that the majority of stem-like cells expressed the receptor, but the number of cells expressing IL-6R decreased with differentiation of the epithelial cells. These results indicate that the JAK-STAT signaling pathway could be constitutively active (within the stem-like population) through IL-6 and its receptor subunit (gp80).

**Activation of STAT3 is mediated through IL-6**

To investigate whether the JAK-STAT signaling pathway is regulated by IL-6 in prostate cancer, we examined the relative levels of activated STAT3 (pSTAT3-tyrosine 705). Primary cultures were generated from 2 patients with BPH and 5 patients with prostate cancer (samples 4, 15, 19–21). Western blot analysis results revealed that the level of activated STAT3 was variable, but pSTAT3 was present in both benign disease and cancer (Fig. 3A). Of note, 3 of 5 cancers examined had high levels of pSTAT3; all three had a Gleason score between 7 and 9. We next investigated whether we could abrogate activation of STAT3 using the chemical inhibitor P6. P6 is a specific pan-JAK inhibitor that has been reported to act within the low nanomolar range (IC$_{50}$, 1–15 nmol/L; ref. 39). Downregulation of pSTAT3 was observed, in all primary cultures examined, and was optimal at 5 µmol/L after 16 hours treatment (samples 17, 22, and 23; Fig. 3B). Using P6 as a positive control for pSTAT3 abrogation, we next treated primary cells [from patients with hormone-naive prostate cancer (HNPC)] with a neutralizing antibody to IL-6, CNTO 328 (siltuximab), to determine whether pSTAT3 was similarly downregulated. Siltuximab was effective at blocking activation of STAT3, but only after 6 days of treatment ($P < 0.05$; samples 22–24; Fig. 3C). There was variability between samples, most likely due to the differing levels of endogenous IL-6 secretion between individuals (Fig. 1). Steiner and colleagues similarly observed downregulation of
STAT3 Blockade Reduces Cancer Stem Cell Frequency

pSTAT3 levels after 6 days treatment with 10 μg/mL siltuximab (40).

These results confirmed that pSTAT3 is constitutively active and activation of the pathway is mediated through IL-6.

Blockade of STAT3 activation suppresses colony-forming ability

We next wanted to establish whether prostate stem-like cells are dependent on JAK-STAT signaling for survival. To establish this, we determined the potential of stem-like and progenitor cells to initiate colonies (at clonal density) following suppression of the pathway. Cells derived from patients with Gleason grade 7–9 prostate cancer, including those who had undergone androgen-ablation, were treated with either 5 μmol/L P6 (for 16 hours) or 10 μg/mL CNTO 328 (for 6 days), sorted for CD133+B2 expression and plated to determine colony-forming efficiency (CFE) in the absence of drug (Fig. 4A and B). P6 was only effective at reducing the CFE of stem-like cells in the Gleason 9 group (samples 11, 23, and 31; Fig. 4A), although this decrease in CFE did not reach significance. An increase in the number of colonies was observed, with treatment, in both the Gleason 7 group (samples 10, 25–30) and in patients who had undergone androgen-ablation therapy (samples 16, 32–35). P6 was similarly not effective at reducing the CFE of the progenitor population in any of the groups of patients examined, and in the majority of cases an increase in CFE was observed. Although P6 effectively blocked activated STAT3, the functional potential of both the stem-like and progenitor cells was not affected. In contrast, treatment of 3 patients with high Gleason grade cancers (samples 23, 35, and 36) with CNTO 328, significantly reduced the CFE of the stem-like cells (60% reduction), but had no effect on the number of colonies found by the progenitor cells in 2 of 3 samples tested (Fig. 4B).
15.9% of cells were viable after 16 hours in the presence of P6 and 91% of cells were viable in the presence of CNTO 328 (results not shown). These results show that IL-6 is required for survival of stem-like cells in prostate cancer, but that the JAK-STAT pathway may not be involved as P6 had little effect on reducing colony-forming activity. To address this, we treated cells with a novel pSTAT3 inhibitor, LLL12, which binds specifically to the phosphoryl tyrosine 705 (pTyr705)–binding site of the STAT3 monomer (41). Treatment with LLL12 prevented phosphorylation of STAT3 at a concentration of 1 μmol/L. P6 was used as a positive control and similarly suppressed pSTAT3 (sample 37; Fig. 4C). We then determined the functional effect of suppressing pSTAT3 following treatment with LLL12. Primary cultures were derived from patients with Gleason 8/9 disease and included patients who had undergone androgen-ablation therapy (samples 23, 33, 35, and 36). Statistical analyses were conducted using the Student t test; *, P < 0.05.

STAT3 blockade abolishes tumor initiation

To provide further preclinical evidence, a murine xenograft model was used to determine the in vivo effects of pSTAT3 suppression. This xenograft was recently derived, in our laboratory, from a castrate-resistant patient (Supplementary Fig. S1). pSTAT3 status was determined (by flow cytometry) on dissociated tumor cells [depleted of mouse lineage− hematopoietic and endothelial cells (Lin−/CD31−)]. The majority of
cells expressed activated STAT3 (Fig. 5A) and were subsequently treated for 12 hours with 0.5 to 10 μmol/L LLL12. LLL12 caused marked cell death, with approximately 15% of cells viable following treatment (results not shown). Equal numbers of viable cells were then injected subcutaneously at limiting dilutions (10^5–10^6 cells) to determine tumor frequency. A, dot-plot of pSTAT3 expression. B, ELISA for pSTAT3 and ISTAT3 on primary cells treated with increasing concentrations of LLL12. C, Kaplan–Meier estimator of cumulative survival with and without LLL12 treatment. The log-rank test was used to determine differences between Kaplan–Meier curves; *, P < 0.05. D, dot-plot of xenograft cells following ex vivo treatment with 1 μmol/L LLL12. Tumor cells were labeled with CD44, CD24, and pSTAT3. One percent of cells expressed pSTAT3 (red).

### Discussion

In this study, we provide evidence that STAT3 activation is required for the survival of stem-like cells in prostate cancer, and that stem-like and progenitor cells, from human patients with prostate cancer, have constitutively activated STAT3. Blockade of activated STAT3 by either anti-IL-6–blocking activity, or direct inhibition, significantly suppresses clonogenic ability in vitro and tumorigenicity in vivo. Our results showed that LLL12 is potent at suppressing tumor initiation of human prostate cancer cells in vivo.

### Table 2. Effect of LLL12 on tumor frequency

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10^5</th>
<th>10^4</th>
<th>10^3</th>
<th>10^2</th>
<th>10^-1</th>
<th>Tumor initiation frequency (95% CI)</th>
<th>Percentage of cells forming tumors (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2/2</td>
<td>4/4</td>
<td>3/4</td>
<td>2/4</td>
<td>0/4</td>
<td>1:161 (1:27–1:984)</td>
<td>0.62 (0.1–3.7)</td>
</tr>
<tr>
<td>0.5 μmol/L LLL12</td>
<td>—</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
<td>1:71 (1:15–1:339)</td>
<td>1.4 (0.3–6.5)</td>
</tr>
<tr>
<td>1 μmol/L LLL12</td>
<td>—</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
<td>0/2</td>
<td>1:3,253 (1:537–1:19,709)</td>
<td>0.031 (0.005–0.19)</td>
</tr>
<tr>
<td>5 μmol/L LLL12</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10 μmol/L LLL12</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
show that blockade of the JAK-STAT signaling pathway represents an important therapeutic approach for prostate cancer.

Activation of STAT3 has been shown to occur through binding of the IL-6 family of cytokines to the gp130 receptor in leukemia (12), HNSCC (13), multiple myeloma (14), breast (15, 16), and prostate cancer (11). Furthermore, high circulating levels of IL-6 have been found in patients with advanced cancer, including prostate cancer (2–4). More recently, a microarray gene expression analysis of selected cell populations (including stem-like cells from prostate cancer biopsies) found over-representation of several components of the JAK-STAT signaling cascade, including IL-6 (32). The results of this study support those findings, as the highest levels of IL-6 were observed in cancer stem-like and progenitor cells. We also found that IL-6 levels did not correlate with tumor grade or disease progression, in contrast to reports that IL-6 levels are significantly greater in patients with castrate-resistant prostate cancer than in those with hormone-sensitive disease (6, 42). It is proposed that this increase is due to the abolished suppression of IL-6 expression by androgens (43). As immune cells are likely to make a significant contribution to circulating IL-6 levels, it is impossible to compare this finding with those in our model. Moreover, primary cultures are mostly composed of basal-like cells that do not express active androgen receptor, and will therefore be most likely indirectly influenced by androgens (28).

Our results indicate that the JAK-STAT signaling pathway is constitutively active in the stem-like population in prostate cancer as IL-6 and the gp80 receptor were both expressed. This was confirmed by detection of activated STAT3, in all samples analyzed. We also established that signaling is mediated through IL-6 in prostate as a blocking antibody against IL-6 (CNTO 328) abolished STAT3 activation. The consequences of STAT3 blockade included the suppression of colony-forming ability, suggesting that cancer stem-like and progenitor cells require pSTAT3 for survival. We found that the small-molecule inhibitor, LLL12, was more potent at suppressing colony formation, than either P6 or CNTO 328. Blockade of activated STAT3, P6 was relatively ineffective at suppressing colony-forming activity. Indeed, we observed an expansion in colony numbers (in the majority of patients treated with P6). Although LLL12 treatment significantly reduced cell viability, we found that P6 had little effect on viability. Pedranzini and colleagues showed that P6 was only effective at reducing cell proliferation of myeloma cell lines after 3 days treatment (44). Although we observed a similarly modest reduction in cell viability with CNTO 328 treatment, colony recovery of the stem-like cells was significantly reduced, suggesting that this population is more dependent on IL-6 for survival. However, direct blockade of pSTAT3, with LLL12, effectively suppressed colony formation of both stem and progenitor populations (in patients with high-grade cancer and advanced disease). Our data are supported by others in glioma and colon cancer, who propose that STAT3 is a survival factor for cancer stem-like cells (20, 45). Although the effect with LLL12 was consistent, a larger study should be undertaken to confirm our results.

Siltuximab (CNTO 328) had little effect on survival in recent phase II trials for chemotherapy-treated patients with castration-resistant prostate cancer (46, 47). A dramatic increase in plasma IL-6 was observed following treatment with siltuximab (46) although C-reactive protein (a marker of systemic inflammation) declined after treatment. It is possible that the observed increase in plasma IL-6 was due to a feedback loop in which blockade of pSTAT3 resulted in a concomitant increase in IL-6 levels. Unfortunately, this did not result in increased survival, suggesting that either pSTAT3 is not required for survival or that there is heterogeneity in expression (of pSTAT3, IL-6, or IL-6R) between patients and the different populations of cancer cells, as shown in our study and by others (5).

Finally, a murine xenograft model was used to determine the in vivo effects of pSTAT3 suppression. The xenograft tumor was derived from a patient with castrate-resistant disease and was chosen for this study as LLL12 was effective at reducing colonies from high-grade, advanced tumors, and we observed that the majority of cells expressed activated STAT3. Our results confirm the in vitro data, as tumor initiation was abolished at concentrations of LLL12 that effectively blocked activated STAT3 and supports findings that STAT3 is a survival factor in a number of solid tumors. These data also show that the phenotype of the tumor-initiating cell is reminiscent of an intermediate cell as both CD44 and androgen receptor is expressed. Further work has to be undertaken on sorted cell populations to confirm this. We also found that tumors generated following ex vivo treatment with either 0.5 or 1 μmol/L LLL12 were largely differentiated as the majority of cells expressed CD24 and lacked expression of pSTAT3. We propose that those tumors would not effectively serially transplant and would be more amenable to combination therapy.

In conclusion, we propose that targeting the JAK-STAT pathway is worthy of consideration for advanced prostate cancer. Despite the disappointing results with siltuximab, the evidence from this study and others supports further investigation. It is vital, however, when designing clinical trials that patients are stratified for their likely response to STAT3 blockade.

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

Authors’ Contributions

Conception and design: P. Kroon, C. Li, P.-K. Li, N.J. Maitland, A.T. Collins

Development of methodology: P. Kroon, C. Li, A.T. Collins

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Kroon, P.A. Berry, M.J. Stower, G. Rodrigues, V.M. Mann, M. Simms, D. Bhasin, S. Chettiar

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Kroon, N.J. Maitland, A.T. Collins

Table 3. Pairwise test to determine differences in tumor frequencies

<table>
<thead>
<tr>
<th>Pairwise test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μmol/L LLL12</td>
<td>0.522</td>
</tr>
<tr>
<td>1 μmol/L LLL12</td>
<td>0.016</td>
</tr>
</tbody>
</table>

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