JAK-STAT blockade inhibits tumor initiation and clonogenic recovery of prostate cancer stem-like cells

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STAT3 blockade reduces cancer stem cell frequency

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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 prostate cancer patients 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 ρSTAT3 inhibitor, suppressed the clonogenicity of the stem-like cells in patients with high grade disease. In a murine xenograft model utilized to determine the in vivo effects of ρSTAT3 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 ρSTAT3 for survival, rationalizing STAT3 as a therapeutic target to treat advanced prostate cancer.
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-6 (IL-6) is involved in the aetiology of prostate cancer. IL-6 serum levels are elevated in patients with advanced prostate cancer (2), and levels correlate with tumour 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 tumours in vivo (7, 8), demonstrating the potential of IL-6/IL-6-receptor interactions as a cancer therapy. IL-6 receptors are composed of an IL-6 specific receptor subunit (gp80) and a signal transducer, gp130 (9). IL-6 utilizes Janus kinase-signal transducers and activators of transcription (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 leukaemia, (12) head and neck squamous cell carcinoma (HNSCC) (13), multiple myeloma (14), breast (15, 16) and prostate cancer (11). Introduction of an activating mutant of STAT3 is sufficient to induce transformation of immortalised cells (17), suggesting that the JAK-STAT signalling pathway, through STAT3 activation, is sufficient in mediating tumourigenesis. 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 leukaemia (21), breast (22), brain (23, 24), lung (25), colon (26), pancreas (27) and prostate (28). In prostate cancer, cells with a basal phenotype; CD44+/α2β1hi/CD133hi (28), CD49fhi-Trop2hi (29, 30) and
CD44$^+$CD49$^fhi$Trop2$^+$ (31) have many of the properties of cancer stem cells. Gene expression profiling of CD44$^+$/αβ$^{{hi}}$/CD133$^+$ cancer cells, from primary cultures, revealed significant over-representation of several components of the JAK-STAT signalling pathway (32) providing further evidence of the role of this signalling 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 prostate cancer patients. 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 signalling pathway is constitutively active in the most primitive cells, and lastly that inhibition of the pathway significantly decreases colony forming ability \textit{in vitro} and tumour-initiation in immune-compromised mice.
Materials and Methods

JAK-STAT Inhibitors and CNTO328

The pan-JAK inhibitor 2-tert-butyl-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinoline-7-one, pyridine 6 (P6) was obtained from Calbiochem, San Diego, CA, USA. The monoclonal antibody CNTO328 (siltuximab), was obtained from Johnson & Johnson, Malvern, PA, USA. CNTO328 is a chimeric (murine-human) IgG1 monoclonal antibody against IL6 which blocks ligand binding. LLL12 was developed as previously described (33). LLL12 binds directly to the phosphoryl tyrosine 705 (pTyr705) binding site of the STAT3 monomer.

Tissue collection, isolation, and culture of tumour cells

Human prostate tissue was obtained, with patient consent and full ethical approval, from patients undergoing radical prostatectomy and channel transurethral resection (TURP) for prostate cancer, and patients undergoing transurethral resection of the prostate for benign prostatic hyperplasia (Table 1). Grade and stage of tumour were confirmed by histological 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 (epidermal growth factor (EGF), bovine pituitary extract) (Invitrogen Ltd., Paisley, Scotland) 2ng/mL stem cell factor (First Link (UK) Ltd, Birmingham, UK), 100 ng/mL cholera toxin (Sigma-Aldrich Company Ltd, Dorset, UK) and 1ng/mL GM-CSF (First Link). Cells were cultured in the presence of irradiated (60 Gy) STO (mouse embryonic fibroblasts) cells. After expansion, $\alpha_2\beta_1^{hi}$/CD133$^+$ (stem-like), $\alpha_2\beta_1^{hi}$/CD133$^-$ (transit amplifying, TA) and $\alpha_2\beta_1^{low}$ (committed basal, CB) cells were isolated by MACS (Miltenyi Biotec Ltd., Surrey, UK), as described previously (35).
**Generation of Xenografts and isolation of tumour cells**

All animal work was approved by the University of York Animal Procedures and Ethics Committee and performed 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 tumour was graded Gleason 4+5 and the patient was placed on androgen ablation therapy. At xeno-transplantation 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/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 NK cells (36). Mice were bred in our facility, and were housed in individually ventilated cages. Once tumours reached 1.5cm\(^3\), which was considered a humane endpoint, the mice were sacrificed and the tumours were either re-implanted into further mice or the tissue was digested for further experiments. Tumour latency was initially 93 days, but reduced to 45 days with serial transplantation. To maintain the tumour xenograft as ‘near-patient,’ tumours were re-established from frozen cells after 5 passages in mice.

To generate single cells from xenografts, tumours were minced into small cubes (3mm\(^3\)) and incubated in RPMI 1640 (Invitrogen, Paisley, UK) containing 5% foetal calf serum (FCS; Invitrogen) and collagenase type 1, at 200IU/ml (Lorne Laboratories, Reading, UK) for 20 hours, at 37°C. Cells were washed in Dulbecco ‘A’ PBS (Oxoid Ltd, Basingstoke, UK) and further disrupted by trituration through a 21G blunt needle (Scientific Laboratory Supplies LTD, Hessle, UK). The cell suspension was then incubated in 0.05% trypsin/EDTA for 30 min.
at 37°C, passed through a 70-µm cell strainer and a Ficoll gradient (Ficoll-Paque Plus; GE Healthcare Life Sciences, Little Chalfont, UK) to further enrich for viable cells. Mouse lineage+ and endothelial cells (CD31+) were depleted by indirectly labeling with biotinylated anti lineage+ antibody cocktail (Miltenyi Biotec) and CD31(ER-MP12; 1:20 dilution, AbD serotec, Oxford, UK) for 10 min 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 RT-PCR**

Total RNA was extracted from sorted α2β1hi/CD133+, α2β1hi/CD133−, and α2β1low cells, derived from malignant and non-malignant primary cultures, using Qiagen RNease 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 was carried out using TaqMan gene expression pre-synthesized reagents and master mix (Applied Biosystems, Warrington, UK). Reactions were prepared following manufacturer’s protocols. 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 min at 50°C, 10 min at 95°C, followed by up to 50 cycles of: 95°C for 15 seconds, 60°C for 1 min. Data analysis was carried out using Microsoft Excel software. Expression values are presented relative to the endogenous control gene, RPLP0.

**IL-6 Enzyme Linked Immunosorbent Assay (ELISA)**
Sorted $\alpha_2\beta_1^{hi}/CD133^+, \alpha_2\beta_1^{hi}/CD133^-$, and $\alpha_2\beta_1^{low}$ cells, from malignant and non-malignant prostate primary cultures, were plated (in triplicate) onto 96-well collagen-coated plates (BD-Biocoat, BD Biosciences, UK) at a density of $1\times10^3$ cells, together with $4\times10^4$ 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 condition media (CM) were measured using 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 performed, 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, Abingdon, United Kingdom), according to the manufacturer’s protocol. Briefly, primary prostate cells ($1\times10^4$) were plated overnight, in triplicate, onto a black collagen-I coated 96-well plate, washed and fixed with 4% paraformaldehyde (w/v). Cells were subsequently incubated with primary antibodies followed by HRP- and AP-conjugated secondary antibodies. Relative fluorescence units (RFU) of phosphorylated STAT3 and total STAT3 were then measured.

**Flow cytometry**

Lin$^-/CD31^-$ tumour cells (isolated from xenografts) were incubated with antibodies to CD44 (clone C26; BD Pharmingen) and CD24 (clone 32D12;Miltenyi Biotec Ltd), or an isotype
contol (Miltenyi Biotec), for 10 min 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 labelling with cell surface CD44 and CD24 (as above). Cells were then fixed with 1.5% formaldehyde (v/v) for 5 min at room temperature, permeabilised with ice-cold methanol for 10 min at 4°C, and finally incubated with anti-phospho-STAT3 antibody (Tyr705 (1:10), Cell Signalling Technology, Beverly, MA) or androgen receptor (Clone 441, Santa Cruz Biotechnology, Inc, Dallas, Texas, USA), for 30 min at 4°C. All cells were analysed on a Cyan ADP flow cytometer (Dako Cytomation, Glostrup, Denmark) and data processed using Summit v4.3 software (Beckman Coulter Ltd., High Wycombe, UK).

**Histochemistry, Immunohistochemistry & Immunocytochemistry**

Histochemistry was performed on formalin-fixed, paraffin-embedded mouse xenografts. Sections were stained with haematoxylin and eosin and classified according to Gleason grading (37). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded normal adult prostate. Antigen retrieval with citric acid (pH 6.0), at 97°C for 30 min, was followed by incubation with antibodies to IL6-R (rabbit polyclonal C-20, 1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), CK5 (mouse monoclonal XM26, 1:100, Vector Laboratories Ltd, Peterborough, UK) or an isotype control. For the detection of IL6-R, anti-rabbit conjugated to Alexa488 was used and anti-mouse Alexa586, for the detection of CK5 (Molecular Probes, Invitrogen). Sections were counterstained with DAPI and mounted in Vectashield (Vector Laboratories Ltd, Peterborough, UK) and visualised using a Zeiss LSM510 meta confocal microscope.
Immunocytochemistry was performed on sorted $\alpha_2\beta_1^{\mathrm{hi}}/\mathrm{CD133}^{+}$, $\alpha_2\beta_1^{\mathrm{hi}}/\mathrm{CD133}^{-}$, and $\alpha_2\beta_1^{\mathrm{low}}$ primary cells. The cell isolates were allowed to adhere for 2-3 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 50mM NH$_4$Cl and visualised using anti-rabbit Alexa 488 and DAPI.

**Western blot for detection of phosphorylated STAT3**

Cell lysates were prepared, from primary prostate cultures, by adding lysis buffer (Cytobuster$^\text{TM}$ Protein Extraction Reagent, Novagen, Merck Chemicals Ltd., Nottingham, UK) to culture plates for 5 min at room temperature. Complete protease inhibitor (EDTA-free Protease Inhibitor Cocktail Tablet, Roche, Burgess Hill, UK) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail, Sigma) were then added. Equal amounts of protein were resolved on a 10-12% SDS-PAGE gel and transferred to PDVF Immobilon-P membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% semi-dried milk for 1 hour and then incubated with primary antibodies against phospho-STAT3 (Tyr705) (1:500), STAT3 (1:1000) (Cell Signalling Technology, Beverly, MA) and $\beta$-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 IgG immunoglobulins-HRP or goat anti-rabbit IgG immunoglobulins-HRP secondary antibodies. Membranes were visualized using BM Chemiluminescence Western Blotting Substrate (POD) (Roche) and ECL Hyperfilm (GE Healthcare Life Sciences, Amersham, UK).

**Clonogenic Recovery**
Primary prostate cultures were treated with either 5μM P6 for 16 hours, 10 μg/mL CNTO328 for 6 days or 1μM LLL12, for 24 hours. CD133⁺ (stem-like) and CD133⁻ (progenitor) cells were then isolated, counted and plated on to 35mm 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 > 32 cells (5 population doublings) usually between 14-28 days after treatment.

**Tumour Initiation**

Mouse depleted tumour cells (Lin⁻/CD31⁻), were treated overnight with 0.5 μM – 10 μM LLL12 or vehicle control. The cells were counted, and injected subcutaneously into 6- to 8-week old male Rag2⁻/⁻γC⁻/⁻ mice at limiting dilutions, together with 2 x 10⁵ irradiated STO feeder cells in Matrigel. The mice were monitored until the tumours reached 1.5cm³ or up to 120 days.

**Statistical Analysis**

Student’s t-test and Kaplan-Meier survival analyses were calculated using Sigma Plot. The log rank test was used to compare Kaplan-Meier curves. χ² tests (to determine tumour 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 tumour tissues of 11 patients with prostate cancer at Gleason grade 6 and above (who had not undergone treatment prior to biopsy; samples 1-11, Table 1) 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, benign prostatic hyperplasia (BPH).

qRT-PCR analysis revealed that the more undifferentiated, stem-like (CD44+/α2β1 hi/CD133+), and transit amplifying cells (CD44+/α2β1 hi/CD133−) from tumour biopsies, expressed 7- and 6-fold higher levels of IL-6 than the more differentiated (committed basal; CD44+, α2β1 low) cells (Figure 1A). Due to patient variability this difference in IL6 expression did not reach statistical significance. The increase in IL-6 expression, in the stem-like and TA population was not observed in patients with either benign disease or those who had undergone androgen ablation therapy. Within the group of patient that 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 (Figure 1B). Similarly, significantly higher levels of IL-6 (p<0.05) were secreted from the stem-like population (from patients with cancer) compared to those with benign disease and the more differentiated cells. In contrast, cultures from patients with benign disease secreted more IL-6 from the TA population, compared to either stem-like cells and 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 that have not undergone androgen-ablation therapy, whereas secreted levels of IL-6 were significantly higher in patients with
cancer, irrespective of treatment. These data also demonstrate that tumour grade did not influence the levels of expression or secretion.

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

We next looked at expression of the IL-6 receptor (gp80) using a combination of immunohistochemistry and immunofluorescence of sorted cell populations (Figure 2). Analysis of prostate tissue sections demonstrated strong expression within the luminal cells, with weak to absent expression within the basal layer (Figure 2). However, closer examination, at higher magnification, revealed that rare cells within the basal layer, marked with CK5 (a basal cell marker), co-expressed the receptor (Figure 2A inset; arrows). To confirm whether those rare cells were the stem-like population, basal cells (derived from 2 BPH and a cancer patient) were sorted into 3 populations (SC, TA and CB) and subsequently stained with antibodies to gp80 (Figure 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 signalling 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 signalling pathway is regulated by IL-6 in prostate cancer, we examined the relative levels of activated STAT3 (p-STAT3-tyrosine 705). Primary cultures were generated from 2 patients with BPH and 5 patients with prostate cancer (samples 4, 15, 19 - 21). Western blot results revealed that the level of activated STAT3 was variable, but pSTAT3 was present in both benign disease and cancer (Figure 3A). 3/5 cancers examined had high levels of pSTAT3; all 3 had a Gleason score between 7 and 9. We next investigated whether we could abrogate activation of STAT3 using the chemical inhibitor 2-tert-butyl-9-
fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinoline-7-one, pyridine 6 (P6). P6 is a specific pan-JAK inhibitor that has been reported to act within the low nanomolar range (IC$_{50}$ 1-15nM; (39). Downregulation of pSTAT3 was observed, in all primary cultures examined, and was optimal at 5 μM, after 16 hours treatment (samples 17, 22, 23; Figure 3B). Using P6 as a positive control for pSTAT3 abrogation, we next treated primary cells (from hormone naive (HNPC) patients) with a neutralising antibody to IL-6, CNTO 328 (siltuximab) to determine whether pSTAT3 was similarly down regulated. Siltuximab was effective at blocking activation of STAT3, but only after 6 days of treatment (p <0.05) (samples 22-24; Figure 3C). There was variability between samples, most likely due to the differing levels of endogenous IL-6 secretion between individuals (Figure 1). Steiner and colleagues similarly observed down regulation of pSTAT3 levels after 6 days treatment with 10μg/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 signalling for survival. In order to establish this we determined the potential of stem-like and progenitor cells to found colonies (at clonal density) following suppression of the pathway.

Cells derived from patients with Gleason grade 7-9 prostate cancer, including those that had undergone androgen-ablation, were treated with either 5 μM P6 (for 16 hours) or 10 μg/mL CNTO 328 (for 6 days), sorted for CD133$^{+/−}$ expression and plated to determine colony forming efficiency in the absence of drug (Figure 4A, B). P6 was only effective at reducing the CFE of stem-like cells in the Gleason 9 group (samples 11, 23, 31; Figure 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 patients that 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, 36) with CNTO 328, significantly reduced the CFE of the stem-like cells (60% reduction), but had no effect on the number of colonies founded by the progenitor cells; in 2/3 samples tested (Figure 4B). Both P6 and CNTO328 had only a modest effect on cell viability as 78±15.9% of cells were viable after 16 hours in the presence of P6 and 91±9.5% of cells were viable in the presence of CNTO328 (results not shown). These results demonstrate 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 ρSTAT3 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 μM. P6 was used as a positive control and similarly suppressed ρSTAT3 (sample 37; Figure 4C). We then determined the functional effect of suppressing ρSTAT3 following treatment with LLL12. Primary cultures from high Gleason grade cancers (samples 23, 33, 35, 36) were treated with 1 μM LLL12 for 24 hours. We observed that, unlike CNT0328 and P6, LLL12 treatment significantly affected cell viability. The number of cells remaining after treatment was approximately 40±26% (results not shown). The viable cells were then plated to determine whether CFE was similarly affected. Unlike treatment with P6, we found a significant and marked reduction in the number of colonies, in all patients tested.
(n=4) and in both populations (Figure 4D). These data suggest that targeting constitutive
STAT3, in prostate cancer maybe an attractive therapeutic option.

**STAT3 blockade abolishes tumour initiation**

To provide further pre-clinical evidence, a murine xenograft model was utilized to determine the *in vivo* effects of ρSTAT3 suppression. This xenograft was recently derived, in our laboratory, from a castrate resistant patient (supplementary Figure 1). ρSTAT3 status was determined (by flow cytometry) on dissociated tumour cells (depleted of mouse-lineage positive haematopoietic and endothelial cells (Lin'/CD31')). The majority of cells expressed activated STAT3 (Figure 5A) and were subsequently treated for 12 hours with 0.5 – 10 μM LLL12. LLL12 caused marked cell death, with ~ 15% of cells viable following treatment (results not shown). Equal numbers of viable cells were then injected subcutaneously, at limiting dilutions to determine tumour frequency (Table 2). Strikingly, LLL12 treatment (at 10 and 5 μM) effectively abolished tumour initiation. At these concentrations ρSTAT3 is effectively blocked (Figure 5B). Tumours were only observed from the DMSO-control group; at a frequency of 1:161 and from *ex vivo* treatment with 0.5 and 1 μM LLL12 respectively (Table 2, Figure 5 C). Pairwise tests, to determine if there were differences in tumour frequencies with treatment, determined that 1 μM LLL12, but not 0.5μM LLL12 significantly reduced the ability to initiate tumours (p = 0.016) (Table 3). At this concentration it is unlikely that ρSTAT3 is sufficiently blocked (Figure 5B). Characterisation of tumours, from *ex vivo* treatment with either 0.5 μM or 1μM LLL12, showed that treatment induced differentiation to a more luminal phenotype as the majority of cells expressed CD24. Interestingly only 1% of the cancer cells expressed pSTAT3 following treatment with both 0.5 and 1μM LLL12 (Figure 5D, indicated in red).
These results demonstrate that LLL12 is potent at suppressing tumour initiation of human prostate cancer cells \emph{in vivo}.
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 prostate cancer patients, have constitutively activated STAT3. Blockade of activated STAT3 by either anti-IL-6 blocking activity, or direct inhibition, significantly suppresses clonogenic ability \textit{in vitro} and tumourigenicity \textit{in vivo}. Our results demonstrate that blockade of the JAK-STAT signalling 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 signalling 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 tumour 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 to 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 signalling 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 analysed. We also established that signalling 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 \( \rho \text{STAT3} \) for survival. We found that the small molecule inhibitor, LLL12 was more potent at suppressing colony formation, than either P6 or CNTO 328. Despite 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). Whereas LLL12 treatment significantly reduced cell viability, we found that P6 had little effect on viability. Pedranzini and colleagues (2006) 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 CNTO328 treatment, colony recovery of the stem-like cells was significantly reduced, suggesting that this population is more dependent on IL6 for survival. However, direct blockade of \( \rho \text{STAT3} \), with LLL12, effectively suppressed colony formation of both stem and progenitor populations (in patients with high grade cancer and advanced disease). Our data is supported by others in glioma and colon cancer, which 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 (CNTO328) 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 post 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 the IL-6R), between patients and the different populations of cancer cells, as demonstrated in our study and by others (5).

Lastly, a murine xenograft model was utilized to determine the in vivo effects of pSTAT3 suppression. The xenograft tumour 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 tumours, and we observed that the majority of cells expressed activated STAT3. Our results confirm the in vitro data, as tumour 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 tumours. These data also demonstrate that the phenotype of the tumour 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 tumours generated following ex vivo treatment with either 0.5 or 1μM LLL12 were largely differentiated as the majority of cells expressed CD24 and lacked expressed of pSTAT3. We propose that those tumours 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.
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References


Table 1. Clinical and pathologic characteristics of patient population

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Table 2. Effect of LLL12 on tumour frequency:

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<th>% of cells forming tumours (95% CI)</th>
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<td>1:71 (1:15 - 1:339)</td>
<td>1.4 (0.3 - 6.5)</td>
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<td>1 μM LLL12</td>
<td>1:3253 (1:537 - 1:19,709)</td>
<td>0.031 (0.005 - 0.19)</td>
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<td>10 μM LLL12</td>
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Table 2. Pairwise test to determine differences in tumour frequencies

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<td>1 mM LLL12</td>
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Figure 1. IL-6 is highly expressed within the stem-like cells in prostate cancer. A.
Quantitative RT-PCR analysis of IL-6 on selected cell populations derived from patients with BPH (n=5), hormone naive (HNPC, samples 1-4) and hormone sensitive and castrate resistant prostate cancer (HT, samples 12-16). The results are normalised to the housekeeping gene RPLP0 and the fold difference is relative to the average CT-value of the CB population. B.
ELISA for secreted IL-6. The concentration of IL-6 was determined in conditioned medium collected over 48 hours, from 1000 pre-selected SC, TA and CB cells. Conditioned medium was collected from BPH (n=6), HNPC (samples 5-11) and HT patients (samples 12, 14, 17-18). Individual samples are represented by different symbols and the bar depicts the average within each group. Statistical analysis was performed using the students t-test, * P < 0.05, ** P < 0.005

Figure 2. IL-6 receptor is expressed by luminal cells and rare stem-like cells.
Immunofluorescence of a paraffin section of normal prostate stained with antibodies to CK5 (red), a basal marker, and IL-6 receptor (green). The nuclei were counterstained with DAPI (blue). B. IL-6 receptor staining of selected cells (SC, TA and CB). The percentage of cells of each phenotype, expressing the receptor was quantified by counting ten random fields. Three patient samples (two BPH and one cancer; sample 10) were analysed and images were taken by confocal microscopy, at x63 magnification

Figure 3. Activation of STAT3 is mediated though IL-6 and Janus kinases. A. Western blot showing differential STAT3 phosphorylation in a series of primary cultures derived from patients with cancer (samples 4, 15, 19 - 21) and benign disease (n=2). The results are expressed as the ratio of pSTAT3 to total STAT3. Beta-actin was used as a loading control. B. Western blot analysis of a primary culture (HNPC) treated with P6 (0.5 μM or 5 μM) (for 16
hours) for ρSTAT3 and total STAT3, with beta-actin as a loading control. The levels of each were quantified from 3 primary cultures, derived from patients with hormone naive prostate cancer (samples 17, 22-23), and are expressed as the ratio of ρSTAT3:tSTAT3. C. Western blot analysis of primary cancer cells treated with 10 μg/mL CNTO 328 (+) or an isotype control (-) for 2, 4 and 6 days. The right hand panel depicts the ratio of ρSTAT3:tSTAT3 from three patients with HNPC (samples 22-24). Grey bar (isotype control). Black bar (CNTO 328). Quantification of western blots was carried out using Image J software. Statistical analysis was performed using the students t-test, * P < 0.05.

Figure 4. STAT3 Blockade suppresses colony forming ability. Primary prostate cells derived from patients with either Gleason grade 7 HNPC (samples 10, 25-30), Gleason 9 HNPC (samples 11, 23, 31) or HT (which included hormone sensitive and castrate resistant patients (samples 16, 32-35)) were treated for 16 hours with 5 μM P6 (A) or 10 μg/mL CNTO 328 for 6 days (samples 23, 35, 36) (B). CD133+ (stem-like) and CD133− (progenitor) cells were subsequently isolated and plated (at clonal density) in the presence of irradiated STO feeder cells to determine colony forming efficiency. Colonies were scored if they had undergone ≥ 5 population doublings (32 cells); approximately 14 days after plating. The results are expressed as relative to the colony forming efficiency (CFE) of the non-treated cells (1%). Each circle represents an individual patient and the bar is the average of each group. C. Western blot analysis of a representative primary culture (sample 37) treated overnight with increasing concentrations of LLL12. D. Relative CFE following treatment of stem-like and progenitor cells with 1 μM LLL12, for 24 hours. Primary cultures were derived from patients with Gleason 8/9 disease, and included patients that had undergone androgen ablation therapy (samples 23, 33, 35-36).
Statistical analyses was performed using the Students t-test *P<0.05.

**Figure 5. STAT3 blockade abolishes tumour initiation.** Lin−/CD31− xenograft tumour cells, derived from an 83 year old patient with castrate resistant prostate cancer, were treated overnight with either vehicle control or increasing concentrations of LLL12 (0.5 – 10 μM). Tumour cells were then engrafted into BALB/c/Rag2−/−γC−/− mice at limited dilutions (10^5 – 10^1 cells) to determine tumour frequency. **A.** Dot-plot of pSTAT3 expression. **B.** ELISA for pSTAT3 and tSTAT3 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 1μM LLL12. Tumour cells were labelled with CD44, CD24 and pSTAT3. 1% of cells expressed pSTAT3 (red).
Figure 1
Figure 4
Figure 5
JAK-STAT blockade inhibits tumor initiation and clonogenic recovery of prostate cancer stem-like cells

Paula Kroon, Paul A. Berry, Michael J. Stower, et al.

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