Studies on the Role of Interleukin-6 in Prostate Cancer Development and in the Induction of Chronic Prostatic Inflammation by *Propionibacterium acnes*

by
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ABSTRACT

Prostate cancer is one of the most common cancers in men worldwide. Inflammation is commonly observed in prostatic tissues, and correlative studies suggest that the inflammatory cytokine interleukin-6 (IL-6) may contribute to prostate carcinogenesis. However, the source of IL-6 production in the prostate tumor microenvironment has yet to be determined.

In this thesis project, the cellular origin of IL-6 in both primary and metastatic prostate cancer was examined by a novel chromogenic *in situ* hybridization (CISH) assay. These studies indicated that, contrary to previously published studies, neither primary nor metastatic prostate adenocarcinoma cells express IL-6 mRNA. In contrast, IL-6 expression was very heterogeneous, nearly exclusively restricted to the prostate stromal compartment, and enriched in areas of acute inflammation. In metastatic disease, tumor cells were negative in all lesions and IL-6 expression was restricted to endothelial cells within the vasculature of bone metastases.

We further report initial evidence that IL-6 may be involved in prostate tumor growth as evidenced by a series of allograft studies in C57BL/6J wildtype and IL-6 knockout (IL6-/-) mice. Compared to wildtype mice, IL6-/- mice had a significant reduction in take rate and tumor size of allografts of the TRAMP-C2 prostate tumor cell line. This trend was not observed for the colon MC38 cell line. Interestingly, IL-6 ELISA analyses showed a significant increase in circulating IL-6 levels in mice with TRAMP-C2 tumors.

We then turned to a mouse model to further study the role of IL-6 in inducing and/or sustaining long-term chronic inflammation in the mouse prostate, as accumulating evidence indicates that this may be an important factor in the early

development of prostate cancer. For these studies we aimed to use a clinically relevant human-derived strain of bacteria to induce inflammation in the mouse prostate. We cultured tissues from a series of radical prostatectomy specimens and found that the pro-inflammatory anaerobe *Propionibacterium acnes* (*P. acnes)* can be readily cultured from these tissues. Cultured *P. acnes* isolates were typed using multilocus sequence typing (MLST), the results of which suggested that prostatectomy-derived *P. acnes* isolates are not simply the result of contamination from skin flora. These data, along with studies in wildtype mice indicating that one of the strains of *P. acnes* (PA-2) can induce long-term prostatic inflammation, suggest that this is a clinically relevant species to study. We inoculated IL6-/- mice with the prostate derived PA-2 *P. acnes* and found that unlike wildtype mice that develop chronic inflammation that persists up to a year post-inoculation, mice with IL-6 depletion did not sustain chronic inflammation beyond a 2 month time point.

In summary, in this thesis we aimed to examine the many possible roles of IL-6 in prostate cancer development and progression. We determined that paracrine rather than autocrine IL-6 production is likely associated with any role for the cytokine in prostate cancer progression. Furthermore, we report that systemic IL-6 levels may play a role in prostate tumor growth and that IL-6 could also be involved in the development of long-term bacteria-induced chronic inflammation in the prostate.

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DEDICATION

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I. INTRODUCTION

1.1 Overview of Prostate Cancer

Prostate cancer is the leading type of non-skin cancer diagnosed in American men, and about 220,800 men will be diagnosed in 2015 [1]. Though compared to 2011, the total annual death number has decreased from 33,720 to 27,540, it is still estimated that 1 man in 38 will die from prostate cancer [2].

Screening for prostate cancer includes the prostate-specific antigen (PSA) blood test and digital rectal examination (DRE) [3]. Abnormal results from prostate cancer screening will prompt a follow-up diagnostic test such as trans-rectal ultrasound (TRUS) guided biopsy. The PSA blood test has been approved by the FDA since 1986 as a prostate biomarker, and it has been widely used in men age 50 or more. Currently, patients who have more than 4 ng/mL PSA value are suggested to undergo prostate biopsy to confirm if tumor is present [4]. However, there are various factors in addition to prostate cancer that can affect circulating PSA levels. For example, men with benign prostatic hyperplasia (BPH), urinary tract infection, or prostatitis can also have an elevated PSA level. In fact, studies have suggested that PSA is not a good biomarker to evaluate prostate cancer development as many men with more than 4 ng/ml PSA do not have prostate cancer, while some men with lower PSA still developed prostate cancer [5]. Elevated PSA values leading to the incidental finding of low grade cancer on prostate biopsy have been associated with overtreatment [4, 6-8].

Once prostate cancer is diagnosed, there are several standard treatments currently in use. If the biopsy and other diagnostic tests show low grade (e.g. Gleason score 6

or less) early-stage (e.g. clinically localized to the prostate) prostate cancer, active surveillance may be suggested. This involves delaying treatment and carefully monitoring the patient until test results (PSA, and/or biopsy) show prostate cancer progression [6, 7]. The major treatments for clinically localized disease include surgery, including radical prostatectomy, or radiation, which can either be external beam radiation or brachytherapy (radioactive seed implantation). Some patients with more aggressive disease will undergo combined hormonal therapy and radiation. However, with the presence of prostate cancer metastasis, hormone therapy (e.g. androgen deprivation and/or antiandrogen treatment) is generally the treatment of choice [9-12].

Prostate cancer metastasis

The presence of prostate cancer metastasis is a crucial factor to determine prognosis since currently there is no cure for metastatic prostate cancer. A large scale study analyzed routine autopsies performed on 19,316 men between 1967 and 1995 and reported that 8.2% (1589) of men older than 40 years had prostate cancer that was either previously known or was discovered at autopsy. Among the 1,589 patients who had prostate cancer, 556 patients had cancer metastasis and the major sites of metastatic disease were bone (90% of patients), lung (46%), liver (25%) , pleura (21%), and adrenals (13%) [13].

In the US, annually about 350,000 patients die with bone metastases by numerous cancer types such as lung, renal carcinomas, melanoma, multiple myeloma, neuroblastoma, breast, and prostate cancer [14]. However, why bone and bone marrow are the most frequent prostate cancer metastasis site is not entirely understood. In previous studies, the inflammatory cytokine interleukin-6 (IL-6) has been reported

to potentially be associated with bone metastasis [15]. In fact, patients with bone metastasis tend to have elevated serum levels of IL-6 [15, 16]. IL-6 may also be produced by the bone marrow microenvironment to trigger bone remodeling and to promote osteolysis [14, 17].

Once metastatic tumors develop, most patients are treated with hormone therapy. Since 1941, studies have shown the relationship between androgens and prostate tumor growth [18]. Therefore, androgen-deprivation therapy (castration or medical castration with LHRH analogues) alone or combined with other methods, including inhibiting androgen production and/or blocking androgen action, became the key treatment for metastatic prostate cancer [9, 11, 12, 19]. Even though patients typically initially respond to castration, the vast majority will progress to castration-insensitive disease at a variable rate [9, 11, 19, 20].

1.2 Prostate Inflammation and Prostate Cancer

Cancers associated with chronic inflammation

In the first version of their landmark paper on “cancer hallmarks”, Dr. Hanahan and Dr. Weinberg proposed six characteristics that all cancer cells share: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless reproductive potential, sustained angiogenesis and tissue invasion and metastasis [21, 22]. A decade later, the same authors added to their list of cancer hallmarks the contributions of “tumor-promoting inflammation” on the tumor microenvironment [23].

How is inflammation involved in the neoplastic process? Initiation of cancer is related to somatic changes. Some bacterial and/or viral infections can trigger DNA

alterations (such as viral integration and disruption of tumor suppressor genes) and cause irreversible DNA changes to initiate neoplastic states [24-26]. Next, inflammation, chemical irritants, hormones or chronic irritation can increase cellular proliferation, recruit pro-tumorigenic inflammatory cells, and create reactive oxygen species that can cause oxidative DNA damage. Eventually, host normal growth control is lost [26].

In summary, inflammation can contribute to tumor development in the early neoplastic process by supplying bioactive molecules to the tumor microenvironment, facilitating genomic alterations and instability and promoting angiogenesis. In the later carcinogenic process, inflammation can also help neoplastic cells to spread and metastasize by facilitating extracellular matrix (ECM) remodeling that promotes invasion, and by evading host defense mechanisms [22, 23, 27-30].

Two types of inflammation-related cytokines, pro-inflammatory and anti-inflammatory cytokines, are tightly controlled under normal inflammatory states. However, during chronic inflammation, inflammatory cytokines can act as initiating factors that contribute to the persistence of inflammation. The longer the inflammation persists, the higher the association with carcinogenesis [31, 32]. Acute inflammation, on the other hand, is not considered to be a risk factor for the development of neoplasia. In acute inflammation, the host immune response is transient, although many of the same molecular mediators are generated in both acute and chronic inflammation [31, 33].

Chronic inflammation is caused by a variety of factors including infectious agents (bacterial, viral, and parasitic infections), chronic noninfectious inflammatory diseases (esophageal reflux, fecal bile acids), non-digestible agents (asbestos, coal,

and silica dust) and/or other environmental factors [31]. In the chronic inflammatory state, sustained tissue damage, damage-induced cellular proliferation, and tissue repairing can be observed [34]. The major cellular components in chronic inflammation are macrophages [26, 33], along with other inflammatory leukocytes (lymphocytes and monocytes). Macrophages and leukocytes can fight infections by generating high levels of reactive oxygen and nitrogen species [35]. In addition, these inflammatory cells can also work with transcription factors and various signaling molecules, which are essential in regulating cancer development and inflammation, to create a microenvironment niche for malignant progression [36].

Multiple studies have reported that malignant diseases are initiated by infections [31, 37-39]. Epidemiological data suggests that up to 20% of all cancer cases, a global total of 1.2 million cases per year, are associated with chronic inflammation, chronic infection, or both [31, 37, 40]. One of the strongest association between infection, chronic inflammation, and malignancy is that of the Gram negative bacterium *Helicobacter pylori* as an etiological agent in gastric cancer development [31, 37] Strong experimental evidence shows that *H. pylori* infection can cause active chronic gastritis, which is followed by a high incidence of gastric adenocarcinoma. In fact, patients infected with *Helicobacter pylori* have at least a twofold increased risk in the development of gastric adenocarcinoma [41, 42]. Crohn's disease and colon carcinogenesis is another example to illustrate the association between chronic inflammation and cancer. Patients with 8 years or more of prolonged chronic ulcerative colitis or Crohn's disease have a five to seven fold increased risk of developing colon cancer [43]. Other infectious viral agents, such as human papilloma virus, hepatitis B virus (HBV), Hepatitis C virus (HCV) or Epstein-Barr virus (EBV) are also well known virus-associated malignancies and, interestingly, in addition to

the role of viral integration in the activation of oncogenes, chronic inflammation may play a role as a co-factor in many of these cancers [26, 44, 45].

Prostate inflammation and prostate cancer

As previously discussed, because prostate cancer is one of the most common cancers in men worldwide [1], it is critical to determine the etiological factors that contribute to prostate cancer development that may in turn shed new light on the development of better prevention and treatment strategies [2, 46].

The pathogenesis of prostate cancer not only involves hereditary aspects, but also includes environmental components [40]. Family history, race, and advanced age are considered to be the well-recognized risk factors for prostate cancer progression. However, research has suggested that men with a "westernized" lifestyle have an increased risk to develop prostate cancer. Compared to the Western population, the incidence and mortality rates for prostate cancer are much lower in Asian countries (U.S. rates are 50 to 60 times higher than Chinese population) [47]. When men in Asian countries immigrate West, the incidence of prostate cancer increases rapidly within the first generation [48]. This supports the notion that environmental factors in addition to hereditary factors are involved in the pathogenesis of prostate cancer [40, 46].

Potential environmental exposures involved in prostate cancer pathogenesis include infectious agents, dietary carcinogens, and hormonal imbalances [40]. Histopathological and molecular histopathological studies suggest that most adult prostates contain some degree of either acute or chronic inflammation [40]. Chronic inflammation has gained recent attention in prostate cancer development because chronic inflammation is associated with a putative risk factor lesion called

proliferative inflammatory atrophy (PIA) [2, 40]. In PIA lesions, the prostatic luminal cells show a marked increase in the proliferative fraction and alterations in a number of key molecular pathways involved in prostate cancer can be found. For example, there is down regulation of tumor-suppressor genes: NKX3.1 [49], CDKN1B [50, 51] and upregulation of a number of stress response genes (e.g. GSTP1, GSTA1, Cox-2), [40].

Chronic inflammation may be involved in prostate carcinogenesis by disrupting immune responses and altering the tumor microenvironment [52]. The cause of prostatic inflammation is often not known, but various potential etiological agents are considered for the initial inciting event, including urine reflux, hormonal changes, chemical and physical trauma, dietary factors, oestrogens, corpora amylacea and infection [46]. Infection-induced inflammation especially has drawn a lot of attention. As chronic inflammation is known to be an "enabling characteristic" of cancer, prostatitis due to prostatic infections may be related to prostate carcinogenesis or progression [21]. Many approaches such as epidemiology, rodent models, advanced molecular techniques and histopathological studies, have been used to study the association between inflammation and prostate cancer, and aim to provide insights into the cause of prostate inflammation and its relevance to prostate carcinogenesis [40].

Prevalence of prostatic inflammation

It is estimated that about 16% of men in US have / had symptomatic prostatitis in their lifetime [46, 53], although the link between histological evidence of prostatic inflammation and the clinical syndrome of "prostatitis" is not always clear. Furthermore, studies like the REDUCE (Reduction by Dutasteride of prostate Cancer

Events) trial that collected patient biopsies and analyzed them for inflammation levels, show that about 80% or more of adult prostate tissues have some degree of inflammation [53]. According to the National Institutes of Health (NIH) consensus classification system, prostatitis syndromes can be separated into four categories: acute bacterial prostatitis, chronic bacterial prostatitis, chronic prostatitis / CPPS (including inflammatory and non-inflammatory), and asymptomatic inflammatory prostatitis [46, 54].

Asymptomatic prostatic inflammation accounts for a much higher percentage of prostatitis. There are several ways that men may be diagnosed with asymptomatic prostatic inflammation, such as testing for other genitourinary tract issues (infertility test), examining prostate biopsies when prostate specific antigen (PSA) is elevated, examining transurethral resections of benign prostatic hyperplasia (BPH), and removal of the prostate at surgery or at autopsy [46, 54]. Bacterial prostatitis accounts for only an estimated 5-10% of prostatitis cases. Interestingly, prostate cancer is often multifocal, and multifocal cancer has been suggested to be associated with infectious agents [55]. *Escherichia coli* and *Enterococcus* spp are considered to be the most common causative microorganisms in bacterial prostatitis [56]. Patients with bacterial prostatitis may have symptoms that include urinary frequency and dysuria as well as some other systemic disease.

1.3 Bacterial Infection, a Possible Cause for Prostatic Inflammation

Multiple studies have defined causal relationships between malignant diseases and infections [31, 39, 57]. As prostate cancer histologic specimens frequently show unexplained acute and chronic inflammation and inflammation-associated lesions, studies have attempted to identify possible infectious agents in the prostate of prostate

cancer patients [46]. The development of prostatic inflammation may be related to microbial infection, as previous studies have demonstrated the presence of multiple microbial species, such as the presence of bacterial, protozoal, and/or viral species, in the prostates of prostate cancer patients [46]. Yet, after so many years, we are still looking for infectious agents that are definitively linked to prostate cancer development [46].

Accumulating evidence has demonstrated that infectious agents may induce potentially tumor-promoting prostatitis. In support of this, it has been reported that bacterial species such as *E. coli* and *Pseudomonas* spp. can be cultured from prostatectomy samples [58, 59]. As mentioned previously, *E. coli* and *Enterococcus* spp. are considered as the most common bacteria involved in bacterial prostatitis. By both culture-dependent and/or culture-independent methods, *E. coli* has been identified in both BPH and prostate cancer tissues [60, 61]. Sexually transmitted infection (STI)-related microorganisms have also been reported as prostatitis-related microbial species, including *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Treponema pallidum*, *Gonococcal* spp. and *Mycoplasma* spp. [46]. In addition, other organisms such as *Proteus mirabilis*, *Klebsiella* spp. and *Serratia* spp. have also been reported as possible prostatitis infectious agents. Interestingly, many of the organisms identified are consistent with genera associated with inflammation-associated conditions including bacterial prostatitis and/or urinary tract infections [46, 60].

Additional studies have attempted to demonstrate a causative association between prostatitis and prostate cancer development in human studies and in animal models. The uropathogenic strain of *E. coli* 1677 was transurethrally inoculated into C57BL/6J mice, and induced epithelial proliferation [62]. Other groups have shown that with *E. coli* inoculation, the mouse prostate had reactive dysplasia and oxidative

DNA damage. In addition, the prostate epithelium in the infected cohort showed a decrease in prostate cancer tumor suppressors such as NKX3.1 and PTEN, which corresponds to human prostate cancer development [63, 64]. Interestingly, in a study of virulence factors present in *E. coli* isolates from acute bacterial prostatitis, more than 70% (13 strains out of 18) were reported to contain at least one genotoxin. These genotoxins such as cytolethal distending toxin (CDT) and the newly described colibactin are suggested to potentially contribute to carcinogenesis due their ability to induce DNA damage such as DNA double strand breaks [65]. In summary, *E. coli* remains a potential infectious agent of interest in prostate cancer etiology [46].

Other microorganisms have also been indicated to be involved in inducing prostatitis in humans and in animal models, such as *C. trachomatis* and *Propionibacterium acnes* (*P. acnes*). Both of these species can be cultured from prostate tissues. *C. trachomatis*, for example, can be found in prostatitis [66], BPH [67] and prostate cancer [68]. However, despite several molecular and epidemiological studies, there is no definitive evidence to support the correlation of *C. trachomatis* with prostate carcinogenesis [46, 69-71].

An introduction to *Propionibacterium acnes* (*P. acnes*)

Even though the human prostate is generally considered to be a bacterial flora-free organ in the non-infected state, accumulating studies have suggested that bacterial infection might act as an initiator to trigger chronic inflammation that may in turn contribute to prostate cancer development. Currently, a gram-positive bacterium, *Propionibacterium acnes* (*P. acnes*), is implicated as an additional causative agent associated with prostatic inflammation. *P. acnes* is a pro-inflammatory bacterium that is ubiquitously found on human skin and is the suspected etiological agent in the skin

disease acne vulgaris [55] [72]. Furthermore, *P. acnes* is also often implicated in association with other inflammatory conditions including endocarditis, sarcoidosis and post-surgical infections [73].

P. acnes was first reported to be cultured from 35% of radical prostatectomy tissues by Cohen and colleagues in 2005, and the presence of *P. acnes* was associated with the presence of chronic inflammation in these specimens [74]. Although not all studies have shown a direct correlation, *P. acnes* has been shown to induce immunostimulatory activity when infecting prostate cell lines [75-77]. Moreover, mouse studies have shown that *P. acnes* infection of the prostate can trigger a long-term inflammatory response [78]. Other studies indicate that plasma antibodies to *P. acnes* are correlated to cancer risk [72, 79-81]. Along this line, we can hypothesize that *P. acnes* may act as a stimulator to trigger prostate inflammation. Other studies have also suggested that *P. acnes*-mediated inflammation is associated with prostate cancer development [60, 75, 82].

Yet, any causal relationship between *P. acnes* and prostate cancer still remains controversial [83]. As a predominant bacterial flora of human skin, the presence of *P. acnes* in prostatectomy tissues is often considered to be a culture contaminant from the skin of medical staff and/or the patient instead of a true infection [73, 84-86]. One aim of this thesis project was to use culture-independent molecular methods such as multilocus sequence typing (MLST) analysis to determine if *P. acnes* fall within typical skin/acne clusters or if they are unique to the genitourinary tract. Moreover, we aimed to examine the role of *P. acnes* in the induction of chronic inflammation in the prostate of a mouse model, and further explored this in relation to IL-6 in a knockout mouse model.

1.4 Role of Interlukin-6 and Prostate Cancer

Interleukin 6 (IL-6) is a pleiotropic cytokine that is produced by a panoply of cell types including macrophages, lymphocytes, fibroblasts, synovial cells, endothelial cells, glia cells and keratinocytes and mediates numerous physiological functions. As such, the presence of IL-6 in tissues is not abnormal; however, unrestrained production of IL-6 drives chronic inflammation related diseases such as inflammatory bowel disease, autoimmune disorders, arthritis, hepatitis, pancreatitis, and even cancer [87, 88].

There are two types of IL-6 receptors: a membrane bound form (mbIL-6R), and a soluble form (sIL-6R). IL-6 binding with mbIL-6R can turn on classical IL-6 signaling. Yet mbIL-6R is only expressed in certain type of cells, such as hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes [89]. Compared to mbIL-6R, sIL-6R is secreted by many cell types. Upon IL-6/sIL-6R complex formation, IL-6 trans-signaling is activated, which is critical to transition of acute to chronic inflammation [90, 91]. Previous research has shown that binding of IL-6 to the IL-6-receptor (both sIL-6R and mbIL-6R) can recruit a common component gp130 to trigger three major signaling pathways: the Janus tyrosine family kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, the extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3-K) regulated phosphor-Akt pathway [6, 92].

IL-6 and prostate cancer pathogenesis: paracrine or autocrine

IL-6 is reportedly produced by a number of epithelial cancers including lung, breast, hepatocellular, colorectal and prostate [16]. Early evidence showed that

elevated serum levels of IL-6 are related to hormone refractory or metastatic prostate cancer and that patients with elevated systemic IL-6 levels have a poor prognosis [93]. Studies also suggest that serum IL-6 may serve as a marker of prostate cancer morbidity [15, 94-97]. These data indicate possible roles of IL-6 in prostate cancer development and progression [98, 99]. Yet, whether IL-6 acts in an autocrine or paracrine manner is still debatable in prostate cancer. Two androgen independent prostate cancer cell lines DU145 and PC3 were found to express IL-6, while the androgen-sensitive prostate cancer cell line LNCaP cells does not secrete IL-6 [100, 101].

Several studies have suggested that IL-6 serves as an autocrine growth factor in both primary and metastatic prostate cancer. For primary prostate cancer, enzyme-linked immunosorbent assay (ELISA) of protein extracts from frozen primary tumor and benign tissues showed that about 50% of tumor samples have elevated IL-6 levels compared to benign tissues [101]. Other studies using immunohistochemistry (IHC) suggest that IL-6 can be expressed by both primary benign and malignant prostate epithelium [102, 103]. Another group utilized an IL-6 IHC assay to study 26 metastatic prostate cancer cases, and they suggested that IL-6 is secreted primarily by prostate cancer bone metastases and to a lesser extent by soft tissue metastases [104]. In addition, during androgen deprivation therapy, IL-6 along with oncostatin M (OSM) were suggested to activate androgen receptor (AR) and contribute to recurrent prostate cancer [105]. Cumulatively, the data above suggest a possible role for autocrine signaling by IL-6 in prostate cancer development and progression [101, 102, 106].

On the other hand, IL-6 signaling has also been reported as a paracrine cytokine in prostate cancer development. Different cell types such as mesenchymal stem cells

and prostate stroma cells show up-regulation of IL-6 expression [107, 108]. In the same respect, when osteoblast-like cells were cultured with medium from prostate cancer cell lines, increased IL-6 expression from the osteoblast-like cells could enhance osteoclastogenesis [109]. Another recent study identified a role for paracrine signaling from IL-6 up-regulation in mesenchymal stem cells and promotion of adipogenesis and prostate cancer cell migration and invasion. Again, this study suggested that the prostate cancer microenvironment can regulate IL-6 production in promoting tumor progression [110]. In summary, elevated IL-6 may promote metastasis by remodeling the bone microenvironment in a paracrine fashion.

In the context of this thesis, we aimed to comprehensively evaluate the expression and cellular origin of IL-6 in both primary and metastatic prostate cancer. We utilized a recently available chromogenic *in situ* hybridization (CISH) assay that we determined was far superior to IHC for the detected of secreted cytokines such as IL-6 [111]. As discussed in the coming chapters, we found that any role for IL-6 in prostate cancer development or progression must involve its action as a paracrine cytokine. Furthermore, we demonstrate that circulating levels of IL-6 may influence prostate tumor growth. Finally, we provide initial evidence that IL-6 may be required to sustain long-term chronic inflammation induced by bacterial infection in the prostate.

II. A PARACRINE ROLE FOR IL-6 IN PROSTATE CANCER PATIENTS: LACK OF PRODUCTION BY PRIMARY OR METASTATIC TUMOR CELLS

2.1 Abstract

Correlative human studies suggest that the pleiotropic cytokine interleukin-6 (IL-6) contributes to the development and/or progression of prostate cancer. However, the source of IL-6 production in the prostate microenvironment in patients has yet to be determined. The cellular origin of IL-6 in primary and metastatic prostate cancer was examined in formalin-fixed, paraffin-embedded (FFPE) tissues using a highly sensitive and specific chromogenic *in situ* hybridization (CISH) assay that underwent extensive analytical validation.

Quantitative RT-PCR (q-RT-PCR) showed that benign prostate tissues often had higher expression of IL-6 mRNA than matched tumor specimens. CISH analysis further indicated that both primary and metastatic prostate adenocarcinoma cells do not express IL-6 mRNA. IL-6 expression was highly heterogeneous across specimens and was nearly exclusively restricted to the prostate stromal compartment – including endothelial cells and macrophages among other cell types. The number of IL-6-expressing cells correlated positively with the presence of acute inflammation.

In metastatic disease, tumor cells were negative in all lesions examined and IL-6 expression was restricted to endothelial cells within the vasculature of bone metastases. Finally, IL-6 was not detected in any cells in soft tissue metastases. These data suggest that, in prostate cancer patients, paracrine rather than autocrine IL-6 production is likely associated with any role for the cytokine in disease progression.

2.2 Introduction

Interleukin 6 (IL-6) is a pleiotropic cytokine that can be produced by an array of cell types and affects diverse physiological processes including immune responses, hematopoiesis, and cellular proliferation and differentiation [112]. Under normal conditions IL-6 levels in cells are typically low, although a number of stimuli result in induction of IL-6 expression and secretion. For example, during acute inflammatory responses to infections, cellular production of IL-6 is essential to the induction of acute phase proteins. While the normal homeostatic response to inflammation is resolution and reversion of IL-6 production to normal low levels, unrestrained production of IL-6 drives chronic inflammation and increased systemic levels of IL-6 have been associated with diseases such as autoimmune disorders, arthritis, hepatitis, inflammatory bowel disease, pancreatitis, and cancer [113].

Early evidence for a role for IL-6 in advanced prostate cancer came from studies examining serum levels of IL-6 in relation to metastatic or hormone refractory prostate cancer [98, 99, 114]. These data showed that serum levels of IL-6 are significantly elevated in prostate cancer patients with hormone refractory disease compared to normal controls or men with prostatitis, benign prostatic hyperplasia (BPH), and localized and recurrent disease [98]. Likewise, serum IL-6 levels were correlated to patients with clinically evident metastases [99] or with extent of bone metastasis [114]. Subsequent studies have consistently shown that elevated systemic IL-6 levels confer poor prognosis [115-117], and may also serve as a marker of prostate cancer morbidity, including cachexia [118, 119]. A key clinical question is precisely when during disease development and progression IL-6 is expressed and what precise cell types are responsible for its production (e.g., prostate tumor cells or another cellular source). A secondary question is whether the elevated systemic levels

of this cytokine actually drive disease progression, or whether elevated levels of IL-6 are a surrogate for tumor burden.

Along these lines, multiple studies showed that some prostate cancer cell lines can secrete IL-6 *in vitro*. The androgen-independent prostate cancer cell lines DU145 and PC3 have been shown to secrete IL-6, whereas androgen-sensitive LNCaP cells do not [100, 101, 118]. In primary prostate cancer, protein extracts prepared from prostate cancer tissues showed elevated IL-6 levels compared to benign tissues in approximately 50% of cases when analyzed by enzyme-linked immunosorbent assay (ELISA) [101]. Furthermore, a number of studies using immunohistochemistry (IHC) to detect IL-6 in prostate tissues have reported IL-6 production by both benign and malignant prostate epithelium [102, 103]. An additional study utilizing IHC to detect IL-6 in a series of metastatic tissues from 26 prostate cancer patients reported that IL-6 is produced in the majority of prostate cancer bone metastases and to a lesser extent in prostate cancer soft tissue metastases [104]. Another series of studies proposed that IL-6 along with a related member of the IL-6 family of cytokines, oncostatin M (OSM), may activate androgen receptor (AR) in the absence of androgen, providing a potential mechanism whereby IL-6 contributes to recurrent prostate cancer growth following androgen deprivation therapy (reviewed in [106]). Moreover, Tawara et al. proposed that in the bone microenvironment, stromal cells can also produce IL-6 to facilitate tumor metastasis [17]. Cumulatively, these studies have led to the hypothesis that IL-6 serves as an *autocrine* growth factor in both primary and metastatic prostate cancer [101, 102, 106].

Additional studies have suggested a role for *paracrine* IL-6 signaling in prostate cancer progression. Recent work using a human prostate dissociation and tissue recombination system identified a role for paracrine expression of IL-6 or OSM

specifically in the stromal compartment in concert with cell-autonomous oncogenic events, such as PTEN loss of function, in the promotion of an aggressive prostate cancer phenotype [120]. Another recent study identified a role for paracrine signaling from IL-6 up-regulation in mesenchymal stem cells and promotion of adipogenesis and prostate cancer cell migration and invasion [110]. Again, this study indicated a specific role for IL-6 production from the stromal compartment in facilitating prostate cancer progression [110].

Cumulatively, the evidence to date indicates that IL-6 may act as a key mediator in several steps in prostate carcinogenesis including initiation, progression, metastases, and the development of castration resistance and/or resistance to chemotherapy. What is less well understood is what cell type(s) are responsible for production of the cytokine in the tumor microenvironment in patients, and by extension, whether IL-6 in prostate cancer patients functions through autocrine or paracrine mechanisms.

2.3 Materials and Methods

Patient population and clinical samples.

All specimens were acquired under Institutional Review Board (IRB) approved protocols at the respective institutions. RNA samples from matched tumor and benign tissues were obtained from 10 radical prostatectomy specimens using the standard operating procedure (SOP) protocols for the Prostate Cancer Biorepository Network (PCBN) as previously described in detail [121]. Each case consisted of fresh frozen tumor and benign tissues obtained at radical prostatectomy. For RNA isolation, tissues containing cancer were dissected such that they contained at least 70-90% tumor cells. Recently collected formalin-fixed paraffin-embedded (FFPE) primary clinical prostate cancer tissues (<1 year old) were obtained from 21 prostatectomy specimens in

addition to 12 biopsy or autopsy metastatic tissue samples from 9 cases at Johns Hopkins Hospital and 20 bone metastatic tissue samples from 10 cases at the University of Washington Medical Center for use in chromogenic *in situ* hybridization (CISH) assays. One block containing the highest grade/index cancer and adjacent benign was chosen for CISH analysis from each prostatectomy case. The clinical and pathological details of the patient samples are listed in **Table 1**. Tissue microarrays (TMAs) containing metastatic tissues (bone and soft tissue metastases) from 21 cases (University of Washington Medical Center) and 15 cases (Johns Hopkins Hospital) were used in IL-6 IHC experiments.

Cell lines.

LNCaP, VcaP and CWR22Rv1 were obtained from the American Type Culture Collection (ATCC). PC-3, DU-145, MCF7, and NCI-H460 cells were obtained from the NCI-Frederick. PrEC and PrSC cells were obtained from Lonza (Basel, Switzerland). LAPC4, RWPE-1, and C4-2B cells were obtained from J.T. Isaacs (Johns Hopkins University) and LNCaP-abl cells were obtained from Z. Culig (University of Innsbruck). All of the cell lines used were authenticated via short tandem repeat (STR) profiling of 9 genomic loci with the Powerplex 1.2 system (Promega) before use.

Quantitative real-time reverse transcription PCR (q-RT-PCR).

RNA was treated with Dnase I (Rnase-free, Ambion) followed by cDNA synthesis using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) following the standard protocol for 'First-Strand Synthesis Using Random Primers'. Quantitative PCR was performed with SYBR Green Supermix (Bio-Rad) and 0.4 μM IL-6 primers (IL6-F 5'-GGTACATCCTCGACGGCATCT-3' and IL-6-R 5'-GTGCCTCTTTGCTGCTTTCAC-3') or 1.0 μM GAPDH primers (GAPDH-F

Table 1. Clinical Characteristics of Patient Samples Used in the Study

qRT-PCR

Patient #	Age	Tumor Grade	pStage
1	46	3+4=7	T3AN0MX
2	61	4+3=7	T3AN0MX
3	56	4+3=7	T3AN0MX
4	61	4+4=8	T2N0MX
5	49	3+3=6	T2N0MX
6	51	4+4=8	T2N0MX
7	53	3+4=7	T2N0MX
8	65	5+5=10	T3BN0MX
9	56	3+4=7	T2N0MX
10	69	4+5=9	T3AN0MX

CISH (radical prostatectomy)

Patient #	Age	Tumor Grade	pStage
1	63	3+3=6	T2N0MX
2	54	3+4=7	T2N0MX
3	60	4+5=9	T2N0MX
4	70	3+4=7	T2N0MX
5	57	3+3=6	T2N0MX
6	67	3+4=7, tertiary 5	T2N0MX
7	65	3+3=6, tertiary 4	T2N0MX
8	51	3+4=7	T2N0MX
9	63	5+4=9	T2N0MX
10	68	3+3=6, tertiary 4	T2N0MX
11	65	3+4=7, tertiary 5	T2N0MX
12	56	3+4=7	T2N0MX
13	59	3+4=7	T3AN0MX
14	68	3+4=7	T2N0MX
15	61	3+3=6	T2N0MX
16	58	3+4=7	T2N0MX
17	65	3+3=6	T2N0MX
18	68	4+3=7, tertiary 5	T3AN0MX
19	62	3+4=7	T3AN0MX
20	58	4+3=7	T3BN0MX
21	60	3+4=7	T2N0MX

5’-CGCTCTCTGCTCCTCCTGTT-3’ and GAPDH-R 5’-CCATGGTGTCTGAGCGATGT-3’) in a real-time detection system. PCR conditions were as follows: 2 min at 94 °C, 40 cycles of 30 sec at 94 °C, 30 sec at 60 °C, and 30 sec at 72 °C, followed by a melt curve analysis. GAPDH was used as a housekeeping gene for normalization. The fold differences in expression levels of IL-6 in tumor samples were determined using the $2^{-\Delta\Delta C}{}_T$ method, relative to GAPDH and to the matched benign tissue.

Chromogenic *in situ* hybridization (CISH).

CISH was performed using the RNAscope® 2.0 FFPE Brown Reagent Kit or RNAscope® 2-plex assay kit (Advanced Cell Diagnostics, Inc.). Briefly, FFPE tissues were first baked at 60°C for 1 hr followed by deparaffinization in two changes of 100% xylene for 5 min each and two changes of 100% alcohol for 3 min each. Next, the slides were treated with endogenous peroxidase blocking pretreatment reagent for 10 min at room temperature. The slides were then added to boiling buffer for 30 min at 99-104°C in a water bath and then treated with protease digestion buffer for 30 min at 40°C. The slides were incubated with a custom RNAscope target probe designed against IL-6 mRNA (probe region 27-876, NCBI reference sequence Accession #NM_000600.3) or peptidyl prolyl isomerase B (PPIB), also known as cyclophilin B, as a positive control mRNA (probe region 139-989, NCBI reference sequence Accession #NM_000942.4) for 2 hr at 40°C, followed by signal amplification. DAB was used for colorimetric detection for 10 min at room temperature.

Immunohistochemistry (IHC).

IHC was performed using the Power Vision+ Poly-HRP IHC kit (Leica Biosystems). Slides were steamed for 45 min in antigen retrieval solution (Dako

#S1700) and incubated with rabbit polyclonal anti-IL-6 antibody (#6672; Abcam, lot #GR106735-5 at 1:1000 dilution) for 45 minutes at room temperature. Poly-HRP-conjugated anti-rabbit IgG antibody was used as secondary antibody. Staining was visualized using 3,3′-diaminobenzidine (Sigma), and slides were counterstained with hematoxylin.

Western blot.

Fresh frozen tumor and benign tissue samples were obtained from 3 patients using the standard operating procedure (SOP) protocols of PCBN [121] as described above. Tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Sigma) and phosphatase inhibitors (Cell Signaling). Lysates were then centrifuged at 14,000 X *g* for 10 min at 4°C. Proteins were electrophoresed and transferred to nitrocellulose membranes for immunoblotting. Membranes were probed with IL-6 antibody (#6672 1:500 dilution; Abcam, lot #GR106735-5) and beta-actin (13E5, 1:1000; Cell Signaling). The blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Recombinant mouse IL-6 (R&D Systems) was used as a positive control for Western blot.

Controls for CISH, Western blot, and IHC.

MCF7 (breast cancer cell line) cells were transfected with the IL-6 cDNA clone expression vector (Origene, SC125236) using lipofectamine (Life Technologies). NCI-H460 cells were treated with monensin (Golgi-Stop™, BD Biosciences) at a dilution of 1:1000 for 4 hrs.

2.4 Results

Variable expression levels of IL-6 transcript are present in primary prostate adenocarcinoma when compared to matched benign tissues as assessed by q-RT-PCR.

To determine if IL-6 expression levels in primary prostate cancer are associated with prognostic factors, we obtained a series of RNA samples from fresh-frozen cancer and matched benign tissues from 10 radical prostatectomy specimens. This series was enriched for cases of higher grade (Gleason ≥ 7) cancer (Table 1). Somewhat surprisingly, IL-6 message levels were not significantly elevated in tumors compared to matched benign tissues, as only 3 of 10 prostatectomy samples were observed to have higher expression of IL-6 in tumor compared to benign tissue (Figure 1). Conversely, there were more cases that showed higher levels of IL-6 in the benign regions than in those containing cancer (Figure 1). Based on this apparent discrepancy with published data [101, 102], we queried the Oncomine database [122] for datasets of prostate carcinoma vs. normal tissues and an analysis of 17 such datasets showed only one dataset with a greater than 2-fold increase in cancer versus benign tissues and a p-value ≤ 0.05 (Table 2). Due to our observation of a low frequency of IL-6 overexpression in tumor compared to benign tissues as well as the elevated IL-6 levels observed in benign tissues in our q-RT-PCR studies (Figure 1B) we next sought to determine the cellular origin of IL-6 in prostate specimens.

Development of a highly sensitive and specific IL-6 chromogenic in situ hybridization (CISH) assay.

For the development of an IL-6 CISH assay, we used the highly sensitive RNAscope® 2.0 assay from Advanced Cell Diagnostics (ACD). This assay consists of

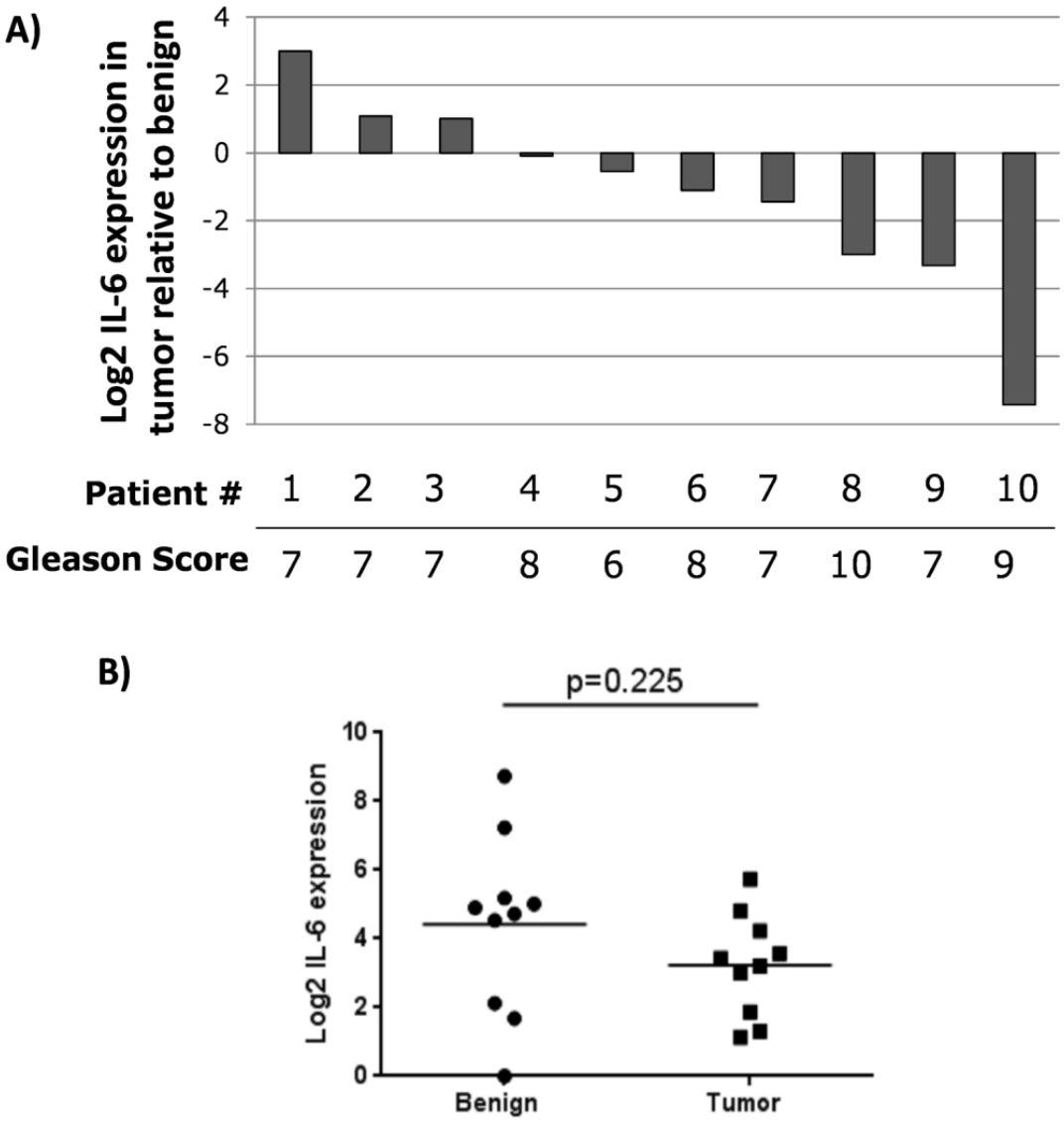

Figure 1. IL-6 mRNA expression in benign and malignant prostate tissues as assessed by q-RT-PCR.

RNA extracts were prepared from matched tumor and benign tissues from radical prostatectomy specimens. IL-6 mRNA expression was then determined by q-RT-PCR. **(A)** Log2 IL-6 mRNA expression in tumor relative to matched benign in 10 prostatectomy specimens. IL-6 mRNA expression levels were normalized to GAPDH and then tumor was compared to benign using the $2^{-\Delta\Delta C}{}_{T}$ method followed by log2 transformation. **(B)** Log2 relative IL-6 mRNA expression in benign samples versus tumor samples.

Table 2. Results of Oncomine Database Query for IL-6 Expression

Data Set	Benign # samples	Tumor # samples	fold change	p < 0.05?
Holzbeierlein Prostate	2	33	2.046	Yes
Taylor Prostate	29	131	1.344	Yes
Magee Prostate	4	8	10.21	No
TCGA Prostate	61	45	1.044	Yes
Varambally Prostate	6	7	1.632	Yes
Tomlins Prostate	23	30	-1.252	No
LaTulippe Prostate	3	23	3.687	No
Yu Prostate	23	65	1.235	No
Grasso Prostate	28	59	1.713	Yes
Singh Prostate	50	52	1.01	No
Welsh Prostate	9	25	-1.175	No
Lapointe Prostate	41	62	-1.278	No
Wallace Prostate	20	69	-1.828	No
Luo Prostate 2	15	15	1.005	No
Liu Prostate	13	44	-1.098	No
Arredouani Prostate	8	13	-1.638	No
Vanaja Prostate	8	27	-1.806	No

a hybridization probe set complimentary to a length of the protein coding region of IL-6 mRNA (see methods) in which the hybridization event is subjected to signal amplification and chromogenic detection. To establish the specificity of the CISH approach, we transfected MCF-7 cells (which do not express IL-6 mRNA) with an IL-6 cDNA clone expression vector (Origene, SC125236). As shown in Figure 2A, the transfected cells stained strongly positive using the CISH assay and thus served as a genetically defined positive control. To further verify the specificity of the probe set, we next quantified IL-6 mRNA levels in a panel of prostate cancer cell lines (LAPC4, CWR22Rv1, PC3, LNCaP, C4-2B, VcaP, DU145, and LNCaP-abl), benign prostate cell lines (PrSC, PrEC) and an HPV transformed prostate cell line (RWPE-1) via q-RT-PCR. Providing further evidence for the specificity of the CISH assay, we found that there was complete concordance between the two assays; i.e. all lines that were positive for IL-6 by q-RT-PCR were positive by CISH for IL-6 mRNA expression (Figure 2B, C). Of note, although previous studies reported that the PC3 cell line secretes IL-6 [100, 101, 118], we did not find the line to be positive for IL-6 mRNA expression in the present study (Figure 2B, C). We hypothesize that this could be due to differences in cell culture conditions for this highly inducible cytokine. While we did not establish the overall detection limit of the CISH assay, it has been reported to have a detection limit of a single mRNA molecule [123].

Prostate adenocarcinoma cells in prostatectomy specimens do not express IL-6 mRNA.

We next utilized the IL-6 CISH mRNA assay on FFPE tissues from a series of 21 radical prostatectomy specimens of varying Gleason grades and tumor stages (see **Table 1**). In each case, we examined full tissue sections containing the highest grade/index cancer and adjacent benign tissue. We verified the RNA integrity in the

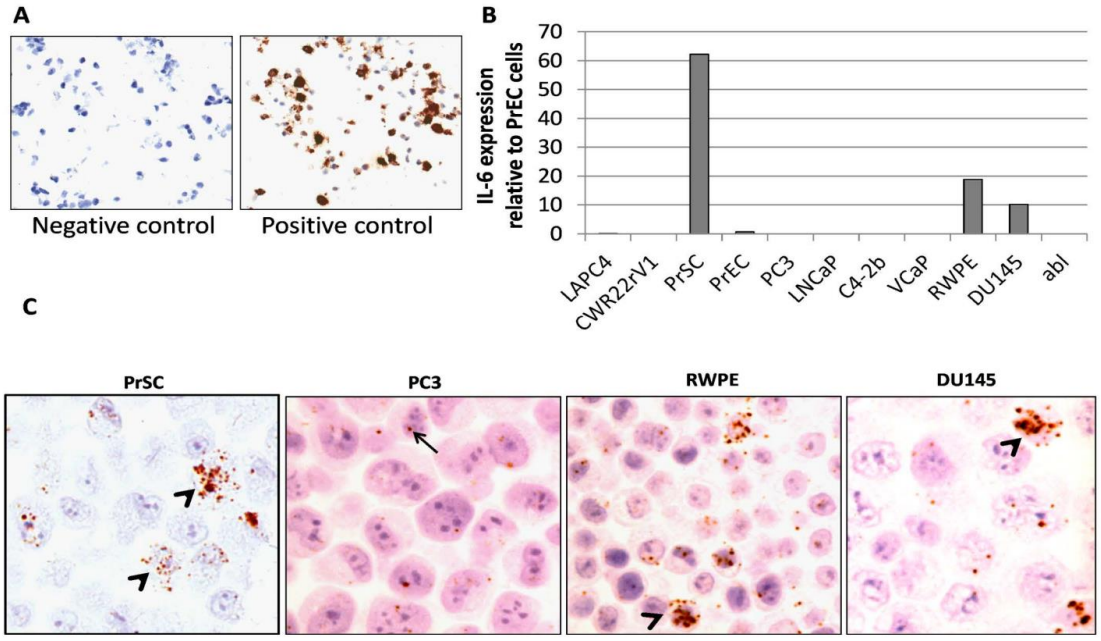

Figure 2. Validation of IL-6 chromogenic in situ hybridization (CISH) assay.

(A) IL-6 transfected (positive control) or non-transfected (negative control) FFPE MCF7 cells assayed by IL-6 CISH. **(B)** IL-6 q-RT-PCR on 11 prostate cell lines. IL-6 mRNA expression levels were normalized to GAPDH and then to PrEC cell expression level using the $2^{-\Delta\Delta C}{}_{T}$ method. **(C)** IL-6 CISH was performed on the same 11 prostate cell lines and representative examples are shown. Brown staining (arrowheads) represent positive IL-6 mRNA expression. Single dots in the nucleus of cells are interpreted as the genomic copy of the gene (PC3 cells, arrow). Both q-RT-PCR and CISH analyses indicated that three of the prostate cell lines (PrSC, RWPE-1, DU145) were positive for IL-6 mRNA, indicating complete concordance between the two assays.

tissue sections used in this study by using a positive control probe set against peptidyl prolyl isomerase B (PPIB) on adjacent sections. PPIB hybridization signals demonstrated expression in virtually all cells present on all slides that were used in this study (Figure 3A), showing that the RNA in these specimens was intact. Surprisingly, we did not detect positive IL-6 mRNA expression in tumor cells in any of the cases examined, regardless of tumor grade (Figure 3A, Table 3). Instead, IL-6 mRNA expression in areas containing prostate cancer was restricted to cells within the stromal compartment of the tumor and primarily in tumor-associated endothelial cells (Figure 3B). The detection of IL-6 mRNA in stromal cells, in combination with positive control staining of transfected MCF-7 cells (Figure 2A) and prostate cell lines (Figure 2C), confirms that the IL-6 CISH probe was functioning as expected and that prostate adenocarcinoma cells in primary tumors do not express IL-6 mRNA.

IL-6 mRNA expression is highly up-regulated in areas of acute inflammation and prostatic atrophy.

As shown in Table 3, an assessment of the distribution of IL-6 mRNA expression in the prostatectomy tissues as analyzed by IL-6 CISH demonstrated that, overall, the expression was highly heterogeneous from case-to-case and by region within a given case. As shown in Figure 4A-B, the number of IL-6 positive cells was highly increased in the stroma in areas of acute inflammation (as evidenced by accumulation of neutrophils within glandular lumens), although the neutrophils themselves were not positive. The cases with the highest numbers of IL-6 mRNA expressing cells were those where acute inflammation was present (see Table 3). Increases in IL-6 positive cells were also seen in areas of proliferative inflammatory atrophy (PIA, Figure 4C-D). Positive cells in areas of prostatic atrophy were generally confined to the stroma surrounding atrophy. In some instances, positive IL-6 mRNA expression was

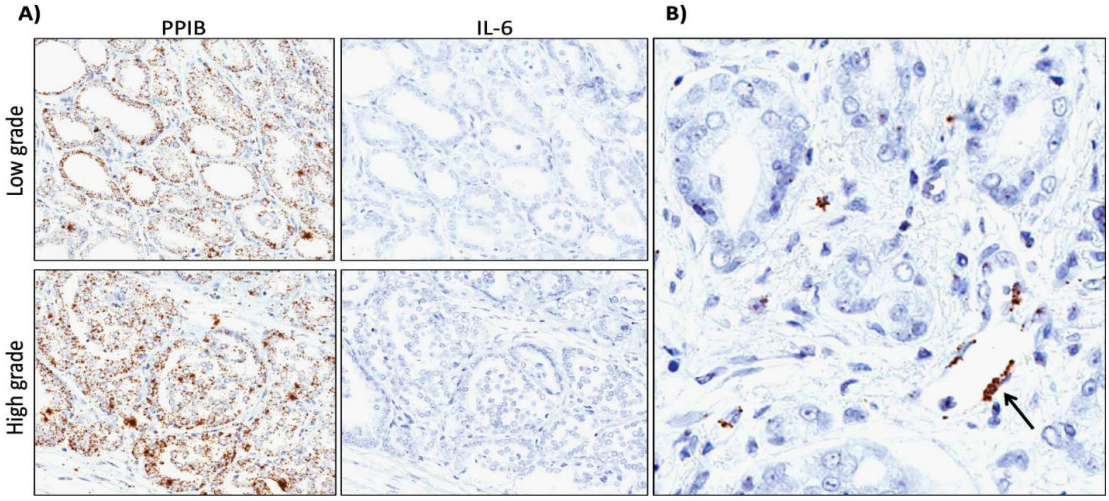

Figure 3. IL-6 mRNA is not detected in prostate adenocarcinoma cells in primary tumors.

Twenty one prostatectomy specimens were selected for IL-6 CISH (see Table 3). PPIB (housekeeping gene) is used as a positive control for the CISH assay. **(A)** Shown are representative examples of low grade (Gleason pattern 3) and higher grade (Gleason patterns 4 and 3) prostate cancer with positive staining for PPIB (positive control) and negative staining for IL-6. **(B)** IL-6 positive cells in endothelium (arrow) in an area of cancer.

Table 3. Assessment of Levels and Distribution of IL-6 mRNA Expression in Prostatectomy Tissues

PATIENT			IL-6 EPITHELIUM			IL-6 STROMA		Acute Inflammation Present?
Patient #	Gleason Score	Clinical Stage	Tumor	Normal	Atrophy	Non-inflamed	Inflamed	
1	3+3=6	T2N0MX	-	-	++	++	++++	Yes
2	3+4=7	T2N0MX	-	-	-	-	+++	Yes
3	4+5=9	T2N0MX	-	-	+	-	-	No
4	3+4=7	T2N0MX	-	-	+	-	+	No
5	3+3=6	T2N0MX	-	-	-	-	+	No
6	3+4=7**	T2N0MX	-	-	-	-	++	No
7	3+3=6*	T2N0MX	-	-	++	-	++	No
8	3+4=7	T2N0MX	-	-	-	-	++++	Yes
9	5+4=9	T2N0MX	-	-	-	-	++	No
10	3+3=6*	T2N0MX	-	-	-	-	-	No
11	3+4=7**	T2N0MX	-	-	-	-	-	No
12	3+4=7	T2N0MX	-	-	+	-	++	No
13	3+4=7	T3AN0MX	-	-	-	-	-	No
14	3+4=7	T2N0MX	-	-	++	-	++	No
15	3+3=6	T2N0MX	-	-	+	-	++	No
16	3+4=7	T2N0MX	-	-	+++	++	++++	Yes
17	3+3=6	T2N0MX	-	-	-	-	+++	Yes
18	4+3=7**	T3AN0MX	-	-	+	-	+	No
19	3+4=7	T3AN0MX	-	-	-	-	-	No
20	4+3=7	T3BN0MX	-	-	++	-	+	No
21	3+4=7**	T2N0MX	-	-	+	-	++	No

- no cells positive for IL-6 expression; + areas with 5-10 positive cells per 20X field; ++ areas with 11-25 positive cells per 20X field; +++ areas with 26-50 positive cells per 20X field; ++++ areas of >50 positive cells per 20X field. * Tertiary pattern 4; ** Tertiary pattern 5.

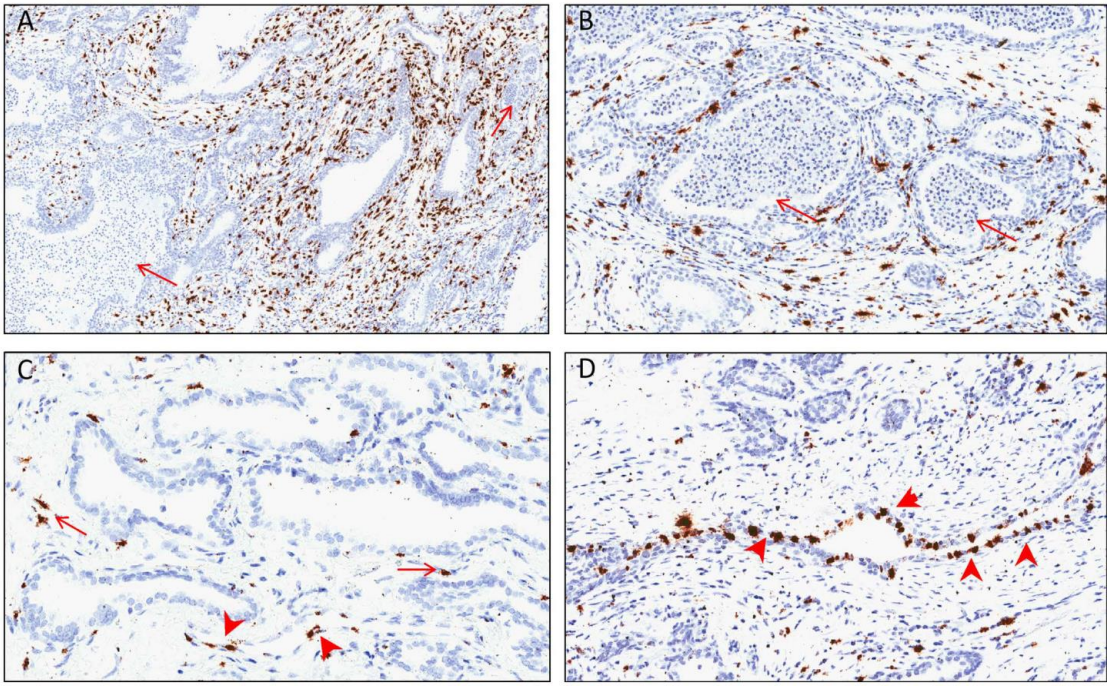

Figure 4. IL-6 mRNA expression is nearly exclusively restricted to the prostate stromal compartment.

(A,B) IL-6 mRNA expressing cells (brown staining) are highly enriched in the stroma in areas of acute inflammation as indicated by the presence of neutrophils in glandular lumens (red arrows). **I** IL-6 positive cells in the stroma (arrows) and endothelium (arrowheads) surrounding prostate atrophy. **(D)** Positive epithelial staining was rare and restricted to prostatic atrophy (arrowheads).

observed in scattered epithelial cells in atrophic glands (Table 3, Figure 4D). Morphologically these appeared to be epithelial cells; however we did not perform a double label with an epithelial cell marker. In a number of cases the IL-6 mRNA expressing cells could be identified as endothelial cells lining small blood vessels (presumably venules or lymphatics, Figure. 4C) as well as prostate smooth muscle cells. Other positive staining cells had an appearance consistent with macrophages. To more definitively identify IL-6 mRNA expressing cells, we performed co-staining (2-plex) for IL-6 and either CD68 (a macrophage marker) or CD31 (an endothelial cell marker). The results of this 2-plex staining clearly indicated that some of the IL-6 positive cells are CD68-positive macrophages and some are CD31-positive endothelial cells (Figure. 5). Interestingly, the majority of the IL-6 positive cells in areas of acute inflammation did not co-stain for CD68 or CD31, suggesting that most of the IL-6 mRNA expressing cells in these areas are of a different stromal cell type, such as fibroblasts, smooth muscle cells, other types of inflammatory cells, etc.

Prostate adenocarcinoma cells in metastatic lesions do not express IL-6 mRNA.

Previous studies suggested that production of IL-6 by metastatic prostate cancer cells may facilitate invasion and metastasis to bone and/or promote resistance to prostate cancer therapies [104, 124]. Therefore, we next examined IL-6 mRNA expression in biopsy samples from patients with castration resistant metastatic prostate cancer (samples evaluated included 4 lymph node specimens and 3 liver biopsy samples) and 25 autopsy samples which included metastases to the liver, lung, bone, and lymph node (see Table 4). Consistent with the data from primary prostatectomy specimens, IL-6 mRNA expression was not observed in prostate cancer cells in any of the metastatic tissues examined. Also consistent with the primary prostatectomy specimens, the metastatic tissues all showed strong hybridization

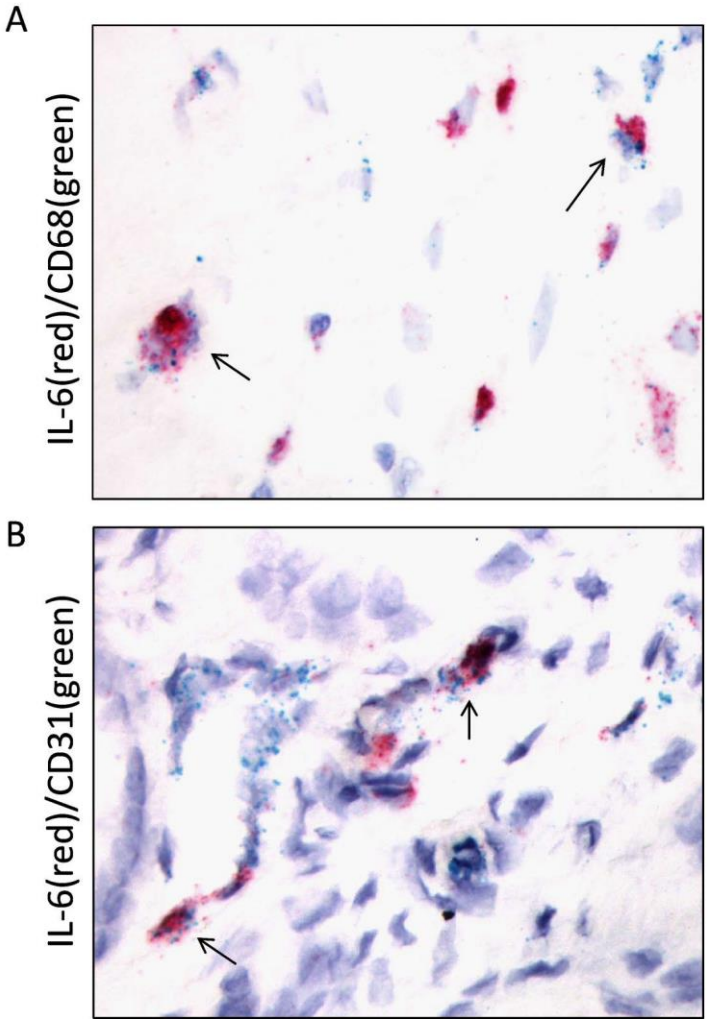

Figure 5. IL-6 mRNA expression in macrophages and endothelial cells.

RNAscope 2-plex assay for IL-6 (red) and CD68 (green) **(A)** or IL-6 (red) and CD31 (green) **(B)** indicated that some IL-6 positive cells in the stroma are macrophages or endothelial cells, respectively (arrows).

signals with PPIB as a positive control (Figure 6A), confirming RNA integrity. Interestingly, IL-6 mRNA staining was observed in endothelial cells in blood vessels in 9 of 21 bone metastases (42.9%, Figure 6B, Figure 7, Table 4) but not in any of the 11 soft tissue metastases analyzed ($p = 0.013$, Fisher's exact test).

IL-6 IHC only works when Golgi export is blocked.

Finally, we performed a series of analyses to examine IL-6 protein production in cell lines and tissues using IHC. To perform these studies, we prepared FFPE blocks from a lung cancer cell line (NCI-H460) that was strongly positive for IL-6 mRNA as assessed by CISH (Figure 8A) and produces physiological levels (e.g., not transfection levels) of IL-6 protein as assessed by Western blot (Figure 9A). Of interest, there appears to be heterogeneity in the cells that are positive for IL-6 expression (i.e., not all cultured cells appear to be positive) via CISH (Figure 8A) and this was consistent with what we observed for the IL-6 mRNA positive prostate cell lines as well (Figure 2C). IHC on the NCI-H460 cells using the polyclonal anti-IL-6 antibody (#6672; Abcam, lot #GR106735-5) showed no detectable signal above background levels (as established by negative control cell lines, Figure 8 B,C). In one sense, those data are not particularly surprising, since IL-6 is a secreted protein and secreted proteins are generally only detectable by flow cytometry analysis when protein export from the Golgi apparatus is blocked using protein transport inhibitors such as brefeldin A or monensin [125]. As such, we next treated NCI-H460 cells with monensin prior to formalin fixation and preparation of FFPE blocks for use with IHC. IHC of "stopped" cell lines treated with monensin showed that NCI-H460 cells now stained positive for IL-6 protein (Figure 8D).

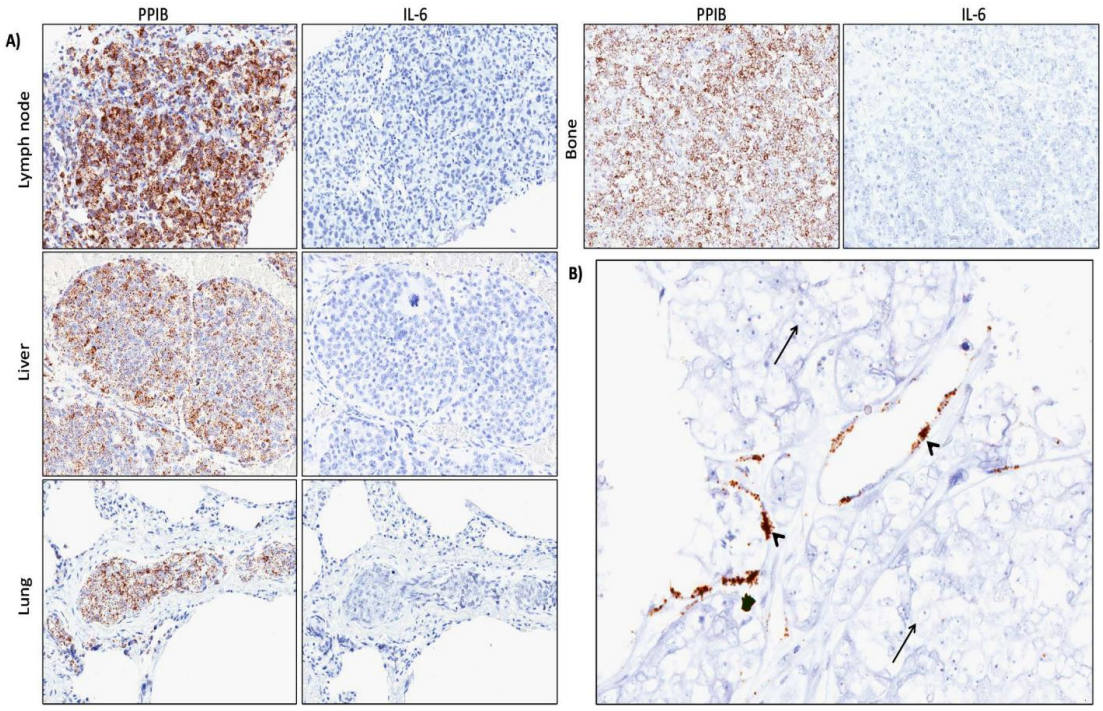

Figure 6. IL-6 mRNA is not detected in metastatic prostate cancer cells.

In this study, metastatic prostate cancer in lymph node and liver biopsy samples and autopsy samples (liver, lung, bone, and lymph node) were assayed by IL-6 CISH. No IL-6 positive mRNA expression was observed in prostate cancer cells in any of the metastatic tissues. **(A)** Shown are representative examples of lymph node biopsy and autopsy liver, lung, and bone metastases with positive staining for PPIB (positive control) and negative staining for IL-6. **(B)** An example of IL-6 mRNA positive blood vessels (arrowheads) and negative tumor cells (arrows) in bone metastases.

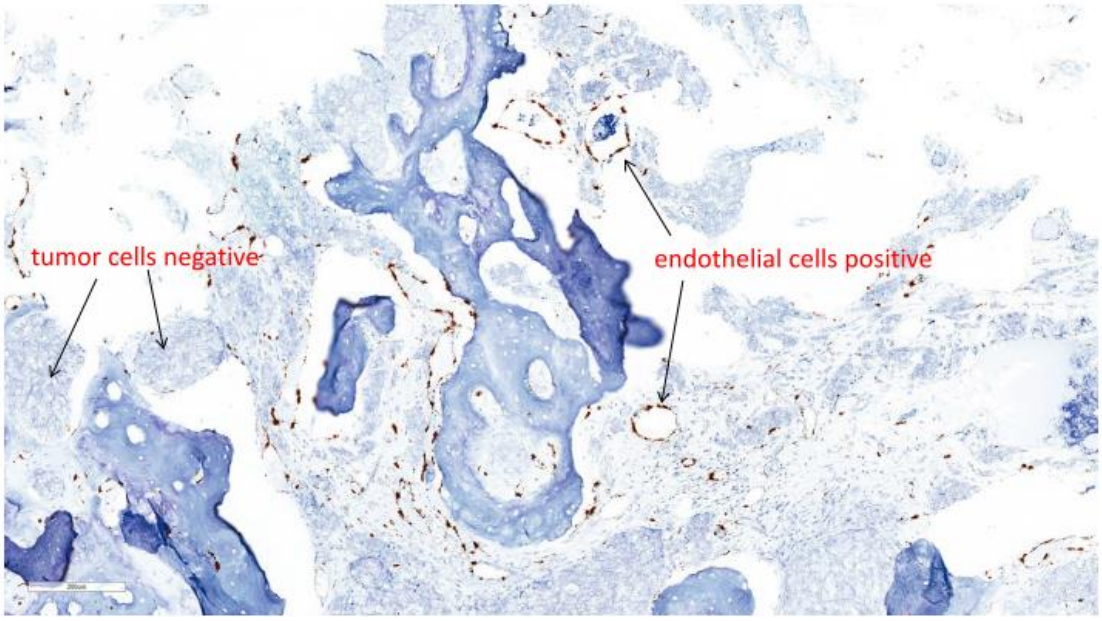

Figure 7. IL-6 mRNA positive blood vessels in prostate cancer bone metastases.

Example of IL-6 positive endothelial cells and IL-6 negative metastatic tumor cells in prostate cancer bone metastases.

Table 4. Assessment of Levels and Distribution of IL-6 mRNA Expression in Metastatic Tissue

Patient #	Tissue type	IL-6 Tumor Epithelium	IL-6 Endothelial Cells
1	Liver biopsy	-	-
2	Left supraclavicular lymph node biopsy	-	-
3	Left supraclavicular lymph node biopsy	-	-
4	Liver biopsy	-	-
5	Liver biopsy	-	-
6	Left supraclavicular lymph node biopsy	-	-
7	Left supraclavicular lymph node biopsy	-	-
8	Lung (autopsy)	-	-
	Liver (autopsy)	-	-
9	Bone (autopsy)	-	-
	Liver (autopsy)	-	-
	Perigastric lymph node (autopsy)	-	-
10	Bone (L2, autopsy)	-	+
	Bone (R Humerus, autopsy)	-	+
11	Bone (L Illiac, autopsy)	-	+
	Bone (L4, autopsy)	-	+
12	Bone (L4, autopsy)	-	-
	Bone (T11, autopsy)	-	-
13	Bone (T12, autopsy)	-	+
	Bone (R Humerus)	-	-
14	Bone (L2, autopsy)	-	+
	Bone (T12, autopsy)	-	-
15	Bone (T4, autopsy)	-	-
	Bone (ribs, autopsy)	-	-
16	Bone (L Humerus, autopsy)	-	-
	Bone (L4, autopsy)	-	-
17	Bone (R Illiac, autopsy)	-	+
	Bone (T9, autopsy)	-	-
18	Bone (sternum, autopsy)	-	+
	Bone (L Humerus, autopsy)	-	+
19	Bone (L5, autopsy)	-	-
	Bone (Left 9th rib, autopsy)	-	-

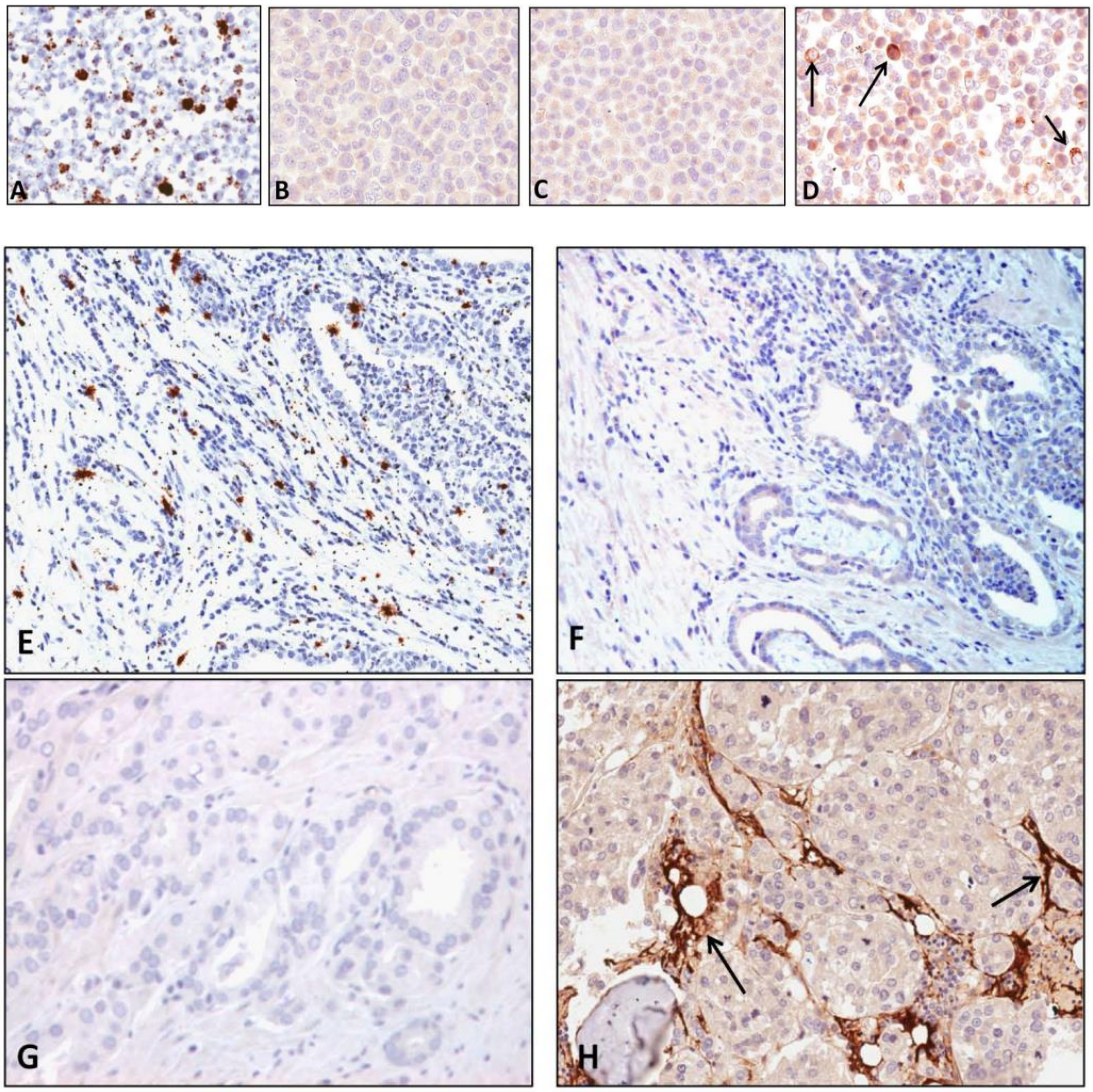

Figure 8. A requirement for protein transport inhibition for IL-6 IHC.

(A) IL-6 CISH performed on the IL-6 positive cell line NCI-H460. IL-6 IHC performed on **(B)** PC3 cells (IL-6 negative as assessed by q-RT-PCR and CISH) and **(C)** NCI-H460 cells. Marked difference in IHC results when NCI-H460 are treated with monensin prior to fixation **(D)**. Arrows point to IL-6 positive cells. The same prostatectomy case containing acute inflammation assayed for IL-6 mRNA by CISH (**E)** and IHC **(F)**. Example of IL-6 IHC on primary prostate cancer **(G)** and prostate cancer bone metastasis **(H)**. All tumor cells were negative in all samples analyzed. Positive staining observed in extracellular spaces (arrows in H) was considered to be non-specific.

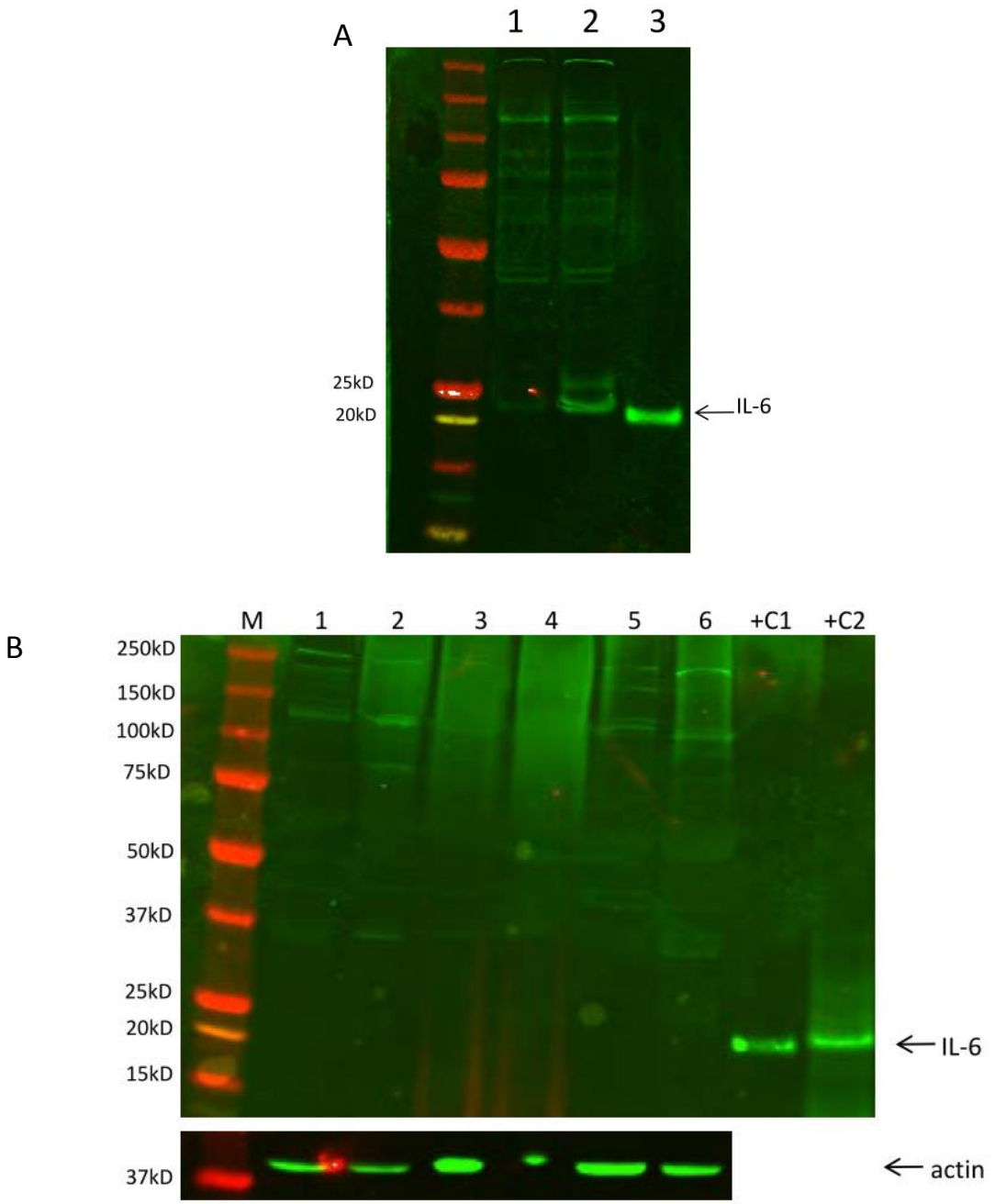

Figure 9. IL-6 Western blot on NCI-H460 cells and selected prostatectomy samples.

(A) Western blot on protein lysates from NCI-H460 cells grown without (lane 1) or with (lane 2) protein transport inhibition with monensin (Golgi-Stop™). 300 ng recombinant IL-6 applied to lane 3 as positive control. **(B)** Western blot on protein lysates from prostate tumor normal pairs (1-6), recombinant mouse IL-6 protein (+C1), and IL-6 transfected MCF7 cells (+C2). Actin was an internal control.

We then performed IL-6 IHC on a series of prostatectomy specimens including cases that were strongly positive in areas of acute inflammation with IL-6 CISH (Figure 8E, F). We did not detect IL-6 protein above background levels in the acutely inflamed areas, either in epithelial or stromal cells using IHC, and likewise we did not observe IL-6 protein in prostate tumor cells using IHC (Figure 8G). Similarly, we did not detect any IL-6 protein in tumor cells via IHC on metastatic lesions from standard slides from the cases used for CISH assays or in two separate metastatic prostate cancer TMAs, including in bone metastases (Figure 8H). The IHC signal in metastases was restricted to extracellular spaces between tumor cell nests (Figure 8H) and no specific signal was detectable above background levels in the tumor cells in any of the cases. The lack of positive signal for IL-6 protein using IHC in areas that were positive for IL-6 mRNA using CISH is likely not due to lack of IL-6 protein production by the IL-6 mRNA positive cells. Moreover, we selected the three patients with higher IL-6 mRNA expression in malignant prostate tissues than matched benign (patient 1, 2, 3. Fig 1A), and we were unable to detect any IL-6 via Western blot on protein lysates from prostate tumor and normal pairs (Figure 9B). Together, these results, coupled with our experiments using monensin in the NCI-H460 cell line, suggest that detection of this particular cytokine in tissue sections using IHC could be severely limited without being able to block protein export prior to fixation.

2.5 Conclusions

The cellular origin of IL-6 in prostate cancer: a focus on the stromal compartment.

In the present study, we analytically validated and used a chromogenic *in situ* hybridization assay to detect IL-6 mRNA in tissue sections to sensitively and

specifically determine the cellular origin of IL-6 in the prostate primary and metastatic tumor microenvironment. The results of our studies indicate that prostatic adenocarcinoma cells do not express IL-6 mRNA. Rather, IL-6 mRNA expression is restricted nearly exclusively to the cells in the stromal compartment including endothelium, and is highly up-regulated in areas of acute inflammation and prostatic atrophy.

Although prostate adenocarcinoma cells do not express IL-6 mRNA as evidenced by the results of our study, this does not eliminate a potential contributory role for IL-6 signaling in prostate cancer development and/or progression. IL-6 production by cells in the stromal compartment may signal in a paracrine fashion through the transmembrane IL-6 receptor (IL-6R) mediated by glycoprotein 130 (gp130) or via a soluble IL-6 receptor (sIL-6R) that signals through membrane-bound gp130. Therefore, IL-6 can potentially signal through any cell that produces IL-6R or gp130. In this respect, multiple studies have demonstrated the presence of IL-6R and/or gp130 in prostate cancer cells (reviewed in [126]).

Interestingly, out of the 11 prostate cell lines derived from cancer or benign tissues that we examined for IL-6 mRNA expression levels in the present study, the cell line that was found to express the highest levels of IL-6 mRNA via qRT-PCR was PrSC cells (Figure 2B). PrSC cells are a prostate stromal cell line; therefore, the high levels of IL-6 mRNA expression in this cell line of stromal cell origin would correlate to our IL-6 CISH results in prostate tissue sections where we found that IL-6 mRNA expression was restricted almost exclusively to the stromal compartment. Whereas dual stains for IL-6 and CD68 or CD31 confirmed that some of the IL-6 mRNA expressing cells in the stromal compartment are prostate-infiltrating macrophages or endothelial cells, respectively (Figure 5), this represented the minority of IL-6

positive stromal cells in the highly positive areas surrounding acute inflammation (Figure 4A-B). We predict that additional cells in the stromal compartment that express IL-6 mRNA may include fibroblasts/myofibroblasts and smooth muscle cells. In this respect, Hobisch *et al*. detected IL-6 secretion into the supernatant of *ex vivo* cultured prostatic fibroblasts and smooth muscle cells, although no positive staining of these types of stromal cells was detected using IHC [103]. It should be noted that those data are quite consistent with the results of the present study.

In areas of acute inflammation in some cases, a large proportion of the cells in the stroma were positive for IL-6 mRNA expression (Figure 4A-B), arguing that a number of cell types in these areas may express IL-6 mRNA in what may be part of a positive feedback loop as has been previously described [127]. The stimulus for acute inflammation that is frequently observed on radical prostatectomy specimens (albeit to a lesser degree than chronic inflammation) is unknown [128], but may be caused in part by bacterial infections [46, 128, 129]. A recent study also identified a role for IL-6 up-regulation by bone marrow-derived mesenchymal stem cells (MSCs) in promotion of adipogenesis and prostate cancer progression [110], and it is possible that some of the IL-6 positive cells identified in the present study may represent MSCs. This would be difficult to assess using the current CISH technologies that are limited to 1-2 markers per assay, as MSCs are typically identified using a number of surface markers such as CD105, CD166, CD44 and CD29 [110]. Future studies utilizing techniques such as flow cytometry may help to further verify this possibility. Another recent study using a human prostate dissociation and tissue recombination system identified a role for paracrine expression of IL-6 in the stromal compartment in concert with cell-autonomous oncogenic events in the promotion of an aggressive prostate cancer phenotype [120]. In all, these studies in parallel with the results of the

present study set the precedence for a potentially important role for paracrine IL-6 signaling originating from the stromal compartment in the prostate tumor microenvironment.

The role of IL-6 in metastatic disease.

Studies have consistently shown that serum levels of IL-6 are elevated in metastatic prostate cancer patients [98, 99, 115-118, 130], and that these levels may correlate to tumor burden [99, 114, 131] and/or may serve as a surrogate marker for morbidity associated with advanced prostate cancer including cachexia [118, 119]. Interestingly, our studies of IL-6 CISH in a series of metastatic prostate cancer biopsy or autopsy samples indicated that metastatic prostate cancer cells do not express IL-6 mRNA. While prostate tumor cells do not express IL-6, we did observe a significant difference between IL-6 positive blood vessels in bone metastases versus soft tissue metastases (Table 4). Our data strongly suggest that the increased systemic levels of IL-6 observed in advanced prostate cancer patients is not from production of the cytokine by metastatic tumor cells. Rather, elevated IL-6 levels may be due at least in part to increased IL-6 production by tumor vasculature. Since the presence of bone metastases is also associated with morbidity, it is plausible that the presence of IL-6 in vasculature in bone metastases could be a source of the elevated serum IL-6 in patients with a high metastatic burden. The lack of IL-6 mRNA expression by primary and metastatic prostate cancer cells observed in our study may help to explain why minimal to no clinical activity has been observed when a monoclonal antibody therapy targeting IL-6 (siltuximab) has been tested in clinical trials thus far [132, 133].

IL-6 and IHC.

IHC-based studies on IL-6 production in prostate tissue sections have reported varying degrees of basal cell staining in benign epithelium as well as more pronounced staining of tumor epithelium [102, 103] that increases in intensity with increasing pathological Gleason grade [102]. Likewise, a previous study using IHC to detect IL-6 production in prostate cancer metastases reported over twice as many bone metastases samples to be positive for IL-6 than soft tissue metastases, and with much stronger staining intensity [104]. Unfortunately, our data do not support those results. With a well-validated, positively controlled IL-6 CISH assay, we did not detect IL-6 mRNA in any of the primary or metastatic prostate cancer cells in our study. We were able to detect IL-6 using IHC in positive control cell line specimens, but only when IL-6 accumulation was augmented by blocking Golgi export using a protein transport inhibitor.

To help determine whether we could account for the discrepant results between a past study [104] and the present one, we compared by IHC the staining obtained with the polyclonal anti-IL-6 antibody batch (#6672; Abcam, lot # 385304) that was used previously to the currently commercially available batch (lot # GR169214-2). These analyses indicated that under identical conditions, the newer version of the antibody does not stain tumor cells in bone metastases, yet, the older antibody batch gave similar results to that reported previously [104] with strong tumor cell staining (C. Morrissey, unpublished data). Taken together with the present study, it would appear that prior results showing high level expression in tumor cells is related to antibody lot variability and no longer appears with the newer currently available antibody from the same vendor. It should also be noted that traditional decalcification/fixation methods used for bone can have a significant impact on RNA integrity; although

formic acid treatment as was used for the metastatic bone samples in the present study has been shown to result in improved nucleic acid recovery and quality over stronger acid treatment [134, 135]. We acknowledge this potential limitation to the present study, and addressed this concern by including the positive control (PPIB) CISH assay for each sample analyzed. Likewise, we detected IL-6 mRNA in the metastatic bone samples (albeit not in the tumor cells but in the blood vessels), indicating that there did not appear to be at least any generalized issues with detection of IL-6 mRNA in the decalcified bone samples.

In conclusion, our results suggest that, in prostate cancer patients, paracrine rather than autocrine IL-6 expression is likely associated with any role for the cytokine in disease development and/or progression.

According to previous studies, IL-6 may be involved in initiating prostate tumorigenesis by promoting cancer progression to a castration-resistant state and in promoting tumor metastasis [136]. Additional studies implicate IL-6 along with STAT3, which is major effector of IL-6, in prostate cancer initiation [17, 137-140]. In the next chapter, we provide some novel animal studies in C57BL/6J wildtype and IL-6 knockout (-/-) mice to determine whether IL-6 has a causative role in supporting prostate tumor growth.

III. A POTENTIAL PRO-TUMORIGENIC ROLE FOR INTERLEUKIN-6 IN PROSTATE TUMOR GROWTH

3.1 Abstract

Introduction: Emerging evidence suggests that chronic or recurrent prostate inflammation may initiate and promote prostate cancer development. IL-6 has been indicated as a mediator of inflammation that can facilitate prostate cancer progression. However, a causative role for IL-6 in prostate cancer growth has not been as well investigated. We now report a series of allograft studies that provide initial evidence that IL-6 may be involved in prostate tumor growth.

Methods: Three mouse cancer cell lines were used in allograft studies with C57BL/6J wildtype and IL-6 knockout (IL6-/-) mice: the prostate cancer cell lines TRAMP-C2 and 100RC2A (novel line derived from a cross of Hi-MYC mice to Tp53+/- heterozygous mice – C. Bethel, K. Sfanos, A.M. De Marzo unpublished data) and a colon cancer line MC38. Tumor sizes were measured at a 3-4 day interval and tumor volumes were calculated as length×width^2×0.52. Serum, allograft tumors and other organs were collected for analysis by ELISA and a chromogenic *in situ* hybridization (CISH) assay.

Results: There was a reduction in TRAMP-C2 and 100RC2A allograft take rates and growth rate in IL6-/- mice versus wildtype mice. This trend was not observed for the MC38 cell line. CISH analysis of the TRAMP-C2 allograft tumors indicated that the tumor cells were not producing IL-6 mRNA. Nevertheless, IL-6 ELISA analyses on the mouse serum showed a significant increase in the circulating levels of IL-6 in wildtype mice with TRAMP-C2 tumors.

Conclusion: Our results are consistent with previous studies in prostate cancer patients that demonstrate that high circulating levels of IL-6 tend to associate with a more aggressive clinical course of the disease. Additionally, our studies provide evidence that IL-6 may be required for prostate tumor growth. The results of our IL-6 -/- animal studies indicate that elevated systemic IL-6 levels may be involved in tumor growth regulation in prostate cancer, and are not simply caused by or indicative of tumor burden.

3.2 Introduction

Emerging evidence suggests that chronic or recurrent prostate inflammation may initiate and/or promote prostate cancer development [40]. Thus, many studies focus on prostate pro-inflammatory and anti-inflammatory cytokines [141]. There are several cytokines involved in prostate cancer regulation, among them IL-6 is frequently investigated in prostate cancer models because of previous literature that reports that there is increased IL-6 expression at early stages of the disease [15, 16, 142]. In the previous chapter, we demonstrated that IL-6 is present in the prostate cancer microenvironment, but must act in a paracrine manner rather than autocrine to support tumor development and/or progression, as it is not produced by prostate tumor cells.

The first indication that IL-6 is involved in prostate cancer progression came from observations that the amount of circulating IL-6 is related to metastatic or hormone refractory prostate cancer. Subsequent studies have consistently shown that elevated systemic IL-6 levels confer poor prognosis, and may also serve as a marker of prostate cancer morbidity, including cachexia [15, 16, 142, 143].

Moreover, IL-6 is considered as a key mediator involved in several steps of

prostate cancer development, including prostate tumor initiation, tumor growth regulation, transition to the aggressive prostate cancer phenotype, progression to the castration-resistant state, and promotion of tumor metastasis [136]. Some studies implicate IL-6 and its major effector STAT3 as pro-tumorigenic agents to initiate prostate cancer. Through the STAT3 pathway, IL-6 has been shown to trans-activate the androgen receptor in prostate cancer cells to facilitate androgen-independence [17, 137-140].

In this study, we introduce novel animal studies using three different mouse cancer cell lines in both wildtype and IL6-/- mice to further study the potential role of IL-6 in prostate tumor growth.

3.3 Materials and Methods

Animals

Animals we used in this study were 8-10 week old C57BL/6J wildtype mice (Harlan), and Interleukin-6 knockout (IL-6 -/-, Jackson Lab, B6.129S2-Il6tm1Kopf/J) mice. Animals were housed in a pathogen-free environment with 12 hour light/dark cycle, and received enough sterile food and water. All procedures were performed under the guidelines of Johns Hopkins Animal Care and Use Committee (ACUC). Animals were sacrificed by CO2 asphyxiation, reference tissues along with serum and allograft tumors were dissected.

Cell preparation

There are three C57BL/6J derived cancer cell lines used in this study: TRAMP-C2, 100RC2A, and MC38. TRAMP-C2 and 100RC2A are prostate cancer cell lines, and MC38 is a colon adenocarcinoma cell line. TRAMP-C2 was purchased

from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in high glucose DMEM (4.5 g/L glucose, ATCC) with 5% heat inactivated FBS (Invitrogen), 5% Nu-Serum IV (Corning, Fisher Scientific), 5μg/ml insulin and 10^{-8} M dihydrotestosterone (Corning, Fisher Scientific). Freezing media contained 5% DMSO (Sigma-Aldrich) in culture medium. 100RC2A cells were initially cultured in our lab from a primary prostate tumor that arose in an animal from crosses between Hi-MYC mice (FVB background, [144]) and p53 knockout mice (C57BL/6J background) (C. Bethel, A.M. De Marzo, unpublished data). 100RC2A was cultured in OPTI-MEM (Invitrogen) with 0.5% heat inactivated FBS, EGF and 1 nM R1881. Murine C57BL/6J CRC tumor cells (MC38) were kindly provided by Dr. Charles Drake, and were cultured in DMEM media with 10% heat inactivated FBS.

For TRAMP-C2, 100RC2A, and MC38, cells double about every 12 h and should be split 1:10 or 1:15 every 3–4 days. Cultured cells were harvested by trypsin and spun down at 1000 rpm for 5min, followed by PBS rinse twice and re-suspension in PBS for allograft injection. Cell numbers were determined by cellometer machine (Cellometer Cytometer, Nexcelom).

Allograft procedure

All procedures were performed under the guidelines of Johns Hopkins Animal Care and Use Committee (ACUC). Each experimental group consisted of 8 to 12 mice. A 1 ml tuberculin syringe was loaded with cells right before injection. 2×10^6 TRAMP-C2 cells / 2×10^6 100RC2A cells with 20ul sterile PBS and 80ul Geltrex (Life Technology), or 1.5×10^6 MC38 cells with 200ul sterile PBS were loaded into a 1ml syringe with attached 26 gauge ½ inch needle with care to eliminate air bubbles.

Anaesthetized 8-10 weeks old males were injected subcutaneously with tumor cells into the right side flank.

Tumor sizes were measured at a 3-4 day interval using electronic calipers and tumor volumes were calculated as length×width^2×0.52. Palpable primary tumors usually develop within four weeks for TRAMP-C2 and 100RC2A, and within a week and half for MC38. Mice were euthanized at 2-8 weeks post-inoculation when animals developed tumors over 2 cm in width or length. Serum, allograft tumor, liver, lung, and kidney were taken and fixed with 10% formalin for 48 hours followed by paraffin embedding. FFPE slides were stained with hematoxylin and eosin for further histological examination.

Chromogenic *in situ* hybridization (CISH).

CISH was performed using the RNAscope® 2.0 FFPE Brown Reagent. Briefly, FFPE tissues were first baked at 60°C for 1 hr followed by deparaffinization. Next, the slides were treated with endogenous peroxidase blocking pretreatment reagent for 10 min at room temperature, and boiled slides for 15-30min with pretreatment 2 solution provided by ACD Bio. The slides then were treated with protease digestion buffer for 30 min at 40°C. The slides were incubated with a custom RNAscope target probe designed against mouse IL-6 mRNA (probe region 300–31–1 - 30019968 at Chromosome 5, Gene ID: 16193) for 2 hr at 40°C, followed by signal amplification. DAB was used for colorimetric detection for 10 min at room temperature.

Phospho-STAT3 cell plug

80-90% confluent HeLa cells were treated with serum starvation overnight. Half of the cells were treating 100ng/ml interferon α (dissolved in PBS) at 37℃ for 5min to induce phospho-STAT 3 expression while other half cells were only treated with the

same amount of PBS as negative control. Cells were formalin fixed and made into FFPE cell plugs.

Immunohistochemistry (IHC)

IHC was performed using the Power Vision+ Poly-HRP IHC kit (Leica Biosystems). Slides were steamed for 45 min in EDTA (Dako #S1700) and incubated with rabbit monoclonal anti-phospho-STAT3 antibody (#9145; Abcam, 1:50 dilution) for 4°C overnight. Poly-HRP-conjugated anti-rabbit IgG antibody was used as secondary antibody. Staining was visualized using 3,3′-diaminobenzidine (Sigma), and slides were counterstained with hematoxylin (Dako).

Enzyme-Linked-Immunosorbent-Assay (ELISA)

A high sensitivity mouse IL-6 ELISA kit (BMS603HS, eBioscience) was used to detect mouse serum IL-6 for both wildtype and IL-6 -/- mice. The procedures were done following the manufacturer's instructions. Samples were diluted 1:3 with diluent buffer to 100 µl. Diluted samples along with 50 µl biotin-conjugated anti-mouse IL-6 antibody were applied into ELISA strip coating mouse anti-IL-6 antibody, and then incubated at 4°C for overnight. Next, 100 µl Streptavidin-HRP was used for secondary incubation to amplify the signal, 100 µl Biotinyl-Tyramide for third incubation, and another 100 µl Streptavidin-HRP for fourth incubation. Finally, 100 µl HRP substrate was added to form colorimetric products. The reaction is terminated by the addition of phosphoric acid. ELISA color development can be monitored by any ELISA reader at 450 nm. Standard curve were transformed into a four parameter logistic (4-PL) curve (Figure 10). All samples were run in duplicate.

Figure 10. Standard curve derived from mouse IL-6 ELISA.

By running high sensitivity mouse IL-6 ELISA (BMS603HS, eBioscience), we were able to detect mouse circulating IL-6 levels with 0.21 pg/ml sensitivity. Standards were converted into a four parameter logistic (4-PL) curve. The concentration equation derived out is $y = d + \frac{a-d}{1+(x/c)^b}$, where y=O.D. value, x=concentration, a= 2.9392101, b=-2.043341, c=34.0862324, and d=0.2035608. R^2 value is 0.9982817. (Figure is adapted from ELISAanalysis.com; ELISA raw data is in Table 5)

3.4 Results

Allograft of three mouse cancer cell lines

Three mouse cancer cells were used to perform allografts studies on C57BL/6J wildtype and IL-6 knockout (IL-6 -/-) mice. Two are prostate cancer cell lines: TRAMP-C2 and 100RC2A (a novel line derived from Hi-MYC mice crossed to Tp53+/- heterozygous mice), and one is a colon cancer cell line MC38.

Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice are a relatively rapid animal model to study prostate cancer that can spontaneously develop in a few weeks and mimics human prostate cancer progression [145, 146]. TRAMP mouse tumors are driven by SV40 large T antigen under the control of the prostate epithelial cell-specific probasin promoter, though the T antigen oncoprotein cannot be detected in the cell lines *in vitro* or *in vivo*. TRAMP mice develop high-grade prostatic intraepithelial neoplasia (PIN) by 8-12 weeks of age, adenocarcinoma by 18 weeks, and metastasis (mostly to lung and lymph nodes and rarely to bone) by 24-30 weeks [146].

There are three cell lines derived from TRAMP mice: TRAMP-C1, TRAMP-C2, and TRAMP-C3. They all can express cytokeratin, E-cadherin, and androgen receptor by IHC analysis and p53 is undetectable. TRAMP-C1 and TRAMP-C2 were tumorigenic when grafted into C57BL/6J hosts, though TRAMP-C3 was not tumorigenic [145]. In this study, we chose to use the TRAMP-C2 cell line.

The 100RC2A cell line was developed in our lab (C. Bethel, K. Sfanos, A.M. De Marzo, unpublished data). This cell line was derived from a cross between the HI-MYC transgenic mice (FVB background) and p53 heterozygous mice (C57BL/6J

background). The line was derived from a mouse that spontaneously developed primary prostate cancer as well as lung metastasis, and it maintained very high levels of MYC oncogene and had heterozygous +/- loss of the tumor suppressor p53. Primary tumor was collected from the prostate of the animal and ten prostate cancer cell lines were cultured. One of the cell lines, 100RC2A, was used in the present study.

IHC of 100RC2A cells showed that MYC, AR, and Nkx3.1 were overexpressed in this cell line while Pten and p53 were negative (C. Bethel, D. Esopi, A.M. De Marzo, unpublished data). This result corresponds to the expression of these proteins in the original tumor.

MC38 is a grade III colon adenocarcinoma cell line, which was chemically induced by Corbett et al. in a C57BL/6J female mouse in 1975. The standard way to evaluate cell tumorigenicity is by performing subcutaneous inoculation of 0.375 to 3 x 10^6 cells with PBS. In the previous studies, MC38 was used as a model tumor to study immunotherapy [147] and chemo-immunotherapy, but not used to further study the function of IL-6 [148].

100RC2A may be rejected by C57BL/6J mice

In the 100RC2A cohort, ten wildtype and eight IL-6 -/- mice were used to perform allografts. 27 days after inoculation, only two wild type mice formed tumors, while all other mice (both wildtype and IL-6 -/-) did not develop tumors. No tumor growth was observed in any of the IL-6 -/- cohort (Figure 11). It is possible that since 100RC2A was derived from mouse with mixed FVB and C57BL/6J genetic background, C57BL/6J mice may reject the 100RC2A cell line allografts.

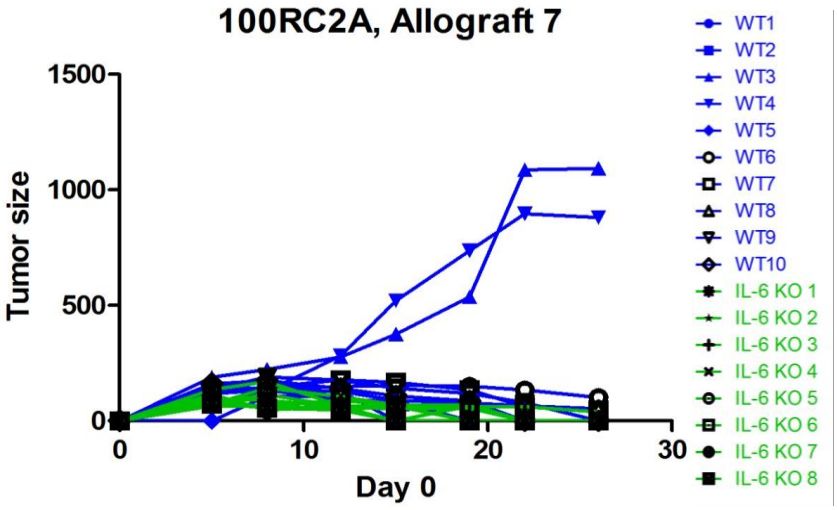

Figure 11. 100RC2A allograft may be rejected in C57BL/6J mice.

100RC2A is novel line derived from a cross of Hi-MYC mice to Tp53+/- heterozygous mice. In the 100RC2A cohort, ten wild type mice (blue) and eight IL-6 -/- mice (green) were used to perform allografts. 27 days after inoculation, only two wild type mice out of ten developed tumors. No tumors were observed in the IL-6 -/- cohort.

IL-6 depletion delays tumor growth in the TRAMP-C2 model

Unlike 100RC2A cells that only developed tumors in two wildtype animals, TRAMP-C2 cells could form tumors in both wildtype and IL-6 -/- mice. At day 25 after subcutaneous injection, tumors became palpable in the wildtype group. 40 days post-injection, a significant tumor size difference was observed between wildtype and IL-6 -/- mice. Allograft tumor growth doubling time in wildtype mice was about 3 to 4 days after 43 days; while IL-6 -/- animals required a relatively long time to develop allograft tumors (Figure 12A).

Animals were sacrifice at Day 57 post injection, and when we compared tumor size at 57 days post-injection, as illustrated in Figure 12B, wildtype mice had significantly larger tumors than IL-6 -/- mice in general. The average tumor size of wildtype mice was 2263.99 mm^3; while IL-6 -/- mice only had an average tumor size of 385.39 mm^3 ($p < 0.001$).

Systemic IL-6 levels are elevated in wildtype mice with TRAMP-C2 tumors

The physiological circulating mouse IL-6 level is less than 10pg/ml [94, 149]. Unless animals are undergoing an infectious and/or inflammatory process, it is hard to detect mouse circulating IL-6. In this study, a high-sensitivity mouse IL-6 ELISA kit (eBioscience) was used, which provides a 2.1pg/ml sensitivity. Table 5 lists detailed information of all the animals we include in the ELISA assay with their tumor size and corresponding serum IL-6 amount. To make sure this ELISA kit did not provide false-positive data, we also tested a C57BL/6J mouse without any treatment as a negative control. All of the samples were run in duplicate. Standard curves were converted into a four parameter logistic (4-PL) curve (Figure 10).

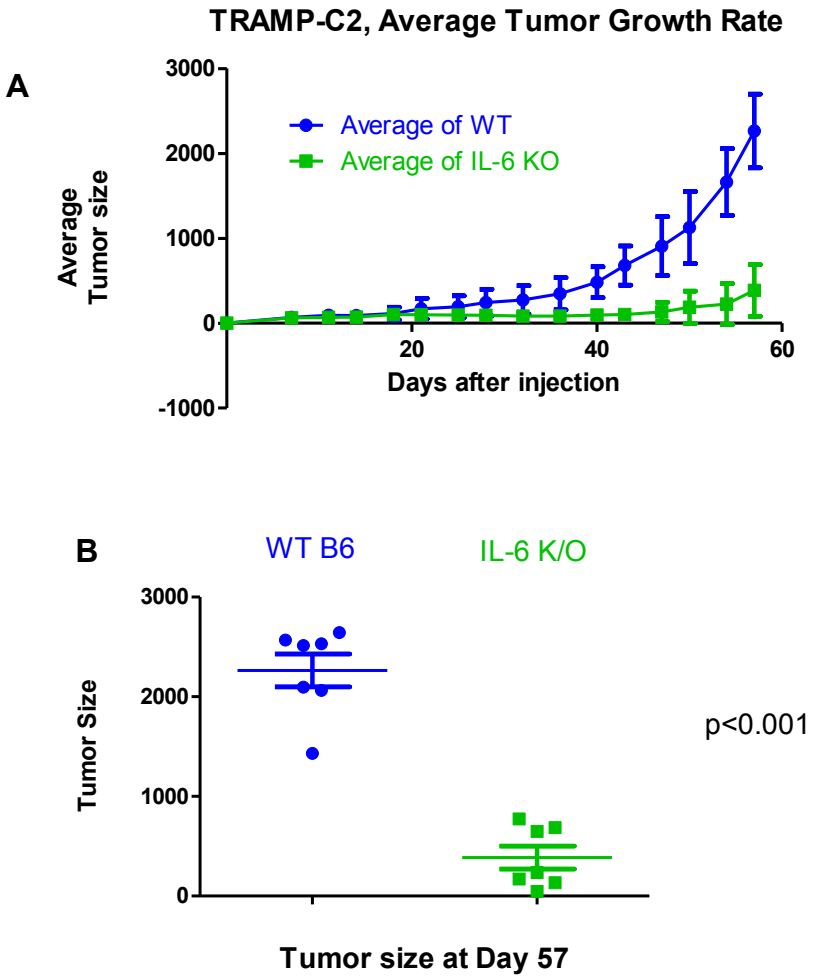

Figure 12. IL-6 depletion may result in delayed tumor development in the TRAMP-C2 allograft model.

(A) TRAMP-C2 cells (2 x 10^6) were subcutaneously injected into the flanks of both C57BL/6J wild type (blue) and IL-6 -/- animals (green). **(B)** After 57 days, TRAMP-C2 tumors grew in both groups, however, with a significant size difference between wild type and IL-6 -/- mice ($P<0.001$). Nine C57BL/6J wild type mice and seven IL-6 -/- animals were included in this study. Tumor volume was calculated as length×width^2×0.52 and is reported as mm^3.

In our ELISA study, we grouped all animals as wildtype and IL-6 -/- cohorts. Among wildtype animals, we observed that mice with TRAMP-C2 allografts had higher IL-6 serum levels (Figure 13, Table 5). Interestingly, the animals with 100RC2A allografts did not have elevated IL-6 in their serum (Table 5, SHY47 and SHY49).

Mouse IL-6 was not expressed by the allografted TRAMP-C2 cells

To determine if the circulating mouse IL-6 detected by ELISA came from the allografted tumor cells or from another source, CISH was applied to all of the allografted tumors. 5.8s rRNA and dihydrodipicolinate reductase gene (dapB, a bacterial gene) were chosen as positive and negative controls, respectively. Each sample was stained with a mouse IL-6 probe along with these positive and negative control probes. Positive signal was detected for 5.8s rRNA (Figure 14A) and dapB was negative (Figure 14B). Interestingly, some IL-6 positive cells could be detected within the tumor stroma (Figure 14D), however, allograft tumor cells did not express any mouse IL-6 mRNA (Figure 14C). This data corresponds to our human study in that prostate tumor cells were not found to express IL-6 mRNA (see Chapter 2).

We have also applied the mouse IL-6 CISH assay to liver, kidney, and spleen, but none of the target organs were positive (data not shown). Therefore, the cellular origin(s) of the elevated circulating IL-6 in this model system remains unclear.

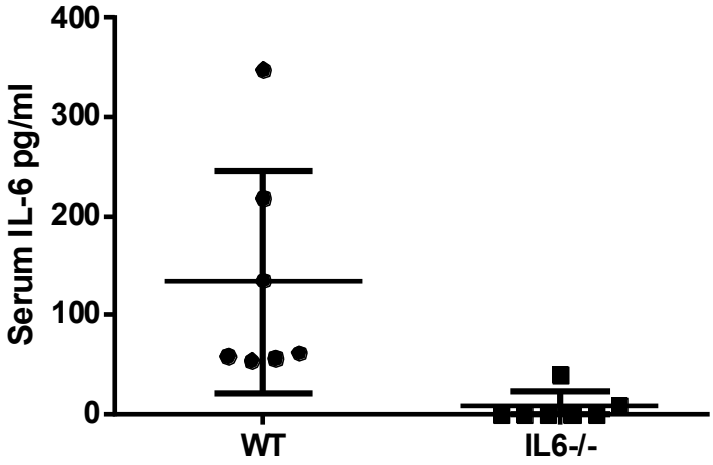

Figure 13. Serum IL-6 levels were elevated in wildtype mice with TRAMP-C2 tumors.

Mice were grouped as wildtype and IL-6 -/- cohorts, and serum was assayed for IL-6 with ELISA. Interestingly, wildtype mice that grew TRAMP-C2 allografts had elevated serum IL-6 levels. Samples were run in duplicate. A subset of the nine C57BL/6J wildtype mice and seven IL-6 -/- mice that received allografts were included in this ELISA study (see Table 5).

Table 5. Mouse IL-6 ELISA.

A high-sensitivity mouse IL-6 ELISA was performed (eBioscience).

Animal Number	Animal Genetic Type	Allograft Tumor type	Tumor size (mm^3)	Serum IL-6 (pg/ml)
SHY87	Wild type	TRAMP-C2	2642.8	218.187
SHY88	Wild type	TRAMP-C2	1431.7	134.706
SHY89	Wild type	TRAMP-C2	2568.5	56.154
SHY90	Wild type	TRAMP-C2	2062.7	61.929
SHY91	Wild type	TRAMP-C2	2513.2	53.766
SHY92	Wild type	TRAMP-C2	2532.1	347.907
SHY93	Wild type	TRAMP-C2	2096.9	58.878
SHY94	IL-6 knockout	TRAMP-C2	774.3	40.302
SHY95	IL-6 knockout	TRAMP-C2	685.9	0
SHY96	IL-6 knockout	TRAMP-C2	47.4	0
SHY97	IL-6 knockout	TRAMP-C2	133.5	0
SHY98	IL-6 knockout	TRAMP-C2	235.3	0
SHY99	IL-6 knockout	TRAMP-C2	171.6	9.567
SHY100	IL-6 knockout	TRAMP-C2	649.7	0
SHY47	Wild type	100RC2A	4018.121	0
SHY49	Wild type	100RC2A	0	0
DBS41	Wild type	Negative control	0	0

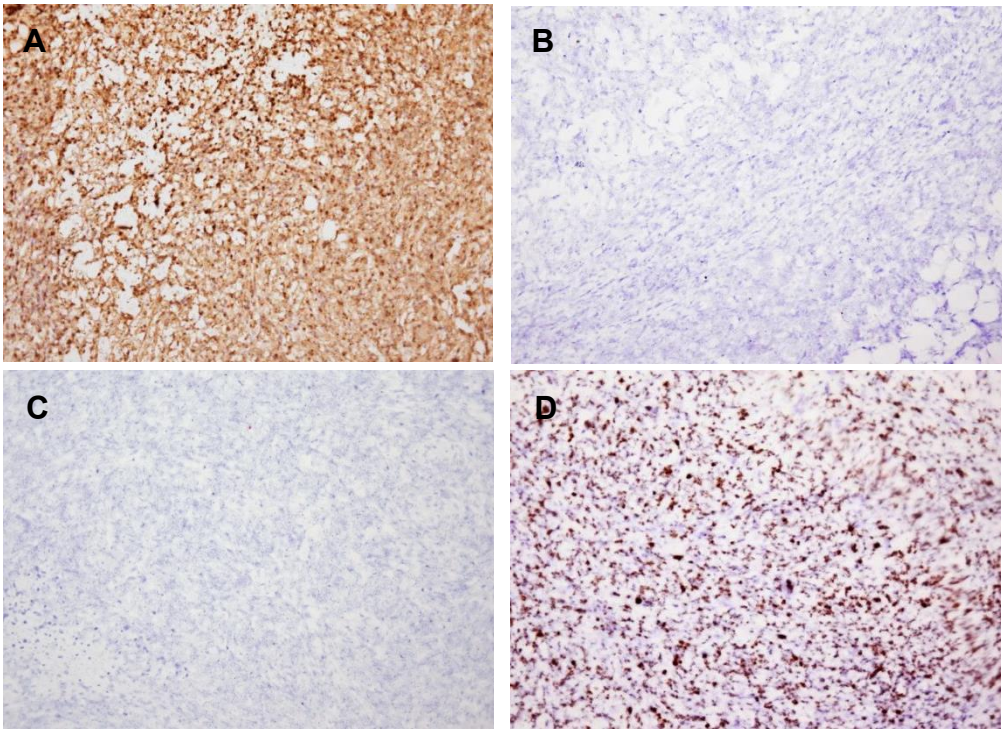

Figure 14. Mouse IL-6 mRNA was not detected in TRAMP-C2 allograft tumors. Tumor specimens were selected for mouse IL-6 CISH (Advanced Cell Diagnostics). Shown are representative examples of allograft tumor section with **(A)** positive control staining for 5.8s rRNA (housekeeping gene, positive control), and **(B)** negative staining for dihydrodipicolinate reductase gene (a bacterial gene). **(C)** Allograft tumor cells do not express mouse IL-6 mRNA. **(D)** Cells expressing mouse IL-6 mRNA are detectable in the stromal compartment but are not TRAMP-C2 cells.

Phospho-STAT3 might not be the downstream target of IL-6 signaling pathway in TRAMP-C2 tumors

There are three pathways: ERK-MAPK, PI3K-Akt, and JAK-STAT that are indicated as possible downstream elements of IL-6 signaling in prostate cancer. Previous research has suggested that STAT3 is the most crucial downstream target of IL-6 for maintenance of the tumor progenitor cell phenotype in prostate cancer development [92]. In order to determine if IL-6 is possibly signaling through phorpho-STAT3 (p-STAT3), we chose to assess p-STAT3 levels in our TRAMP-C2 allograft studies.

To optimize an IHC assay for p-STAT3, we transiently stimulated HeLa cells with interferon α to induce expression of p-STAT3 in these cells to use as a positive control (Figure 15 A, B). Unexpectedly, using this optimized p-STAT3 IHC assay, we did not observe any significant difference in p-STAT3 levels in TRAMP-C2 allografts grown in C57BL/6J wildtype versus IL-6 -/- mice (Figure 15 C, D). p-STAT3 expression was detected in TRAMP-C2 tumors in mice from both genotypes. One possible reason is that, even though suppression of IL-6 signaling may inhibit permanent activation of STAT3, p-STAT3 may also have IL-6-independent effects that can be activated by other cytokines [150].

MC38 tumor development is IL-6 independent

1.5 x 10^6 MC38 colon adenocarcinoma cells were subcutaneously injected into ten C57BL/6J wildtype and thirteen IL-6 -/- mice. MC38 allograft tumors grow faster than 100RC2A and TRAMP-C2. At 7 days post injection, palpable allograft tumors could be measured in both groups. Unlike what was observed in the TRAMP-C2 model where until 25 days post-injection, palpable tumors could only be observed in

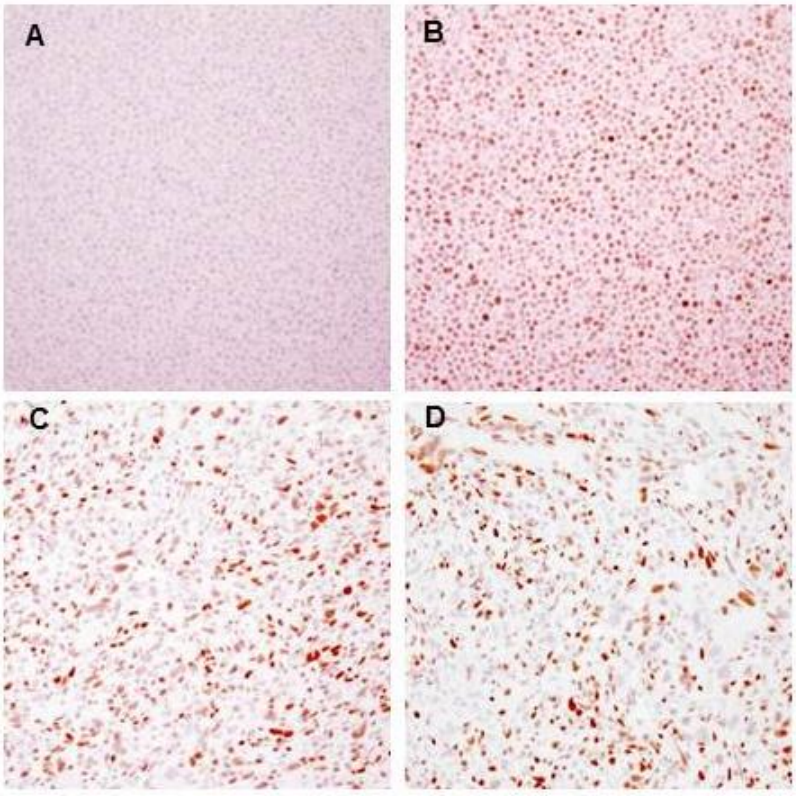

Figure 15. Phospho-STAT3 activation was independent of the IL-6 signaling pathway in TRAMP-C2 tumors in IL-6 -/- mice.

(A, B) p-STAT3 antibody (Tyr705, Cell Signaling, 9145) was used for IHC. Formalin-fixed, paraffin- embedded (FFPE) HeLa cells showed no detectable p-STAT3 on the untreated cells **(A)**, but strong positive signals on IFN-alpha-treated cells at 100 ng/ml **(B)**. **(C, D)** TRAMP-C2 allograft tumors from both wild type **(C)** and IL-6 -/- mice **(D)** were positive for pSTAT3 staining. Representative examples of allograft tumor sections are shown.

wildtype mice, MC38 allograft tumors had a tumor growth doubling time of about 3-4 days around 7 days after injection in both genotypes of mice. In fact, mice had to be sacrificed at day 17 post-inoculation due to tumors that were doubling every other day and growing close to 2 cm in width or length at that point (Figure 16A).

Interestingly, in the MC38 model, tumor development appears to be independent of IL-6. We did not observe any significant tumor growth difference between the two groups. Unlike the TRAMP-C2 allografts, MC38 allograft tumors did not grow larger in the wildtype mice. In fact, IL-6 -/- mice even developed a bit larger tumors (about 17% larger, but with $p=0.354$) at day 17 post-injection (Figure 16B).

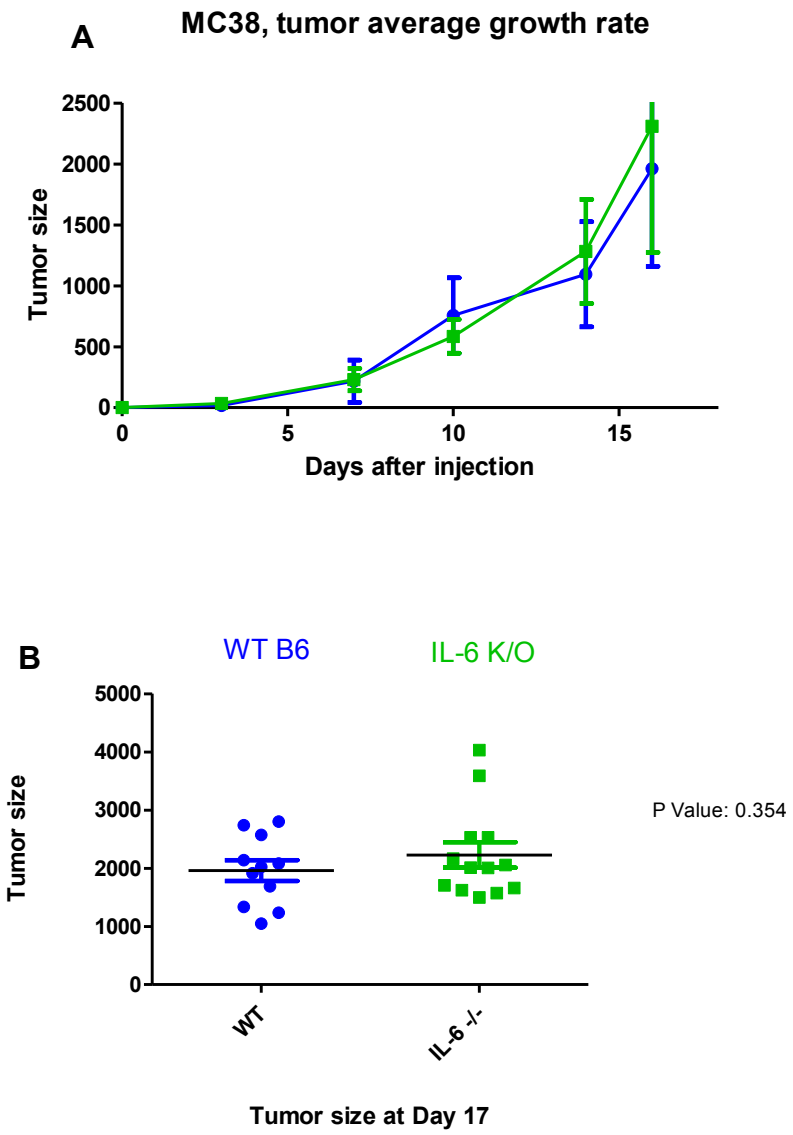

Figure 16. MC38 allograft tumor development is IL-6 independent.

(A) MC38 colon adenocarcinoma cells (1.5 x 10^6) were injected into ten C57BL/6J wildtype (blue) and thirteen IL-6 -/- animals (green). Interestingly, in this model, tumor development appears to be independent of IL-6. **(B)** At day 17 post-inoculation, MC38 cells grew in both mouse genotypes with no significant size difference. The average tumor size of wildtype mice was 1961.695 mm^3; while IL-6 -/- mice had an average tumor size of 2308.551 mm^3, which is about 17% larger than wildtype mice. Tumor sizes were measured in 3-4 days periods, and tumor volumes were evaluated as length×$width^2$×0.52 and are reported as mm^3.

3.5 Conclusions

TRAMP-C2 allograft as a novel model to study pro-tumorigenic role of IL-6

In non-diseased states, mouse circulating IL-6 levels are less than 10 pg/mL. During infection and/or inflammatory processes, serum IL-6 levels will be elevated. In our ELISA study, the cohort of wildtype mice with TRAMP-C2 tumors had elevated serum IL-6 levels, which indicated that mouse circulating IL-6 levels were associated with tumor growth in the TRAMP-C2 allograft model (Figure 13). This result is consistent with previous studies in prostate cancer patients that high circulating levels of IL-6 tend to associate with a more aggressive clinical course of the disease, which suggests that this TRAMP-C2 allograft model might be valuable to study a pro-tumorigenic role for IL-6 [15, 16, 142].

In addition, TRAMP-C2 allografts in IL-6 -/- animals resulted in delayed tumor growth (Figure 12). However, IL-6 depletion did not affect colon adenocarcinoma MC38 cell growth (Figure 16), which indicates this tumor line grows in an IL-6 independent manner. In summary, our studies provided preliminary evidence that IL-6 may be required for prostate tumor growth. The ELISA data further suggests that elevated systemic IL-6 levels as opposed to local IL-6 production in the tumor may be involved in prostate tumor growth regulation. Unfortunately, at this time there are no other prostate cancer cells lines from the appropriate mouse background strain to test in this model. Future efforts will involve testing other C57BL/6J-derived prostate cancer cell lines (if and when they become available), as well as other cancer types such as the breast cancer cell line EO771, in this model system.

Phospho-STAT3 may not be the downstream target of IL-6 signaling pathway in TRAMP-C2 allografts

There are multiple downstream pathways that might be targeted by IL-6 and IL-6R. Previous research has shown that binding of IL-6 to the IL-6-receptor can activate three major signaling pathways: the Janus tyrosine family kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, the extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3-K) regulated phosphor-Akt pathway [92].

Among these, phospho-STAT3 (p-STAT3) has been considered as one of the most important targets of IL-6. In fact, studies have shown that IL-6 along with p-STAT3 are important for prostate cancer development [17, 137-140]. In this study, we performed p-STAT3 IHC to determine if the IL-6 signaling pathway can directly recruit STAT3. However, in our study, p-STAT3 did not appear to be acting in an IL-6 dependent pattern. Instead p-STAT3 was activated in both wildtype and IL-6 -/- animals (Figure 15).

In future studies we aim to determine if there are other possible downstream factors involved in this IL-6 depletion model that contribute to prostate cancer development.

IL-6 might be a therapeutic target to prevent prostate cancer growth

Anti-interleukin-6 agents are a recent class of therapeutics that have been widely used to treat many diseases successfully including in patients with refractory cutaneous lupus and urticarial vasculitis, rheumatoid arthritis, B-lymphoproliferative disorder, plasma cell leukemia, lymphoma, and myeloma [52, 151-158]. The very first

approved antibody against IL-6R is tocilizumab (Actemra) which can directly act against the IL6-receptor [159, 160]. Since the 1990s, a mouse monoclonal antibody to IL-6 (murine mAbs BE-4 and BE-8) has been used to study blockade of IL-6 as a therapeutic target against prostate cancer in mice. More recently, a chimerised monoclonal antibody targeting IL-6 named CNTO 328 (Siltuximab) was developed to inhibit the binding of IL-6 to the IL-6-receptor [157]. Though IL-6 antagonists have been reported to prevent disease progression in some instances [52, 151-158], the effect of the antagonist in prostate cancer treatment is still questionable. CNTO 328 used in advanced prostate cancer clinical studies did not show an improvement [143, 161-163].

In clinical trials, CNTO 328 has again shown disparate results. Some have reported that Siltuximab can significantly inhibit tumor growth, decrease serum PSA levels, and increase survival in hormone-dependent LuCaP prostate cancer xenografts [161]. However, other reports indicate that with CNTO 328 treatment, tumor volume was reduced although the difference did not reach statistical significance [143]. A phase II trial of CNTO 328 in 53 patients showed median progression-free and overall survival were 1.6 and 11.6 months, respectively, although the CNTO 328 cohort did not show an improvement compared to patients with chemotherapy in this study [162]. Another phase II trial reported that when combined with mitoxantrone/prednisone, CNTO 328 did not improve progression-free survival versus mitoxantrone/prednisone alone [163]. Further studies need to be conducted to improve IL-6 antagonist effects.

In our study, we demonstrate that IL-6 depletion may delay TRAMP-C2 allograft tumor growth but not MC38 allograft tumor growth. Our data provided intriguing initial evidence that IL-6 may be involved specifically in prostate tumor growth as opposed to other cancer types, although more prostatic and other types of tumor cell

lines would need to be examined before a definitive pattern can be determined. In addition, ELISA data suggested that serum IL-6 levels were associated with tumor growth in the TRAMP-C2 allograft model, which is consistent with previous studies that prostate cancer patients with higher serum IL-6 tend to associate with a more aggressive clinical course. Along this road, our TRAMP-C2 allograft model might be valuable to study a pro-tumorigenic role for IL-6 and/or to test effects of IL-6 antagonists toward prostate cancer therapy.

IV. MULTILOCUS SEQUENCE TYPING (MLST) ANALYSIS OF PROPIONIBACTERIUM ACNES ISOLATES FROM RADICAL PROSTATECTOMY SPECIMENS

4.1 Abstract

Inflammation is commonly observed in radical prostatectomy specimens, and evidence suggests that inflammation may contribute to prostate carcinogenesis. Multiple microorganisms have been implicated in serving as a stimulus for prostatic inflammation. The pro-inflammatory anaerobe, *Propionibacterium acnes*, is ubiquitously found on human skin and is associated with the skin disease acne vulgaris. Recent studies have shown that *P. acnes* can be detected in prostatectomy specimens by bacterial culture or by culture-independent molecular techniques.

Radical prostatectomy tissue samples were obtained from 30 prostate cancer patients and subject to both aerobic and anaerobic culture. Cultured species were identified by 16S rDNA gene sequencing. *P. acnes* isolates were typed using multilocus sequence typing (MLST).

Our study confirmed that *P. acnes* can be readily cultured from prostatectomy tissues (7 of 30 cases, 23%). In some cases, multiple isolates of *P. acnes* were cultured as well as other *Propionibacterium* species, such as *P. granulosum* and *P. avidum*. Overall, 9 of 30 cases (30%) were positive for *Propionibacterium* spp. MLST analyses identified 8 different sequence types (STs) among prostate-derived *P. acnes* isolates. These STs belong to two clonal complexes, namely CC36 (type I-2) and CC53/60 (type II), or are CC53/60-related singletons.

In conclusion, MLST typing results indicated that prostate-derived *P. acnes*

isolates do not fall within the typical skin/acne STs, but rather are characteristic of STs associated with opportunistic infections and/or urethral flora. The MLST typing results argue against the likelihood that prostatectomy-derived *P. acnes* isolates represent contamination from skin flora.

4.2 Introduction

In the previous chapters, we have illustrated potential functions of IL-6 as a paracrine cytokine that may support prostate tumor growth. In our next studies, we aimed to examine how IL-6 may be involved in sustaining long-term bacterial-induced chronic prostatic inflammation that may in turn contribute to prostate cancer development. To do this, we sought to develop a mouse model of bacteria-induced prostatitis using a clinically relevant strain of bacteria. In this chapter, we describe a series of studies aimed at typing strains of *P. acnes* isolated from radical prostatectomy specimens to be used for this specific purpose.

Histologic specimens of prostate cancer tissue frequently exhibit unexplained acute and chronic inflammation and inflammation-associated lesions [40, 46, 129]. The development of prostatic inflammation may be related to microbial infection, as previous studies have demonstrated the presence of multiple microbial species in the prostates of prostate cancer patients [60, 74]. Interestingly, many of the organisms identified are consistent with genera associated with inflammation-associated conditions including bacterial prostatitis and/or urinary tract infections [60]. *Propionibacterium acnes* (*P. acnes*) is a bacterium of particular interest in relation to prostate cancer. *P. acnes* is a pro-inflammatory bacterium that is considered to be the etiological agent in the skin condition acne vulgaris, and has also been reported in association with other inflammatory conditions including endocarditis, sarcoidosis and

post-surgical infections [73]. *P. acnes* was first reported in association with prostate inflammation and cancer in 2005 [74]. Interestingly, this study reported that prostatectomy specimens from which *P. acnes* could be cultured were more likely to be inflamed, leading to the hypothesis that *P. acnes*-mediated inflammation may contribute to prostate carcinogenesis [74]. Several subsequent studies have also reported on the presence of *P. acnes* in prostate specimens [60, 75, 82]. Although not all studies have shown a positive association, the correlation between acne and/or plasma antibodies to *P. acnes* and prostate cancer incidence and outcomes has also been examined in multiple epidemiological studies [79-81]. In addition, *in vitro* studies have demonstrated that *P. acnes* is capable of inducing a strong inflammatory response in prostate cell lines [75-77].

Initially, sequencing of *P. acnes tly* and *recA* genes was used to categorize *P. acnes* strains into phylotypes I, II, and III [164, 165]. A more recent strategy for typing bacterial strains is called multilocus sequence typing, or MLST, which has dissolved the population structure of the species *P. acnes.* MLST generates "sequence types (STs)" based on DNA sequencing and the determination of different alleles of internal fragments of housekeeping genes [166, 167]. Related STs can form "clonal complexes" (CCs) based on their similarity to a central allelic profile. The MLST typing scheme for *P. acnes* again identified three divisions of *P. acnes* strains (I, II, and III) [166]. Division I was further subdivided into I-1a, I-1b, and I-2, and further into CCs. MLST analysis performed on 210 isolates of *P. acnes* from healthy individuals, patients with moderate to severe acne, and patients with various opportunistic infections (abscess, wounds, endocarditis, bursitis, hip prosthesis, etc.) demonstrated that severe acne isolates were predominantly classified into CCs belonging to group I-1a and I-1b strains, i.e. CC3, CC18, and CC31, whereas isolates

associated with opportunistic infections were predominantly classified into CCs belonging to group I-2, II and III strains, i.e. CC36, CC53/60 and CC43 [166].

P. acnes is an ubiquitous skin bacterium and is also reported to be a common culture contaminant. It is therefore often difficult to determine if the presence of *P. acnes* in surgical specimens (including radical prostatectomy specimens) has arisen from contamination from the skin of the patient and/or the medical staff or whether it represents a true infection of clinical significance [73, 84-86]. The present study was undertaken to perform MLST analysis of *P. acnes* isolates from radical prostatectomy specimens in order to determine if the sequence types of these isolates are similar to the sequence types associated with healthy or diseased human skin or other anatomic locations and disease conditions.

4.3 Materials and Methods

Prostate tissue samples.

All specimens were collected under a Johns Hopkins Internal Review Board (IRB) approved protocol. Post-prostatectomy tissue samples were obtained from 30 patients undergoing treatment for prostate cancer at the Johns Hopkins hospital. The clinical and pathological parameters of the patient samples are listed in Table 6. A total of 10 tissue cores from peripheral prostate were collected into 2 ml of sterile PBS using a Bard Biotpy gun and needles as previously described [60].

Bacterial culture.

The prostate tissues were first minced using sterile razor blades. Minced tissues were then equally divided into 5 mL of culture broth in polystyrene tubes for aerobic and anaerobic culture (BD Biosciences). For aerobic culture, minced tissues were

Table 6. Clinical and Pathological Parameters of Patient Samples for Bacterial Culture

Parameter	Value
Total number of patients	30
Mean age (range)	57 (43-74)
Gleason Score (number of patients)	
6	8
3+4=7	11
4+3=7	6
8	2
9	3
TNM stage	
T2	14
T3	16

cultured in Luria-Bertani (LB) broth (BD Biosciences) at 200 rpm at 37 ^{o}C in a shaking incubator for a minimum of 1 week. Most positive aerobic cultures were positive for growth within 24-48 hrs. For anaerobic culture, minced tissues were cultured in Brain Heart Infusion broth (BD Biosciences) in anaerobic pouches (GasPak EZ Anaerobe Gas System, BD Biosciences) at 37 ^{o}C for at least 2 weeks. Most positive anaerobic cultures had visible growth within 1 week.

Strain identification.

Bacteria from cultures positive for growth was harvested and gDNA was isolated using the modified protocol for Gram positive bacteria and the QIAamp DNA mini kit (Qiagen) or the MasterPure™ Gram Positive DNA Purification Kit (Epicentre). A universal primer set designed against the bacterial 16S rDNA gene, Ecoli9-F and Loop27-R was used for PCR as previously described [168]. The PCR cycling parameters were as follows: 94^{o}C for 2 min, 35x cycle of 94^{o}C for 30 s, 53^{o}C for 30 s and 72^{o}C for 1 min and 72^{o}C for 5 min. Purified PCR products were sent for Sanger Sequencing at the DNA Analysis Facility at Johns Hopkins. Sequencing results were analyzed by Standard Nucleotide BLAST search against reference bacterial genomic sequences (NCBI).

Multilocus sequence typing (MLST).

For bacterial isolates that were identified as *P. acnes*, MLST was performed per the typing scheme described by Lomholt and Kilian in 2010 [166]. Nine housekeeping genes were amplified by PCR and used for sequence analysis (cel, coa, fba, gms, lac, oxc, pak, recA, and zno) [166]. In two *P. acnes* strains (from patients #20 and #22), we were unable to amplify the oxc allele and the zno allele. This was presumably due to mismatches in the primer sets for these strains using the Lomholt and Kilian MLST

scheme. In these cases, the following replacement primers were used: oxc2-F 5'-AGGCGTGCTGCCGGAAAAG-3', oxc2-R 5'-CACCACCGGCGTCAGGATT-3', and zno2-R 5'-TCATATGCCGCGTCGACCTC-3'. The PCR cycling parameters for all housekeeping genes except for recA were as follows: 96^{o}C for 40 s, 35x cycle of 94^{o}C for 35 s, 55^{o}C for 40 s and 72^{o}C for 40 s and 72^{o}C for 7 min. The PCR cycling parameters for recA were as follows: 95^{o}C for 3 min, 35x cycle of 95^{o}C for 1 min, 55^{o}C for 30 s and 72^{o}C for 90 s and 72^{o}C for 10 min. Purified PCR products were sent for Sanger Sequencing at the DNA Analysis Facility at Johns Hopkins or Genomic Services at Beckman Coulter Genomics. Sequence type (ST) of each isolate was determined using a publically available MLST database (http://pacnes.mlst.net) [166]. All sequences will be submitted to GenBank (Acession #'s TBD).

The identification of clonal complexes (CCs) and their founders based on allele profiles was achieved by eBURST analysis at http://eburst.mlst.net/ using the eBURST version 2 clustering algorithm, which was developed and is hosted by Imperial College London and is based on principles originally described by Feil *et al.* [169].

4.4 Results

Bacterial culture.

The results of bacterial culture from prostatectomy tissues are shown in Table 7. Half of the patient samples were negative for bacterial growth. As determined by 16S rDNA sequence analysis, in the remaining cases, *P. acnes* was the most frequently cultured species, isolated from 23% of patient samples. Other species cultured from prostatectomy tissues included *P. avidum* (7%), *P. granulosum* (3%), *Staphylococcus epidermidis* (17%), and *Corynebacterium glucuronolyticum* (7%). As shown in Table 8, in several cases either more than one species of bacteria or more than one strain of *P. acnes* was cultured. Interestingly, in two cases, *P. acnes* was isolated from an aerobic culture.

There were no significant correlations between *P. acnes* culture status and patient age, Gleason score, or tumor stage. Interestingly, there was a significant correlation between tumor grade (pT2 vs. pT3) and cases that were positive for culture of *S. epidermidis* (Fisher's exact test, $p = 0.045$, Table 9).

MLST analysis of prostatectomy-derived P. acnes isolates.

In all, 9 different strains of *P. acnes* from the present study were cultured and subject to MLST analyses (Table 8). In addition to these isolates, we also performed MLST typing on a prostatectomy-derived *P. acnes* isolate from a previous study [60]. The results of MLST analysis are shown in Table 10. We observed 8 different STs among the prostatectomy-derived *P. acnes* isolates (Table 10). In most cases (6 of the 8 STs), the allelic profile of the *P. acnes* strains could not be matched completely with the known STs defined in the MLST database (differing at 1-2 alleles) [166]. We therefore assigned new STs (ST61 and ST79-83). An eBURST analysis revealed that

Table 7. Bacteria Isolated from Prostatic Tissue of 30 Unselected Patients with Prostate Cancer

Organism	No. Patients* (%)
No bacterial growth	15 (50%)
Corynebacterium glucuronolyticum	2 (7%)
Propionibacterium acnes	7 (23%)
Propionibacterium avidum	2 (7%)
Propionibacterium granulosum	1 (3%)
Staphylococcus epidermidis	5 (17%)

* More than one species was cultured from two cases

Table 8. Prostate Tissue Samples Positive for Bacteria Growth and Species Identification by 16S rDNA Sequence Analysis

Patient #	Gleason Grade	Stage	Aerobic Bacteria*	Anaerobic bacteria*
5	3+3=6	T2	*P. acnes* (99%, NC_017535)	*P. acnes* (99%, NC_017535)
8	3+4=7	T3A	-	*S. epidermidis* (100%, NC_004461)
9	3+4=7	T2	-	*P. acnes* (99%, NC_017535)
10	3+4=7	T3B	*P. acnes* (99%, NC_017535)	1) *P. acnes* (100%, NC_017535) 2) *P. avidum* (99%, NZ_JH165055)
11	3+4=7	T2	-	*P. granulosum* (99%, NR_025276)
15	4+4=8	T3A	*S. epidermidis* (100%, NC_004461)	*P. acnes* (99%, NC_017535)
16	3+4=7	T3A	-	*P. avidum* (99%, NZ_JH165055)
19	3+4=7	T2	-	*P. acnes* (99%, NC_017535)
20	3+3=6	T2	-	*P. acnes* (100%, NC_017535)
22	4+3=7	T3A	-	*P. acnes* (99%, NC_017535)
23	4+3=7	T3A	-	*C. glucuronolyticum* (97%, NZ_GG667131)
24	5+4=9	T3B,N1	*S. epidermidis* (99%, NZ_GG696777)	
25	4+5=9	T3A	*S. epidermidis* (100%, NC_004461)	
26	4+3=7	T2		*C. glucuronolyticum* (100%, NZ_GG667131)
27	4+3=7	T3A		*S. epidermidis* (100%, NC_004461)

***** Closest match to GenBank reference genomic sequence (% similarity, Accession #).

Table 9. Association Between Prostate Cancer Pathological Stage and Bacterial Culture Status

Stage	No . cases *P. acnes* +	No. cases *P. acnes* -	p value*	No. cases *S. epidermidis* +	No. cases *S. epidermidis* -	p value*
pT2	4	10		0	14	
pT3	3	13	0.6746	5	11	0.0447

* As determined by Fisher's exact test

Table 10. MLST Profiles of Prostate-Derived P. acnes Isolates

Patient #	MLST profile (cel-coa-fba-gms-lac-oxc-pac-recA-zno)	ST[+]	CC[++]	Division[+]
5 (aerobic)	3-9-7-11-7-3-5-6-9	61	Singleton	II
5	3-13-8-11-7-3-11[#]-6-9	79	Singleton	II
9	5-9-3-3-4-3-5-2-9	36	36	I-2
10 (aerobic)	3-13-7-11-7-7-5-6-9	80	53	II
10-1	3-13-11[#]-11-7-3-5-6-9	81	Singleton	II
15	3-9-7-11-7-3-5-6-9	61	Singleton	II
19	5-9-3-3-2-3-5-2-9	38	36	I-2
20	3-13-8-11-7-7-5-6-14	82	53	II
22	3-13-7-11-7-7-5-6-14	83	53	II
PA-2*	5-9-3-3-4-3-5-2-9	36	36	I-2

[+] Based on [166]

[++] As determined by eBURST analysis

* *P. acnes* isolate from previous prostate cancer study [60]

[#] Represents a new allele

prostatectomy tissue-derived *P. acnes* strains belong to two CCs: CC36, the representative CC of group I-2 strains, and CC53/60, a major CC of group II strains (Figure 17). The newly assigned STs (ST61 and ST79-83) are all type II strains; they are either part of or closely related to CC53/60.

Comparison of prostatectomy-derived P. acnes CCs and STs to previously characterized strains.

We compared the results of MLST analysis of prostatectomy tissue-derived *P. acnes* strains to previous MLST studies that have been conducted on 210 *P. acnes* isolates from human skin, severe acne, and opportunistic infections [166] and 75 human skin and acne-associated isolates from a cohort in the Unites States included as part of the Human Microbiome Project (Figure 18) [170]. MLST analysis was previously performed on these strains [171]. Human skin isolates are distributed across the spectrum of CCs, and are most predominantly strains of group I-1a (CC3, CC18, and CC28). Strains isolated from opportunistic infections [166] most often belong to CC36 and CC53/60. Likewise, prostatectomy-derived *P. acnes* isolates were identified as CC36 and CC53/60 strains as well as CC53/60-related singletons. Interestingly, as shown in Figure 19, prostatectomy-derived *P. acnes* isolates do not overlap with CCs determined to be associated with isolates from severe acne [166].

4.5 Discussion and Conclusions

The association between *P. acnes* and disease conditions has been difficult to confirm largely because *P. acnes* is the most predominant species found on human skin and is reported to be a common culture contaminant. Even the strongest association between *P. acnes* and a disease condition - as a causative agent in acne vulgaris - remains controversial [83]. Although multiple studies have now reported on the ability

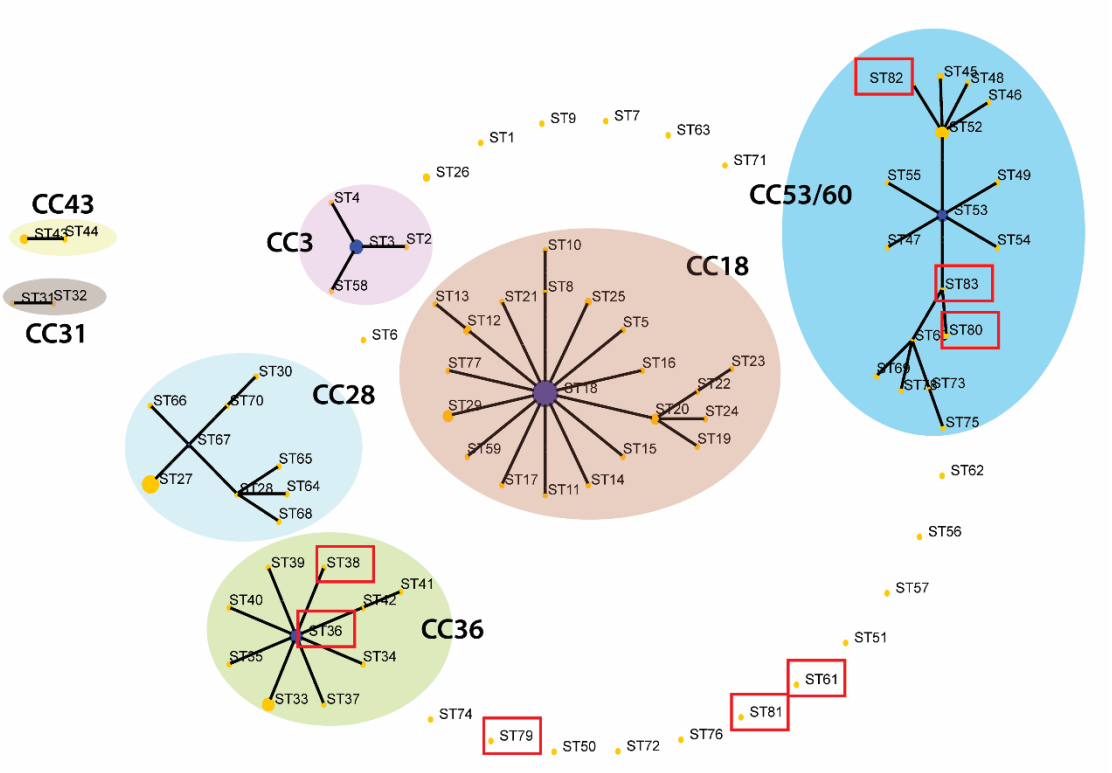

Figure 17. *P. acnes* ST distribution based on MLST allele profiles.

Population snapshots of *P. acnes* generated by eBURST analysis based on the MLST allele profiles. ▭ Prostatectomy-derived *P. acnes* strains are associated with these STs.

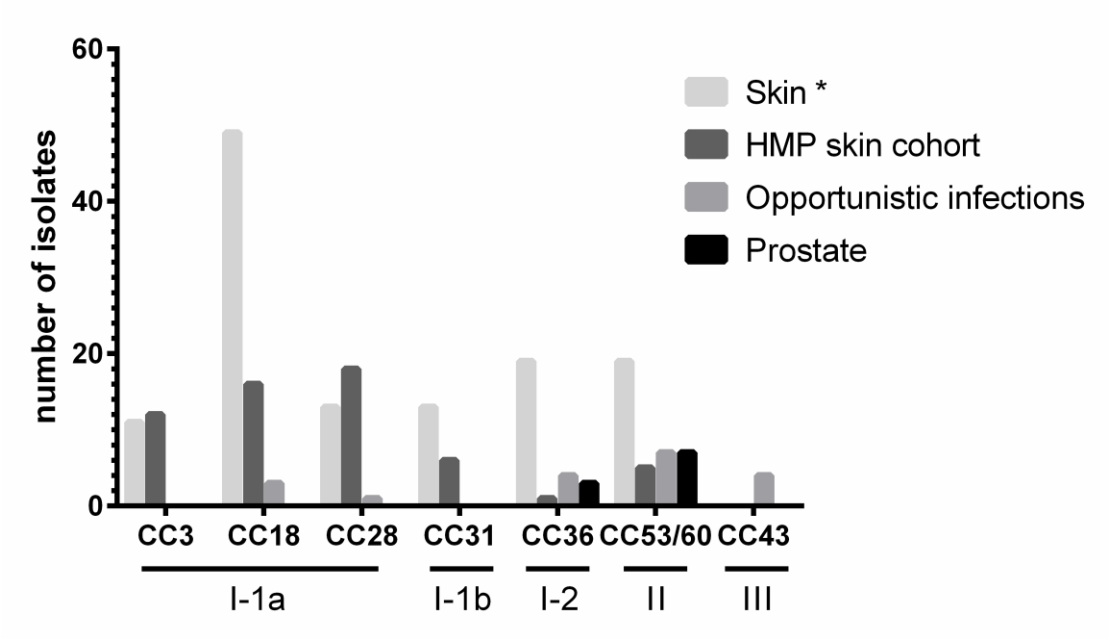

Figure 18. Comparison of prostatectomy-associated *P. acnes* strains.

Compare prostatectomy-associated *P. acnes* strains to previously described strain collections of isolates from skin and opportunistic infections [166] and skin isolates from a Human Microbiome Project (HMP) skin cohort. Singletons ST61, ST79 and ST81 are related to CC53/60 and are grouped in this CC for the purpose of this figure.

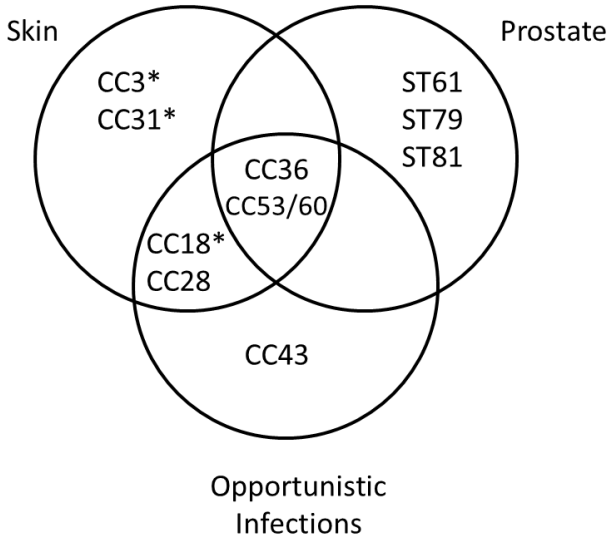

Figure 19. *P. acnes* isolates cultured from prostatectomy specimens are associate with opportunistic infections, but not with severe acne.

Venn diagram of the association between CC of *P. acnes* strains from prostatectomy tissues in the present study compared to isolates from opportunistic infections and skin from a previous study [166]. Severe acne isolates are associated with these CCs. ST61, ST79 and ST81 are singletons and related to CC53/60.

to culture *P. acnes* from prostate cancer tissues [60, 74], the question still remains as to whether the presence of this species represents a true prostatic infection or contamination from patient skin, the medical team, or the surgical environment.

The presence of bacteria in prostatectomy tissues

There are few studies that have been performed to characterize the normal microbial constituents of the adult male urethral flora. Many of these studies have relied on urine culture and the most commonly recognized species thought to inhabit the male urethra include *Staphylococcus* sp., *Corynebacterium* sp., *Enterococcus* sp., and streptococci [172, 173]. Interestingly, in addition to *Propionibacterium* sp., two of these urethra flora-associated species were cultured from prostatectomy tissues in the present study (*Staphylococcus* sp. and *Corynebacterium* sp.). Studies that have utilized PCR-based molecular techniques have also identified *P. acnes* in the urine of adult males [174, 175]. In the study by Shannon *et al.* [174], urethral *P. acnes* isolates were found to be associated with phylogenetic clusters IB and II (analogous to I-2 and II in [166]). In all, the results of the present study indicate that the bacterial isolates obtained from prostatectomy specimens may reflect urethral flora as opposed to skin flora. This would support the theory that these bacterial strains may infect the prostate, as the proposed route that bacteria may infect the prostate is via the urethra. On the other hand, the presence of these species in prostatectomy tissues could also represent contamination of the prostatectomy specimen from urethral flora, and this remains a topic of future studies.

Prostate-derived *P. acnes* isolates are unlikely from skin contamination

In the present study, we aimed to begin to address this question by employing a newly established MLST scheme to compare the STs/CCs of prostatectomy

tissue-derived *P. acnes* isolates to previous collections of *P. acnes* isolates from healthy skin, severe acne, and opportunistic infections. The results of these analyses indicated that the prostatectomy-derived *P. acnes* isolates included in this study do not overlap with STs/CCs associated with severe acne, but instead overlap with CCs associated with opportunistic infections.

Healthy skin-associated isolates are somewhat uniformly distributed among CCs (with the exception of ST18 strains that are thought to represent an "epidemic clone" of *P. acnes* that is frequently associated with severe acne and prevalent on human skin [166]), and prostatectomy tissue-derived *P. acnes* isolates did fall within the same CCs as some isolates from healthy skin. However, if the prostatectomy tissue-derived *P. acnes* isolates were simply reflective of normal skin flora, they would be expected to fall within a broad spectrum of CCs (and especially within the I-1a group) and not confined to distinct CCs in the I-2 and II groups. Instead, none of the prostatectomy tissue-derived *P. acnes* isolates belong to group I-1a. Moreover, some prostatectomy tissue-derived *P. acnes* isolates are unique and represent new STs within group II.

Association between prostate cancer pathological stage and bacterial culture status

We discovered an interesting significant correlation between the ability to culture *S. epidermidis* from prostatectomy tissues and advanced stage (T3) prostate cancer (Table 9). *S. epidermidis* has been previously associated with chronic bacterial prostatitis and is therefore implicated in the pathogenesis of prostatic inflammation [176, 177]. The isolation of *S. epidermidis* from prostatectomy tissues has also been previously reported [60, 74]. On the other hand, it is known that necrotic tumors can become infected with bacteria from endogenous sources, especially when they occur

next to a site where bacteria flora resides (such as the urethra) [178]. Additional studies must be conducted to determine if this association holds up in a larger sample size and whether the relationship is causal or consequent in regards to tumorigenesis.

Since the MLST typing results indicated that prostate-derived *P. acnes* isolates do not fall within the typical skin/acne sequence types, but rather are characteristic of sequence types associated with opportunistic infections and phylogenetic clusters associated with urethral flora, we decided to use one of these prostatectomy-derived strains of *P. acnes* (called PA-2) to inoculate into IL-6 -/- mice and study its effect on the induction of bacterial prostatitis.

This work was published in The Prostate (73(7): p. 770-7, 2013).

V. IL-6 MAY BE REQUIRED TO SUSTAIN BACTERIA-INDUCED LONG-TERM CHRONIC INFLAMMATION IN THE PROSTATE

5.1 Abstract

We have previously developed a mouse model of chronic prostatic inflammation using a human prostatectomy-derived strain *Propioniobacterium acnes* (PA-2) using wildtype C57BL/6J mice. In this model, we found that PA-2 infected animals developed acute and then chronic inflammation that was restricted to the mouse dorsal prostate lobe. Surprisingly, chronic inflammation in this model was found to persist up to at least a year post-inoculation.

Interestingly, when we performed chromogenic *in situ* hybridization (CISH) on wildtype mouse dorsal prostate 2 weeks post-inoculation with *P. acnes*, we observed IL-6 mRNA expression in areas of acute inflammation. This was highly analogous to the IL-6 expression pattern in association with acute inflammation that we observed in the human prostate (see Chapter 2). These data indicated that IL-6 might be involved in the induction of bacterial prostatitis. Furthermore, as previously described in detail, we suspect that prostatic inflammation may contribute to the carcinogenic process.

In this study, the same *P. acnes* strain (PA-2) was used to inoculate IL-6 -/- mice so that we could conduct a direct histopathological comparison to what we previously observed in wildtype mice. IL-6 -/- mice were inoculated with PA-2 and animals we sacrificed 2 weeks, 8 weeks, 6 montha, and 1 year post-inoculation. We found that the dorsal prostate of IL-6 -/- mice initially developed severe inflammation that subsided significantly by 2 months post-inoculation. Surprisingly, unlike wildtype animals, IL-6 -/- mice did not sustain chronic inflammation at 6 months or beyond. These data

would support the hypothesis that IL-6 may be required to sustain long-term chronic prostatic inflammation.

5.2 Introduction

P. acnes as a possible agent for tumor-promoting prostatitis.

In a previous study, our team developed a mouse model of chronic prostatic inflammation using a human prostatectomy-derived strain of *P. acnes* (PA-2) [179]. Wildtype C57BL/6J mice were inoculated with PA-2 at 10 weeks of age and animals were sacrificed at 1, 2, and 8 weeks, 6 months, and 1 year post-infection. Acute and then chronic inflammation was observed specifically in the dorsal prostate in this model after PA2 inoculation. Unlike bacterial prostatitis caused by species such as *E. coli* that can induce an acute inflammatory response within 24 hours post-infection, *P. acnes* infection-induced inflammation is somewhat delayed, with no inflammation observed until 1 week post-inoculation. In this model, chronic inflammation can persist for at least a year post-inoculation in wildtype mice. To further confirm if *P. acnes* infection can induce cellular proliferation, Ki-67, a proliferation-associated marker, was assayed by IHC. An increased number of Ki-67 positive epithelial cells were present in inflamed areas of the dorsal prostate at 1 and 2 weeks post-inoculation, and even after inflammation had subsided somewhat at 8 weeks, increased numbers of Ki-67 positive epithelial cells could be observed [179]. This model is valuable because it may serve as an *in vivo* model to study additional inflammation-associated prostatic diseases [179]. Once again, the model suggested that *P. acnes* can infect the mouse dorsal prostate lobe and induce long-term chronic inflammation.

IL-6 knockout mice as a model to study the possible role of IL-6 in chronic prostate inflammation.

Uncontrolled IL-6 production has been implicated in many disease processes. In HIV patients, for example, an infected cohort had higher serum IL-6 levels compare to healthy individuals [180]. Yet, some animal studies also suggest that mice with IL-6 depletion are more prone to infection [40]. IL-6 knockout (IL-6 -/-) mice have been utilized in multiple studies to determine how IL-6 plays a role in resistance to bacterial infections. One group showed that when IL-6 -/- mice were infected with *C. albicans*, the mice couldn't initiate a Th1 immune response, and this resulted in more infection [181]. In another study, *L. monocytogenes* was introduced into wildtype and IL-6 -/- mice, and the knockout animals showed uncontrolled bacterial growth and also had a higher mortality rate at 1 week post-inoculation [182].

Elevated systemic IL-6 levels have also been associated with prostate cancer [113]. Our studies described in Chapter 2 and Chapter 3 of this thesis demonstrate the possible paracrine role of IL-6 in prostate cancer development, and its potential pro-tumorigenic role in sustaining tumor growth. Yet, IL-6 may also be involved in other steps of prostate cancer development, such as sustaining chronic inflammation to transform cancer into an aggressive phenotype, or promoting cancer progression and tumor metastasis. Here, we aimed to inoculate PA-2, a human prostatectomy derived *P. acnes* strain, into IL-6 -/- mice to study the possible role of IL-6 in sustaining bacterial-induced chronic prostate inflammation [181].

5.3 Materials and Methods

Animals

Animals used in this study were 8-10 week old C57BL/6J wildtype (Harlan) or Interleukin-6 knockout (IL-6 -/-, Jackson Lab, B6.129S2-Il6tm1Kopf/J) mice. Animals were housed in a pathogen-free environment with 12 hour light/dark cycle, and received enough sterile food and water. All procedures were performed under the guidelines of Johns Hopkins Animal Care and Use Committee (ACUC). Animals were sacrificed by CO2 asphyxiation and serum, reference tissues, and mouse prostate lobes were harvested.

Prostatectomy isolated *P. acnes* strain: PA-2

In this study, a human prostate derived *P. acnes* strain, PA-2 was used. This bacterium was isolated from minced prostatectomy tissue with anaerobic culture in Brain Heart Infusion broth (BD Biosciences) in anaerobic pouches (GasPak EZ Anaerobe Gas System, BD Biosciences) at 37 ^{o}C for at least 2 weeks.

Inoculation of *P. acnes* via Transurethral Catheterization

2.5 cm length sterile polyethylene catheters (BD Bioscience) were used to performed transurethral catheterization on mice anaesthetized with ketamine/xylazine. PA-2 was inoculated into IL-6 -/- mice at a dose of approximate of 10^7 colony forming units (CFU) suspended in 20 µl sterile phosphate buffered saline (PBS). Control animals were treated with an equal volume sterile PBS.

Chromogenic in situ hybridization (CISH).

CISH was performed using the RNAscope® 2.0 FFPE Brown Reagent Kit. Briefly, FFPE tissues were first baked at 60°C for 1 hr followed by deparaffinization

in two changes of 100% xylene for 5 min each and two changes of 100% alcohol for 3 min each. Next, the slides were treated with endogenous peroxidase blocking pretreatment reagent for 10 min at room temperature. The slides were then added to boiling buffer for 30 min at 99-104°C in a water bath and then treated with protease digestion buffer for 30 min at 40°C. The slides were incubated with a custom RNAscope target probe designed against mouse IL-6 for 2 hr at 40°C, followed by DAB for colorimetric detection and hematoxylin staining.

Prostate Histopathology and Inflammation Grading

All prostate lobes were collected and separately fixed with 10% neutral phosphate buffered formalin for 48 hours and embedded in paraffin, followed by Hematoxylin and eosin (H&E) staining. At 1 year post-inoculation group, mouse dorsal prostates were fixed with PAXgene (765112, Qiagen) to prevent destructive nucleic acid and protein crosslinking and degradation to performed further RNA microarray study.

Dorsal prostate was dissected with lateral lobe, but anterior, ventral lobes were dissected separately. For inflammation grading, we used the same method as previous described [179]. Prostate tissues were considered positive for mild inflammation if scattered neutrophilic or mononuclear inflammatory cells were present and involved multiple glands/foci. Prostate tissues were considered to be moderately inflamed if clusters (but not follicles) of lymphocytes and/or macrophages were present and involved multiple glands/foci. Prostate tissues were considered to be severely inflamed if dense nodules/follicles of inflammatory cells were present.

Microarray analysis

RNA was extracted from OCT embedded frozen dorsal prostate using the RNeasy kit (Qiagen). Microarray analysis was performed at the SKCCC Microarray core facility using Illumina Mouse WG-6 arrays. All of the analysis was done using R software and different packages: GMD, RColorBrewer, gplots, preprocessCore, limma, and xslx. The data was filtered using filter criteria (if probe was not detected using a detection cutpoint of 0.05 in all samples then it was excluded from analysis). This filtering decreased number of probes from 45,281 probes to 26,562 probes. Then data was normalized using quantile normalization method. Using ANOVA models in limma package, all different comparisons were performed. Top 250 up and down-regulated genes were analyzed by DAVID Bioinformatics Resources (version 6.7), and build up functional annotation charts. Functional charts were then transformed by KEGG system (Kyoto Encyclopedia of Genes and Genomes) into signaling pathways which may be involved in your input gene IDs.

5.4 Results

P. acnes inoculation induces acute and chronic inflammation at early time points in the IL-6 -/- mouse dorsal prostate

Interestingly, when we performed chromogenic *in situ* hybridization (CISH) on wildtype mouse dorsal prostate 2 weeks post-inoculation with *P. acnes*, we observed IL-6 mRNA expression in areas of acute inflammation (Figure 20). This was highly analogous to the IL-6 expression pattern in association with acute inflammation that we observed in the human prostate (see Chapter 2). This data indicated that IL-6 might be involved in the induction of bacterial prostatitis.

As mentioned, in our previous studies [179], wildtype mouse dorsal prostates became acutely and then chronically inflamed after PA-2 inoculation, and the chronic inflammation persisted for at least 1 year post-infection. In the present study IL-6 -/- mice between 8-10 weeks old were inoculated with either PBS or with 10^7 CFU PA-2. Mice were sacrificed 1 week, 2 weeks, 8 weeks, 6 months, and 1 year post-inoculation. At each time point we had 4 PBS controls and 5 PA-2 treated mice. All mice in the PBS control groups were not observed to have inflammation in their prostate lobes at any time point.

Impaired long-term prostatic chronic inflammation in IL-6 -/- mice

At 1 week post-infection with PA-2 *P. acnes*, IL-6 -/- animals had acute inflammation and to a lesser extent chronic inflammation in the dorsal prostate lobe (Figure 21). At 2 weeks post-inoculation, 100% of the animals developed severe to moderate chronic inflammation, (Figure 21 and Figure 22). At 8 weeks after PA-2 inoculation, 60% of IL-6 -/- mice still sustained mild chronic inflammation (Figure 22). Unlike our previous studies in wildtype animals, we did not observe any

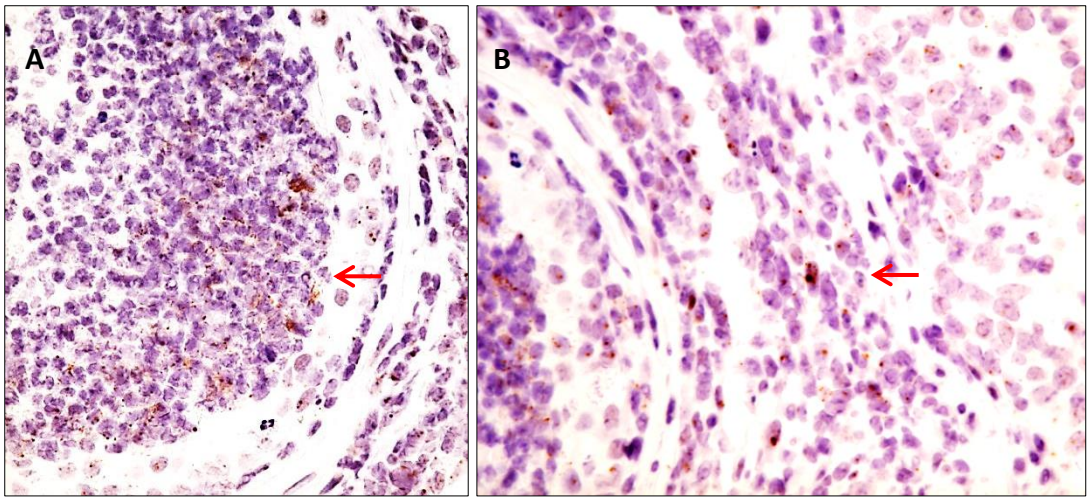

Figure 20. IL- 6 mRNA expression in PA-2 infected mouse prostates.

Wildtype mouse dorsal prostates were collected 2 weeks post-inoculation with PA-2, and fixed for 48 hours in formalin. **(A)** IL-6 mRNA CISH. IL-6 expressing cells (brown staining) were highly enriched in the areas of acute inflammation as indicated by the presence of neutrophils in glandular lumens (red arrows). **(B)** IL-6 positive cells can also be found in the stroma in areas of acute inflammation (arrows).

severely inflamed areas in IL-6 -/- animals 8 weeks after inoculation. Also unlike wildtype animals, at 6 months and 1 year, none of IL-6 -/- animals still sustained chronic inflammation in the dorsal lobe (Figure 22).

Microarray analysis

Mouse dorsal prostate from C57BL/6J wildtype and IL-6 -/- mice were collected at 2 weeks post PA-2 inoculation. RNA was extracted from OCT frozen tissues, and analyzed by microarray. R software and different packages were applied for data analysis. In Figure 23, preliminary analyses show a heatmap of hierarchical unsupervised clustering that illustrates differentially expressed genes. In this heatmap, we compared the top 2500 up-regulated and down-regulated genes in C57BL/6J wildtype and IL-6 knockout mice treated with PBS or with PA-2. We did observe significant differences among each group. Especially in the PA-2 inoculated IL-6 -/- group, this cohort had the most unique gene expression pattern compared to others.

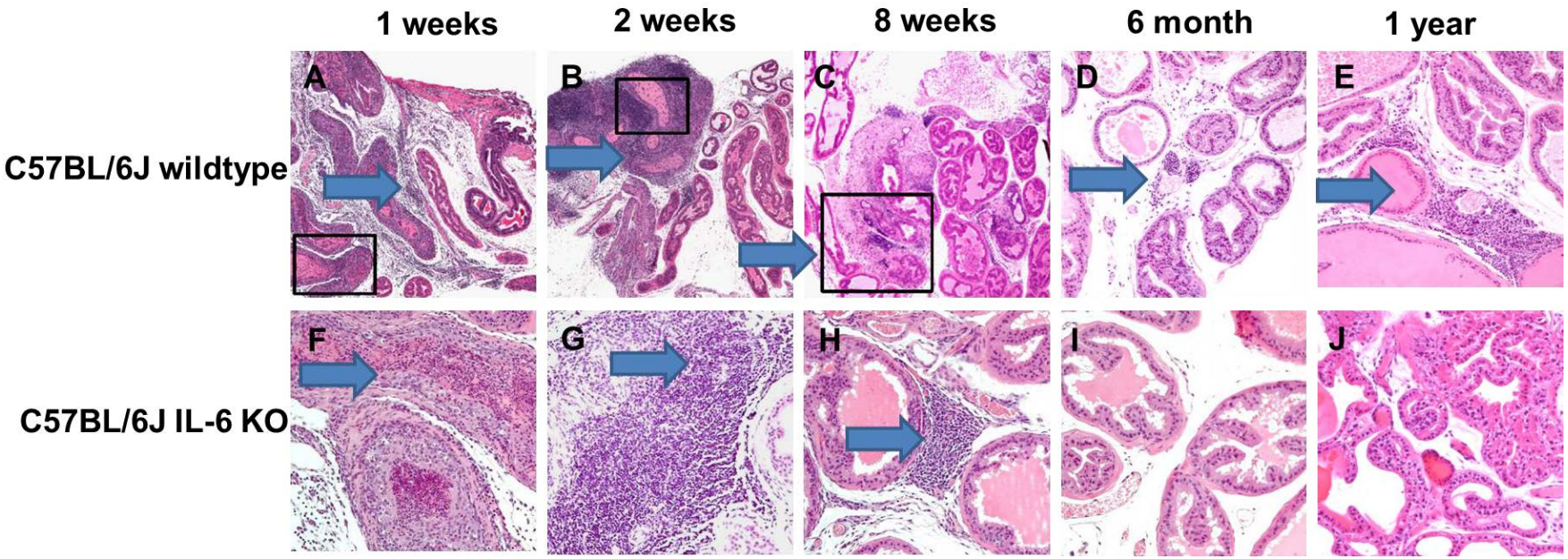

Figure 21. Dorsal prostate of C57BL/6J wildtype and IL-6 -/- mice inoculated with PA-2 at different time points.

(A-E) H&E stained on PA-2 infected wildtype dorsal prostates, which were collected from 1 week (A), 2 weeks (B), 8 weeks (C), 6 months (D), and 1 year (E) post-inoculation. Acute inflammation was observed at 1 week and 2 weeks post-inoculation as accumulation neutrophils at glandular lumens. Chronic inflammation can be observed through all time points starting from 1 week post-inoculation. Chronic inflammatory cells were observed mainly in the stoma (arrows). **(F-J)** Dorsal prostates from IL-6 -/- mice 1 week (F), 2 weeks (G), 8 weeks (H), 6 months (I), and 1 year (J) after inoculation with PA-2. Acute inflammation was observed at 1 week followed by predominantly chronic inflammation at 2 weeks post-inoculation. Chronic inflammation was observed to a lesser extent at 8 weeks

post-inoculation and absent at 6 months and 1 year post-inoculation. Arrows indicate acute and/or chronic inflammation. (Inflammation data from wildtype mice are adapted from Shinohara et al. 2013)

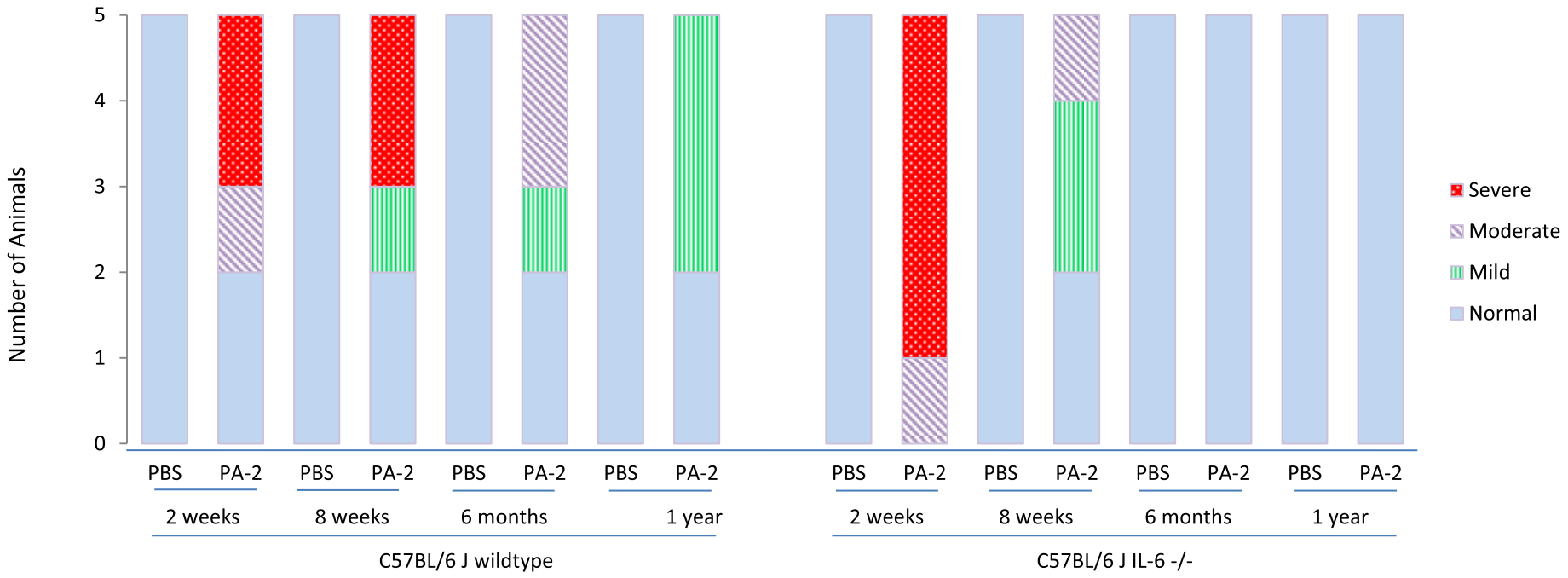

Figure 22. Long-term chronic inflammation is not sustained in IL-6 -/- mice.

(A, B) Prevalence of inflammation in wildtype (A) and IL-6 -/- (B) dorsal prostate at 2 weeks, 8 weeks, 6 months, or a year after PA-2 inoculation or PBS (control). (Prevalence inflammation data of wildtype mice are adapted from Shinohara et al. 2013)

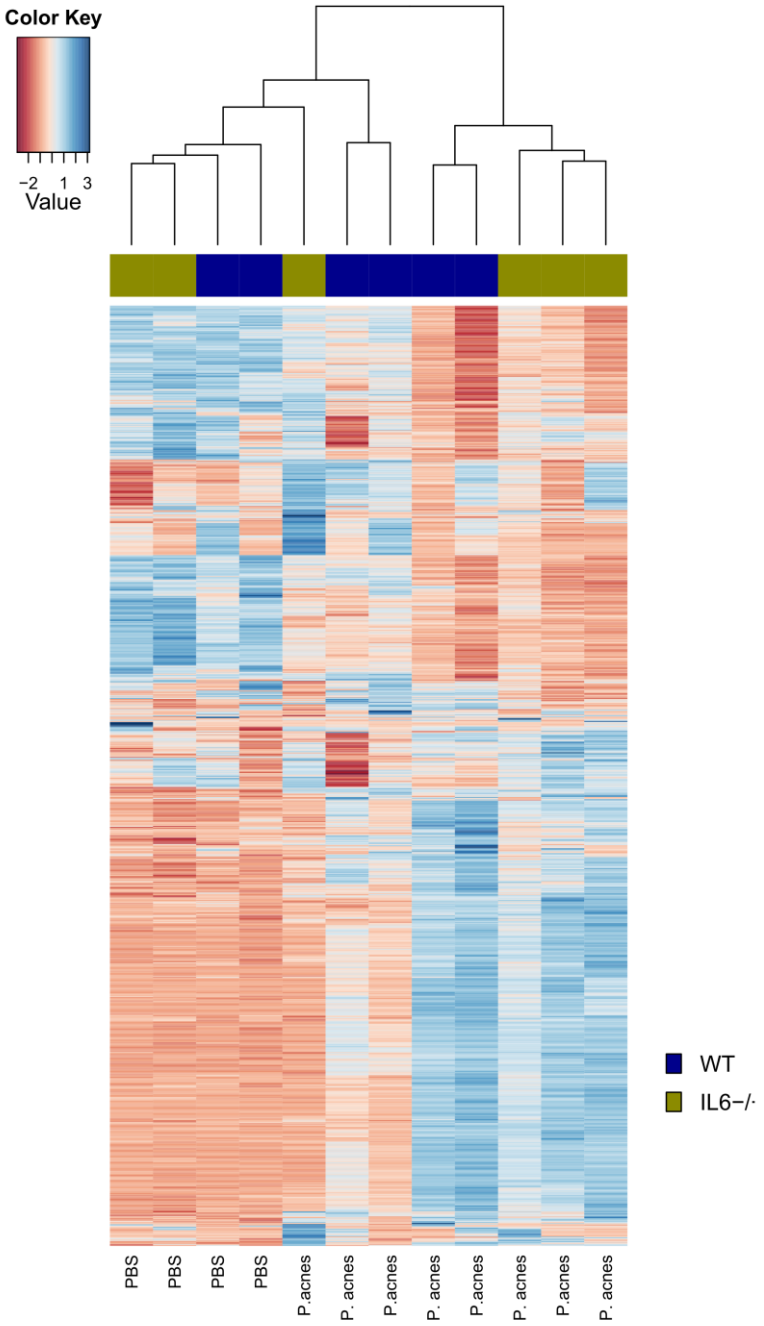

Figure 23. Hierarchical unsupervised clustered heatmap illustrating differentially expressed genes among wildtype and IL-6 -/- mice with different treatments.

Top 2500 genes were chosen from each group among total 26,562 probes, including wildtype PBS control, wildtype with PA-2, IL-6 -/- PBS control, and IL-6-/- with PA-2. Down-regulated genes were labeled as red while up-regulated genes were labeled as blue. R and other package software were applied for data analysis.

Lack of long-term chronic inflammation with IL-6 depletion might potentially involve B-cell related genes

In Figure 24, another heatmap was created by analyzing gene expression in C57BL/6J wildtype and IL-6 -/- mice upon PA-2 inoculation. In this preliminary analysis, the top 25 up-upregulated and down-regulated genes based on an adjusted p value of < 0.05 were listed. The data showed B cell related genes, such as immunoglobulin kappa chain complex and immunoglobulin heavy chain complex, had higher expression in PA-2 infected wildtype mice than in IL-6 -/- mice. Interestingly, we did not observe that any T-cell related genes that had differential expression in this list. IL-6 may act as a key factor in the transition from acute to chronic inflammation and to promote B cell differentiation [52, 91, 141]. Along this line, based on our microarray data, it might indicate that IL-6 might be involved in the recruitment of B cells instead of T cells in long-term chronic inflammation. This hypothesis would need to be followed up by, for example, staining for B cells by IHC and doing a quantitative analysis in the FFPE tissues from the PA-2 treated animals.

In addition, we also noticed that one IL-18 related gene (erythroid differentiation regulator 1) had higher expression in wildtype mice. For IL-6 knockout animals, GSTP1, HSP90AB1, and some degradation related genes (lysozyme 1 and lipolysis stimulated lipoprotein receptor) were found to have higher expression levels compared to wildtype animals.

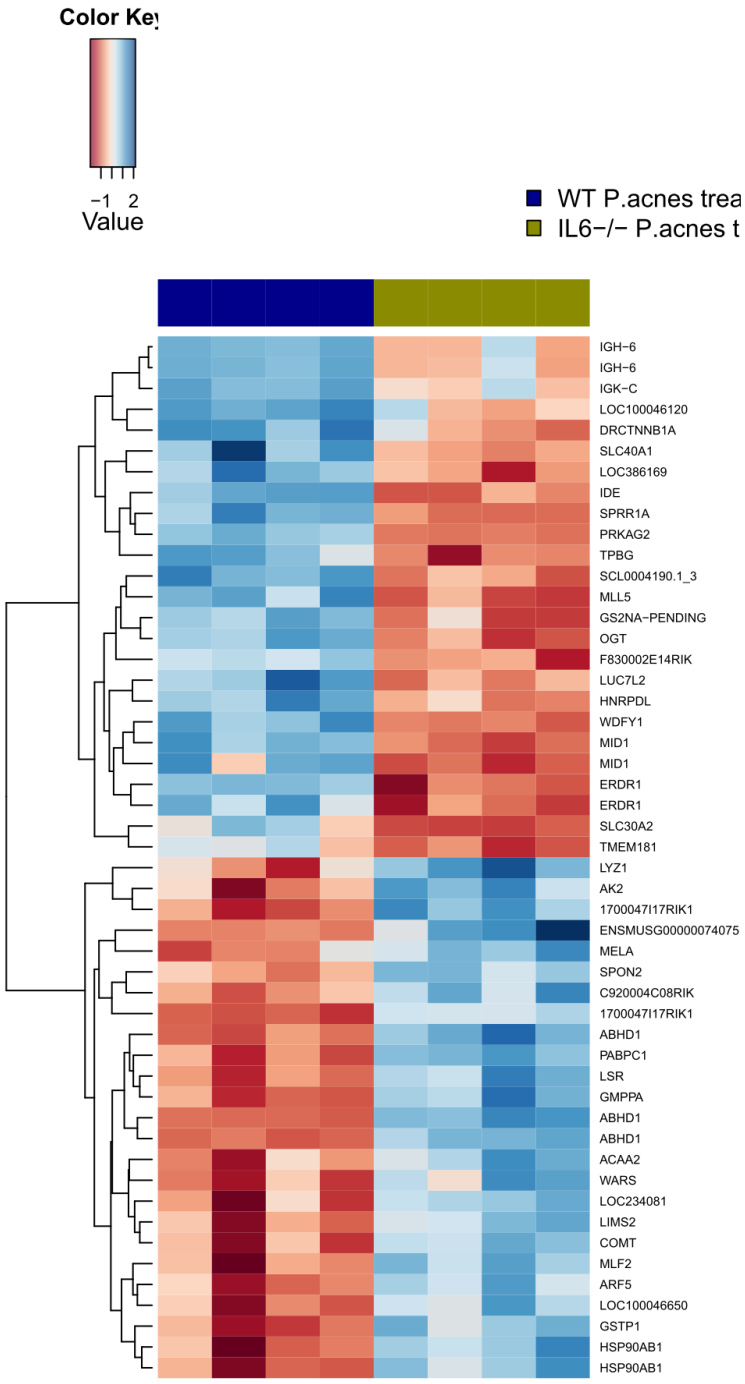

Figure 24. Heatmap of upregulated and downregulated genes in PA-2 treated C57BL/6J wildtype versus IL-6 -/- mice.

Top 250 up-regulated and down-regulated genes were selected to perform this heatmap. Down-regulated genes were labeled as red while up-regulated genes were labeled as blue.

5.5 Conclusions

The potential function of IL-6: to sustain long-term prostatic chronic inflammation

IL-6 is produced at inflammation sites, and has been considered as an acute and chronic inflammation-related cytokine. In prostate cancer, IL-6 is also important since serum IL-6 levels are related to metastatic or castration-resistant prostate cancer (CRPC). IL-6 may also be involved in metastatic progression by regulating epithelial mesenchymal transition (EMT) [52]. IL-6 together with IL-1 and tumor necrosis factor α function as important inflammatory mediators. IL-6 in combination with its soluble receptor sIL-6R has been indicated in regulation through JAK-STAT, ERK-MAPK, and PISK-Akt signaling pathways to produce acute phase proteins, and to promote leucocyte infiltration, which is a key step in the transition from acute to chronic inflammation [91].

IL-6 deficient mice have been used to determine the role of IL-6 in response to infection and/or inflammation [182, 183]. LPS and sterile turpentine were used to mimic systemic inflammation and local inflammation (subcutaneous injection) [183]. Interestingly, in this study, it was found that IL-6 was not required to induce inflammation in response to LPS. In addition, in IL-6 -/- mice, three times higher TNF-α levels were detected, which indicated TNF-α may have functional redundancy to compensate for IL-6 depletion [183]. Another parallel study utilized vaccinia virus and *Listeria monocytogenes* to infect animals. IL-6 -/- mice failed to control bacterial infection, and the T-cell-dependent antibody response also failed to clear vesicular stomatitis virus infection [182].

However, how IL-6 depletion would affect prostate cancer-related inflammation

remains unclear. In our study, a novel bacterial-induced chronic prostatitis animal model was developed to study the potential role of IL-6 in prostatic infection / chronic inflammation. As shown in Figure 21F, initially IL-6 -/- animals develop acute inflammation at 1 week and 2 weeks post-inoculation with PA-2 *P. acnes*. In addition, chronic inflammation became dominant at 2 weeks post-inoculation in IL-6 -/- mice. In fact, 100% of mice developed chronic inflammation at this time point, with 80% of animals with severe prostatic inflammation and 20% of mice with moderate inflammation (Figure 22). Interestingly, at 8 weeks-post inoculation, only mild chronic inflammation was observed (Figure 21G). At 6 month and a year time points, no more prostatic chronic inflammation existed, which may indicate that in response to PA-2 induced inflammation, IL-6 may serve to sustain chronic inflammation rather than in the induction and initiation of acute inflammation.

Impaired long-term prostatic chronic inflammation may be related to B cell dysfunctions

As our studies indicate that IL-6 may be required to sustain bacterial induced chronic prostatitis, determining how IL-6 is affecting chronic inflammatory cells is a logical next step. IL-6 has been considered as one of the key factors in the transition from acute inflammation into chronic inflammation [91]. Previous research has indicated that IL-6 can affect B cells in profound ways, such as regulating B cell differentiation, promoting plasma cell differentiation, and increasing antibody production [52, 141]. In fact, when performing B-cell depletion by the monoclonal antibody rituximab in 9 patients with Systemic Scleroderma symptoms, their serum IL-6 concentration was decreased [184]. This early evidence may suggest a relationship between IL-6 and B cells. In fact, preliminary analysis of a gene

expression microarray performed on PA-2 treated wildtype versus IL-6-/- mice (Figure 24), indicated B cells associated genes (immunoglobulin kappa /heavy chain complex) but not any T-cell regulated genes were up-regulated only in the wildtype group, which suggests that IL-6 might be involved in B cell regulation in this mouse prostatitis model.

IL-6 also has been implicated as a crucial neutrophil regulator. Upon stimulation by orchestrating chemokines and / or by leukocyte apoptosis, IL-6 can activate the STAT-3 pathway and trigger neutrophils trafficking [185]. Interestingly, when we compared the microarray data of wildtype and IL-6 -/- animals with PA-2 inoculation, the wildtype group had higher expression of leukocyte migration related genes, such as extracellular matrix, integrin-linked kinase, and B-cadherin genes (Figure 24), which indicated a possible role for IL-6 in bacterial induced chronic inflammation via regulating leukocyte migration to inflamed areas.

Moreover, Figure 24 also indicates that the IL-6-/- mice had more GSTP1 and HSP90 gene expressions after PA-2 inoculation compared to wildtype mice. As a suspected tumor suppressor gene, GSTP1 promoter methylation is considered as a prognostic marker of prostate cancer [186].

Taken together, IL-6 might regulate bacteria-induced prostatitis, perhaps by promoting B cell infiltration, and/or by recruiting more leukocyte migration to inflamed areas. This might explain why mice with IL-6 depletion exhibit a lack of long-term chronic inflammation upon bacterial infection.

VI. CONCLUSIONS AND PERSPECTIVES

Elucidating the roles of IL-6 in prostate cancer development in both human and animal models

Interleukin 6 (IL-6) is a pleiotropic cytokine that is produced by an array of cell types and exerts diverse physiological effects including immune responses, hematopoiesis, and cellular proliferation and differentiation. For example, IL-6 production primarily by macrophages and monocytes is essential to the induction of acute phase proteins during acute inflammatory responses to infections [87, 88]. As such, the presence of IL-6 in tissues is tightly under control; however, unrestrained production of IL-6 drives chronic inflammation that is associated with diseases such as autoimmune disorders, arthritis, hepatitis, inflammatory bowel disease, pancreatitis, and cancer [113].

IL-6 signals through two receptor (membrane-bound and soluble) pathways via a common receptor and signal transducer: gp130. In the classical signaling pathway, IL-6 ligand targets the IL-6 membrane bound form of the receptor (mbIL-6R) to recruit downstream elements. In the trans-signaling pathway, IL-6 activates the targets cells by binding with soluble form (sIL-6R) [141]. IL-6 can act as both a pro-inflammatory and anti-inflammatory cytokine. It has been proposed that when the classic IL-6 signaling pathway is turned on, anti-inflammatory responses will be activated in target cells. On the other hand, the trans-signaling pathway can trigger target cells to develop pro-inflammatory activities [141].

In different epithelial cancers such as lung, breast, hepatocellular, and colorectal, studies have reported that IL-6 widely functions as an autocrine cytokine [16]. For

example, in colon cancer, IL-6 has been reported to promote T-cell adherence on endothelial cells by increasing its adhesion molecule expression [187, 188]. In malignant ovarian cancer, endothelial cells also require IL-6 to reduce chemotherapy induced apoptosis and promote cellular survival [141, 189]. In prostate cancer, cell line models showed that IL-6 can transform cells in an AR ligand-independent pathway [190]. In addition, patients with advanced prostate cancer and/or metastatic or hormone refractory prostate cancer have been shown to have elevated IL-6 in their serum compared to control groups [98].

Paracrine role of IL-6 in prostate cancer development

Additional studies have suggested that IL-6 acts as a paracrine cytokine in prostate cancer progression. In the stromal compartment, IL-6 along with OSM can lead to cell-autonomous oncogenic events to promote aggressive prostate cancer [120]. Moreover, stromal cells in the bone metastatic tumor microenvironment have also been suggested to express IL-6 [17].

In chapter 2 of this thesis, early evidence from IL-6 qPCR analysis showed only 3 of 10 prostatectomy samples had more IL-6 expression in adenocarcinoma tissues compared to match benign tissues. We went on to conduct a comprehensive analysis of *in situ* IL-6 mRNA expression in 21 primary prostatectomy FFPE tissues, and 32 metastatic tumor samples. Using a positively controlled assay and with IL-6 CISH we did not observe any positive IL-6 mRNA expression from adenocarcinoma cells in any of the primary cases examined regardless of Gleason scores. Instead, IL-6 expression was restricted to areas with scattered peri-tumoral inflammatory cells and/or the stromal compartment of the tumor, which may include tumor-infiltrating inflammatory cells and tumor-associated endothelial cells.

Moreover, when we examined metastatic prostate cancer biopsy or autopsy samples (including lung, liver, lymph node, and bone), consistent with the primary prostate cancer data, no IL-6 mRNA positive expression was observed in metastatic cancer cells in any tissues. Interestingly, IL-6 mRNA expressing cells could be identified as endothelial cells lining small blood vessels surrounding metastatic tumor in 9 of 21 bone metastases (42.9%) but not in any other metastases types. As previously mentioned, serum IL-6 levels have been associated with metastatic or hormone refractory prostate cancer [98, 99, 114]. In this study, we conclude that circulating IL-6 is likely not produced by metastatic tumor cells, but may come from tumor vasculature.

Finally, we went to great lengths to demonstrate that unless we first treated with Golgi inhibitor to block IL-6 secretion, we were not able to detect IL-6 protein by IHC or western blot in cell lines. Likewise, when we applied IL-6 IHC in the primary and metastatic prostate cancer samples, no IL-6 protein could be detected. We conclude that chromogenic *in situ* hybridization (CISH) is a better way to detect IL-6 (and presumably other cytokines) in prostate and other tissues and that IHC can be a problematic method for detecting secreted proteins like IL-6 [111].

Pro-tumorigenic role of IL-6 in prostate cancer development

IL-6 is thought to be a critical mediator that may promote prostate cancer development in several ways, such as initiating prostate tumorigenesis, modulating tumor growth, promoting aggressive disease, and supporting tumor metastasis [136]. Emerging evidence indicated that IL-6 and its major effector STAT3 may act in a protumorigenic role to facilitate prostate cancer growth via trans-activating androgen receptor in prostate cancer cells [17, 137-140]. In chapter 3, we report on a series of

allograft studies aimed to determine if IL-6 may be critical to facilitate prostate cancer growth.

Three mouse cancer cell lines were incorporated in allograft studies. Two prostate cancer cell lines (TRAMP-C2 and 100RC2A) and one colon cancer line (MC38) were injected into C57BL/6J wildtype and IL-6 -/- mice. TRAMP-C2 allografts showed a reduction in tumor growth and lower tumor take rates with IL-6 depletion; while in MC38 allografts, tumors grew in an IL-6 independent manner. Further efforts will be needed to examine other C57BL/6J-derived cancer cell lines, like the breast cancer cell line EO771 and additional prostate lines, to determine if the reduced tumor growth in IL-6-/- mice is specific to prostate tumor cells.

Interestingly, in our TRAMP-C2 model, the wildtype mice that grew allografted tumors had elevated serum IL-6 levels. This result is consistent with human studies, which suggests this TRAMP-C2 allograft model might be valuable to study a pro-tumorigenic role for systemic levels of IL-6 [15, 16, 142].

Finally, we aimed to determine possible downstream elements involved in IL-6 signaling to facilitate prostate tumor growth. Phospho-STAT3 (p-STAT3) was our first target, since p-STAT3 has been considered as one of the most important IL-6 activating factors in prostate cancer. Surprisingly, p-STAT3 IHC showed very subtle differences between wildtype and IL-6 depleted animals. In future studies we aim to test other possible molecules, like IGF-1R, ErbB2, phospho-Akt, phosphor-ERK1/2, and phospho-Stat1. In fact, IGF-1R [191] and ErbB2 [192] have been suggested as possible IL-6 activating molecules. For example, IL-6 could trigger prostate tumorigenesis through IGF-1R [191].

Propionibacterium acnes (_P. acnes_) may act as an initial inciting factor to trigger prostate cancer-related inflammation

Several factors have been suggested to initiate prostate inflammation such as urine reflux, hormonal changes, dietary factors, estrogens, corpora amylacea and pathogen infections [46]. Yet, the initial inciting factors to trigger prostate cancer-related inflammation remain unclear. In chapter 4, we studied a gram-positive bacterium *Propionibacterium acnes (P. acnes)*, which is known as an ubiquitous human skin bacterium, and could be the initial factor to induce prostatitis.

P. acnes can be ubiquitously found on human skin and is associated with the skin disease acne vulgaris [55] [72], endocarditis, sarcoidosis, post-surgical infections, and other inflammation related diseases [73]. In our study, we isolated eight *P. acnes* strains from 30 radical prostatectomy tissues. This result corresponded to the first reported which claimed *P. acnes* could be cultured from 35% of radical prostatectomy tissues [74]. Further, by performing MLST typing to cluster prostate derived *P. acnes*, it suggested that prostate-derived *P. acnes* were more associated with opportunistic infections and/or urethral flora and do not fall within typical skin/acne strains.

Future efforts of this project will be to further elucidate a causal role for *P. acnes* in acute or chronic prostatitis [72]. As such, we have designed a *P. acnes*-specific 16s rRNA probe for CISH. We aim to apply this CISH assay to prostatectomy specimens with varying degrees of acute and chronic inflammation to determine if we can detect *P. acnes* in association with these or other prostate lesions (PIA, PIN, cancer, etc.).

Role of IL-6 in bacteria-induced chronic prostatitis

IL-6 also plays important physiological roles at mediating the transition from to

chronic inflammation by regulating T-cell and B-cell differentiation [52, 141]. As both an anti-inflammatory and pro-inflammatory cytokine, IL-6 may also be involved in prostate inflammation regulation.

Our group previously developed a model of long term prostatic inflammation using a prostate-derived strain of *P. acnes* (PA-2) inoculated into C57BL/6J wildtype mice [179]. Wildtype mice develop primarily acute inflammation at 1 week post-inoculation that turns to primarily chronic inflammation that persists at 2 weeks, 8 weeks, 6 months and even at least 1 year post-inoculation. In chapter 5, we used the same strain of *P. acnes* to infect the prostate of IL-6 -/- mice. Interestingly, with IL-6 depletion, mice did not sustain any chronic inflammation after a 2 month time point.

Dorsal prostates from treated wildtype and IL-6 -/- mice were collected for microarray at 2 weeks post-inoculation. Preliminary microarray results suggested that PA-2 treated wildtype animals had higher B-cell associated gene expression than IL-6 -/- mice (Figure 24). In addition, functional annotation analysis suggested that leukocyte trans-endothelial migration and local adhesion related genes had higher expression in wildtype compared to IL-6 -/- mice.

IL-6 might be critical for maintaining B cell functions in bacterial induced chronic prostatitis

IL-6 has been suggested to play an essential role in the B cell differentiation by turning B cells into Ig-secreting cells [52, 141]. When analyzing the top 25 up-regulated and down-regulated genes in PA2 treated mice by microarray, we found B-cell-associated genes (immunoglobulin kappa /heavy chain complex) but not any T-cell regulated genes were up-regulated only in the PA-2 treated wildtype group (Figure 24). These preliminary data raise the intriguing hypothesis that that IL-6

might be involved in B cell regulation in this mouse prostatitis model. Future studies will be needed to verify the findings and to test this hypothesis.

IL-6 was initially introduced as human B-cell differentiation factor (BCDF) or B cell stimulation factor 2 (BSF-2), which can trigger B cell maturation to have more immunoglobulin secretion [193]. IL-6 has also been reported to play a role in B cell proliferation and isotype switching [194]. Unstrained IL-6 can trigger B-cell elevation and cause autoimmune, mucosal inflammation and other inflammation-related diseases [194, 195]. On the other hand, IL-6 -/- mice have been shown to fail to develop autoimmune encephalomyelitis [194]. IL-6 -/- cannot support proper lymphocyte differentiation and proliferation under myelin oligodendrocyte glycoprotein treatment, thus resulting central nervous system demyelination [194, 196, 197]. Similarly, in our study we also found IL-6-/- animals failed to sustain long-term chronic prostatic inflammation after bacterial infection.

Taken together, we suggest that IL-6 may play an important role in bacteria-induced prostatitis, perhaps by regulation of B cells, and that that IL6-/- mice have a worse ability to recruit leukocytes via transendothelial migration, and/or with lower efficiency of recruiting certain types leukocytes to inflamed areas. This could explain the lack of long-term chronic inflammation in our PA2 treated IL-6 -/- model.

The problem of choice: current challenge and future prospects of IL-6 antagonist therapy

As we have mentioned, IL-6 may act as a key mediator in several steps in prostate carcinogenesis, which makes it as a good therapeutic target. Current anti-interleukin-6 agents have been widely used to treat many diseases successfully, including: B-lymphoproliferative disorder, lymphoma, plasma cell leukemia,

myeloma refractory cutaneous lupus and urticarial vasculitis, and rheumatoid arthritis [52, 151-158, 198]. The very first approved antibody is an anti-IL-6R antibody called tocilizumab or Actemra [159, 160]. Later on, a chimerised monoclonal antibody CNTO 328 (Siltuximab) was developed, which acts by directly binding with IL-6. CNTO 328 can interfere the binding of IL-6 to the IL-6-receptor [157]. More recently, a soluble form IL-6R neutralizing monoclonal antibody was developed, which can block the sIL-6R pathway to inhibit local inflammatory responses [199].

Even though IL-6 antagonists have been reported to improve some diseases [52, 151-158], in prostate cancer the effects of IL-6 antagonists still remain unclear. For example, studies showed CNTO328 treatment can increase apoptosis and decrease pStat3 expression, yet no significant outcome has been observed in advanced prostate cancer clinical studies [143, 161-163, 198]. Based on these studies, it has been suggested that chemotherapy might be required in combination with IL-6 antagonist treatment to get better prostate cancer therapy efficiencies. [198]

In conclusion, in our studies, we have found a lack of IL-6 mRNA expression by primary and metastatic prostate cancer cells, and this may help to explain why minimal to no clinical activity has been observed when a monoclonal antibody therapy targeting IL-6 (siltuximab) has been tested in clinical trials thus far [132, 133]. In addition, current IL-6 antagonist studies are based on theory that IL-6 signaling has a role in metastatic disease, but does not address a potential pro-tumorigenic role for IL-6 in facilitating cancer progression. Patients are treated with IL-antagonists only after diagnosis with advanced prostate cancer, rather than treating with IL-6 antagonists earlier in the carcinogenic process. This may explain why the effect of the IL-6 antagonist therapy in prostate cancer treatment is still questionable.

Summary

In this thesis, we examined several possible roles for IL-6 in prostate cancer including whether it function in an autocrine or paracrine manner in primary and metastatic cancer, its role in facilitating tumor growth, and a potential role in sustaining long-term chronic inflammation. As such, in chapter 2 we determined that paracrine rather than autocrine IL-6 signaling must account for any role that IL-6 plays in both primary and metastatic prostate cancer. In addition, allograft studies also suggested the pro-tumorigenic role of IL-6 in facilitating prostate cancer growth (chapter 3). Moreover, by MLST analyses, we were able to cluster 8 prostate-derived *P. acnes* isolates and identify these isolates are more associated with opportunistic infections and/or urethral flora instead of being skin flora contamination by their ST characteristics (chapter 4). Finally, we utilized a clinically relevant human-derived strain of *P. acnes* to induce chronic prostatitis in IL-6 -/- mice. Unlike wildtype mice that develop chronic inflammation that persists up to a year post-inoculation, mice with IL-6 depletion did not sustain chronic inflammation beyond a 2 month time point. Our microarray data further indicated that IL-6 may play an important role in bacteria-induced prostatitis by influencing B cell differentiation and by facilitating leukocytes migration to inflamed areas. In summary, in this thesis we elucidated possible roles of IL-6 in prostate cancer development and progression.

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trial of CNTO328 (siltuximab), a monoclonal antibody against Interleukin-6, in chemotherapy-pretreated patients with castration-resistant prostate cancer. Clinical Cancer Research, 2010. **16**(11): p. 3028-3034.

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VII. CURRICULUM VITAE

SHU-HAN, YU

Curriculum Vitae
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EDUCATION

2003-2007	**Bachelor of Science**, Department of Plant Pathology and Microbiology, College of Bioresources and Agriculture, National Taiwan University (**GPA**: 3.99/4.00**, Ranking**: 1/27 (top 2%)
2007-2009	**Master of Science**, Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University
2010-2015	**Doctor of Philosophy**, Pathobiology Graduate Program, School of Medicine, Johns Hopkins University Mentor: Dr. Karen Sfanos, Dr. Angelo De Marzo

RESEARCH EXPERIENCE

2004-2006	National Science Council Undergraduate Research program: Identification and selection of arsenic resistant bacteria from the groundwater of the blackfoot disease area in the Chia-Nan plain, southwestern Taiwan.
2007-2009	Protein Analysis Lab: Function and subcellular localization change of phosphoproteins induced by *Helicobacter pylori* infection.
2009-2010	Research Assistant: Assisting LC-MS/MS
2010-present	Molecular Pathobiology of Prostate Cancer Lab: Studies on the role of Interleukin-6 in prostate cancer development and in the induction of chronic prostatic inflammation by *Propionibacterium acnes*.

RESEARCH INTERESTS

Role of IL-6 in Prostate Cancer

To study the possible role of an inflammatory cytokine interleukin-6 (IL-6) in prostate development, we analyzed human FFPE prostate tumor samples by on chromogenic *in situ* hybridization (CISH) assay. Interestingly, both primary and metastatic prostate adenocarcinoma cells did not express IL-6 mRNA which indicated paracrine rather than autocrine IL-6 production is likely associated with any role for the cytokine in prostate cancer progression.

In addition, initial evidence also suggested that IL-6 may be involved in early prostate tumor development by conducting serial allograft studies to C57BL/6J wildtype and IL-6 knockout (IL6-/-) mice. Compared to wildtype, IL6-/- mice had significant reduction take rate and growth rate in prostate cell TRAMP-C2 allograft. This trend was not observed for the colon MC38 cell line, indicating tumor-type specificity may be applied to the phenomenon. Interestingly, IL-6 ELISA analyses showed a significant increase in the circulating IL-6 in wildtype mice with TRAMP-C2 tumors.

Prostate Infectious Agents

In our studies, we have shown that the pro-inflammatory anaerobe *Propionibacterium acnes* (*P. acnes)* can be cultured from radical prostatectomy tissues. Cultured *P. acnes* were identified by 16S rDNA sequencing followed by multilocus sequence typing (MLST). MLST analyses identified 8 different sequence types (STs) among prostate-derived *P. acnes* isolates which suggested that prostatectomy-derived *P. acnes* isolates do not fall within the typical skin/acne STs, but rather are characteristic of STs associated with opportunistic infections and/or urethral flora.

RELATED TECHNIQUES

Prostate cancer inflammation

PCR, Bacterial 16s rRNA Sequencing, Multilocus Sequencing Typing (MLST), Inoculation of Bacteria to Mouse Prostate via Transurethral Catheterization, Hematoxylin and Eosin Staining, Plasmid Construction,

	RNA preparation / Microarray
Role of IL-6 in Prostate Cancer	Cell Culture, Transfection, Immunohistochemistry (IHC), In-situ hybridization (CISH), Allograft Animal Studies (subcutaneous injection), ELISA, Animal Breeding, Western Blot, qPCR,
Prostate Infectious Agents	Purification and Amplification Microbial Genomic DNA from Human Prostatectomy Samples, Sample Preparation for Illumina HiSeq2000 sequencing machine, Metagenomics
Others	Confocal Microscopy (Leica LCS5), Immunofluorescence, Site-Directed Mutagenesis (by DpnI), Two Dimensional Electrophoresis (2D), Cell Fraction (membrane, cytoplasm, nucleus), Large Scale Plasmid Purification, Flow Cytometry, Hypoxia Chamber Operation, Apoptosis assay (TUNEL)

HONORS and AWARDS

2013/04 **Poster Award, 2rd place**

- Graduate Student Association 2013 Poster Section, Group A (2^{nd} to 4^{th} years), School of Medicine, Johns Hopkins University

2013/03 **Poster Award, 3rd place**

- 2013 Prostate Research Day, Johns Hopkins University

2012/05 **Excellent in Translational Research, Young Investigator Award**

- 14th Annual Department of Pathology Young Investigators' Day, School of Medicine, Johns Hopkins University

2012/03 **Poster Award, 2nd place**

- Graduate Student Association 2012 Poster Section, Group A (2nd to 4th years), School of Medicine, Johns Hopkins University

2010-2012 **Government Scholarship, Ministry of Education, Taiwan**

- Awarded by the Ministry of Education, ROC

2010/07 **Young Scientist Award**

- 17th East Asia Joint on Biomedical Research conference

2006/10	**Certification of Dean's List Award, National Taiwan University** - Top 10 students selected from those who received the "Presidential Award"
2004-2007	**Presidential Awards, Department of Plant Pathology and Microbiology, NTU** - Top 5% of students: Mar 2007, Oct 2006, Mar 2006, Oct 2005, Mar 2005, Oct 2004, Mar 2004
2005-2006	**Undergraduate Research Fellowship, Republic of China National Science Council**

PUBLICATIONS

Peer Review Publication

Yu SH, Zheng Q, Esopi D, Macgregor-Das A, Luo J, Antonarakis SE, Drake CG, Vessella R, Morrissey C, De Marzo AM, Sfanos KS. A Paracrine Role for IL-6 in Prostate Cancer Patients: Lack of Production by Primary or Metastatic Tumor Cells. Cancer Immunol Res. 2015. (In revision)

Sfanos KS, Canene-Adams K, Hempel H, Yu SH, Simons B, Schaeffer A, Schaeffer E, Nelson G, De Marzo A. Bacterial Prostatitis Enhances 2-amino-1-methyl-6-phenylimidazo[4,5-β]pyridine (PhIP)-Induced Cancer at Multiple Sites. Cancer Prev. Res. 2015 (Submitted)

Yu SH, Zheng Q, Yegnasubramanian S, De Marzo AM, Sfanos KS. Infectious Agents, Inflammation and Aggressive Prostate Cancer. 2015. (In preparation)

Yu SH , Vaghasia AM , Drake CG, De Marzo AM, Sfanos KS. A Potential Pro-tumorigenic Role for Interleukin-6 in Prostate Cancer Development. 2015. (In preparation)

Le A, Stine ZE, Nguyen C, Afzal J, Sun P, Hamaker M, Siegel NM, Gouw A, Kang BK, Yu SH, Cochran RL, Sailor KA, Song H, Dang CV. Tumorigenicity of hypoxic respiring cancer cells revealed by a hypoxia-cell cycle dual reporter. PNAS. 2014. 111:12486-12491.

Shinohara DB, Vaghasia AM, Yu SH, Mak TN, Brüggemann H, Nelson WG, De Marzo AM, Yegnasubramanian S, Sfanos KS. A mouse model of chronic prostatic inflammation using a human prostate cancer-derived isolate of Propionibacterium acnes. Prostate. 2013. 73: 1007-1015.

Mak TN, Yu SH, De Marzo AM, Brüggemann H, Sfanos KS. Multilocus sequence typing (MLST) analysis of Propionibacterium acnes isolates from radical prostatectomy specimens. Prostate. 2012. 73:770-777. (co-first author)

Thesis and Dissertation

Function and subcellular localization change of phosphoproteins induced by *Helicobacter pylori* infection. 2009. Master Thesis. Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University.

Studies on the Role of Interleukin-6 in Prostate Cancer Development and in the Induction of Chronic Prostatic Inflammation by *Propionibacterium acnes*. 2015. Doctoral dissertation. Pathobiology Program, School of Medicine, Johns Hopkins University.

Conference / Presentations

Yu SH, Zheng Q, Esopi D, Macgregor-Das A, Luo J, Antonarakis SE, Drake CG, Vessella R, Morrissey C, De Marzo AM, Sfanos KS. A Paracrine Role for IL-6 in Prostate Cancer Patients: Lack of Production by Primary or Metastatic Tumor Cells. 2015. Prostate Research Day. Johns Hopkins University.

Yu SH , Vaghasia AM , Drake CG, De Marzo AM, Sfanos KS. A Potential Pro-tumorigenic Role for Interleukin-6 in Prostate Cancer Development. 2015. Prostate Research Day. Johns Hopkins University.

Yu SH, Zheng Q, Luo J, Macgregor-Das A, Antonarakis E, De Marzo AM, Sfanos KS. Interleukin-6 expression is restricted to the prostate stromal compartment and is not expressed by either primary or metastatic prostatic adenocarcinoma cells. 105th American Association for Cancer Research Annual Meeting. IM01-08 tumor immunology. 2014. San Diego.

Yu SH, et al. Interleukin-6 is not produced by prostate adenocarcinoma cells in primary tumors and is restricted to prostate-infiltrating immune cells and endothelium. 16th Annual Department of Pathology Young Investigators' Day. 2014. School of Medicine, Johns Hopkins University.

Yu SH, et al. Interleukin-6 is not produced by prostate adenocarcinoma cells in primary tumors and is restricted to prostate-infiltrating immune cells and endothelium. 15th Annual Department of Pathology Young Investigators' Day. 2013. School of Medicine, Johns Hopkins University.

Yu SH et al. Multilocus sequence typing (MLST) analysis of Propionibacterium acnes isolates from radical prostatectomy specimens.Graduate Student Association. 2012. Poster Section, Group A (2nd to 4th years), School of Medicine, Johns Hopkins University.

Yu SH, Mak TN, Brüggemann H, De Marzo AM, Sfanos KS. Prostate Cancer and Inflammation: A Potential Role for *Propionibacterium acnes*. 14th Annual Department of Pathology Young Investigators' Day. 2012. School of Medicine, Johns Hopkins University.

Yu SH, Yang LC, Chow LP. Phosphoproteomics approach to analyze subcellular localization change of phosphoproteins induced by *Helicobacter pylori*. 17th East Asia Joint on Biomedical Research conference. 2010. Taipei, Taiwan.

GRANT / PROPOSAL WRITING

2012 Howard Hughes Medical Institute International Student Research Fellowships, for 2013 JOHNS HOPKINS UNIVERSITY Internal Application

INVITED TALKS

2010, July	Yu SH, Yang LC, Chow LP. Phosphoproteomics approach to analyze subcellular localization change of phosphoproteins induced by *Helicobacter pylori*. 17th East Asia Joint on Biomedical Research conference.
2012, March	Yu SH, Mak TN, Brüggemann H, De Marzo AM, Sfanos KS. Prostate Cancer and Inflammation: A Potential Role for *Propionibacterium acnes*. 5th Annual Multi-Institutional Prostate Cancer Program Meeting, Ft. Lauderdale.
2015, Feb	Yu SH. The role of Interleukin-6 in prostate cancer. Pathobiology Graduate Program Recruit, Student Presentation. School of Medicine, Johns Hopkins University.

LEADERSHIP AND TEACHING EXPERIENCES

2011-2012	Student Representative of Taiwanese Student Association, Johns Hopkins University
2011-2013	TA for "*Medical Mandarin classes*": Moderated JHU medical student discussion groups in the course, and graded homework and exam.
2013	TA for "*Basic Mechanism of Disease, ME300.713*": Scheduled professors for lecture, created exam questions, and moderated and answering questions for JHU graduate student discussion groups in the course.

MEETINGS (only lists recently attended meetings)

2012/02	Johns Hopkins Prostate Cancer Day, Baltimore
2012/03	Multi-institute Prostate Program Meeting, Florida
2012/04	American Association for Cancer Research, Chicago
2013/03	Johns Hopkin Prostate Cancer Day, Baltimore
2013/04	American Association for Cancer Research, Washington DC
2014/02	Johns Hopkin Prostate Cancer Day, Baltimore
2014/04	American Association for Cancer Research, San Diego

2015/02 Johns Hopkin Prostate Cancer Day, Baltimore

MEMBERSHIP IN PROFESSIONAL SOCIETIES

2011-present American Association for Cancer Research

2013-present Women in Cancer Research, American Association for Cancer Research