INHIBITION OF UROTHELIAL CARCINOMA BY TYPE I INTERFERON ACTIVATION OF THE INNATE AND ADAPTIVE IMMUNE RESPONSE

Devin Plote

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INHIBITION OF UROTHELIAL CARCINOMA BY TYPE I INTERFERON
ACTIVATION OF THE INNATE AND ADAPTIVE IMMUNE RESPONSE

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INHIBITION OF UROTHELIAL CARCINOMA BY TYPE I INTERFERON
ACTIVATION OF THE INNATE AND ADAPTIVE IMMUNE RESPONSE

A DISSERTATION

Presented to the Faculty of
The University of Texas MD Anderson Cancer Center
UTHHealth Graduate School of Biomedical Sciences
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Requirements for the Degree of
DOCTOR OF PHILOSOPHY

By

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August, 2019
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INHIBITION OF UROTHELIAL CARCINOMA BY TYPE I INTERFERON ACTIVATION OF THE INNATE AND ADAPTIVE IMMUNE RESPONSE

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Bacillus Calmette-Guerin (BCG) is the first line therapy for bladder cancer patients with non-invasive disease. However, roughly 40% of patients exhibit no response, tumor recurrence or tumor progression following BCG treatment. Type I interferon (IFN-I) has potent anti-tumor effects against urothelial carcinoma (UC) and may be an alternative treatment option for patients who do not respond to BCG standard of care. However, the mechanisms that mediate the IFN-I-stimulated immune responses against UC are not fully elucidated. Herein, we evaluated the anti-tumor mechanisms of IFN-I in UC by use of adenoviral interferon-α (Ad-IFNα/Syn3) in human patients, and poly(I:C) or lentiviral IFNα (LV-IFNα) in mice. To this end, I evaluated the IFN-I enhanced immune response by observing increases in expression of immune cell and checkpoint markers in tumors pre- and post- IFN-I treatment. I also characterized the tumor-immune landscape, identified important antitumor effector cells, and described the pathways elicited to recruit the immune response. I found that IFN-I increased the intratumoral levels of Ly6G cells, CD8 T cells, and NK cells, and that the anti-tumor benefit of IFN-I was dependent on IL-6 signaling and multiple immune cell types. I sought to establish therapeutic synergy between IFN-I therapy and PD-1 pathway checkpoint inhibition, and found combination therapy increases survival but is not wholly synergistic, and has additional effect in increasing the immune infiltrate, angiogenesis, and enriching gene signatures of metabolism, extracellular matrix organization, and MAPK/AKT signaling. Altogether, these studies highlight the importance of targeting multiple aspects of the immune response against tumors, and provide a preclinical conceptual example for using type I IFN activation to increase the therapeutic benefit of PD-1 blockade for bladder cancer patients.
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Chapter 1: Introduction

The term “cancer” was first described by Hippocrates in the late centuries Before Common Era, but incidences of cancerous lesions have been documented back to the Ancient Egyptians. Hailing to the treatment practices of those archaic times, surgical intervention and removal of tumors has been the ultimate method of therapy for cancer since 1500 B.C.E. (1). The surgery itself could be dramatically aggressive, resulting in loss of life, low life expectancy, and low quality of life, if preserved. Not until the early 20th century did other treatment modalities such as radiation and hormone therapy begin to enter the medical field, with indications for the treatment of cancers, particularly for tumors incapable of being safely resected by surgery. The premise of chemotherapy was introduced by coincidence during World War II, a by-product of defense research to protect soldiers against mustard gas. Since its discovery, chemotherapy has been catapulted into the front line of cancer treatments; agents which first began as general DNA damagers have evolved into pathway- and gene-specific targeted drugs that can be used alone, in combination with other chemotherapy agents, or used in an adjuvant capacity in combination with other therapy modalities. The most recent addition to the arsenal of cancer therapeutics has been the rapidly expanding field of immunotherapy. With the goal of capitalizing on the body’s own defense system, immunotherapy has been highlighted as the future of cancer therapy, meant to succeed where most other modalities have failed in treatment and prevention of recurrence. In the following chapter, I aim to provide an overview of the current field of bladder cancer and its diagnosis and treatment, our immune system and how it recognizes and respond
to tumors, with attention paid to mechanisms of tumor-immune suppression, and finally a discussion on the immunotherapies used to overcome tumor-immunosuppression and treat cancer, focusing on the current status of therapies in all tumors and specifically in bladder cancer.

1.1: Bladder Cancer

Bladder cancer is the second most widely prevalent cancer type of the genitourinary malignancies, ranking as the tenth most common malignant disease worldwide (2). Indeed, 80,470 new cases of bladder cancer and 17,670 estimated deaths attributed to bladder cancer were predicted to occur in the U.S. in 2019 (3), making it the fourth most common cancer type in men and the twelfth most common type in females. These estimated incidence and death rates have increased by 4.4% and 7.3%, respectively, since 2016. Bladder cancer affects men more frequently than women at a ratio of about 3:1, and incidences increase with age (2). While genetic predisposition to bladder cancer and other environmental factors related to diet and occupational exposure to chemical and water contaminants have shown to be linked to bladder cancer diagnoses (4), cigarette smoking is the primary risk factor for bladder cancer. Unsurprisingly epidemiologically, regions where smoking rates are highest have the highest rates of bladder cancer occurrence.

Most patients are initially diagnosed due to observance of blood in their urine (hematuria)(5), subsequently confirming the presence of suspected cancerous lesions by cystoscopy, biopsy, and histopathology. As with most cancer types, prognosis for bladder cancer depends on the stage and
histopathology of the disease. Early staged, minimally-invasive tumors generally present with good prognosis following the standard of care of tumor resection, Bacillus Calmette-Guerin (BCG) therapy, and lifelong monitoring (6); patients with late staged, advanced and potentially metastatic disease have little treatment options as alternative to aggressive chemotherapy and cystectomy, or surgical removal of the bladder (7). Despite having a good prognosis, patients still have high rates of disease recurrence and even progression, making bladder cancer one of the most expensive of all cancer types due to the constant surveillance and need for reception procedures. For the more aggressive and muscle-invasive cancers, radical cystectomy, including prostatectomy in men and hysterectomy in women in addition to bladder removal, is the gold-standard of therapy. These procedures result in severely impaired quality of life and an abysmal 10-year survival rate of <34% when disease involves the surrounding lymph nodes (8). With a fairly stable bladder cancer incidence due to the lasting effects of global tobacco usage, and the potential of costly life-long monitoring and lowered quality of life, there is great interest in development of effective alternative treatment options to improve the care and survival of bladder cancer patients.

This section aims to provide a discussion of key differences in bladder cancer phenotypes, Muscle-Invasive bladder cancer (MIBC) and Non-muscle Invasive bladder cancer (NMIBC) with a focus on NMIBC, and provide a summary of the current treatment practices and the utility of biomarkers in early detection and therapy response.
1.1.1: Defining the Characteristics of Muscle-Invasive vs. Non-Muscle Invasive Bladder Cancer Phenotypes

**Pathophysiology.** Most incidences (about 90%) of bladder cancer are classified as urothelial carcinomas, and originate from the epithelial cells lining of the bladder called the urothelium (5). Tumors differ greatly in their histology owing to their diverse cells of origin or to divergence of cell clones (clonality), which results in multifocal disease within the standard two-pathway development model of bladder cancer (papillary, non-invasive lesions or non-papillary, invasive lesions). Bladder cancer is staged according to the classical Tumor, Node, Metastasis (TNM) system in which minimally invasive tumors (stages Tis, Ta, and T1) represent tumors that have remained on the urothelial mucosal surface, potentially beginning to invade the submucosal lamina propria, or carcinoma *in situ* (CIS) (**Figure 1**). These tumors may also characteristically project out into the bladder lumen, becoming papillary in nature. Tumors can additionally be classified as low-grade or high-grade depending on their invasive status into the lamina propria and muscularis propria. Stage T2, T3, and T4 tumors have invaded past the first two tissue layers (urothelium and lamina propria) into the muscular bladder wall and beyond. Early staged tumors (Tis-T1/T2) are typically classified as non-muscle invasive bladder cancers (NMIBC), and late stage tumors (T2-T4) are usually appropriately named muscle-invasive bladder cancers (MIBC) (5, 9). Approximately 75% of new diagnoses of bladder cancer are NMIBC, and the other 25% of cases are MIBC or metastatic disease (4). About 15-40% of high-grade Ta staged NMIBC tumors, and 30-50% of high-grade T1 staged NMIBC tumors progress to highly invasive disease; however only about
20% of initially diagnoses NMIBC patients progress to MIBC with the potential for metastasis (10). Urothelial carcinomas can also be classified by variant

**Figure 1: Stages, grades, and subtypes of bladder cancer.** Staging of bladder cancer according to the TMN system is shown according to the tissue layer of the tumor invasion. Non-muscle invasive (NMIBC) tumors are the least tissue invasive tumors, typically papillary in nature and have lower stages (Tis-T1/T2) and lower graded disease. Muscle-invasive (MIBC) tumors are more aggressive with higher stages (T2-T4) and higher grades, including invasion past the bladder muscular wall and into fat and the bloodstream. Grading schemes at the bottom of the figure reflect World Health Organization (WHO) and International Society of Urological Pathology (ISUP) disease stratification classifications. PUNLMP, papillary urothelial malignancy of low malignant potential. This figure was taken with permission from Sanli, O, Dobruch J, Knowles MA, Burger M, Alemozaffar M, Nielsen ME, Lotan Y. 2017. Bladder Cancer. *Nat Rev Dis Primers* (5). License number 4580430584829.
histologies including micropapillary, sarcomatoid, squamous, and glandular differentiation (11); many of these subtypes are aggressive and thus important to identify in order to avoid misdiagnoses and inappropriate treatment regimes.

**Molecular Characteristics.** In addition to the TNM classification and invasion status, bladder tumors can be subtyped by their molecular landscape and characteristics. In humans and also in mouse models of NMIBC, precursors to tumors present as hyperplastic lesions where the two most common genetic alternations found are loss of heterozygosity of chromosome 9, particularly 9q, and mutations in FGFR3 (12, 13). The deleted areas on chromosome 9 encompass loci for multiple tumor suppressors including CDKN2A, TSC1, and PTCH1, and are often found deleted in other tumor types (14, 15). FGFR3 activating mutations can trigger activating mutations in PIK3CA and downstream cell cycle regulatory pathways such as RAS/MAPK signaling, resulting in increased cell growth and division (15, 16). Whole exome-sequencing of NMIBCs revealed high frequencies of inactivating mutations in chromatin-modifying proteins KDM6A, ARID1A, CREBBP, and EP300, present at significantly higher levels than in other cancer types, signifying a key role for epigenetic regulation in the development of these tumors (17-19). In contrast to NMIBC, flat dysplastic lesions or CIS are precursors to MIBC tumors, with generation of MIBCs in humans and mouse models requiring the inactivation of tumor suppressor genes Trp53, Rb1, and Pten. These alterations affect cell cycle regulation, genetic instability and immortality, and regulation of the PI3K/AKT/mTOR pathway (20), ultimately resulting in increased disease aggressiveness, invasion, and worse clinical outcomes. In addition to the difference in overall patterns of mutated
genes in these two major tumor groups, there is also a significant difference in their mutational burden. According to exome sequencing studies, NMIBCs have showed an average of 169-195 mutations, less than two-thirds of 302 mutations found in MIBCs (17, 18).

**Molecular Subtyping.** Molecular subtyping of bladder cancer goes deeper than just characteristics identifying NMIBC and MIBC. As briefly touched on above, urothelial carcinoma can have differing cells of origin, generating heterogeneous tumors with varying clinical outcome suggesting there can be subtyping within subtypes. Transcriptional profiling has provided the best subtyping definitions, though to date, the major studies performed use different nomenclatures and so bioinformatics tools have been enlisted to find alignment and overlap of these profiles (Figure 2). The initial profiling study performed by Lund encompasses tumors of all grades and stages (NMIBC and MIBC) and defined the following five subtypes: urobasal A, genomically unstable, (immune) infiltrated, squamous cell carcinoma-like, and urobasal B (21). Low-grade Ta tumors were classified as urobasal A, consisting of high levels of markers of urothelial differentiation, cell adhesion, early cell cycle, and FGFR3-related genes. Stage T1 and high-grade tumors contained more genomically unstable and infiltrated tumors, classified by late cell cycle gene expression and markers of urothelial differential, or high levels of immune cell and stromal markers, respectively. These higher-staged NMIBC tumors showed more overlap with subtypes of MIBC, which other independent transcriptional analyses have segregated into two major tumor groups: “luminal” and “basal” (18, 22, 23). The luminal and basal clusters are identified by mutually exclusive expression of differentiation markers that were
Figure 2: Molecular subtyping of bladder cancer. Several subtypes of NMIBC and MIBC have been defined based on transcriptional characteristics. 

**a)** UROMOL classification scheme of NMIBC into three class subtypes: Class 1 is characterized by high expression of early cell cycle and uroplakin genes, which are involved in urothelial differentiation; Class 2 expresses late cell cycle genes and shows increases in keratins as well as uroplakins; Class 3 tumors show high expression of keratins, a sign of undifferentiated (basal) cells, and high levels of long non-coding RNAs. 

**b)** Characterization of bladder cancer subtypes defined by the University of North Carolina (UNC), MD Anderson Cancer Center (MDA), The Cancer Genome Atlas (TCGA), and Lund University (Lund) projects. UNC, MDA, and TCGA included MIBC alone; Lund included both NMIBC and MIBC. Key markers and therapeutic targets are shown aligning to Lund nomenclature. 

similar to the intrinsic basal and luminal subtypes of breast cancer (24). MIBC luminal tumors often display papillary morphology and express markers of urothelial differentiation such as KRT20, uroplakins, E-cadherin, early cell cycle genes, and FGFR3. Basal tumor markers reflect their cells of origin in the basal layer of the bladder, expressing CD44, KRT5, KRT14, markers of EMT, with some showing low expression of the claudin gene family or squamous differentiation markers (25). Data from studies performed by UNC, MD Anderson, and TCGA show that their MIBC clusters overlap with the Lund study’s subtyping nomenclature of unstable and infiltrated tumors (mostly luminal MIBC), and the more basal-like groups of urobasal B and squamous cell carcinoma-like (basal MIBC) (21).

Of the major currently published transcriptional studies, the UROMOL study focused solely on NMIBC, transcriptional profiling 460 patients with either low-grade or high-grade Ta, T1 and CIS tumors (26). Comparing their nomenclature to the original Lund findings, UROMOL Class 1 tumors were very similar to urobasal A tumors. Consisting of mostly Ta tumors, patients who were Class 1 showed the best prognosis of all groups. Class 2 contained more high-grade and T1 tumors, more likely to be at risk for recurrence and progression to MIBC, and aligned most commonly with genomically unstable and infiltrated Lund profiles (21). It is thought that Class 2 tumors, may represent tumors of origin for luminal-like MIBC tumors that retain more markers of urothelial differentiation, based on their characteristic expression of late cell cycle, EMT, and stem-cell related genes (5). Class 3 tumors had both common gene signatures like FGFR3 of the urobasal A subtype, but also displayed a basal expression pattern similar to basal MIBCs including expression of KRT5+, KRT14+, and CD44+ (26).
Some researchers in the field believe that molecular subtyping of bladder cancer is at the forefront in the future of disease diagnostics and treatment planning, predicting that the use of transcriptional profiling will overtake the traditional reliance on pathology for bladder cancer diagnosis. However, despite the broader categories of luminal and basal and even more defined categories like infiltrated and genomically unstable, within each subtype are more specific tumors types with actionable therapeutic targets, suggesting that future dependence on molecular classifiers for cancer diagnosis could be a journey down the rabbit hole. This prospect it a heavily debated topic among the field, and for the immediate future, efforts are aimed at combining conventional histopathology, immunohistochemistry, and molecular classification to subtype bladder cancer diseases and assign therapy (27-30).

In summary, bladder cancer is broadly assigned to a two-pathway system: non-muscle invasive (NMIBC) or muscle-invasive (MIBC). These two classifications differ in their stage and grade, as well as their molecular landscapes and molecular subtypes. These characteristics influence disease pathology, progression, and prognosis. NMIBC tend to be papillary, earlier staged, lower grade, and minimally invasive, characterized by FGFR3 mutations and higher expression of urothelial differentiation markers, but overall lower mutational burden. MIBC tumors by comparison are more dysplastic, aggressive, higher staged, and potentially metastatic. They are characterized by TP53, RB1, PTEN, and PI3K/AKT/mTOR pathway mutations, have higher mutational loads, and can be broadly molecularly subtyped into luminal and basal MIBC. The differences in these two urothelial tumor classifications lead to significant variation in clinical implications, as well as treatment and disease management.
In the future, there needs to be a consensus on nomenclature and characteristics of bladder cancer subtypes to aid in standardization of patient diagnoses and development of comprehensive treatments (31). The studies described in the subsequent chapters will be focused on NMIBC disease.

1.1.2: Standard Treatment Practices for Urothelial Carcinomas

**Diagnosis and prognosis.** As previously mentioned, most bladder cancer patients are diagnosed due to incidence of hematuria that is followed by evaluation by cystoscopy (32). Abnormal findings potentially indicating papillary, solid, muscle-invasive, or carcinoma *in situ* lesions require histological confirmation obtained from biopsy or transurethral resection of the tumor area (33). Commonly, urine cytology is measured in conjunction with cystoscopy to detect missed cancer: shed abnormal cells can indicate the presence of cancerous lesions (34). Expectedly, bladder cancer prognosis and disease management depend on classification of either NMIBC or MIBC lesions, staging, and grading (7, 35, 36). In low grade NMIBC tumors (Ta, T1), recurrence is usually common, but with low risk of progression; in high grade NMIBC tumors (majority T1, CIS), often regarded as precursors to development of invasive cancer, progression rates range from 40-83% if tumors are left untreated (37). MIBC tumors (T2, T3, T4), even after aggressive chemotherapy and radical cystectomy have recurrence rates of 20-30% for T2, 40% for T3, and >50% for T4 staged lesions (38). These patients also have shown a 5-year overall survival rate of less than 30% (39); additionally of note, initially diagnosed NMIBCs that
have progressed to MIBC show worse overall prognosis and cancer-specific survival as compared to primary diagnosed MIBC (40).

**Management and treatment.** All newly diagnosed bladder tumors undergo endoscopic resection, typically as part of diagnostic cystoscopy. This procedure, transurethral resection of bladder tumor (TURBT), is performed to remove all visible tumor and provide an appropriate sample for accurate pathologic staging (33). Multiple guidelines are available for recommended management of bladder cancer, but in general, NMIBCs are typically managed by TURBT and risk-based intravesical therapy with Bacillus Calmette-Guerin (BCG), while MIBCs are more insistently treated with radical surgery with or without pre-surgical systemic therapy of chemotherapy, radiation, or immunotherapy (7, 35, 41, 42).

**TURBT.** TURBT is performed by passing an electrified-wire loop resectoscope through the urethra during local or general anesthesia, resecting the tumor in a piecemeal fashion until all visible tumor is removed (43). Patients with low-risk disease, identified by solitary, Ta tumors that are <3 cm in size, are often treated by an initial TURBT followed by routine surveillance by cystoscopy for up to 5 years, with future recurrences treated with additional TURBT (42). Intermediate- and high-risk patients, identified by larger, multifocal or recurrent Ta tumors, T1 tumors, or CIS, follow initial TURBT with lifelong surveillance, potential repeat TURBT, and adjuvant immunotherapy with intravesical BCG (42). Unfortunately, understaging disease is a serious risk for patients, caused by incomplete resection due to tumor multiplicity, size or location, and results in inadequate treatment and higher probability of disease relapse and progression (44, 45). Thus, it is important and recommended for high-grade disease to be resected
again when incomplete resections have occurred, or when the tumor invades past the urothelium, to improve staging accuracy and patient survival (46, 47). Traditional TURBT as treatment for more advanced disease is not highly practiced due to chance of bladder perforation and the inevitable decision for removal of the bladder (48).

**Intravesical therapy.** Bladder cancer patients with intermediate- and high-risk NMIBC disease typically undergo adjuvant intravesical therapy with BCG. BCG, or Bacillus Calmette-Guerin, is an attenuated mycobacterium originally developed as a vaccine for tuberculosis (49). It was found to provide antitumor activity in urothelial carcinoma by intravesical instillation into the bladder by decreasing recurrence and inhibiting tumor progression (50). BCG acts as an immunotherapy, in that it stimulates a local inflammatory response characterized by an influx of granulocytes, monocytes and dendritic cells, and induced expression of inflammatory cytokines such as TNF-\(\alpha\), GM-CSF, IFN\(\gamma\), IL-2 among others (51). This inflammatory response is believed to activate a cytotoxic cell-mediated killing of tumor cells, producing the efficacy of BCG in preventing recurrence and progression (52). In North America particularly, patients treated with BCG exhibited a 68% complete response rate compared to 47% of patients receiving chemotherapy (53). Adjuvant therapy with BCG includes maintenance therapy (follow up instillations after initial treatment course) for 1-3 years depending on disease risk to further reduce incidence of tumor progression. Patients who have tumor recurrence/relapse, disease progression, or have intolerable adverse effects while on maintenance therapy or after treatment course of BCG are deemed “BCG unresponsive” or “BCG failures” (54).
these patients, there are few alternative therapeutic options to radical cystectomy, although bladder preservation strategies including chemotherapy, investigational immunotherapeutic agents, and clinical trials may be considered (55).

**Cystectomy.** For patients with high grade NMIBC, BCG unresponsive NMIBC, and MIBC, cystectomy (surgical removal of the bladder), or radical cystectomy with lymph node dissection is the gold standard of treatment. Radical cystectomy in men includes prostatectomy, and in women includes hysterectomy, and partial excision of the vagina and urethra (5). Following radical cystectomy, urine is diverted from its normal pathway by a segment of intestine into either a newly constructed neobladder (intestine segment with anastomosed ureters) which can still provide continence, or into a non-continent ileal conduit diversion brought to the skin as a stoma for use with an urostomy bag (56). Survival outcomes post-radical cystectomy depend on final pathological staging of the removed bladder and tumor. The 10-year recurrence-free survival is 76% for patients staged T1-T3a, 61% for T3b, and 45% for T4 when lymph nodes are not involved in cancer progression/stage, but when lymph nodes are involved survival drops to 34% regardless of stage (8). Radical cystectomy can seriously affect patient quality of life, and so much effort is being put towards perioperative care and Enhanced Recovery after Surgery (ERAS) protocols.

**Systemic therapy.** Neoadjuvant and adjuvant chemotherapy are used mostly in MIBC patients both prior to and directly following radical cystectomy to varying results. Typically, platinum based chemotherapy is efficacious used in a gemcitabine-cisplatin combination, producing a decrease in risk of death after surgery and a reduction in diseases recurrence (57, 58). In the neoadjuvant
setting, arguments for cisplatin-based chemotherapy use include its potential to
downstage bulky and invasive tumors which can improve surgical outcomes, but
its use can also delay other therapies and allow disease progression if patients
do not respond (11). Patients who are diagnosed with metastatic disease initially
or following cystectomy are treated with standard of care cisplatin-based
chemotherapy combinations such as methotrexate, vinblastine, doxorubicin,
cisplatin (M-VAC), cisplatin, methotrexate, vinblastine (CMV), or gemcitabine-
cisplatin plus paclitaxel (59). However, most patients with metastatic disease,
while initially responsive to systemic chemotherapy, will ultimately progress with
a median survival of 14 months and an overall 5-year survival rate of 5-20% (60).

Typically given in combination with chemotherapy rather than single
modality for optimal survival improvement (61), radiation therapy or
chemoradiation can be used in bladder preservation protocols when radical
surgery has substantial quality of life consequences. These treatments are
subsequent to TURBT (together deemed trimodal therapy) and can result in 5-
year cancer-specific survival rates between 50-82%, although roughly one-fourth
of patients will eventually need to undergo radical cystectomy due to lack of
response (62).

Systemic immunotherapy has more recently come into treatment practices
in MIBC with the recent US Food and Drug Administration approval of
atezolizumab (anti-PD-L1 mAb) and nivolumab (anti-PD-1 mAb) for treatment of
advanced and metastatic urothelial carcinoma (63). Initial results as a second-
line treatment have been promising enough that clinical trials are ongoing for
first-line care, and patients in earlier stages of urothelial cancer.
**Investigational therapy.** Due to frequency of recurrence of NMIBC despite BCG induction and maintenance treatments, and the life-changing decision for cystectomy, new treatments are being investigated for use in BCG unresponsive patients. Oncolytic viruses and adenoviral-mediated interferon-α (Ad-IFNα2b) are being explored as intravesical therapies to not only target cancer cells, but also to stimulate the host immune system to fight tumors cells as well (64, 65). Early clinical trials of Ad-IFNα2b gene therapy has shown safety and efficacy with 43% of patients exhibiting complete response at an average of 31 months, and 35% of patients free of high-grade recurrence at 1 year (66, 67). Many bladder tumors harbor molecular alterations that are potentially druggable, such as FGFR3 or alterations in the PI3K-AKT and MAPK pathways, and many clinical trials are enrolling and investigating the therapeutic potential of targeted therapies in bladder carcinoma (NCT02465060, NCT03410693, NCT03047213). As mentioned above, systemic immunotherapy with checkpoint inhibition in treatment of MIBC and NMIBC is a current area of investigation. The mechanism and functional importance of these will be further discussed in proceeding sections.

Together, once diagnosed, it is necessary for proper stratification of bladder cancer patients in order to receive the most effective therapy. However, the most effective treatment scheme may come at high cost both literally, due to high disease recurrence and progression rates, and figuratively, as patients can face serious challenges to their quality of life depending on their disease and the appropriate therapeutic action. For MIBC patients, standard of care radical cystectomy in combination with chemotherapy or immunotherapy provides the
best survival outcomes. For NMIBC patients, tumor resection followed by intravesical BCG are standard practice generating ~70% complete response rates; but for those that are deemed BCG unresponsive, no effective second-line therapy exists as an alternative to cystectomy (68-70). These patients make up a target population for investigational therapies that can improve prognosis, aid in the preservation of bladders and nullify life-altering surgical intervention.

1.1.3: Potential of Biomarkers for Early Detection, Diagnosis, and Response

**Early detection and diagnosis.** Early screening of high-risk patients as a potential option for early detection for bladder cancer is not a viable option owing to the lack of genetic markers and heredity of the disease. However, due to the invasiveness and cost of cystoscopy and biopsy as diagnostic tools, there is a strong desire among the field to identify novel, noninvasive diagnostic methods. Cytology is often performed in the diagnosis of urothelial carcinoma, to serve as a backup mechanism for detecting malignant cells originating from the bladder that a cystoscopy may overlook (71); within the premise of cytology lies the potential for urine-based tumor markers for early cancer detection and diagnosis. Several proteomic based tests have been approved by the FDA for diagnosis and surveillance, but many of these tests are below 80% sensitivity and range from 60-90% in specificity (72-74). Liquid biopsy detection of urinary markers by cell free DNA (cfDNA) have been used prognostically in identifying bladder cancer recurrence, progression and metastasis (75, 76). Urinary detection of DNA methylation status has been used for risk stratification and to differentiate bladder cancer patients from controls at sensitivities > 90% (77, 78). MicroRNAs
(miRNAs) and expression of other genes in voided urine are also being studied for diagnostic testing to mixed successes, with sensitivities ranging from 71-94% and specificity from 51-100% (72, 79). However, despite the emerging evidence of the utility of urine molecular diagnostic markers, currently there are no urinary based tumor markers that are definitively able to detect bladder tumors based on sensitivity and specificity (80, 81).

**Markers of response.** Many therapies for bladder cancer, like BCG, have been used for decades, and yet there is still no tool to aid in stratification of patients as likely responders and non-responders to therapy. Kamat and colleagues describe a urinary cytokine based assay that could predict the likelihood of tumor recurrence with 85.5% accuracy based on changes in a panel of nine induced cytokines after BCG therapy (82). Research on urinary exosomes as a marker for therapeutic response has been investigated in prostate cancer, but remain to show convincing evidence of correlation with response (83). To date, risk stratification models based on clinicopathological features from biopsied specimens remain the most widely used tools available to predict therapeutic response in bladder cancer (84). Future utilization of biomarkers in the detection of cancer (predictive) and response to therapy (prognostic) will require validation to minimize false-negative results, but their potential shows great promise in directing the field toward improved patient care and treatment strategies.

1.1.4: Summary
Bladder cancer is a malignancy most often associated with older men, with a fairly stable incidence outlook as tobacco usage and exposure has been ingrained in daily life worldwide. Bladder cancer can be subtyped by several different phenotypic and genotypic aspects, but clinical stratification is based on tumor invasion status into the muscular wall of the bladder (NMIBC vs. MIBC) and pathological grading. While the standard of treatments for NMIBC are costly, though effective for the majority of patients, there is room for improvement particularly for those who have failed BCG. Standard treatments for patients with MIBC greatly affect quality of life and survival outcomes, so the continued investigation into immunotherapy and bladder preservation strategies is crucial. Improving the treatment and survival of patients with bladder cancer will require further development of early detection tools and more effective local and targeted therapies moving forward. Exploring the role of the immune response to foreign signals and tumors, and the mechanisms tumors use to evade the host defense system will be reviewed in the next section.
1.2: The Immune Response and Tumor Immunity

The human immune system as we know it today is a product of evolution, originating from an archaic defense mechanism which used protein receptors to recognize common features of dangerous pathogens (85). The first line of defense for animals, known as the “innate” immune system, involves fast-responding molecules and cells that rush to the site of an infection. The innate response is often sufficient to contain and eliminate sources of danger to the host, and is therefore a very important first step in host protection. Higher organisms later developed a secondary immune response, a predominantly cellular mechanism intricately designed to target pathogens based on specificity, and be able to mount a quicker defense response in the incidence of recurrent infection, otherwise known as “adaptive” immune memory. Although evolutionarily created to identify and fend off invasion and infection by microorganisms, the same principles of recognizing a “foreign” molecule that initiates a cellular and molecular immune response can be applied to cancer.

Cancer is known for its loss of normal cell regulatory function and gain of innumerable genetic alterations (86). These alterations and dis-regulations have been known to result in the expression of antigens, leading to presentation of “foreign” peptides recognized by MHC molecules on surveilling immune cells (87). In a normal immune response, this peptide presentation and recognition by an immune cell would result in elimination of the tumor cell. Unfortunately, despite the ability of our inherent defense system to recognize and kill cancer cells, the durability and persistence of tumors presumably meant that something was hindering our immune cells from destroying it. Definitive proof of these
immune inhibitory mechanisms was found in mice, when Dunn and colleagues discovered that cancer cells had the unique ability to delete T cell targets and thus avoid being detected and attacked (88). Tumors can also suppress an immune response by activating negative regulatory pathways that exploit immune homeostasis and inhibit immune cell effector functions (89, 90). Deemed tumor-immune “evasion” or “escape”, these mechanisms allow tumors to survive in an environment that would normally prove hostile.

The following section provides a background on the normal mechanisms of immune response when foreign antigens are discovered, including responses of the innate and adaptive arms of the immune system, and will also provide a discussion on the specialties of tumor-immunology and the mechanisms of tumor-immunosuppression.

1.2.1: Overview of Innate vs. Adaptive Immune Response to Foreign or Tumor Signals

**Innate response.** As touched on above, the innate immune system biologically evolved as the first line of defense against pathogens. Innate immunity usually encompasses the myeloid derived lineage of phagocytic and inflammatory white blood cells (neutrophils, monocytes, macrophages), complement, cytokines, and acute phase proteins, all of which are designed to provide immediate response to signals of infection or damage (91). These responses are hard-wired from germline encoded genes to recognize molecular patterns common to microbial, toxic, or allergenic structures that are not present in the host (92). Membrane-associated proteins called pattern-recognition receptors (PRRs) recognize
essential microbial components known as pathogen associated molecular patterns (PAMPs) and host tissue distress signals known as damage-associated molecular patterns (DAMPs) (93). PRRs are constitutively expressed on large numbers of cells, and once activated, initiate specific signaling pathways to eliminate the foreign invader (94). PRRs can be differentiated by their expression patterns and ultimate function, generally either classified as cytoplasmic receptors (NLRs, RLRs) or membrane-bound receptors (TLRs). Toll-like receptors (TLRs) are one class of transmembrane PRRs that have been evolutionarily conserved across species, existing as either extracellular or intracellular signaling proteins that recognize microbial lipids and nucleic acids (Table 1) (94). Most prominently expressed on antigen-presenting cells (APCs) including macrophages and dendritic cells (DCs), stimulation of receptors with a TLR ligand (microbial component) initiates a signaling cascade driven by either MyD88 or TRIF adaptor molecules, resulting in the production of pro-inflammatory cytokines and type I interferons (IFN), respectively (95, 96). IFN and cytokines serve to further recruit and activate more immune cells and lead to pathogen destruction, to be discussed more in depth below. TLRs were among the first targets validated for cancer immunotherapy based on the clinical efficacy of BCG therapy, an agonist of TLR2 and TLR4, in patients with early-stage bladder cancer (97). Exploitation of TLR signaling continues to be explored and utilized in many cancer therapies today.

The other major class of PRRs, cytoplasmic receptors including RIG-I like receptors (RLRs), cytosolic DNA sensors, and nucleotide-binding oligomerization domain [NOD]- leucine-rich repeat containing receptors
(NLRs), are associated with recognition of bacterial and viral components in

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<tr>
<td>1</td>
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<td>cell surface</td>
<td>TIRAP, MyD88</td>
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<tr>
<td>2</td>
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<td>Heat shock proteins (HSPs), proteoglycans</td>
<td>cell surface</td>
<td>TIRAP, MyD88</td>
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<td>HSP60, 70; Fibrinogen</td>
<td>cell surface</td>
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<td>Flagellin</td>
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<td>Lipopeptides (mycoplasma)</td>
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<td>7</td>
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<td>viral DNA, CpG DNA, CpG ODNs</td>
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the cytosol (98). NLR family members can sense intracellular bacterial peptidoglycans, cellular stress products, microbial products, and noninfectious crystal particles (99). The most common family members of NLRs, NOD1 and NOD2 and NLRPs, form oligomers once activated to further initiate production of other inflammatory cytokines, active NF-kB inflammatory signaling, and induce the inflammasome complex which leads to the cleaving and secretion of IL-1 family members (100, 101). RLRs are RNA helicases that recognize cytosolic dsRNA and are particularly important in viral infections. Unlike TLRs, RLRs are expressed by most cell types. The most described RLR helicases are retinoic-acid-inducible protein I (RIG-I), melanoma differentiation associated gene 4 (MDA5), and LGP2, which when stimulated initiate a cascade to increase production of inflammatory cytokines and type I IFN, like NLRs and TLRs (98). Similarly, cytosolic DNA sensors like STING and cGAS detect viral and cytoplasmic DNA and initiate a type I IFN inflammatory response.

Surveilling phagocytes based in common entry tissues and PRR-expressing epithelial cells are among the first responders to foreign pathogens. Upon PRR recognition, they initiate an NF-kB-mediated signaling cascade of cytokines to stimulate inflammation within the infected tissue, causing more DCs, macrophages, and other innate cells to produce cytokines and chemokines to attract circulating leukocytes and begin the first stages of the cellular arm of the innate response (102). These cytokines are considered pro-inflammatory, and are important in staging the innate defense, but once mounted they must also be regulated to eventually subside to prevent extensive tissue damage (103). Common inflammation-induced cytokines and
Chemokines include IL-1, IL-6, IL-12, TNF, IFNγ, IL-4, IL-10, IL-18, CXCL8, CCL4, RANTES, TGFβ and later GM-CSF and M-CSF, with functions ranging from monocyte differentiation, increasing vascular permeability, further immune cell recruitment, lymphocyte activation, and the suppression of response (104, 105). The early response cytokines TNF and IL-1 increase expression of cell-adhesion molecules such as selectins and integrins to attract monocytes and neutrophils to the site of infection (106). Chemokines CXCL8, CCL4, and RANTES are chemotactic cytokines, serving to recruit neutrophils, basophils, monocytes, T cells and other immune cells by their sensing of these small molecules in a gradient fashion with highest concentrations at the source of infection (107). IL-4, IL-10, and TGFβ generally serve as anti-inflammatory cytokines by negatively regulating the innate response. IL-10 and TGFβ suppress DC differentiation, inhibiting their ability to activate T cells, and can also directly inhibit T cell functions; they negatively regulate pro-inflammatory cytokine production and cytotoxic cell function, and induce differentiation of regulatory T cells, but have a role in tissue repair (105, 108). IL-4 is initially produced by mast cells and is important in protecting the host during parasitic infection (109). IL-4 has mixed roles in the inflammatory response: its important in the differentiation of monocytes into DCs and B cell activation, but following the cellular activation of the innate response, IL-4 also induces T cell differentiation into Th2 type cells (110, 111). IL-12 and IL-6 have roles in activating lymphocytes and inducing Th1 type immune differentiation (105, 112). TNF-α and IL-1β in combination with IL-6 are also important for initiating the acute phase response and opsonization-mediated pathogen killing (113).
TLRs, RLRs, and STING sensors are the major pattern recognition receptors that are stimulated to signal type-I interferon (IFN-I) production. Type I interferons (IFN-α and IFN-β), induced from sensing for foreign (viral) nucleic acids by TLR3,4,7,9, RIG-I and MDA-5, are produced by almost all cell types; however the major production of IFNs-I comes from plasmacytoid DCs (pDCs) (114). Both IFN-α and IFN-β induce resistance to viral replication in all cells, hence the name “interferon”, through the JAK/STAT signaling cascade which culminate in the transcriptional regulation of many interferon-stimulated genes (ISGs) (115). Signaling through PI3K/AKT, NF-kB, and MAPK are also triggered by activation of the IFN receptor, resulting in induction in cell division, proliferation, differentiation, and survival (116, 117). IFNs-I exert effects on the immune response directly by stimulating innate cells to activate other cells, and indirectly through the induction in chemokines, such as CXCL9, CXCL10 and CXCL11 for immune cell recruitment, and the induction of cytokines important in regulating innate and adaptive cells (118-120). IFNs-I support the migration, maturation, and differentiation of monocytes into DCs thereby upregulating antigen presentation, as well as macrophage cytotoxicity and phagocytosis (121, 122). They also increase MHC class I molecule expression to enhance antigen recognition by antigen presenting cells (APCs; DCs and macrophages) and encourage DC migration, together with priming and activation of the adaptive immune response (123-126), to be discussed further in the next section.

As touched on above, IL-6 is a very pleotropic cytokine induced by PRR recognition of PAMPs and DAMPs, NF-kB signaling, and IFN-I signaling from
immune cells, but also from fibroblasts, endothelial cells, and other cells (127). Like other cytokines (IL-4), IL-6 is known for both pro-inflammatory and anti-inflammatory actions. Differences in its roles may be linked to the different ways IL-6 signals at the plasma membrane: 1) classical signaling involves membrane IL-6Rα binding with receptor protein gp130, but not every cell type has membrane-bound IL-6Rα, and so 2) soluble IL-6/IL-6Rα (sIL-6R) is utilized and known as IL-6 trans-signaling (128). Binding of IL-6 to IL6-R and gp130 initiates JAK/STAT or MAPK signaling pathways, the former simultaneously inducing a negative feedback loop to suppress IL-6 by SOCS genes (129). IL-6 contributes to immune defense in a pro-inflammatory role by inducing the acute phase response, hematopoiesis (granulopoiesis), B cell differentiation, and interferon-like activity (130, 131). It also promotes differentiation of CD4 T cells in to Th17 T cells and suppresses CD4 TReg formation, and induces CD8 cytotoxic T cell differentiation (132-134) (Figure 3). If left unchecked, these pro-inflammatory responses contribute to development of chronic inflammation and autoimmune diseases (135, 136). Anti-inflammatory effects of IL-6 include a role in wound healing and liver regeneration (137), and negative regulation of pro-inflammatory TNFα, GM-CSF, IFNγ, and MIP-2 cytokine production in acute lung inflammation (138). In relation to cancer and disease pathology, IL-6 is a known instigator of autoimmune disease such as rheumatoid arthritis and colitis caused by chronic inflammation (139), and has been linked to anti-tumorigenic and pro-tumorigenic actions with poor clinical outcomes in cancer patients (140). IL-6 has controversial roles in immune activation, chronic inflammation and cancer, but it remains a strategic bridge connecting the
Figure 3: IL-6 in inflammation, immune response, and pathogenesis. IL-6 is a pleiotropic cytokine. It induces acute phase response proteins such as CRP, serum amyloid A, fibrinogen and hepcidin from hepatocytes. IL-6 also plays an important role in adaptive immunity by stimulating B cell and T cell differentiation and function. IL-6 can also promote proliferation of many immune and non-immune cells. RANKL, receptor activator of nuclear factor kB (NF-kB); VEGF, vascular endothelial growth factor. This figure was taken with permission from Tanaka T, Narazaki M, Kishimoto T. 2015. IL-6 in inflammation, immunity, and disease. Cold Spring Harbor Perspectives in Biology (136). License number
innate and adaptive immune responses.

Natural Killer cells (NK cells) serve as a special component of the innate immune system. NK cells have similarities to both innate and adaptive cells: they develop from the same progenitor cells as B and T cells, and have the ability to kill cells by direct contact after binding cell-surface receptors, however, NK cells are fast-responding and have invariant, germline-encoded receptors that recognize molecules on infected cell surfaces, and as such are considered innate cells (141). NK cells are activated in response to the triggering of their activation receptors by cognate ligands (142), IFNs-I, and cytokines produced from macrophages and DCs. Once activated, they then identify infected cells based on aberrations or loss of MHC class I expression, and directly kill cells by cytotoxic granule release (perforin) or production of IFNγ (143-145). Normal class I MHC expression is an inhibitory signal to NK cells, as this is the sign of self-host cells (146, 147). Loss of this inhibitory signal (normal class I MHC) was proved to be insufficient for NK cell killing, and that an additional activating signal was necessary (148); NKG2D and FcγRIIIa (CD16) activating receptors provide the necessary secondary “kill” signal (149, 150). Further, NK cells can be “primed” by DCs to be more effective killers (151), and can also exhibit a form of immunological memory, similar to adaptive cells (152-154). Though able to provide immune defense by fast response and pathogen receptor recognition, NK cells have many functional features that make them an intermediate to the precisely targeted adaptive arm of the immune system.

Innate responses are able to prevent infection from establishing and spreading by discriminating between foreign pathogens (PRRs) and self, but are
mostly non-specific. Most of these PAMP signaling pathways by PRRs overlap with sensing of tissue damage (DAMPs), including tumor-derived antigens (155). Thus, the innate immune system has a role in recognizing and responding to cancerous cells. Tumor neoantigens, RNA, and DNA can be sensed by TLRs, RIG-I (RLR), and STING, triggering the macrophage and DC production of cytokines, interferons, chemokines, and antigen presentation with T cell priming (156, 157). The result is cancer cell death by eliciting apoptosis pathways, interferon signaling, or primed cytotoxic cells (158-160). Increased ligand expression provided by cellular stress or DNA damage of tumors cells help activate NK cells via their activating receptors (161) and cause direct tumor cell death by cytotoxic granules (perforin, granzymes), Fas-ligand or TRAIL, secreting IFNγ, and through antibody-dependent cellular cytotoxicity (162).

Not only is the innate immune response of key importance in the initial stages of host defense, but it is also essential to the adaptive response, as the cells stimulated by microorganisms during infection, or tissue damage-associated tumor antigens, go on to activate antigen-specific defense directly and through cytokines and chemokine migration. PRR signaling that leads to macrophage and DC maturation, antigen presentation, and type I interferon production, in combination with NK cell pathogen recognition and activation, provide mechanisms of containment, whistle-blowing, and killing. These necessary first steps provide sufficient time for the induced T and B cell adaptive responses with high tumor cell specificity and cytotoxicity, explained further in the next section.
Adaptive response. As previously mentioned, the immune response consists of two arms: the innate component, an immediate though non-specific response consisting of myeloid cell pathogen sensing and cytokine and chemokine release for cellular recruitment and activation; and the adaptive component, a delayed but intricately more specific response largely reliant on the lymphocytic lineage of cells. Many of the responses of innate immunity are necessary for the adaptive immune system to get started. In brief, dendritic cells that are stimulated by PRR recognition increase their surface expression of MHC class II receptors and co-stimulatory molecules which will allow them to activate naïve T cells once they migrate to peripheral lymphoid tissues and present antigen (163, 164). Once the lymphocytes are activated, the DCs die, and the T cells begin to proliferate and mature into cytotoxic CD8 effector T cells (165). The cytokines produced by the innate cells also encourage differentiation of naïve CD4 cells into Th1, Th2, Th17, T_{FH}, or T_{reg} effectors (166, 167), which then after DC activation, are able to 'help' stimulate the antigen receptors of B cells to an effector state, or differentiate into plasma cells, and produce secreted antibodies against pathogens (168-170). Once activated, effector T cells reenter circulation and migrate back to the site of infection, following chemokine gradients and adhesion molecules (171). Serving as the cell-mediated responders of adaptive immunity, CD8 T cells can directly kill aberrant cells while CD4 T cells help to activate macrophages, neutrophils, and secrete protective and activating cytokines to aid in defense (172-174). Activated plasma B cells secrete antibodies specific to the foreign antigen to eliminate pathogens by humoral neutralization, opsonization, or complement activation (175). Thus, the adaptive response relies heavily on
innate immunity to be initiated and guided in order to achieve precise immune protection and disease resolution.

Originally, T and B lymphocytes develop from a common progenitor stem cell in the bone marrow (HSC), but T cell progenitors must migrate to the thymus for further stages of development into T cells. In the thymus, T cell progenitors acquire antigen-specific receptors, known as TCRs (T cell receptor), by random somatic rearrangement of DNA segments that specifically code for antigen binding regions of the receptor through a process called VDJ (variable-diversity-joining) recombination. In total, VDJ recombination can produce up to $10^8$ different TCRs to match the wide variety of potential antigens (176), which are then further selected by their TCR reactivity to self-antigens (negative selection) and expression of CD4 or CD8 (positive selection based on class I (CD8) or class II (CD4) MHC molecules). Once T cells pass through selection and complete development, they home to secondary lymphoid organs such as the spleen or lymph nodes, and proceed to continuously circulate through these organs, lymph, and blood, awaiting the potential of antigen recognition and shedding their naïve status (177). Stimulated DCs, with antigen in tow, traffic from the origin of pathogen recognition to the inflamed or “reactive” lymph node and encounter scanning T cells, while concomitantly expressing CD80 and CD86 co-stimulatory molecules (178-180). When an antigen-naïve T cell recognizes its cognate antigen presented on the MHC molecule of an APC, the T cell undergoes several changes: If the peptide is presented on an MHC class I molecule, which is broadly expressed on all cells, and activating cytokines such as IL-12 and IFN-β are present, naïve CD8 T cells will differentiate into cytotoxic CD8 cells and undergo clonal expansion. If the antigen peptide is presented on
an MHC class II molecule, naïve CD4 T cells will differentiate into various CD4 helper T cell subsets, dependent on the cytokines present in the environment, to be elaborated on in the next paragraph. Additionally, T cell activation upregulates the expression of CD69 surface molecules to ensure the T cell stays in the supportive environment of the lymph node, with antigen, co-stimulation, and cytokines present, promoting proliferation of high (and moderate) antigen-affinity T cells specific to that cognate antigen, called clonal expansion (181, 182). During clonal expansion, the majority of expanded T cells down regulate CD62L and CCR7, allowing them to exit the lymph node and migrate to the sites of antigen source by way of chemokine gradients, integrins, and selectins, and perform their effector functions (183). For CD8 T cells, those effector functions include TNFα and IFNγ cytokine production, and most especially, cytotoxic-mediated cell death of the target cell recognized by its specific antigen peptide/MHC class I complex (184). This cytotoxic function is accomplished by cell-cell contact and delivery of perforin and granzyme toxic granules into the cytosol of the infected cell, lysing and killing the target cell (185, 186).

When DCs present antigen peptide on MHC class II receptors, naïve CD4 T cells are activated to differentiate into multiple subtypes of effector and regulatory CD4 cells. After stimulation with antigen, CD4 cells begin to produce IL-2 to further promote their proliferation, and proceed towards differentiation into Th1, Th2, Th17, Treg, or TFH depending on the cytokines present at the activation site (187). IL-12 and IFNγ produced by macrophages and NK cells induces the differentiation of Th1 cells (188). Th1 cells are effector cells that support cell-mediated immune response; they express transcription factor T-bet, and produce
IL-2 and IFN\(_\gamma\) to support macrophage and T cell proliferation and cytotoxicity (189), and protect the host from bacteria and viruses. IL-4 from basophils, mast cells, and eosinophils encourage differentiation into Th2 cells (190). Th2 cells are characterized by expression of transcription factor GATA3, are thought to support more humoral and allergic responses and defense against parasites, and produce suppressive cytokines IL-4, IL-5, and IL-13 (191). Th17 cells arise when IL-6 and TGF\(\beta\) are present and are more pro-inflammatory cells, expressing the transcription factor ROR\(\gamma\)T and producing IL-6 and IL-17 cytokines (192). They help to recruit neutrophils to the site of infection. T\(_{FH}\) cells, or follicular helper cells, express the transcription factor Bcl6, and exist in the lymphoid follicles to help stimulate B cells to produce high-affinity antibodies (193). Unlike the other effector cells, cytokines involved in T\(_{FH}\) differentiation are not completely known, but IL-6 is known to be important (194). T\(_{FH}\) cells can produce cytokines that are similar to those of Th1 and Th2 cells, signaling to B cells which antibody isotype to secrete (195). Effector CD4 cell responses act in defense by producing cytokines and chemokines that activate and recruit other immune cell subsets to their effector states. Because of this broad reaction, CD4 cells need to be more tightly regulated than the specific response of CD8 cells, and so only certain types of antigen presenting cells have MHC class II molecules. T\(_{reg}\), or regulatory T cells, also serve in this purpose. T\(_{reg}\) cells suppress the immune response, and are key in preventing autoimmunity, by secreting IL-10 and TGF\(\beta\) (196). There are two main groups of regulatory T cells: natural and inducible. Natural T\(_{regs}\) are committed to a regulatory fate while still in the thymus, and have high levels of surface CTLA-4 expression; inducible T\(_{regs}\) differentiate from naïve CD4 T cells in
the periphery under the influence of the environment (105). Induced T\textsubscript{regs} arise when TGF\textbeta alone is present in the lymph tissue, and like natural T\textsubscript{regs}, induced T\textsubscript{regs} express the FOXP3 transcription factor which prevents the transcription and production of IL-2 (197). CD4 and CD8 T cell differentiation are critical in host defense and entirely dependent on the type of cytokine signals produced from pathogen-activated innate cells, underlining the cohesiveness of the immune response.

As mentioned before, B lymphocytes differentiate from the same common progenitor as T lymphocytes, however B cells remain in the bone marrow. Like T cells, B cells also generate surface antigen-specific receptors, called B cell receptors (BCR), by way of somatic VDJ recombination (198). Unlike TCRs though, BCRs are expressed as an immunoglobulin molecule (Ig) with heavy and light chains that make up the antigen binding domain (199). The recognition of antigen by BCR not only can activate the naïve B cell directly, in conjunction with TLR stimulus (200), but it can also be internalized, processed, and presented back on MHC class II molecules for further antigen-specific CD4 T cell stimulation (201). The stimulated CD4 T cell then binds to the B cell by CD40 ligand interaction, and produces activating cytokines for B cell proliferation and differentiation into antibody-producing plasma cells (202-204). IL-4, IL-5, TGF\textbeta, or IFN\gamma are selectively secreted depending on the type of pathogen detected to induce specific antibody class production (205). Once secreted, antibodies function to kill the foreign pathogen by neutralization, opsonization, or complement activation (206). Neutralization involves antibody binding to bacterial toxins and viruses to prevent their interaction with normal host cells (207, 208).
Antibodies activate the complement immune response by binding the first complement proteins to the Fc region of the antibody, ultimately resulting in opsonization and phagocytosis, killing of the pathogen (209). The Fc region of antibodies can also activate other immune cells like NK cells to induce pathogen elimination (210, 211).

Cellular and molecular components of the innate and adaptive immune responses work together to resolve pathogen infections, tissue damage, and foreign bodies. For most acute diseases, once the danger is resolved, the inflammatory stimulus is removed and effector cells undergo apoptosis and phagocytosis. Some effector cells remain and differentiate into memory cells (B and T), who have the benefit of faster response times if the original pathogen or damage recurs (212, 213). Together, the immune response is the most powerful line of defense and management of infections, tumors, and disease. The inherent processes of both the innate and adaptive arms provide major targets for treatment strategies in effort to optimize the events that naturally occur. However, despite its effectiveness in protection and pathogen elimination, microorganisms and malignancies like cancer have developed ways to subvert the immune response, which will be addressed further in the following section.

1.2.2: Mechanisms of Immunosuppression in Tumor Immunity

As touched on previously, the genetic alterations and changes in cell regulatory processes in cancer cells often produce antigens from mutated genes, overexpressed genes, or cellular distress (DAMPs) (214, 215) that can be recognized by surveilling immune cells. These tumor antigens, either presented
on the cell’s MHC class I molecule or released from the cell in death, are sensed or picked up by APCs (macrophages and DCs) via TLRs, RLRs, or other sensors, triggering a pro-inflammatory response, and antigen processing and presentation (216). Once activated by these “danger” signals, APCs upregulate co-stimulatory ligands and traffic to proximal lymphoid tissue, such as the tumor draining lymph node, to prime the adaptive T cell and B cell response (217, 218).

CD4 T helper 1 cells (Th1), CD4 T helper 17 (Th17) cells, CD8 T cells, and B cells in combination with NK cells and the other innate immune cells all play important roles in tumor inhibition by mechanisms similar to those for foreign pathogens (219, 220). Despite the host’s ability to stage an immune-mediated antitumor response however, incidences of tumor eradication, even with therapeutic assistance, are rare. In order to avoid elimination, tumors have developed strategies to evade immune detection, hinder immune activation, block tumor infiltration, and inhibit tumor-killing mechanisms. This section will provide an overview of the most common mechanisms of immune suppression co-opted by cancer cells to escape eradication, focusing on the immune cell types primarily affected.

**Loss of antigenicity and inhibition of sufficient immune activation by APCs.**

Tumors have developed a wide variety of ways to avoid detection by APCs as well as activated T cells. These evasive mechanisms result in decreased innate and adaptive cell activation and tumor-killing ability, leaving the tumor to continue thriving. Tumor cells have been shown to down regulate the expression of MHC class I molecules, evading detection by both DCs during initial surveillance, and antigen-specific T cells during the adaptive response (221, 222). Loss of MHC
class I molecule expression in tumors is associated with poor disease prognosis and clinical outcome (221). Tumor secreted factors such as VEGF (223), IL-6, and M-CSF (224) also cause defects in DC differentiation and maturation, resulting in less expression of MHC class II molecules and lack of expression of co-stimulatory molecules CD80 and CD86, making them unable to present antigen to T cells and activate the T cell response (225, 226). Lack of co-stimulatory molecules can lead to T cell tolerance, anergy, and T cell death (227). In addition to the direct effects tumors have on DCs and T cells, tumors can acquire the ability to delete their own cells that express antigens T cells recognize, a process called “immune editing” (228). The loss of antigenic epitopes also occurs from many targeted cancer therapy treatments (229, 230). This loss of antigenicity can result in the evolution of tumor cell variants that are not recognizable to immune cells, leading to tumor progression (231, 232). The NKG2D activation receptor expressed on cytotoxic NK and T cells is a known responder to damage and stress-induced ligands in cancer. Tumor cells however can manipulate the expression of these ligands through post-transcriptional and post-translational regulation by misfolding, adenylyating, splicing, or glycosylating the ligands in order to avoid immune recognition (233). Consequently, down regulation of antigens and inhibition of immune activation by loss of antigen presentation and co-stimulation represent two mechanisms utilized by tumors to evade the immune system.

**Immune exclusion.** Despite the existence of cancer therapies that are tumor-antigen specific, or involve blockade of tumor-suppressive chemical mechanisms, there remain many tumor types that exhibit little to no antitumor
response (234). This lack of response could be linked to physical obstacles prohibiting activated immune cells from infiltrating the tumors (235). The microenvironment of a tumor (TME) is characterized by not only by cancer cells and immune cells, but also blood vessels, fibroblastic cells, and extracellular matrix (ECM) (236). Studies have shown that stromal cells in the TME such as fibroblasts (cancer-associated fibroblast, CAFs), myeloid-derived suppressor cells (MDSCs), and tumor associated macrophages (TAMs) and the tumor vasculature all play a role in preventing immune infiltration. Reactive nitrogen species produced by MDSCs can trap T cells in the surrounding tumor stroma by nitration of chemokine CCL2 (237). CAFs can also trap T cells in the tumor periphery by secreting dense ECM proteins that inhibit T cell migration (238). High expression of Fas ligand (FasL) on endothelial cells in the tumor vasculature can induce CD8 cell apoptosis, preventing their infiltration, while simultaneously not excluding T_{reg} cells because of their inherent high expression of apoptosis inhibitor c-FLIP (239). This mechanism is known as immune privilege. Immune cells can also be excluded from poorly vascularized and hypoxic tumors, caused by rapid tumor growth and expression of HIF-1α (240). Normalization of tumor vasculature by VEGF inhibition or deleting the signaling regulator Rgs5 can enhance T cell infiltration (241, 242). Until the problem of TME barriers can be overcome, the full benefit of immune cell therapies may not be realized. Thus, prevention of immune infiltration is a key mechanism exploited by tumors and their co-opted stromal cells in suppressing immune-mediated elimination.
**Immunosuppressive ligands.** T cell activation is a complex process not only involving antigen recognition, co-stimulation, proliferation and differentiation, but it also induces inhibitory pathways that can lead to eventual attenuation of T cell responses (243). In normal tissue homeostasis, negative-feedback responses called “immune checkpoints” are crucial to maintain self-tolerance and protect host tissue from potential damage induced during the immune response to infection (244). However, tumors and their microenvironment often have dysregulated expression of immunosuppressive receptors and ligands that regulate T cell effector functions, providing a mechanism of immune evasion. The high expression of antigens in cancer can also lead to the deterioration of T cell effector functions caused by continuous activation stimuli, a state termed “exhaustion” (245). Therefore, it makes sense that exhausted T cells are also characterized by expression of multiple inhibitory receptors. Immune inhibitory receptors such as PD-1, CTLA-4, TIM-3, LAG-3 and their ligands, among others, are expressed on tumor cells, T cells, and other immune cells in the TME including DCs, macrophages, fibroblasts, immature MDSCs, and Tregs (244), making the potential of receptor-ligand binding, and immune escape extremely likely.

The most well studied immune checkpoints are CTLA-4 and the PD-1/PD-L1 pathways. Programmed cell death (PD-1) receptor is induced upon T cell activation and is intended to limit T cell effector functions by decreasing their production of inflammatory cytokines and cell survival proteins (246, 247). It’s ligands, PD-L1 and PD-L2, are most often expressed in peripheral tissues by tumor cells and myeloid-derived cells in the tumor microenvironment (248). The PD-1/PD-1/PD-L2 pathway gene expression can be upregulated on tumor cells
and cells in the TME as a consequence of constitutive oncogenic pathway signaling (249), and in response to therapy, HIF-1α expression and hypoxia (250), immune-associated cytokines such as IL-10, TGFβ and IFNγ (251, 252), and tumor-secreted factors such as VEGF and PGE2 (253). PD-1 can also be induced on NK cells and B cells as well as Tregs, limiting their lytic and effector activities (254, 255) or promoting their proliferation and immune suppressive function (256), respectively, exhibiting another mechanism of immune resistance. Unlike PD-1, cytotoxic T lymphocyte associated protein-4, or CTLA-4, predominantly regulates T cell activation, but is also important in maintaining immune tolerance and avoiding autoimmunity, and thus can be found more broadly in host tissues and secondary lymphoid organs, as well as the tumor microenvironment (244). CTLA-4, which is expressed upon T cell activation, has very high homology to CD28 co-stimulatory receptor, but binds to B7 (CD80/86) receptors on APCs with much higher affinity than CD28; thus, it is believed to outcompete CD28 for receptor binding, resulting in lack of T cell activation (257, 258). To produce its inhibitory effects, CTLA-4 interferes with the TCR signaling chain, and by removing the active pool of CD80/CD86 from APC surfaces (247, 259). Despite inhibiting CD8 effector T cells, the major effects of CTLA-4 signaling come from down-modulation of CD4 helper T cell activity (Th2) and increasing Treg immunosuppressive functions (260, 261).

Other checkpoint receptors have been more recently discovered and investigated, including TIM-3, LAG-3, BTLA, A2aR, KIRs and others. These molecules are expressed on T cells, NK cells, and TME-associated endothelial cells, macrophages, APCs, and Tregs, induced from inflammatory signals and T
cell activation (244, 262, 263). They have been shown to inhibit NK, CD8, and CD4 helper T cell activity, cytotoxicity, and promote anergy and T_{reg} proliferation and function (264-266).

Therefore, immunosuppressive receptors and ligands represent one more mechanism co-opted by cancer cells to combat the immune response.

**Immunosuppressive cells.** In addition to the loss of antigenicity and ability to be recognized, creation of barriers to prevent immune infiltration, and induction of inhibitory receptors, tumors can co-opt stromal and immune cells in the TME to aid in suppressing effector cell functions and evade extinction. Often encompassed in the total tumor infiltrating lymphocytes, T_{regs} play an important role in immunosuppression by secreting anti-inflammatory cytokines such as IL-10, IL-35, and TGFβ, competing for activating cytokines like IL-2 with effector cells (267), and by direct contact with CTLA-4 (268), adenosine (269), and cytotoxic cytokines to inhibit DCs and effector T cell function (270, 271). T_{reg} differentiation is induced by antigenic stimulation in the presence of TGFβ (272), and naturally formed T_{regs} can traffic to the tumor site via tumor and macrophage secreted CCL22 (273). TGFβ itself can disrupt T cell activation directly as well as by limiting the mobility and survival of DCs (274), and by promoting polarization of tumor associated macrophages that sequester tumor antigen (275), contributing to inhibition of T cell priming. Macrophages and undifferentiated monocytes in the TME can be influenced by environmental cues to preferentially differentiate into M2 macrophages (276). M2 macrophages are induced by IL-4 and IL-13, typically produced by Th2 helper cells, and are deemed
immunosuppressive because they contribute to the production of IL-10 and arginase, inhibitors of T cell activation (277). Immature myeloid cells known as MDSCs are recruited to the TME by GM-CSF, IL-6, and CCL2, often produced from tumor cells (278). Major MDSC-mediated mechanisms of immune suppression are promoted by TGF-β (279) and include nitrosylation of the TCR and T cell surface proteins to inhibit T cell effector function (280), and expression of arginase to deplete arginine nutrients from the TME (281). Suppressive subsets from the myeloid lineage have overlapping abilities to express immunoregulatory molecules such as arginase, iNOS, and indoleamine 2,3-dioxygenase (IDO) to inhibit CD8 cell proliferation or induce apoptosis (282). IDO and arginase are metabolic enzymes that can catabolize essential amino acids, tryptophan and arginine, used by T cells for inducing proliferation, cell cycle growth, and effector functions (283-285). iNOS also catabolizes arginine to form nitric oxide (NO), a reactive nitrogen species used by MDSCs, utilized for nitrosylation of the TCR to interfere with TCR binding peptide-MHC complexes and promote hyposensitivity and tolerance (286), and nitration of chemokines used in T cell recruitment (237).

Many of the cellular interactions in the TME are complex, showing redundancy in suppressive action, or cytokine profiles having positive feed-back loops supporting the differentiation and function of immunosuppressive cells. In addition to the few mechanisms described here, tumors have many other immune resistance mechanisms, a product of their constant need for evolution to maintain survival. It has been shown that tumors can become resistant to cytotoxic cytokines, shown by the development of tumor-IFNγ insensitivity from
the absence or dysfunction of IFN\(_\gamma\) receptor signaling pathway (287). Tumor cells can also lose the expression of genes necessary for cytotoxic signaling such as JAK family members and genes of the IFN pathway, promoting resistance to immune-mediated killing (288, 289).

1.2.3: Summary

The innate and adaptive immune system are evolutionarily advanced in their ability to cooperate to resolve danger within their host. Humans have developed intricate pathways to detect foreign pathogens and cancer, following up sensing with action, by activating the effector cells and molecules of immune protection. However, tumors have developed ways to counteract and suppress the defensive activity of our immune system at most turns. The most interesting aspect of tumor-immune suppression is that most of the cells and molecules utilized by tumors to promote their own survival are inherently purposed for self-tolerance to prevent autoimmunity. Despite this fact, many of the suppressive mechanisms tumors have acquired such as checkpoint inhibitors and immunosuppressive cells can be therapeutically targeted to repolarize the TME into an antitumor phenotype, which will be reviewed in the following section.

1.3: Immune Therapy: Overcoming Tumor-Immunosuppression

As reviewed above, humans are equipped with extensive protective mechanisms that enable the detection and recognition of foreign pathogens and damage, or cancer associated markers. This identification process initiates a
cascade of signaling, recruiting an immediate cellular and chemical response to instigate inflammation at the source of trouble, killing microorganisms and cells in a non-specific but timely matter; while behind the curtain, an intricately more specific immune response ramps up to deliver a precise second wave attack. Despite the involved, sophisticated, and specific process that is the immune response, pathogens and cancer in particular have simultaneously evolved ways to suppress and evade it in order to continue surviving. These include tumor-induced impairment of antigen presentation and negative regulation of APC function and development; creation of a physical barrier to infiltration T cells by utilization of fibroblasts and stromal cells in the tumor microenvironment; promoting upregulation of pro-apoptotic and immunosuppressive cytokines, ligands, and receptors to inhibit cellular mediators of cytotoxic response; and drafting and recruiting of immunosuppressive cells from the microenvironment and circulation to further inhibit effector cells of the immune system (234, 290).

The vast mutational landscape and heterogeneity of tumors between patients, and even between different areas of the same tumor, make personalized medicine targeting specific gene mutations challenging. Given the potent immunosuppressive capacity of tumor cells, infiltrated suppressive immune cells, the surrounding tumor stroma, and the propensity of immune cells to respond to cancer antigens in similar ways across patients, targeting the suppressive pathways tumors co-opt for self-promotion presents an attractive intervention for cancer treatment. Many investigators have pointed their therapeutic inquiries towards T cells as the major effector cells of the tumor response, developing antibodies that can antagonize suppressive molecules and cells, cytokines and molecules to agonize T cell effector functions and reverse
tolerance and anergy, or adoptively transferring activated cells into the tumor environment itself (291). Others have taken on a more holistic approach in targeting cells or pathways of the innate immune response that enable the efficacy of the adaptive response (292).

This section aims to provide current knowledge on immune therapies utilized in the treatment of cancer, including immune checkpoint blockade, stimulators of the innate immune response, and efforts to combine therapies directed at components of the innate and adaptive immune arms. Studies discussed will be broadly in multiple tumor models, but with special attention to those currently used for bladder cancer.

1.3.1: Therapeutic Targeting of Immune Checkpoints

Inhibitory checkpoints. Immune checkpoints are biologically important for maintaining self-tolerance and limiting bystander tissue damage during the non-specific innate immune response and hyper-activated adaptive immune response (293). However, these anti-inflammatory processes can be commandeered by tumors to evade immune-mediated destruction. In the treatment of cancer, immune checkpoint blockade removes these inhibitor signals to NK, macrophage, and T cell (effector cell) activation and function, enabling antitumor immune cells to overcome regulatory mechanisms established by tumors, and eliminate them (294). Inhibitory checkpoints like CTLA-4, the PD-1/PD-L1 axis, TIM-3, and LAG-3 all have different mechanisms to make immune cells, particularly T cells, quiescent, and therefore different therapies have been developed to address them.
CTLA-4, as discussed previously, has very similar homology to CD28, and therefore shares the same ligands CD80 (B7.1) and CD86 (B7.2), necessary for APC-mediated co-stimulation and activation amplification of T cells (295). However, CTLA-4 has over 100x more affinity for both CD80 and CD86, and its engagement with either ligand down-modulates the amplitude of T cell responses, typically at the site of T cell priming in secondary lymphoid organs (261, 296, 297). Expression of CTLA-4 is upregulated upon T cell activation on the cell surface and at the immunological synapse (298), and is thought to be more predominantly expressed on CD4 T helper cells, insinuating that heightened T cell responses seen with CTLA-4 inhibition are due to promoted activity of CD4 T cells on other immune subsets (CD8 T cells) (299). CTLA-4 expressed on Tregs also has a role in attenuating the effector T cell response (300). Therefore, blockade of CTLA-4 with inhibitory antibodies has been shown to enhance tumor rejection by inhibiting Treg function and Treg killing (301, 302), enhance CD28 co-stimulation and T cell activation, and thus expansion of tumor antigen-specific CD8 T cells (303). It does this by blocking CTLA-4 competition for costimulatory ligands by antagonistic antibody binding to the interaction domain on B7 (304). Anti-CTLA4 therapy also leads to the expansion of effector CD4 populations (305), as well as exhausted CD8 populations (306). In humans, anti-CTLA-4 therapy with ipilimumab (307) antibody produced a 3-year survival rate of 21% in metastatic melanoma patients (308), and similarly a 5-year survival rate of 20% was observed patients treated with tremelimumab (anti-CTLA-4 mAb) (309). Unfortunately, as anti-CLTA-4 therapy is effectively lowering the activation threshold and taking the brakes off of effector T cells, many patients experience immune-related adverse events while on therapy (310). This
is also potentially due to CTLA4 blockade-associated TCR repertoire broadening which increases T cell functional reactivity (311).

The PD-1/PD-L1/PD-L2 axis serves primarily to maintain tolerance and dampen T cell responses in the periphery (312), and similar to CTLA-4, its expression as both receptor and ligands is induced upon B and T cell activation, and as a byproduct of the immune response on many cell types in the TME (313). PD-L1 and PD-L2 are widely expressed on non-lymphoid tissues and actively upregulated in response to inflammatory cytokines like IFNs (314). Unlike CTLA-4, PD-1 signaling directly interferes with the TCR signaling cascade to regulate T cell activation (315). Persistent PD-1 signaling also induces metabolic restriction, inhibiting glycolysis while simultaneously promoting fatty-acid oxidation (FAO) and lipid catabolism, perpetrating T cell exhaustion (316, 317). Blockade of PD-1 signaling can reinvigorate effector cells and antigen-specific T cells by preventing the attenuation of TCR signaling, effectively jump-starting exhausted T cells to proliferate (318). It has also recently been shown to reverse negative metabolic reprogramming induced by PD-1 signaling (319).

Because of the propensity of PD-1 ligands expressed in tumor tissue and by immune cells of the tumor stroma, it has been suggested that the effectiveness of PD-1 therapy is reliant on the bulk of effector cells to be already present in the TME (320). Early large scale clinical trials with anti-PD-1 antibody Nivolumab have shown successful antitumor responses in patients, with objective response rates of 17%, 27%, and 31% in NSCLC, RCC, and melanoma, respectively. However, exhausted T cells have a distinct epigenetic profile that can limit T cell reinvigoration, and thus PD-1 blockade may not be sufficient to functionally restore T cells once they meet a certain exhaustion threshold (321, 322).
Other inhibitory checkpoints are currently being explored preclinically and in clinical trials as the “next generation” of checkpoint blockade therapy. Lymphocyte activation gene 3 (LAG-3) is highly homologous to the CD4 T cell co-receptor, and as such its ligand is the MHC class II molecule (323); however new work has found a potential additional ligand as LSECtin (324). As previously mentioned, LAG-3 is expressed on T cells, B cells, NK cells, and DCs in response to activation, and serves as a negative regulator of T cell expansion and DC activation as it competitively binds with MHC II (325, 326). T cell immunoglobulin and mucin-3 (TIM-3) is marker for exhaustion in combination with other checkpoints, expressed on activated T cells, NK cells, Tregs, DCs, and monocytes, and negatively regulates Th1 type immunity. TIM-3 functionally binds to galectin-9 (265), PtdSer (327), HMGB1 (328), and CEACAM-1 (329) to promote immune cell dysfunction and apoptosis by negatively regulating TCR signaling, and other mechanisms not fully clarified (330, 331). VISTA, B7-H3, and TIGIT all represent other recently identified immune inhibitory receptors that negatively regulate T cell activity, and are currently being investigated further to understand their mechanisms of action (299).

**Co-stimulatory checkpoints.** Immune activation is mostly regulated by two major receptor families: the immunoglobulin-like (Ig) superfamily, consisting of co-stimulatory receptors CD28 and inducible T cell co-stimulator (ICOS), and the TNFR superfamily, consisting of co-stimulatory receptors OX40, CD27, 4-1BB, CD40, and GITR (332, 333). A general theme in the function of these stimulatory receptors, and potential antibodies that may be used to agonize them therapeutically, is that the resulting effects will be 1) activation of APCs, 2)
reduction in Treg suppressive activity and 3) co-stimulation of CD4 and CD8 T cells and NK cells, based on the cells that express the receptor (334).

The Ig superfamily comprises receptors that express a variable immunoglobulin-like domain that binds to cognate ligands expressed on APCs; for ICOS, the ligand is B7H (335). ICOS is upregulated on activated T cells, B cells, and ILC2 cells and serves to enhance type I and II immune responses, T_{reg} maintenance, T_{FH} differentiation (294). It regulates the production of IL-4 (336), antibody isotype switching (337). ICOS signals through PI3K/AKT signaling and also enhances calcium signaling (PLC_{γ}) (338), suggesting it has a role in cellular metabolism, protein translation, and apoptosis (339). In preclinical studies, agonist antibodies to ICOS have shown antitumor potency and activation of effector immune responses similar to antagonistic blockade of its other Ig superfamily inhibitory members (340). However, antagonist antibodies to ICOS also show positive effects in dampening T_{reg} functions (340). Studies are ongoing, but much more needs to be learned about the dual effects of ICOS and its ligand to better understand its therapeutic use in patients.

The TNFR family members are appealing candidates for targeted therapies, with the greatest attention being laid on OX40 and 4-1BB. 4-1BB (CD137) is an enhancer of T cell co-stimulation, through signaling of TRAF1 and TRAF2 (341). 4-1BB or its ligand 4-1BBL are expressed on activated T cells, NK cells, monocytes, DCs, and B cells. Ligation on T cells results in upregulation of anti-apoptotic genes and protection from activation induced death, promoting the differentiation of memory T cells (342). In preclinical trials, agonistic 4-1BB antibody enhances antitumor T cell responses and enables tumor rejection (343),
however in clinical studies in patients, there is evidence of serious immune related adverse events, and so dosing, timing schedules, and combination approaches are still under investigation (344). OX40 is present on activated T cells, Tregs, NK cells, NKT cells, and neutrophils, and its ligand is expressed on APCs and T cells as well. Its ligation has been shown to inhibit Treg suppression, and sustain and enhance CD4 T cell responses, as well as CD4 and CD8 T cell survival and memory generation (345, 346). It regulates survival signaling though Bcl-2/Bcl-xL and also enhances PI3K/AKT signaling (347). Similar to 4-1BB, preclinically OX40 agonist antibodies increase anti-tumor activity (348), and DCs with enhanced OX40 expression can enhance tumor rejection in a CD8, CD4, and NK T cell dependent manner (349). Therapeutic agonism of OX40 in patients has not been completed on a large scale, but it has been shown to induce proliferation of effector T cells and augment tumor-immune responses (350).

GITR, CD27, and CD40 and their ligands are expressed on T cells, B cells, NK cells, APCs upon activation, and through their own signaling schemes likewise are important for promoting T cell and NK cell activation and proliferation, humoral immune response (CD40), APC maturation, inhibiting Treg function (GITR), and generating T cell memory (CD27) (351-354). While proving to be exciting new targets in the field of cancer therapy, the fundamental biology of these molecules remains underdeveloped, and is being outpaced by clinical investigations, so there is much still to be learned regarding their function and signaling paths.

1.3.2: Innate Immune Stimulation
**Toll-Like Receptor Agonists.** Immune checkpoint blockade and adoptive cell transfer of antigen-specific T cells have owed their therapeutic success to the improved function, recruitment, and activation of T cells, or more broadly, the adaptive immune response. Historically, the best responders to these therapies are patients with tumor subtypes that are highly CD8 T cell infiltrated, have chemokine signatures and other indicators of chronic inflammation, have high mutational burdens, and typically include high expression of immune inhibitory factors such as PD-L1, IDO, or Tregs, among others. However, macrophages, DCs, NK cells, and other cells of the innate immune system, via their roles in antigen recognition, presentation, T cell co-stimulation and direct tumor cell killing, are essential for the initiation, maintenance and programming of antitumor immune reactions, making them attractive targets for therapeutic exploitation. Toll-like receptor stimulation, as one of the best defined PRR pathways, can serve as a key activator of an antitumor response, aimed at waking up the host immune response when spontaneous T cell priming has not occurred (292).

Down regulation of antigen expression and suppression of APC maturation and priming are some strategies that tumors have cultivated in order to evade innate immune recognition. As previously discussed under the innate immune response, TLRs expressed on immune cells are type I transmembrane proteins that have fundamental roles in the detection of diverse microbial signatures. TLRs 2, 4, 5, 6 detect proteins, peptidoglycans, lipids (LPS), and bacterial flagellin extracellularly, while TLRs 3, 7, 8, 9 detect single-stranded or double-stranded RNA, and CpG modification of DNA residues (CpG-ODN) intracellularly (Table 1). Several ligands agonizing theses TLRs have already been approved, and used in cancer therapy: TLR2/4 agonist BCG, TLR4 agonist
monophosphoryl lipid A (MPL), and TLR7 agonist imiquimod (355-357). Others such as TLR5 agonist Entolimod, and TLR7 agonist 852A are still being investigated in preclinical and clinical trials (358, 359).

Focusing more on the Th1 immunity-inducing TLRs, nucleic acid sensing TLRs (3,7,8,9) are relevant to cancer detection as they can recognize DAMPs from cellular debris of necrotic or dying tumor cells (360). Upon ligand binding, these TLRs induce signaling cascades through NF-kB and interferon regulatory factors (IRFs) to promote the transcription of inflammatory cytokines and IFNs-I (361). TLR7 agonist imiquimod has shown antitumor effects in basal cell carcinoma, with more limited activity in melanoma and breast tumors (356, 362). It is believed to aid in recruitment of tumor-infiltrating plasmacytoid DCs (pDCs) and macrophages by cytokines TNFα, IL-12, and IFNα, leading to further infiltration of helper T cells (363). Imiquimod is being further tested for use in noninvasive bladder cancer (364) and as an adjuvant to cancer vaccines in several solid tumors (365). Resiquimod, a dual TLR7/8 agonist, has been shown to more potently induce cytokine expression than TLR7 therapy alone (366).

Clinical studies in skin tumors have shown improved recruitment of effector T cells, antigen-specific CD4 T cell responses, and tumor cell elimination (367, 368). TLR9 ligation by unmethylated cytosine-guanosine (CpG) DNA induces type I IFN production, activating DCs, NK cells, and tumor-specific CD8 T cells and generating tumor regression (369). CpG ODNs (oligonucleotides) are synthetic agonists of TLR9 which are most actively being explored in solid and hematologic cancers as both monotherapies and adjuvants (370). CpG ODN and TLR9 agonist SD-101 has demonstrated the ability to overcome tumor resistance
to checkpoint blockade therapy and increase effector T cell infiltration in preclinical studies (371); clinical trials in humans are ongoing. TLR3 stimulation by recognition of double-stranded RNA dsRNA induces the secretion of type I IFNs, similar to TLR9, but also can lead to direct activation of apoptosis of tumor cells (372). In addition to stimulating TLR3, is activates RLR sensing, instigating a two-pathway production of IFN-1 (373). Poly(I:C) is a synthetic dsRNA that is being explored preclinically and clinically in the treatment of many solid cancers, including bladder cancer (373, 374). Poly(I:C), and derivatives of it have been shown to inhibit tumor growth and promote tumor infiltration of activated immune cells (375-377), however most of its uses and successes in patients are as an adjuvant therapy (378).

Though not a TLR, STING PRR agonists are also being exploited in cancer therapy. To review, like TLR9, cytosolic enzyme cyclic GMP-AMP synthase (cGAS) senses foreign DNA within the cell cytosol and synthesizes dinucleotide cyclic GMP-AMP (cGAMP) (379). cGAMP is then able to bind and activate STING, initiating pro-inflammatory signaling that induces IFN-1 transcription and NF-kB mediated production of IL-6, IL-15, TNF, and IL-1β (380-382). STING signaling is important for the generation of tumor-specific CD8 T cell responses and tumor regression (383). STING agonistic cyclic dinucleotides such as cyclic di-GMP and 2’3’-cGAMP are utilized in therapeutic investigation because of their affinity, stability, and specificity for STING (384). Preclinical studies have found that STING agonists can suppress cancer metastasis, increase CD4 and CD8 T cell recruitment, and stimulate IL-12 production by MDSCs, furthering the activation of inflammatory cells (385, 386). Initial clinical
studies of STING agonist (DMXAA) in humans proved unsuccessful due to poor CDN binding to STING (387), but new agonist structures are currently being investigated in clinical trials alone and in combination with another immunotherapy.

Recent studies have identified TLR expression on cancer cells themselves, linking their expression with diseases progression, metastasis, and shortened survival (388, 389). The potential dual agonistic and antagonistic role in cancer inhibition or progression, as well as their potential serious adverse effects, including cytokine storm, have drawn caution to the use of TLR stimulants in immunotherapy (390). Despite some hesitancy, the use of TLR agonists has shown definitive antitumor benefits by activating immune cells in the TME, and inducing the expression of pro-inflammatory cytokines that facilitate immune infiltration and inhibit oncogenic signaling in the tumor. In many cancers, though TLR agonists show efficacy, more work needs to be performed to better define their mechanisms of action to ensure confidence for their use in humans.

**Cytokine therapy: Interferon-alpha.** Interferons have a pleiotropic role in the stimulation of antitumor immunity. As previously mentioned, almost all cells are capable of producing and responding to IFN-I, however pDCs are able to secrete higher levels of type I IFN than any other cell type. Often, type I IFN induction is the product of stimulation of TLR, RLR, or STING signaling pathways. Activation of IFN receptors leads to a multifaceted response including but not limited to: promotion of NK cell function (391), support for DC maturation, migration, and antigen presentation/priming to activate T cells (392), stimulating chemokine production for immune recruitment (118-120), and direct cell killing through
TRAIL-mediated apoptosis (393); but counter-productively, it can induce immune suppressive enzymes such as IDO (394), and increase expression of PD-L1 on stromal and cancer cells (395), contributing to tumor-immune evasion. Preclinically, IFN-I production has been shown to be critical for CD8α+ DC cross-presentation to CD8 T cells, generating antigen-specific immunity and tumor rejection (396, 397). Exogenous IFNα and IFNβ delivery to tumors by either association with transferred monocytes or conjugation to antibodies resulted in impeded growth and metastasis (398, 399). There is also evidence that the production or delivery of IFN-I can help mitigate tumor-induced suppressive immune cells (400), thereby overcoming some mechanisms of immune avoidance. Although the process of immunoediting, in which tumors cycle through elimination, equilibrium, and evasion with the immune response, can produce mechanisms of resistance to immune-mediated tumor eradication, type I IFNs intervene in all of these phases (401, 402).

In bladder cancer specifically, IFNα has been investigated as a salvage therapy in NMIBC after BCG failure due to its anti-proliferative activity on tumor cells. Both IFNα and IFNβ have also shown to inhibit tumor growth by inhibiting angiogenesis and expression of VEGF and basic fibroblast growth factor (FGF) in human xenograft orthotopic mouse models of bladder cancer (403-405). However, when using recombinant IFN protein in experimental strategies, antitumor-related results were not durable due to unsustainable IFNα levels (406). To overcome these limitations, intravesical gene delivery of IFNα through the use of adenoviral encoding IFNα (Ad-IFNα/Syn3, i.e. Instiladrin) was developed (407), and early clinical trials have shown its safety and efficacy in
treating BCG Unresponsive NMIBC (66, 67, 408). Clinical studies evaluating the use of intravesical Ad-IFNα/Syn3 in the bladder are ongoing. IFNs-I direct and indirect effects on tumor cells, the TME, and the immune system have been described broadly in multiple cancer models. Because IFNα’s mechanisms are diverse, its precise role in the immune response to cancer has only begun to be understood in bladder cancer, and they could be distinct between tumor types. Elucidating IFNα’s immune-mediated antitumor mechanism in bladder cancer is therefore critical for interpreting patient response, identifying effective combination therapies, and improving the treatment of bladder cancer.

1.3.3: Current Status of the Therapeutic Utility of Single and Combination Immune Therapy

**Immune checkpoints.** Remarkable advances in immunotherapy treatment for cancer have occurred in recent years. Approaches aimed at co-activating different tumor inhibitory pathways with immunotherapy, radiotherapy, targeted molecular therapy, and chemotherapy are widely used. Most immune checkpoints are non-redundant, leaving the possibility of combination checkpoint blockade, or antagonist/agonist approaches open. Based on understanding of how CTLA-4 and PD-1 act to attenuate T cell activity (activation and effector function, respectively), it is believed that anti-CTLA-4 and anti-PD-1 therapies can act at different stages of the cancer-immune response (87). Single agent checkpoint inhibitors have seen successful activity in advanced and metastatic malignancies, as discussed previously, with roughly 20% survival rates (anti-
CTLA-4) and 30% response rates (anti-PD-1) in patients with melanoma, non-small cell lung cancer, and renal carcinoma (308, 409-412). However, treatment of patients with combination checkpoint inhibitors produced longer median progression-free survival (PFR) rates than either therapy alone (413, 414). Other inhibitors and stimulators of checkpoints, notably TIM-3, LAG-3, ICOS, 4-1BB, and OX40 among others, are currently being investigated as single agents, and in combination with anti-PD-1/PD-L1/CTLA-4 therapies (415). Progress that has been achieved through monotherapies is notable, however certain aspects of the TME may limit tumor responses, for example by upregulation of additional checkpoint molecules to limit single agent treatment efficacy (294). Targeting immune checkpoints improves patient median survival and also can provide long-term durable responses, and combinatory immunotherapies may lead the pack in increasing the number of patients who continue to see clinical benefit over time.

**Innate immune stimulators.** For poorly immune-infiltrated tumors, providing inflammatory signals to facilitate recruitment of activated effector cells is beneficial in fighting tumor progression. This can be seen with single agent recombinant viral vector therapy, exogenous cytokines, and TLR agonists, used to incite chemokine production and an inflammatory response (416). However, therapeutic potential of PRR agonists have focused on their adjuvant use in activating an immune response, and most successes in cancer have come in combination therapy with checkpoint inhibition, adoptive cell transfer, or cancer vaccines, rather than as a single agent. Treatment with exogenous cytokines can promote cytotoxic activity of effector CD8 T cells and NK cells, and differentiation of CD4 T cells into T helpers; however, some cytokines can expand T_{reg
populations and promote nonspecific activation of immune cells with associated toxicities (417). To limit these adverse effects, current clinical trials are investigating modified inflammatory cytokines like IFN-1, IL-15, and IL-2 in combination with checkpoint inhibitors, utilizing more targeted delivery tactics rather than systemic approaches. Immunotherapeutic strategies that simultaneously target the innate and adaptive immune response are suggested to reduce immune tolerance, and are effective in eliminating large tumors (418, 419). The combination of TLR agonists and checkpoint blockade is thought to augment T cell activation and to potentially overcome resistance to checkpoint blockade by priming APCs to enhance the adaptive immune component (361). Indeed, combination of TLR9 agonist CMP-001 with anti-PD-1 Ab pembrolizumab was reported to reverse PD-1 inhibition resistance with no maximum tolerated dose (420). Clinical studies of the combination of TLR agonists with checkpoint inhibitors have also shown increased levels of APCs in the TME and suppression of head and neck cancers (421). Many trials are ongoing with the combined use of TLR3, 4, 7, 8, 9 and STING agonists with anti-PD-1 antibody, anti-CTLA4 antibody, or a combination of all three (361, 377). To move forward in improving patient responses, care providers need to have an arsenal of weapons to meet cancer at every turn. Mechanistically speaking, the ability to use an innate stimulator to jump start a lagging immune response but coincidentally may increase tumor evasion mechanisms, followed by an adaptive targeted therapy like checkpoint inhibition to combat the Stimulator’s induced evasion is akin to cutting off cancer’s support legs.

1.3.4: Summary
Humans are equipped with the exquisite defensive tools of the immune system to fight off pathogens, cellular dysregulations, and cancer. Despite our intrinsic innate and adaptive mechanisms, malignancies like cancer have evolved strategies to avoid our immune system and continue surviving. Therapeutic intervention with drugs designed to reverse immune-suppressive mechanisms has proved to be a successful venture in tumor inhibition. Many therapies antagonizing immune inhibitory checkpoints, or agonizing intrinsic innate immune activation or stimulatory checkpoints are either approved or being investigated for use in patients today. It’s important to keep in mind, as these therapies are designed to increase effector cell responsiveness and inhibit suppressive mechanisms that adverse reactions and tissue damage due to highly stimulated cytotoxic cells will occur. Ultimately, the balance of harm versus benefit must be constantly monitored to ensure patient safety, treatment efficacy, and clinical ethics.

For bladder cancer, BCG therapy in NMIBC already capitalizes on TLR stimulation to produce antitumor efficacy, though there is room for improvement for patients who exhibit disease progression, recurrence or no response to BCG. For more aggressive tumors, the approved use of checkpoint inhibitors in bladder cancer has demonstrated improved overall response rates for locally advanced and metastatic disease, and is continually being studied in combination settings (422-424). The use of Ad-IFNα therapy for BCG Unresponsive patients may be able to fill the need of an alternative immune stimulatory treatment, inducing TLR recognition and endogenous IFN-I production, to control and treat NMIBC. However, one of the qualms of BCG is the incomplete understanding of its
mechanism of action; as IFN$\alpha$ is a pleotropic cytokine, elucidating its specific immune-mediated antitumor mechanism is therefore critical for interpreting patient response, identifying effective combination therapies, and improving the treatment of bladder cancer.

I hypothesized that IFN$\alpha$ specifically induced recruitment and activation of immune cells into NMIBC tumors, leading to more robust antitumor responses alone and in combination with checkpoint blockade therapy. First looking at patients to determine the validity of these questions, I found that treatment with Ad-IFN$\alpha$ therapy induced expression of T cell markers and checkpoint markers within NMIBC tumors, and a plethora of inflammatory cytokines compared to before treatment. This finding encouraged further investigation into the MB49 and BBN murine bladder cancer models to determine which immune cells were most crucial in the IFN$\alpha$-mediated antitumor response, and if and how they were recruited to the tumor. The patient data also encouraged finding the utility of combination immunotherapy with IFN$\alpha$ and checkpoint blockade (anti-PD-1) treatment on mouse bladder cancer tumors, to see if there were synergistic effects on tumor inhibition and survival. The results are detailed in the next chapters.
Chapter 2: Effects of Ad-IFNα Therapy in Patients with NMIBC

This work is based upon “Inhibition of urothelial carcinoma through targeted type I interferon-mediated immune activation” by Plote, D, Choi, W, Mokkapati M, Sundi, D, Ferguson, J, Duplisea, J, Parker, N, Yla-Herttuala, S, McConkey, D, Schluns, K, Dinney, C. 2019. Oncoimmunology; presented with permission from Oncoimmunology.

2.1: Introduction

Non-muscle-invasive bladder cancer (NMIBC) comprises ~70% of diagnosed urothelial carcinomas (UC) (9). Although not immediately life-threatening, they have a propensity to recur and progress. Intravesical therapy, mainly in the form of Bacillus Calmette-Guerin (BCG), is administered to prevent recurrence, delay progression, and provide for bladder preservation to avoid the quality of life issues that accompany radical cystectomy (9, 425). Despite its success as frontline immunotherapy, not all patients respond to BCG, and of those who do respond, over half will relapse with BCG Unresponsive NMIBC (68, 69, 426). Unfortunately, no effective second-line therapy for BCG Unresponsive NMIBC exists (68-70, 425). Interferon alpha (IFNα) is a pleiotropic cytokine that inhibits tumor growth directly as well as indirectly through activation of the immune system. These multifaceted anti-tumor properties make IFNα a promising alternative therapy for UC. IFNα monotherapy for NMIBC was previously studied demonstrating good tolerability and dose-related clinical
effectiveness following BCG failure; however, its response durability was insufficient (406). With standard intravesical therapy, patients are unable to retain the instilled cytokine for more than 1- to 2 hours, limiting local tumor exposure. To overcome these limitations, intravesical gene delivery of IFNα through the use of adenoviral encoding IFNα (Ad-IFNα/Syn3, i.e. Instiladrin) was developed and early clinical trials have shown its safety and efficacy in treating BCG Unresponsive NMIBC (66, 67, 408). Clinical studies evaluating the use of intravesical Ad-IFNα/Syn3 in the bladder are ongoing (66, 67, 408).

Despite the clinical efficacy that has been achieved with Ad-IFNα/Syn3 in BCG unresponsive patients, its mechanisms of action are still not well defined in UC. It has been previously demonstrated that IFNα gene therapy inhibits the growth of human tumor xenografts by an anti-angiogenic effect and tumor necrosis factor-related apoptosis ligand (TRAIL)-mediated cytotoxicity (393, 403-405, 407). However, since these previous preclinical data were generated in studies of nude mice, the immune mechanisms underlying the anti-tumor activity of IFNα in UC have not been elucidated. It has been well described that a type I IFN response indirectly induces a cascade of inflammatory cytokines and chemokines to encourage recruitment of effector immune cells to the site of host distress (115). It also directly stimulates immune cells that can then further activate the adaptive cellular response, including memory CD8 T cells. I hypothesize that Ad-IFNα/Syn3 utilizes this induction of cytokines and increased immune cell recruitment and tumor infiltration to bestow its antitumor effects in patients with NMIBC.

In this portion of the study, I sought to identify what changes occurred in patients and their tumors before and after treatment with Ad-IFNα/Syn3. I wanted
to determine if patient tumors were more highly infiltrated with effector cells after treatment, and if there was a correlated relationship with the presence of inflammatory cytokines. Using RNA from eight Phase I Trial patients biopsied before and after treatment with Ad-IFNα/Syn3, I found that only 2/8 patients showed robust increases in expression of several T cell related genes, though four other patients displayed increase in at least one T cell marker gene. This response rate was further confirmed by IHC staining of the same patient specimens, in that 1/5 patients with banked tissue samples showed an increase in CD3+ cells following treatment with Ad-IFNα/Syn3. Utilizing urine samples from 39 Phase II Trial patients, I identified if treatment with Ad-IFNα/Syn3 affected the expression of inflammatory cytokines and chemokines present in the urine, as a marker of the immune response, and if the presence of these cytokines was correlated with patient response to therapy, or clinical response (CR). I found that compared to pre-treatment samples, Ad-IFNα/Syn3 significantly increased the urinary levels of IFN and CXCL10, while also increasing TRAIL, CCL2, and IL-6 four days after instillation with Ad-IFNα/Syn3. The increase in urinary IL-6 was also correlated with patient CR.

The potential for a non-invasive biomarker detection of bladder cancer, and treatment response is a vigorously sought-after discovery. Urine is already used in urological practice for bladder cancer screening, as a secondary measure of malignant cell presence (cytology) (5). Utilizing urine cellular and molecular analysis as a predictive or prognostic marker of patient response to therapy represents an ideal non-invasive procedure for both patients and physicians. Previous studies have demonstrated that cytokines levels in urine can be used to determine response to BCG therapy, experimentally (82);
however, there is no definitive evidence of cellular biomarkers of therapeutic response in bladder cancer, or as a measure of response to Ad-IFNα/Syn3. I investigated the potential use of flow cytometry on urine samples to identify therapeutically-driven changes in both immune cell populations, and epithelial cell populations. Preliminary results revealed that it is possible to identify T cell populations and epithelial population in urine by flow cytometry, and that further investigation into more functional markers is possible. Utilizing CyTOF analysis may also prove to be a greater biomarker identification tool for future analysis of patient samples.

2.2: Results

2.2.1: Ad-IFNα Therapy in BCG-Unresponsive NMIBC Patients Induces an IFN-I Response in the Bladder and Increases Expression of T cell and Checkpoint Markers

Because our previous preclinical data and Phase I trials has provided evidence that localized, sustained IFN could be therapeutically beneficial to BCG-Unresponsive NMIBC patients (66, 407, 408), a Phase II trial with intravesical Ad-IFNα/Syn3 was conducted in 39 patients (67). To confirm that localized Ad-IFNα/Syn3 treatment induced a sustained IFN-I phenotype, we measured cytokine concentrations in patient urines. Ad-IFNα/Syn3 instilled on Day 1 significantly increased urine levels of IFNα2, CXCL10 on Day 4, with additional increasing trends in TRAIL, CCL2, IL-6, and G-CSF on Day 4. Significant increases in IFNα2, CXCL10 were still present by Day 12 (Figure 4A).

Interestingly, the correlation of increased urinary IFNα2 and IL-6 levels from
Day4:Day1 (D4:D1) was significant for 13 patients who exhibited a complete response (CR) to Ad-IFNα/Syn3 therapy, and not significant for patients deemed “non-responders” (NR, 26 of 39 patients) (Figure 4B). There was also positive correlation for increased G-CSF levels in relation to increased IFNα2 and CR at Day 4, but it did not reach statistical significance (Figure 4B). There was no positive correlation with any other cytokine from Day12:Day1 (D12:D1) in relation to increased IFNα2 and CR (Figure 5). Interestingly, there are significant positive correlations of IFNα2 level vs. CXCL10 and TRAIL on D4:D1 for 26 of 26 patients who did not achieve CR, and IFNα2 vs. CXCL10, CCL2, and IL-6 on D12:D1 from 24 of 26 of these patients (Figure 5). This may be related to a prolonged inflammatory response that may have deleterious effects on the patient and tumor as noted in other tumor models (427), but is an area for further investigation in NMIBC. Whole transcriptome RNAseq was conducted with matched pre-treatment and post-treatment tissue specimens from 8 patients with BCG-unresponsive NMIBC, treated with Ad-IFNα/Syn3 in the Phase I trials (66, 408). Gene expression of PD-L1, CTLA-4, and several T cell markers were markedly increased in two of eight (25%) matched tumor pairs following treatment with Ad-IFNα/Syn3 (Figure 4C). Less dramatic upregulation of one or more immune biomarkers was evident in 4 of the 6 additional tumors. In addition, histology sections from 5 of the 8 tissue samples were also stained for CD3+ T cells. IHC analysis of these tumors showed an increase in CD3 T cells, localized in the tumor stroma, after treatment with Ad-IFNα/Syn3 in 1 of 5 samples (Figure 4D), and undetectable changes in the CD3+ populations in the other 4 samples (data not shown), exemplifying IFNα’s ability to enhance intratumoral T cells with variability in patients.
More thorough molecular analysis of the 8 matched pre- and post- Ad-IFNα/Syn3 treated patient tumor specimens from Figure 4C showed similar trends to the Phase II urine samples of cytokine levels, as well as other published knowledge of IFN-1 induced gene signaling (Figure 6). Many of the IFN response and immune cell mediated cytotoxicity genes such as *CXCL10*, *CCL5*, *CCL4*, *CCL2*, *PRF1*, *CD8A*, and *NFATC1* were increased in at least 4 of the 8 patient samples post- Ad-IFNα, mimicking characteristics of a Th1-type immune response. These results are also seen in the MB49 mouse tumor model, and will be discussed more in Chapter 4. Mirroring upregulation seen in urine samples, 4 of 8 patients also had an increase in *IL-6* gene expression. Interestingly, there was decreased tumor expression of genes related to fatty acid catabolism (*FASN*, *ACLY*, *ACACA*), amino acid transport, and *VEGFA* post-treatment, potentially indicating a role for IFN to decrease metabolic pathways in tumor inhibition. However, despite the heterogeneity of tumor response, none of the 8 patients analyzed achieved CR at 12 months.
Figure 4: Effects of intravesical Ad-IFNα/Syn3 therapy on T cells and immune biomarkers in patients. A) Log$_2$ observed concentration (Day 1 pre-Ad-IFNα therapy [D1], Day 4 post- Ad-IFNα therapy [D4] or Day 12 post- [D12]) of levels for cytokines indicated. Significant p-value (one way ANOVA, multiple
comparisons) **p<0.01 and ***p<0.001 comparing D1:D4 and D1:D12 (Error bars: mean ± SEM; n=39). B) Spearman correlation between log$_2$ expression of IFNα2 levels and respective cytokine indicated from ratio of Day 4 post Ad-IFNα/Syn3 to Day 1 pre-treatment in 39 patient urines. Rank coefficient $r > 0.5$ indicates a positive correlation with IFNα2. Yellow boxes indicate significant p value (Two tailed) *p<0.05, ***p<0.001. CR=Complete Response patients; NR=Non-Responder patients. C) RNA from macrodissected matched tumors collected before or after Ad-IFNα therapy was analyzed by whole transcriptome RNAseq (Ion Torrent Ampliseq platform). Top panel: ratio of gene expression in posttreatment to pretreatment specimens. Note: gene expression increased significantly in two of the eight tumor pairs. Red = increased expression, green = decreased expression. Bottom panel: heat map displaying differential gene expression in each tumor pair. D) Immunohistochemistry staining of CD3+ cells in a patient tumor (Tumor 1 (C)), pre- and 3 months post-treatment with one dose of Ad-IFNα/Syn3. Scale bar = 200 µm.
Figure 5: Correlation of inflammatory cytokines measured in patient urine

12 days after Ad-IFNα/Syn3 treatment. A) Spearman correlation between log₂ expression of IFNα2 levels and respective cytokine indicated from ratio of Day 12 post Ad-IFNα/Syn3 to Day 1 pre-treatment in 39 patient urines. Rank coefficient r > 0.5 indicates a positive correlation with IFNα2. Yellow boxes indicate significant p value (Two tailed) *p<0.05, ***p<0.001. CR=Complete Response patients; NR=Non-Responent patients.
Figure 6: Ad-IFNα/Syn3 treatment increases gene expression associated with Th1 type anti-tumor immunity and decreases expression of metabolic markers. Heatmap illustrating normalized (log$_2$) gene expression patterns from patients treated with Ad-IFNα/Syn3. RNA was isolated from FFPE tumor specimens from Phase I and Ib trials of Instiladrin (Ad-IFNα/Syn3). Whole transcriptome RNA sequencing was performed using the Ion Torrent AmpliseqRNA platform analyzed using AmpliSeqRNA plugin with the Torrent Suite Software, and visualized with the Broad Institute’s Morpheus software. Samples were hierarchical clustered according to averaged one minus pearson correlation.
2.2.2: Utilizing Urine as a Non-invasive Diagnostic and Prognostic Resource in the Analysis of the UC Immune and Epithelial Landscape after treatment with Ad-IFNα Therapy

Previous work in UC has utilized urine as a biomarker medium for identifying NMIBC patient responses to BCG therapy, but it was focused on voided levels of inflammatory cytokines rather than measuring changes in the intra-bladder cell populations (82). Recent work has identified the use of urine-derived lymphocytes (UDLs) as a liquid biopsy tool for mapping the TME and identifying patients with actionable targets, like high PD-1 expression, in cases of MIBC (428). To this end, I hypothesized that similar strategies from these studies could be applied for NMIBC patients treated with Ad-IFNα: to utilize flow cytometry to identify immune cells and epithelial cells in voided urine, with the goal of identifying markers of patient response to therapy, and the potential identification of candidates for combination therapy strategies. Using urine voided from patients about to undergo standard TURBT for preliminary investigation, I found that viable immune cells and epithelial cells could be analyzed (Figure 7A). As expected, cytokeratin+ cells expressed higher levels of PD-L1 than CD45+ cells (Figure 7B), following the results seen by many other groups. Contrary to the T cell focused study in MIBC, this flow analysis showed that myeloid cell populations as well as lymphoid cells could be easily distinguished within CD45+ cells (Figure 7C), which is relevant to the mechanisms of action of IFN-I. Interestingly, of the lymphocyte (SSC/FSC) population, roughly 50% were TCRαβ+CD8+, indicating a large proportion of CD4 T cells, with only about 20% of CD8+ lymphocytes by comparison (Figure 7D). These results serve as a proof of
concept that urine can be used to measure the immune response in NMIBC patients. Further investigation into identifying functional aspects of these urinary cells, like activation status and checkpoint expression, is being performed using CyTOF analysis in order to develop a more comprehensive diagnostic protocol for liquid biopsy and detection of patient response.
Figure 7: Patient urine can be utilized to prospectively identify immune and epithelial responses to IFN therapy. Analysis of one representative urine sample from a patient about to undergo routine TURBT. Flow cytometry plots depicting A) percentage of CD45+ immune cells versus pan-Cytokeratin positive epithelial cells, which were more PD-L1 positive than the CD45+ cells (B). C) Forward scatter and side scatter immune cell classification of CD45+ population based on size resolution. D) Frequencies of TCRαβ+CD8−, TCRαβ+CD8+, and TCRαβ−CD8+ cells in the urine sample, gated from CD45+ lymphocyte cells.
2.3: Summary and Discussion

There is an unmet need for effective alternative treatment options for patients who undergo BCG treatment, but unfortunately exhibit tumor recurrence, progression, or no response, and wish to preserve their bladder (avoid cystectomy). Type I IFNs are known to stimulate innate immune cell activation, enhancing recruitment of other inflammatory and effector cells, and to maintain and regulate these cells' functions. It’s immune influencing actions, along with IFN-I’s ability to directly kill cells through TRAIL and caspase mediated apoptosis make the use of IFN-I effective in cancer therapy, and attractive for BCG unresponsive bladder cancer patients.

In this chapter, I report that the use of Ad-IFNα/Syn3 therapy in BCG unresponsive NMIBC patients does induce expression of immune markers within tumors, and incites secretion of inflammatory cytokines and chemokines that may be used to encourage immune recruitment and tumor infiltration. Analyzing tumor specimens from Phase I Trial patients pre- and post-treatment with Ad-IFNα/Syn3 showed increased RNA gene expression and IHC protein expression of T cell markers (CD3, CD4, CD8) in about 25% of patients after instillation with Ad-IFNα/Syn3. Delving deeper into the transcriptional changes in patient tumors comparing before and after treatment, I also found increased expression of cytokine and chemokine genes related to IFN-I response, increased expression of immune cell cytotoxicity genes, and decreased expression of angiogenic and metabolic genes related to fatty acid synthesis and amino acid transport. Likewise mirroring the established effects of IFN-I from literature and the results
seen with RNAseq here, I report that Ad-IFNα/Syn3 treatment increased inflammatory cytokine levels in patient urines from the Phase II Trial, most dramatically CXCL10, TRAIL, CCL2, G-CSF, and IL-6. Unexpectedly, I identified that the increase seen in IL-6 post-therapy was positively correlated with patient CR, inciting the need for further investigation into the role that IL-6 may play in an IFN-mediated antitumor response. This will be further addressed in Chapter 3.

Angiogenesis has long been recognized as a major hallmark of cancer progression and its induction can lead to tumor invasion and progression. IFN-I pleiotropically affects multiple immune cell types, and can directly inhibit tumor growth by decreasing tumor vasculature, which has been previously shown in my group’s IFN gene therapy work (403). Recent reports show that antiangiogenic therapy, such as VEGF/VEGFR2 inhibitors, can up-regulate PD-L1 mediated immunosuppression as a strategy for immune escape (429). The use of antiangiogenic therapy combined with immune checkpoint blockade has been shown to promote higher lymphocyte infiltration and activity in several tumor models (429), and is currently being investigated further in clinical trials. The regulation of metabolic genes by IFN-I induction is an area that is currently under investigation. UC cells rely on glycolysis-dependent metabolism as the main energy source for oncogenesis, overexpressing genes such as GLUT1, HK2, and LDHA/B to generate products of the TCA cycle (430). Bladder cancer also increases expression of fatty acid synthesis metabolic genes in order to store surplus energy generated (431). Here I show that Ad-IFNα/Syn3 treatment downregulates the expression of lipid synthesis genes FASN, ACACA and ACLY, and amino acid transporters SNAT1 and SNAT2, potentially contributing to tumor inhibition. The role of IFN-I and angiogenesis and tumor cell and immune cell
metabolism will be discussed more in depth in Chapter 4 and the global discussion in Chapter 5.

I think that Ad-IFNα/Syn3 treatment in a subset of patients (25-30%) is effective in preventing recurrence when i) patient tumors are initially immune infiltrated (by APCs), so that they can further incite an inflammatory and adaptive response, and when ii) Ad-IFNα is able to stimulate viral nucleic acid sensors (TLRs/STING), in addition to secreted TNF and IL-1β, to increase production of IL-6. IL-6 then acts as a master regulator of immune cell recruitment, activation, expansion, and differentiation, and therefore promotes inhibition tumor growth by immune activity and surveillance (to be discussed more in depth in Chapter 3).

However, a major aspect in patient response is if they had localized, lower staged tumors and had a thorough and complete TURBT prior to instillation with Ad-IFNα/Syn3. As evident from molecular profiling, histopathology, and diverging clonality, tumors are extremely heterogeneous, leading to greatly varying patient response. Response to therapy may be related to baseline immune infiltrate or tumor mutational status, if they have lost or mutated expression of IFN genes or other immune response genes, but more critical to the determination of response is the initial staging of patient tumors; that roughly 30% of patients are inaccurately staged (understaged) at the time of their TURBT, and so are recommended inappropriate therapeutic strategies (44, 432).

Urine may also serve as a future liquid biopsy medium in NMIBC, to identify patients who respond to therapy or who may be candidates for combination immunotherapy, as I tested the possibility of immune and epithelial cell identification and stratification from fresh patient urines. These data suggest
that the use of Ad-IFNα/Syn3 therapy can potentiate immune-driven antitumor responses and tumor inhibition in a subset of BCG unresponsive NMIBCs. The finding that increased expression of T cell markers was coupled with increased expression of inhibitory checkpoint genes also encourages the future testing and use of immune checkpoint blockade and Ad-IFNα combination therapy in UC treatment.
Chapter 3: Interferon-alpha Activation inhibits growth of Murine Urothelial Carcinoma

This work is based upon “Inhibition of urothelial carcinoma through targeted type I interferon-mediated immune activation” by Plote, D, Choi, W, Mokkapati M, Sundi, D, Ferguson, J, Duplisea, J, Parker, N, Yla-Herttuala, S, McConkey, D, Schluns, K, Dinney, C. 2019. *Oncoimmunology*; presented with permission.

3.1: Introduction

Type I interferons (IFN-I), are produced by multiple cell types following the stimulation of PRRs. Upon receptor activation, IFNs-I like interferon-alpha (IFNα) and interferon-beta (IFNβ) elicit many immunostimulatory effects including promotion of antigen processing, presentation, and recognition by professional antigen-presenting cells, and production of cytokines and chemokines, which in turn recruit and activate a cytotoxic T cell response against the tumor (91, 115). As previously mentioned, IFN-I can intervene during all stages of immunoediting, protecting the host against onconeogenesis and aiding the immune response to control existing tumors (282, 433, 434). To this point, studies have shown that absence of *Ifnar1* encourages cellular transformation of embryonic fibroblasts (435), and can increase tumor burden in carcinogen-treated mice (436). Furthermore, many cancers have developed strategies to interfere with IFN actions, downregulating expression of STAT proteins and interferon regulatory factors and sensing genes (IRFs and ISGs) to inhibit IFN signaling and promote tumor progression and metastasis (437-439). However, restoration of IFN
signaling rescues immunosurveillance and an antitumor phenotype (397), underlining its crucial role in the anti-tumor immune response.

Innate immune activation is critical to then further stimulate the antigen-specific effector cell response. IFN-I activated CD8α+ DCs are necessary to cross-prime tumor-specific CD8 T cells in vivo (397, 440). Preclinical studies in immune-poor melanoma showed that an IFN-I response induced by TLR3 agonist poly(I:C) (polynosinic:polycytidylic acid), a synthetic dsRNA that triggers PRR activation on APCs, inhibited tumor growth and increased survival alone and in combination with anti-PD-1 mAb checkpoint blockade (375). In this model, the effectiveness of poly(I:C) was particularly reliant on interferon-gamma positive (IFNγ+) CD8 T and NK cells (375). Further, robust tumor infiltration of NK cells and cytotoxic T cells correlates with spontaneous IFN-I production and good prognosis in melanoma patients (441, 442). IFNs-I also support the differentiation of monocytes into mature macrophages, and promote macrophage cytotoxicity and phagocytosis (121, 122). With the numerous targets and pathways stimulated by IFNα along with the variations in tumor immune landscape, the mechanisms of IFN’s actions could be distinct between tumor types. Because of the diversity of IFNα’s mechanisms in regulating the immune response and tumor control, elucidating IFNα’s immune-mediated antitumor mechanism in bladder cancer is critical for interpreting patient response, identifying effective combination therapies, and improving the treatment of bladder cancer. I hypothesize that type I IFN enhances the activation and recruitment of effector immune cells in non-invasive bladder cancer, leading to robust IFN-driven antitumor responses.
In this portion of the study, I sought to identify the importance of specific immune cell populations in the IFN-driven antitumor response in bladder cancer, and the mechanisms by which IFN-I recruited cells to the TME. To elucidate the immune mechanisms underlying IFN-I’s antitumor activity in UC, I utilized local injection of the TLR3 agonist poly(l:C) into MB49 tumors in syngeneic C57BL/6 mice. Indeed, I found that poly(l:C) induces an IFN-I response, inhibits tumor growth, and increases immune cells in murine MB49 tumors, but the antitumor activity was not specifically reliant on any one immune cell type, unlike the studies performed in melanoma (375). Interestingly however, there was an important anti-tumor role for IFN-I induced IL-6. I also investigated the effects of lentiviral-mediated IFNα (LV-IFNα) in the BBN-induced orthotopic mouse model of non-invasive bladder cancer and found LV-IFNα significantly prolongs animal survival in comparison to lentiviral-empty vector controls (LV-CTL), and increases the frequency of intratumoral NK cells and CD8 T cells compared to control treated groups.

3.2: Results

**3.2.1: Type I IFN Activation by Poly(l:C) Impairs MB49 Bladder Cancer Growth**

To determine how local induction of IFN-I impacts tumor growth in a murine model of bladder cancer, MB49 bladder tumor cells were implanted subcutaneously into syngeneic wildtype (WT) mice, followed by peritumoral injections of established tumors with either poly(l:C) (100 µg) or PBS every three
days; changes in tumor growth were monitored over time (Figure 8A). MB49 cells were chosen owing to characteristics reminiscent of non-muscle invasive, non-metastatic UC (443). Treatment with poly(I:C) delayed MB49 tumor growth and significantly improved overall survival (Figure 8A, B). The antitumor effect of poly(I:C) was mediated through IFNα signaling as poly(I:C) did not induce tumor regression in IFNAR-/- mice (Figure 8C). Poly(I:C)-mediated tumor regression is likely mediated in part through direct effects of IFN-I as murine IFNα increased MB49 cell death in-vitro at doses over 100 IU/mL (Figure 8D). For reference, one dose of poly(I:C) (100 µg) induced an average ~400 pg/mL of intratumoral IFNα, and showed clearance from the serum in 24 hours (Figure 9A, B). Similar to the observed effects with Ad-IFNα/Syn3 in human urine and tumors and in immune-poor melanoma (Figure 4A, C)(375), poly(I:C) treatment of MB49 tumors also led to an induction of IFN-I responsive genes IRF7 and PD-L1 compared with PBS-treated controls, as determined by RT-PCR (Figure 8E). Furthermore, the increase in IRF7 expression significantly correlated with the up-regulation of CD274 (PD-L1) gene expression across all tumor samples (Figure 8E). These data show that poly(I:C) inhibits MB49 tumor growth and prolongs survival in an IFNAR-dependent manner, suggesting important roles for IFNα. These data also confirm in the MB49 model that IFNα has direct anti-tumor action, and that IFN-I induces PD-L1 expression, as previously reported (444). Other murine UC cell lines BBN975, UPPL1541 and UPPL1595 were also used to evaluate the in vivo response to poly(I:C); however these tumor models exhibited spontaneous regression in PBS-treated controls, or inconsistent growth patterns per replicate, and were not deemed as viable tumor growth models (Figure 9C-E).
Figure 8: Poly(I:C) Treatment impairs MB49 tumor growth while upregulating PD-L1 expression on tumors. A) Tumor growth of subcutaneous MB49 tumors treated peritumorally with PBS (closed circles) or poly(I:C) (open square) beginning 7 days post-tumor implantation and continuing every 3 days.

B) Kaplan-Meier analysis showing survival of mice from A. C) MB49 tumor growth curves of poly(I:C) or PBS-treated mice in WT or interferon alpha receptor knockout (IFNAR-/−) mice. D) AnnexinV/PI staining for early (Annexin+PI−) and late (Annexin+PI+) stage cell apoptosis of MB49 cells treated in vitro with increasing doses of murine IFNa. E) Correlation of relative gene expression for CD274 and IRF7 in control and poly(I:C) treated MB49 samples determined by qRT-PCR. Error bars indicate mean ± SEM; n=5 mice per group in tumor growth/survival and n=3 for in vitro. *p<0.05, ***p<0.001 with Student’s t test or Log-Rank test (Kaplan-Meier).
Figure 9: *In vivo* effects of poly(I:C) on IFNα and tumor growth in BBN and UPPL bladder tumor models. A,B) Average concentration (pg/mL) of IFNα in MB49 tumors (A) and mouse serum (B) following one peritumoral injection of poly(I:C). C-E) Tumor growth curves of poly(I:C) or PBS treated mice in with either BBN975 (C), UPPL1541 (D), or UPPL1595 (E) tumors. Error bars indicate mean ± SEM.
**3.2.2: Poly(I:C) Activates Intratumoral Innate and Adaptive Immune Cells**

To investigate how poly(I:C) impacts intratumoral immune responses, we examined established MB49 tumors for gene expression and immune cell infiltration 24 hours after the prior treatment (day 14) with peritumoral poly(I:C) as described. Poly(I:C) significantly induced the expression of IFN-I regulated gene CXCL10, with other trending gene expression increases in IRF7 and the effector cytokines TNFA and PRF1 (*perforin*) (Figure 10A). We also observed a significant increase in the percentage of CD8 T cells and NK cell populations and decrease in percentage in CD4 T cells in tumor infiltrates (Figure 10B).

Additionally, there was a consistent increase in Ly6G+ cells and accompanying decrease in Ly6C+Ly6G− (Ly6Chi) and Ly6C−Ly6G− (Ly6Clo) populations, though these changes were not statistically significant (Figure 10B, C), demonstrating that poly(I:C) alters the composition of intratumoral CD11b+ myeloid cell subsets. The CD8+ T cells in the poly(I:C)-treated tumors showed a trend in increased expression of IFNγ (Figure 10D), which was not statistically significant. This increased IFNγ may be due to an exhausted CD8+ T cell phenotype caused by the IFN-I induced *PD-L1* expression in the tumors (Figure 8E). We could also observe similar effects in poly I:C-mediated changes in T cells in tumor tissue sections. After two treatments of poly(I:C) (i.e. day 11) the total numbers of intratumoral CD8+ T cells increased while CD4 T cells decreased (Figure 10E).

While these changes were not statistically significant, there was a significant decrease in the ratio of CD4:CD8 T cells compared with PBS-treated control mice (Figure 10F). Altogether, these findings suggest poly(I:C) promotes immune cell recruitment and/or expansion.
Though inconsistent for tumor growth studies, UPPL1541 and UPPL1595 tumors were analyzed for their immune infiltration with poly(I:C) treatment as compared with PBS- treated controls. Mixed effects of poly(I:C) were observed in UPPL1541 tumors, whereby poly(I:C) increased the percentage of total intratumoral CD45\(^+\) cells, CD11b\(^+\)Ly6G\(^+\)/lo and decreased the CD11b\(^+\)Ly6C\(^{hi}\)Ly6G\(^-\) population similar to MB49, but T cells were not affected. In UPPL1595 tumors, which showed minimal growth inhibition from poly(I:C) (Figure 9), the CD45\(^+\) population decreased with poly(I:C) treatment; however, there were increases in the CD8 T cells, NK cells, and CD11b\(^+\)Ly6G\(^+\) populations, similar to MB49 (Figure 11A, B). The inconsistent tumor growth kinetics of both UPPL tumors despite both tumor lines exhibiting similar molecular subtypes and mutations (445) may be a factor in their immune infiltrate differences, and led us to believe these models needed to be further investigated before use in our study.
Figure 10: Induction of Type I IFN by poly(I:C) enhances immune cell infiltration and activation. A) Relative gene expression of immune genes from whole tumors treated with PBS or poly(I:C); Error bars indicate mean ± SEM; n=4. B) Percentage of tumor infiltrating immune cells in poly(I:C)-treated tumors compared to PBS- treated controls at day 14, n=4. C) Flow cytometry plot depicting frequencies of Ly6G⁺, Ly6C⁺, and Ly6G⁻Ly6C⁻ cells in a mouse from each group in (B) analysis, gated from CD45⁺CD11b⁺CD11c⁻ cells. D)
Percentage of ex-vivo CD3 stimulated IFNγ+ CD8 T cells from PBS or poly(I:C) treated MB49 tumors at day 14 post-implantation. E) Immunofluorescent staining of tumor-infiltrating CD8+ (red) and CD4+ (green) cells in PBS- or poly(I:C)-treated tumors after 2 treatments at day 11. Image representative of 3 tumor samples per treatment group. Scale bar = 200μm. F) Ratio of CD4:CD8 T cells calculated from tumors in (E); n=3 per group. *p<0.05, **p<0.01 with Student’s t-test.
Figure 11: Poly(I:C) effects on immune cell infiltration in UPPL bladder tumor models. A) Percentage of tumor infiltrating immune cells in poly(I:C)-treated tumors compared to PBS-treated controls in UPPL1595 tumor model. B) Percentage of tumor infiltrating immune cells in poly(I:C)-treated tumors compared to PBS-treated controls in UPPL1541 tumor model. All results from Day 14 tumors. Error bars indicate mean ± SEM; n=5 per group. *p<0.05, **p<0.01 with Student’s t test.
3.2.3: IL-6 is important for Poly(I:C) Anti-tumor Efficacy, But No Specific Immune Cell Population is Required

To understand the role of individual immune cell types in MB49 tumor progression and IFN-1-mediated antitumor responses, we examined tumor inhibition in mice deficient in various innate and adaptive cells. MB49 tumor growth in PBS-treated RAG-/- mice was increased in compared to PBS-treated WT mice; however, tumor growth was equivalent in poly(I:C)-treated RAG-/- and WT mice (Figure 12A). Similarly, depletion of T cell populations with anti-Thy1.2 or anti-CD8 mAbs led to increased tumor growth in PBS-treated mice, but did not affect tumor growth in poly(I:C)-treated mice (Figure 12A). Altogether, these data indicate that while adaptive immune cells moderate growth of MB49 tumors in untreated controls, they were not critical for the poly(I:C)-mediated antitumor response. While IFNs can stimulate NK cells and an IFNγ+ NK cell antitumor response (375), in our MB49 model, depletion of NK cells led to a reduction in tumor growth (Figure 12A) suggesting NK cells are pro-tumor in an untreated MB49 model, and that they do not play a critical role in poly(I:C)-mediated anti-tumor activity. To address the role of neutrophilic MDSCs and monocytes/macrophages, efficacy of poly(I:C) was examined in mice depleted of Ly6G+ or CSFR1+ cells, respectively. Whereas depletion with αLy6G mAb had no effect on tumor growth in either PBS- or poly(I:C)-treated mice, depletion of CSFR1+ cells led to significant tumor regression in control mice but not (poly)I:C-treated mice. These results suggest that in this tumor model, CSFR1+ tumor-associated macrophages, but not Ly6G+ cells have anti-tumor activity but are not critical to the poly(I:C)-mediated anti-tumor response. Interestingly, we observed a modest
abscopal effect following the treatment of the primary MB49 tumor with poly(I:C) (Figure 12B) that was abrogated when mice were depleted of CD8+ T cells (Figure 12C). Collectively, these findings suggest that T cell adaptive immunity is enhanced by poly(I:C) treatment but is not crucial for its antitumor effect.

We also examined the roles for IL-15 and inducible nitric oxide synthase (iNOS) in the IFN-I response due to the central role for IL-15 in driving IFN-mediated T cell and NK cell responses (446), and the reported use of iNOS as an anti-tumor effector produced by Ly6G+ neutrophils (447). In IL-15 receptor α deficient mice (IL15Rα-/-), both PBS and poly(I:C) treatments had similar anti-tumor effects as in WT mice indicating a minimal role for IL-15 in the poly(I:C) response (Figure 13A). Drug-mediated inhibition of iNOS by N-iminoethyl-L-lysine (L-NIL) had no effect on tumor growth when animals were treated with poly(I:C) however, in PBS-treated mice, inhibition of iNOS reduced tumor growth (Figure 13B). Because activation of innate cells can lead to production of IL-12, a cytokine important in the Th1 immune response and IFNγ induction (112, 448), we looked at the gene expression of both IL-12 isoforms and their heterodimeric receptor and found that poly(I:C) does significantly increase IL-12p40 and IL-12Rb1 expression within tumors (Figure 13C). Collectively these results suggest that the regulation of IL-15 or iNOS by IFN-I are not critical to the antitumor response of poly(I:C) in this model system, but there may be a role for IL-12 influencing the IFN-I induced Th1 response.

Due to the positive correlation of IL-6 with IFNα and patient response (Figure 1), we investigated the effect of IL-6 in MB49 tumor growth. The antitumor benefit of poly(I:C) was significantly inhibited in IL-6 knockout mice (IL-
6KO), and survival of poly(I:C) treated IL-6KO mice was also significantly decreased as compared to poly(I:C) treated WT mice. (Figure 12D, E). In addition, poly(I:C) upregulated IL-6 protein as poly(I:C)-treated MB49 tumors had higher levels of IL-6 per mg of tumor as compared to PBS-treated controls, though this increase was not statistically significant (Figure 12F). As in earlier experiments (Figure 10B, C), poly(I:C) altered the myeloid cell landscape by significantly increasing the frequency of Ly6G+ cells and decreasing Ly6Chi and Ly6Clo cells within the tumors (Figure 12G). Interestingly, these changes in the myeloid cell landscape did not occur in tumors present in the IL-6KO (Figure 12G). Among tumor lymphocytes, the changes in NK and T cells observed in poly(I:C)-treated tumors in WT mice were still intact in IL-6KO mice, though the poly(I:C)-mediated increase in CD8 and decrease in CD4 T cells was slightly impaired in IL-6KO (Figure 12H). We also examined additional parameters of lymphocyte activation in the poly(I:C)-treated WT and IL-6KO mice. Within secondary lymphoid tissues, poly(I:C) increased the frequency of Ki-67+ NK cells and CD8 T cells in spleens and draining lymph nodes (dLN) but not in tumors, which was abrogated in IL-6KO mice (Figure 14A, B, data not shown). Similarly, there was an increased frequency of Granzyme B+ CD8 T cells in dLN with poly(I:C) treatment that was impaired in IL-6KO mice (Figure 14C). Overall, the anti-tumor response elicited by IFN-I likely represents the collective activity of multiple cellular components of the adaptive and innate immune response pathways.
Figure 12: Anti-tumor efficacy of poly(I:C) relies on IL-6 signaling and multiple immune subtypes. A) Growth of MB49 tumors treated peritumorally with PBS or poly(I:C) in RAG-/- mice or WT mice depleted of specific immune cell populations with the indicated Ab or given control Ig. Anti-Thy1.2 mAb was used to deplete T cells and anti-CSFR1 mAb was used to deplete monocytes and macrophages; n=5 per group. B) Tumor growth of primary and secondary MB49 tumors in WT mice. Primary (“Established”) tumors were treated peritumorally with either PBS or poly(I:C) beginning 7 days post-implantation. Secondary (“Contralateral”) tumors were implanted 4 days after the primary tumors. Arrow indicates beginning of treatment. C) Tumor growth of the contralateral tumor similar to (B) in CD8 depleted mice. D) Growth of MB49 tumors treated peritumorally with PBS or poly(I:C) in IL-6 knockout (IL-6KO) or WT mice. E) Kaplan-Meier analysis of survival of mice from D; n=5. F) Average concentration of IL-6 (pg/mL) per tumor weight (mg) in MB49 tumors from Day 14 tumors treated with either poly(I:C) or PBS; n=5 per group. G,H) Frequency (percentage) of Ly6G⁺, Ly6Chi, and Ly6Clo cells (G) and frequency of NK, CD4, CD8 T cells from MB49 tumors of WT and IL-6KO mice treated with PBS or poly(I:C) among gated CD45⁺CD11b⁺ cells and CD45⁺ cells, respectively; n=5. Error bars indicate mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 with Student’s t test.
Figure 13: Anti-tumor efficacy of Poly(I:C) is not mediated by IL-15 or iNOS.

A) MB49 tumor growth curves of poly(I:C) or PBS treated mice in WT or IL-15 receptor alpha knockout mice (IL15Rα−/−). B) MB49 tumor growth of mice treated with iNOS inhibitor L-NIL in combination with peritumoral PBS or poly(I:C). For all groups, n=5. Error bars indicate mean ± SEM. C) Log₂ gene expression of IL-12 subunits and receptor subunits from MB49 tumors after 3 treatments of PBS or poly(I:C); n=4 per treatment group, error bars indicate mean ± SEM. *p<0.05 with Student’s t test.
Figure 14: Poly (I:C)-mediated lymphocyte activation is impaired in the absence of IL-6. A,B) Percentage of Ki-67+ immune cells in either spleen (A) or tumor draining lymph node (dLN) (B) of poly(I:C)-treated WT and IL-6KO mice compared to PBS- treated respective controls at day 14, gated from CD45+ cells; spleen n=5, dLN n=3. D) Percentage of GZMB+CD44+ CD8 T cells in spleen and tumor dLN of poly(I:C)-treated WT and IL-6KO mice compared to PBS- treated respective controls at day 14, gated from CD45+CD8+ cells; spleen n=5, dLN n=3. Error bars indicated mean ± SEM. *p<0.05, **p<0.001 with Student’s t test.
3.2.4: LV-IFNα promotes survival and increased intratumoral immune effector cells in BBN-induced murine bladder tumors

To determine how local production of IFN-I by viral-mediated intravesicle instillation impacts tumor inhibition and immune cell responses in the bladder, I examined animal survival and tumor-immune infiltrate in the N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) carcinogen-induced murine orthotopic bladder cancer model (Figure 15). Following 60 days of BBN treatment, in which mice were deemed to have non-invasive tumors or CIS (Figure 15A), mice were intravesically instilled weekly with LV-IFNα or LV-CTL. Differences in tumor growth were difficult to monitor, as ultrasound imaging was not sensitive enough to detect weekly changes; however, treatment with LV-IFNα significantly prolonged survival in comparison to LV-CTL (Figure 15B). Investigating the effect of LV-IFNα on the immune infiltrate of BBN-induced tumors, I observed an increase in the frequency of CD8 T cells in both lentiviral-vector treated groups as compared to PBS controls, but this increase was not statistically significant (Figure 15C). Unlike the MB49 model, there was no increase in Ly6G+ cells and accompanying decrease Ly6C+ populations (Figure 15C), suggesting that LV-IFNα doesn’t preferentially induce a Ly6G+ cell response like poly(I:C).

Altogether, these findings suggest that like poly(I:C) in MB49 tumors, LV-IFNα treatment promotes survival and immune cell recruitment and/or expansion in the BBN-induced tumor model. However, it is important to keep in mind that the inherent differences between the MB49 and BBN-induced tumors brought on by their origins, such as their mutational burden and pathology, may be linked to the diversity in their immune landscapes and responses to IFN-I, as seen here.
Figure 15: LV-IFNα improves survival and increases intratumoral immune cells in BBN-carcinogen induced bladder cancer. A) Experimental strategy for development of BBN-induced bladder cancer mouse model. After 2 months, mice began intravesical instillation with lentiviral-control vector (LV-Control) or lentiviral-IFNα vector (LV-IFNα). B) Kaplan-Meier analysis of survival of mice treated with either LV-Control or LC-IFNα. C) Frequency (percentage) of NK, CD4, and CD8 T cells from the SSC/FSC CD45+ lymphocyte population, frequency of CD11b+ cells from the SSC/FSC myeloid designated population, and Ly6G+ and Ly6C+ cells among gated CD11b+ population from bladder of BBN-tumor induced mice treated with LV-Control or LV-IFNα; n=5. Error bars indicate mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 with Student's t test or Log-Rank test; These experiments were performed in part by Dr. Sharada Mokkapati.
3.3: Summary and Discussion

The use of type I IFN as a cancer therapy has had mixed successes due to its route of administration and short half-life. Despite early therapeutic shortcomings, IFN-I has been shown to have direct tumor killing ability by the induction of TRAIL and apoptosis signaling, and it can incite an inflammatory response, including maturation and activation of innate and adaptive immune cells, promoting their migration to the tumor. Because of the variety of IFNα’s mechanisms in controlling tumor growth and the immune response across different tumor types, determining IFNα’s immune-mediated antitumor mechanism in bladder cancer is critical for interpreting patient response, identifying effective combination therapies, and improving patient outcomes.

In this chapter, I examined the immune mechanisms behind IFN-I-mediated anti-tumor responses in murine models of UC. I found that IFN-I induction by poly(I:C) in MB49 tumors inhibits tumor growth, increases longevity, and activates both the innate and adaptive immune systems. Poly(I:C) increased the intratumoral frequencies of CD8, NK, and Ly6G+ cells, and decreased the frequencies of CD4 and Ly6C+Ly6G− cells, but it’s antitumor efficacy was found to not be dependent on any one of these individual cell types. The poly(I:C) mediated tumor inhibition was, however, found to be dependent on functioning IL-6 signaling, which was necessary for higher levels of intratumoral Ly6G+ and CD8 cells, and proliferation and activation of NK cells and CD8 cells in secondary lymphoid organs. This IL-6 dependence mirrors the positive correlation of IL-6
urinary cytokine expression with patient response (CR) to Ad-IFNα/Syn3 therapy. I also found that LV-IFNα similarly prolonged survival and increased intratumoral T cell and NK cell populations in the BBN-induced bladder cancer mouse model.

Treatment of MB49, BBN, and UPPL tumors with poly(I:C) or LV-IFNα resulted in an IFN-I induced infiltration of highly diverse immune populations representing a multifaceted pro-inflammatory anti-tumor phenotype, contrary to the defined dependence of tumor-inhibitory poly(I:C) on specific immune subsets in studies performed in melanoma (18). Previous studies in experimental bladder cancer have focused on the necessity of the T cell infiltration for an anti-tumor response, and thus have relied on T cell checkpoint targeted immunotherapy (443, 449, 450). However, the importance of both lymphoid and myeloid cell types in the anti-tumor response in my MB49 studies indicates that focusing on a single subset of effector cells may limit the insights to be gained.

I also found an important role for IL-6 signaling in the type I IFN-driven MB49 tumor inhibition as well as in patients treated with Ad-IFNα/Syn3. IL-6 has been shown to have a dichotic role, acting as both a pro-inflammatory and an anti-inflammatory cytokine, in cancer as well as autoimmune diseases, providing an activation signal to immune cells that left unchecked has the potential to produce deleterious effects (451). As previously mentioned, IL-6 regulates innate and adaptive immune defense in a pro-inflammatory capacity by inducing the acute phase response, hematopoiesis (granulopoiesis), B cell differentiation and antibody production, and recruitment of neutrophils and other immune cells by promoting increased expression of trafficking chemokines CCL2, CXCL5, CXCL6 and adhesion molecules CD62L, ICAM-1, and VCAM-1 (130,
It also promotes the activation and expansion of T cells, and differentiation of CD4 T cells into i) T_{FH} cells to aid in B cell antibody switching and ii) pro-inflammatory Th17 T cells, and can suppress inducible CD4 T_{reg} formation and function (132-134, 139). Anti-inflammatory effects of IL-6 include a role in wound healing and liver regeneration (137), and regulation of the production of pro-inflammatory cytokines TNF\(\alpha\), GM-CSF, IFN\(\gamma\), MIP-2 in acute inflammation to prevent deleterious immunopathology and promote inflammatory resolution (138, 452).

In the MB49 model treated with poly(I:C) and in NMIBC patients treated with Ad-IFN\(\alpha\), I believe IL-6 is the master key in regulating the multifocal immune cell anti-tumor response by increasing chemokine production for immune recruitment, particularly the Ly6G\(^+\) cell recruitment, and increasing NK and T cell proliferation and activation, summarized in Figure 16. Backing this conclusion, it has been shown that IL6/-/- neutrophils have impaired respiratory burst and degranulation, and also have impaired leukocyte apoptosis, affecting the transition into adaptive immunity (453). IL-6 also increases CD8 cytotoxic activity in vitro (134), supporting my observation that IL6/-/- mice had decreased GZMB\(^+\) CD8 T cells in the draining lymph node. There are many other effects of IL-6 on immune cells that I did not thoroughly investigate here, such as the propensity and role of CD4 Th17 and T_{reg} cells in the MB49 model, and how IL-6 may affect macrophage activity and monocyte differentiation which was just touched upon by CSFR1 depletion in WT mice (Figure 12A). I would speculate that IL-6 driven differentiation and activation of Th17 cells and macrophages also contributes to the IFN-I antitumor response in bladder cancer by further
promoting neutrophil recruitment, T cell priming, and pro-inflammatory cytokine production (454). The potential link of IL-6 to type I IFN-driven anti-tumor responses in both murine models and in patients sparks the need for further investigation of the role of this cytokine in IFN-I treated bladder cancer.
Figure 16: Suspected role of IL-6 signaling in UC. Stimulated by inflammatory cytokines, TLR signaling and stress, IL-6 is produced by almost all stromal and immune cells. It goes on to activate STAT3 signaling and regulate T cell differentiation (promotes CD4 Th17, inhibits CD4 T_{reg}), proliferation, and activation (GRZB+). IL-6 is also important in the recruitment of innate and adaptive immune cells through induction of the acute phase response (secretion of inflammatory cytokines) and stimulation of chemokine production from stromal cells. I show that IL-6 signaling is needed to increase (Ki67+) NK cell proliferation
in secondary lymphoid tissues. Not depicted here: IL-6 also controls B cell survival, expansion and maturation (Ab production), but I did not examine the B cell compartment in MB49/BBN tumors.
Chapter 4: Combination Therapy of Interferon-alpha Activation with T-Cell Checkpoint Modulation Prolongs Survival

This work is based upon “Inhibition of urothelial carcinoma through targeted type I interferon-mediated immune activation” by Plote, D, Choi, W, Mokkapati M, Sundi, D, Ferguson, J, Duplisea, J, Parker, N, Yla-Herttuala, S, McConkey, D, Schluns, K, Dinney, C. 2019. *Oncoimmunology*; presented with permission from *Oncoimmunology*.

4.1: Introduction

In addition to its roles in promotion of DC, macrophage, T cell, and NK cell maturation, migration/taxis, and function (118-120, 391, 392), and direct cell killing through TRAIL-mediated apoptosis (393), IFNα has been shown to increase programmed death ligand-1 (PD-L1) and programmed cell death protein-1 (PD-1) expression on tumor and immune cell subsets (395, 444). Induction of PD-L1 and PD-1 has led to adaptive immune resistance by promoting T cell exhaustion and immune evasion (244, 289, 395, 444). This consequence may decrease the effectiveness of IFNα as a monotherapy, but suggests that IFNα use in combination with immune checkpoint blockade may lead to improved therapeutic outcomes. Use of anti-CTLA-4 and anti-PD-1/PD-L1 checkpoint blockade therapies have produced clinical responses and tumor regression across many solid and hematologic cancers, often with durable or indefinite results, by increasing absolute lymphocyte counts and T cell activation, inducing expression of ICOS, and depleting T_{reg} populations (90, 455). However,
refractory disease and acquired resistance mechanisms via immunoediting are major problems of checkpoint blockade therapy (455).

Indeed, acquired resistance to PD-1/PD-L1 blockade is associated with loss of genes encoding IFN receptor-associated Janus kinases, JAK1 and JAK2 (288, 456). Additionally, non-responders to checkpoint inhibitors such as anti-Cytotoxic T-Lymphocyte Antigen-4 (anti-CTLA-4) possess tumors with co-deletions of IFNα and IFNβ genes on chromosome 9p21 and defects in IFNγ pathway genes (288). These findings highlight the potential exploitable relationship between not only PD-1/PD-L1 and IFNs, but other checkpoint molecules as well. I hypothesize that though IFN-I induces expression of immune evasion markers like the PD-1/PD-L1 axis, combined use of IFN and anti-PD-1 checkpoint inhibition therapy will synergistically increase the antitumor effects against bladder cancer seen with IFNα alone, via increases in immune recruitment and activation and decreased tumor growth, improving clinical benefit.

Understanding the relationship between IFNα and immune checkpoint inhibition is important for interpreting immunotherapy resistance and improving the treatment of UC and other solid tumors. In this portion of the study, to test therapeutic synergism, I utilized local poly(I:C) administration in combination with systemic anti-PD-1 mAb therapy in C57BL/6 mice with MB49 tumors. I found that their combined use reduced tumor burden comparably to single-agent poly(I:C) treated mice, but combination treatment significantly prolonged animal survival. However, no significant difference was found in the intratumoral immune cell populations between single agent and combination treated tumor groups.
Investigating the differences between either poly(I:C) or anti-PD-1 mAb alone, and combination treatment of MB49 tumors on a molecular level, combination treatment comparatively decreased tumor vasculature and angiogenesis, and increased expression of genes associated with metabolism, extracellular matrix organization, and ERK/MAPK signaling. I also compared the molecular changes seen in PBS-, poly(I:C)-, anti-PD-1 mAb-, and combination-treated MB49 tumors to pre- and post- Ad-IFNα treated patient tumor samples (from Figure 6), and found that signaling pathway genes associated with poly(I:C) treatment, such as IFN response and cytotoxicity are similarly upregulated, and metabolic pathway genes are similarly downregulated to those in patients post-Ad-IFNα treatment, but not with anti-PD-1 or combination treatments.

4.2: Results

4.2.1: Combination Treatment with Anti-PD-1 mAb and Poly(I:C) Reduces Tumor Burden and Prolongs Survival

Given that IFN-I signaling induces expression of checkpoint markers such as PD-L1 (Figure 4, 8) which may lead to decreased effector T cell function (244, 289, 395, 444), we reasoned that therapeutic blockade of the PD-1/PD-L1 pathway could further enhance the anti-tumor efficacy of poly(I:C). Subcutaneous MB49 tumor-bearing mice were treated with poly(I:C) and a PD-1–blocking mAb either as monotherapies or in combination (Figure 17A). Tumor growth was measured overtime until mice became moribund. Treatment with both single-agent poly(I:C) and combination therapy [poly(I:C) with anti-PD-1 mAb] significantly repressed
tumor growth compared with anti-PD-1 mAb alone and IgG/PBS-treated controls (Figure 17B). However, we observed no significant difference in tumor growth inhibition between poly(I:C) monotherapy and combination therapy. Nonetheless, combination therapy significantly prolonged survival compared with poly(I:C) alone (Figure 17C) demonstrating IFN-I can work with checkpoint blockade for enhanced efficacy. In 16 day-old MB49 tumors treated with either single agent or combination therapy, combination treatment significantly increased the level of intratumoral Ly6G+ cells in comparison to poly(I:C) alone, which may contribute to the difference in survival, but there was no significant difference between the CD8, CD4, NK, and Ly6C+ immune cell populations between poly(I:C) and combination treatment groups (Figure 18). However, this observed result was only from one experimental analysis, and would need to be consistently repeated for a more definitive conclusion.
Figure 17: Blockade of PD-1/PD-L1 pathway reduces tumor burden and prolongs survival in poly(I:C) treated mice. A) Experimental strategy for combination therapy for s.c. engrafted MB49 tumors for peritumoral poly(I:C) and anti-PD-1 mAb (i.p.). B) Averaged tumor growth of mice treated with either single agent poly(I:C) or anti-PD-1 mAb, poly(I:C) plus anti-PD-1 mAb, or control IgG plus PBS or control observation. C) Kaplan-Meier analysis of survival of mice from C. Error bars indicate mean ± SEM, n=10; *p<0.05, **p<0.01, ***p<0.001 with Student's t test or Log-Rank test; Graphs representative of 3 separate trials.
Figure 18: Poly(I:C) and anti-PD-1 mAb combination therapy increases intratumoral Ly6G+ cells in MB49 tumors as compared to poly(I:C) alone.

Percentage of tumor infiltrating immune cells in poly(I:C)-, anti-PD-1 mAb-, and poly(I:C) + anti-PD-1 mAb- treated MB49 tumors compared to IgG + PBS-treated control MB49 tumors at Day 16. CD8, CD4, and NK cells are gated from CD45+ SSC/FSC designated lymphocytes; Ly6G and Ly6Chi cells are gated from lineage negative (TCRβ-CD19-NK1.1-), CD11b+ cells. aPD1, anti PD-1 mAb; Combo, combination poly(I:C) + anti-PD-1 mAb treatment. Error bars indicate mean ± SEM, n=5; *p<0.05, **p<0.01, ***p<0.001 with Student’s t test.
4.2.2: Combination Treatment Induces MAPK Signaling, Metabolic Pathways, and Reorganization of Tumor Microenvironment

To examine potential molecular changes between treatment groups, comprehensive gene expression analysis was performed via RNA sequencing and GSEA on total mRNA collected from day 17 tumors treated with PBS, poly(I:C), anti-PD-1 mAb, or in combination. GSEA of the RNAseq data from the poly(I:C), anti-PD-1 mAb, and the combination showed enrichment in viral stress response, IFN signaling, cytokine signaling pathways, and innate immune response in comparison to control tumors (Table 2). However, the single agent anti-PD-1 mAb as well as the combination treatment also enriched pathways promoting cell migration, differentiation, proliferation, and survival through MAPK, MEK/ERK and AKT signaling (Table 2). Combination treatment up-regulated additional pathways related to collagen formation, extracellular matrix formation, and cell-cell signaling (Table 2). Comparing the poly(I:C) and combination treated groups to each other, metabolic pathways for glucokinase regulation and fatty acid oxidation (FAO) and synthesis were enriched in the combination group (data not shown).

Looking more closely at effects on individual genes, we found numerous genes related to IFN pathway signaling (Figure 19A), as well as adaptive and innate effector cell cytotoxicity such as Granzyme B (GZMB) were significantly increased in the poly(I:C) treated group, and more modestly increased in the anti-PD-1 mAb and combination treated groups, in comparison to control IgG/PBS treated tumors (Figure 19A, B). As expected, there were also increases in gene expression for immune suppressive molecules in poly(I:C), anti-PD-1 mAb, and combination treated mice, including ARG1 and ARG2 (Arginase 1 and 2), IDO,
and CD274 (PD-L1) (data not shown). Exploring the GSEA-identified upregulated metabolic pathways, we found poly(I:C) induced expression of glucose transporter GLUT1, and all treatment groups increased expression of glycolysis enzymes HK2 and GCK in comparison to PBS/IgG control (Figure 19A, B). Interestingly, while poly(I:C) decreased tumor expression of genes related to fatty acid catabolism and synthesis such as FASN, ACACA, and ACLY in comparison to PBS control, anti-PD-1 mAb and combination treatment significantly rescued their expression (Figure 19A, B). We also observed increased MAPK signaling genes in all treated groups in comparison to control, and decreased expression of VEGF, MMP9, and EGFR. Similar trends in gene expression were also observed in the 8 matched pre- and post- Ad-IFNa/Syn3 treated patient tumor specimens from Figure 6, despite none of the patients reaching CR. To further investigate the decreased expression of angiogenesis markers seen in the RNAseq of MB49 tumors treated with poly(I:C), anti-PD-1 mAb, or combination, we performed IHC staining of CD31 on tumors from each treatment group taken at their end point (day 38) (Figure 19C). Poly(I:C) alone and in combination with anti-PD-1 mAb significantly decreased microvessel density (MVD) compared to control PBS/IgG treated tumors by ~50% (Figure 19D). Thus, while IFN-I has significant anti-tumor action, combination therapy with checkpoint blockade activates additional pathways regulating the increased stromal influx and reorganization of ECM, inhibition of angiogenesis, glycolysis and fatty acid catabolism, and increased MAPK/ERK/AKT signaling that may be related to prolonged survival.
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* Pathways are up-regulated in both poly(I:C) and anti-PD-1 treated groups
# Pathways are up-regulated in both anti-PD-1 and combination poly(I:C)+anti-PD-1 treated groups
+ Pathways are up-regulated in poly(I:C), anti-PD-1, and combination treated groups
` Normalized enrichment score (NES); up-regulated pathways defined as (NES) > 0
Figure 19: Poly(I:C) and anti-PD-1 mAb combination therapy promotes gene expression associated with survival, metabolism, and Th-1 type anti-tumor immunity and decreases angiogenesis. A) Heatmap illustrating normalized (log2) gene expression patterns from MB49 whole tumor lysates treated with either PBS+IgG Ab, poly(I:C), anti-PD-1 mAb, or poly(I:C)+anti-PD-1 mAb; RNA was isolated from tumors 17 days post-implantation (4 treatments) (Figure 4A). Each column represents one mouse. B) Average relative gene expression of indicated genes associated with effector function, fatty acid oxidative metabolism, glycolysis, and AKT, MEK/ERK pathway from the 4 treatment arms (n=4 per group). C) IHC staining for CD31 (PECAM-1) in end point tumors from 38 days post-implantation (11 treatments); Scale bar = 100µm. Image is representative of 3 tumors per treatment group. D) Quantified microvessel density (MVD) averaged from IHC CD31-stained tumors (6C) (n=3 per group). All values normalized by DeSeq and log2 transformed (heatmap). Error bars indicate mean ± SEM, n=4 per group. *p<0.05, **p<0.01 with Student’s t test.
IFN-I, while capable of inhibiting tumor growth and inciting an immune response to aid in host defense, intrinsically increases mechanisms of tumor resistance by upregulating expression of immune checkpoint markers like PD-L1 on cancer tissue and surrounding cells of the TME. Immune inhibitory checkpoints are important in regulating autoimmunity and collateral tissue damage in an immune response, but in the context of cancer, can inhibit effector T cell responses by inhibiting proliferation and IL-2 secretion and promoting T cell exhaustion and anergy (257). Despite these mechanisms of adaptive immune resistance, therapeutic antibody-mediated blockade of inhibitory checkpoints has produced tumor regression and durable patient responses (415). Because of the inherent PD-1/PD-L1 pathway tumor evasion strategy induced by IFN treatment, I explored the potential of IFN-I therapy combined with PD-1 pathway checkpoint inhibition to provide synergistic treatment benefit to MB49 tumors.

In this chapter, I show that combination IFN-I and anti-PD-1 mAb checkpoint blockade reduces tumor burden and significantly prolongs survival, though there is no significant difference in tumor reduction between poly(I:C) alone and combination treatment. Similarly, there were no significant differences in the frequencies of CD8, CD4, NK, Ly6G, or Ly6C$^{hi}$ cells between poly(I:C), anti-PD-1, or combination treatment, although there was an increasing trend in Ly6G cells with corresponding decrease in Ly6C$^{hi}$ cells with combination therapy that was consistently reproducible. Molecular profiling of the differently treated tumors revealed that poly(I:C), anti-PD-1, and combination treatment all increased expression of genes related to antiviral response, interferon, and
cytokine signaling, and decreased genes related to angiogenesis and vascularization (CD31 IHC). Interestingly, combination treatment enriched gene signatures related to ECM organization and collagen formation, possibly alluding to an increased stromal infiltrate. Further clarification of which GSEA analyzed pathways are conserved or different between each treatment group is described in Figure 20A. I also compared the molecular changes seen in PBS-, poly(I:C)-, anti-PD-1 mAb-, and combination-treated MB49 tumors to pre- and post- Ad-IFNα treated patient tumor samples (from Figure 6). I found that signaling pathway genes, such as IFN response and cytotoxicity related CXCL9, CXCL10, TRAIL, CCL2, CCL5, CD8, and PRF1 are similarly upregulated in poly(I:C)-, anti-PD-1 mAb-, and combination-treated MB49 tumors, like in the post-Ad-IFNα specimens; however, metabolic pathway genes such as FASN, ACLY, and ACACA, are only similarly downregulated with single agent poly(I:C)/IFN-I treatment, not with anti-PD-1 or combination treatments. My initial hypothesis that combined usage of IFN-I therapy with anti-PD-1 checkpoint blockade would synergize to provide greater clinical benefit was not herein definitively proven. While I did see a difference in animal survival, there were no clear factors as to how combination therapy provides greater clinical benefit, based on lack of significant differences in tumor burden, no major differences in the tumor-immune infiltrate, and RNA sequencing analysis shows stronger upregulation of IFN response and immune cytotoxicity genes in poly(I:C) alone than other treatment groups. Overstimulation of inflammatory pathways has been shown to produce a “cytokine storm” that can result in significant pathology and ultimately death (457, 458). In a model of viral infection and
Figure 20: Summary: Gene expression pathways regulated by single and combination treatment of MB49 tumors. A) Enriched signaling pathways identified by GSEA analysis in MB49 tumors treated with either poly(I:C), anti-PD-1 mAb, or poly(I:C)+anti-PD-1 compared to PBS/IgG control. B) Canonical pathways upregulated or downregulated in poly(I:C) and poly(I:C)+anti-PD-1
mAb treated tumors identified by IPA analysis. ISGs, interferon sensing genes; PDGF, platelet derived growth factor; LXR/RXR, liver X receptor/retinoid X receptor; PRRs, pattern recognition receptor; IRF, interferon regulatory factor; THOP, thimet oligopeptidase; GPCR, g protein coupled receptor; MMPs, matrix metalloproteinases.
poly(I:C), mice lacking an adaptive immune response (nude and Rag-/- mice) had higher mortality rates after virus/poly(I:C) dosage due to higher abundances of proinflammatory cytokines TNF and IFNγ in the serum days after infection compared to WT mice (459). Addition of T cells to the non-T cell, TLR3-stimulated system efficiently prevented this cytokine surge, suggesting that active T cell monitoring is necessary to temper an innate response. One hypothesis for why combination treated mice had improved survival over poly(I:C) alone is that the addition of anti-PD-1 mAb is able to reactivate T cells (particularly Tregs) that have become exhausted from IFN-I stimulated PD-L1 expression, which results in tempering of the innate cytokine response invoked by IFN-I signaling. This warrants future analysis in the MB49 model.

GSEA and RNAseq analysis of MB49 tumors treated with either single agent poly(I:C), anti-PD-1 mAb alone, or combination therapy showed anticipated enrichment in genes and pathways related to IFN induction, viral stress response, cytokine production and innate immune activation, as well as MAPK and ERK signaling in comparison to PBS treated control tumors. MAPK/ERK signaling traditionally is associated with cell survival, proliferation, and differentiation. However, there is evidence that ERK signaling has pro-apoptotic functions in response to damage stimuli (460), and this mechanism may add to the immune component of the IFN-I anti-tumor response, providing further survival benefit demonstrated in this study.

Previously published studies identified that blockade of the PD-1/PD-L1 axis reverses T cell exhaustion, re-inducing glycolysis and anabolic metabolism to produce a more active state (317, 461). In this work, I found that poly(I:C),
anti-PD-1 mAb, and combination treated groups have increased levels of enzymes involved in glycolysis and the TCA cycle (GLUT1, HCK, GCK).

Interestingly, I also found in comparison to poly(I:C) treatment alone, the addition of anti-PD-1 mAb enriched for extracellular matrix reorganization, collagen formation, and increased genes related to FAO and fatty acid synthesis such as FASN, ACLY, and ACACA. Increasing mitochondrial FAO metabolism in T cells has been shown to favor the formation of long-lived memory T cells (462, 463). However, the whole tumor RNA analyzed here is likely more reflective of the tumor genome than the immune microenvironment. Since and fatty acid synthesis and glycolysis are necessary for cellular growth and proliferation (464), this analysis suggests that combination treated tumors are upregulating pathways of proliferation in the tumor cells while simultaneously inhibiting tumor growth. Upregulation of expression of genes involved in ECM remodeling and mesenchymal transition has been shown to be a marker of resistance to anti-PD-1 therapy (innate anti-PD-1 resistance, IPRES) (465). The possibility of combination therapy upregulating mechanisms of resistance pathways will need to be further explored in the MB49 model.

Despite no clear synergistic benefit from combination therapy, and no definitive reasoning as to why combination treatment prolongs animal survival, I have hypothesized that addition of anti-PD-1 antibody may contribute to activation of regulatory immune function to subdue chronic inflammation adverse effects, which may improve survival. I have also suggested that "rescue" of fatty acid synthesis and metabolic gene expression by addition of anti-PD-1 mAb may contribute to the differences seen between combination treatment and poly(I:C) alone. Ingenuity Pathway Analysis (IPA) canonical pathway analysis
also shows that upregulated pathways are predominantly immune response related in poly(I:C) treatment, whereas combination treatment upregulates calcium signaling, G-protein signaling that regulates metabolic enzymes, ion channels, and transcriptional machinery, matrix metalloproteases, and wound healing pathways (actin cytoskeleton and intrinsic prothrombin activation pathways) in addition to immune response pathways and leukocyte extravasation signaling (Figure 20B). Combination treatment also more dramatically downregulates upstream regulator ACKR2, involved in regulating inflammatory cytokines. This combination of immune response, metabolic pathways, and ECM modification may be related to greater immune infiltration, or to promoting cancer growth and metastasis. Immune checkpoint inhibitors can regulate T cell migration, and particularly in the case of CTLA-4, can increase immune cell infiltration into tumors (and other tissues) (466). PD-L1 has been found to localize to the central T cell activation cluster and decrease antiviral CD8 T-cell motility; antibodies to PD-1 and PD-L1 restored CD8 T cell motility by limiting interaction time between T cells and DCs (467). While increasing motility, this effect of PD-1 blockade becomes complicated as it reduces the efficacy of TCR signaling, raising the threshold needed for T cell activation (466). These analyses demonstrate that while not clearly synergistic, anti-PD-1 mAb in addition to IFN therapy may provide clinical benefit by increasing immune and TME regulatory functions that are not stimulated by IFN-I activation alone. Thus, though not synergistic in these studies, combination treatment of IFN and immune checkpoint blockade may provide improved therapeutic outcomes in NMIBC patients.
Chapter 5: Global Discussion (Implications) and Future Directions

Bladder cancer is a heterogeneous disease that affects a large proportion of cancer incidences, particularly in men, each year. Most patients are diagnosed with NMIBC, and though this classification of the disease is less severe and life-threatening compared to MIBC, current standard treatment practices are not well-defined, and result in 30-40% of patients exhibiting recurrent and progressive tumors (55, 69). Therefore, finding an effective alternative therapy that is mechanistically understood is essential to improve patient outcomes. Use of type I IFN and adenoviral-mediated IFN (Ad-IFNα) have shown clinical response in this patient population, however i) its mechanisms of action have not been well characterized, and ii) IFN-I has been shown to increase tumor and TME immune-evasion strategies such as upregulation of checkpoint markers, prompting the need to develop potential combination therapies. In this dissertation, I tested the hypothesis that immune-mediated mechanisms of type I IFN therapy with the general hypothesis that localized IFN-I treatment would increase immune cell recruitment and activation in tumors, creating an IFN driven antitumor response and an environment in which checkpoint blockade immunotherapy could counteract immune evasion and T cell exhaustion. In the course of these studies, I learned that local delivery of poly(I:C) and viral-mediated IFN incites inflammatory cytokines and chemokines, increases intratumoral effector immune cells, and inhibits tumor growth, prolonging survival. In characterizing this response, I found IL-6 had an important role in regulating positive responses to type I IFN therapy in both mice and humans, that IL-6 signaling was necessary
for antitumor effects of Ly6G cells and NK cell and T cell proliferation and activation. I further established that local delivery of poly(I:C) in combination with anti-PD-1 checkpoint modulator prolongs survival, which may be a product of anti-PD-1 mAb’s ability to regulate TME cell metabolism, and to reactivate regulatory cells to balance treatment efficacy and inflammatory pathology. I touched on how my findings supported or contradicted the current knowledge in the field, and speculated on the potential underlying mechanism for my results. In this chapter, I will discuss the potential future implications of my work, and address what areas of study need to be further pursued.

IL-6 signaling has been portrayed as both promoting oncogenesis by supporting cancer cell proliferation, survival, and metastasis, and opposing tumor growth by mobilizing immune responses against the tumor. My findings outlined in Chapter 3 indicate that contrary to many publications categorizing high IL-6 levels as a marker for poor prognosis and tumor progression, the induction of IL-6 in mice and patients with bladder cancer treated with IFN therapy correlated with antitumor response and tumor regression. This implies that measureable IL-6 levels may be used as a positive prognostic marker for IFN treatment of bladder cancer in the future. In this dissertation, I only touched on the need of IL-6 in the poly(I:C) antitumor response, inhibiting tumor growth, promoting NK and T cell activation, and increasing intratumoral Ly6G+ cells, but further analysis of IL-6’s mechanisms of action would aid in understanding it’s defined role in the antitumor response for bladder cancer. IL-6 has been shown to inhibit T_{reg} suppression of DC-mediated T cell activation (468), and promote Th17 cell differentiation, a process often connected by IL-6’s ability to suppress the Foxp3-dependent T_{reg} developmental program in favor of Th17 formation in the
presence of TGFβ (469). The effect of IL-6 on the CD4 T cell compartment in MB49 and other bladder cancer models needs to be further pursued as another effector mechanism of IFN-1.

One of the areas brought into question from my studies was how combination treatment increased survival without having significantly smaller tumor sizes or greater expression of immune effector gene expression than poly(I:C) alone. To better elucidate the objective differences, if there are any, between poly(I:C) and combination poly(I:C) + anti-PD-1 therapy, the treatment dosing schedule may be an important area of future research. In this study, I treated mice concurrently with IFN agonist poly(I:C) and PD-1 inhibitor based on previously published work (375), however a delayed application of anti-PD-1, after IFN-I has been able to incite the inflammatory response (and upregulate PD-L1) may provide greater benefit. It has been shown that chemotherapy and radiation therapy given prior to checkpoint inhibition therapy generate antitumor and abscopal responses (470). As these therapies act to stimulate the antitumor response by increasing tumor antigenicity and immune cell activation, the same reasoning can be applied to innate immune stimulating therapy (IFN) being given prior to inhibitory checkpoint antagonists.

Additionally, in the RNAseq data (Figure 19), I show that in comparison to poly(I:C), combination treated tumors have enriched expression of fatty acid metabolism. In Chapter 4, I mentioned that increasing mitochondrial FAO metabolism in T cells has been shown to favor the formation of long-lived memory T cells (462, 463), and FAO is necessary for other effector immune cell function: TLR-stimulated macrophages require increased FAO, and specifically
ACLY function, for cancer cell phagocytosis and antitumor activity (471). However, bladder cancer has been known to rely on glycolysis as well as fatty acid synthesis for oncogenesis (430, 431), and so, dual expression of fatty acid metabolism and glycolysis from the RNAseq analysis may indicate that the tumor itself is proliferating and may be a sign of tumor progression and therapeutic resistance.

For the tumor to overcome an antitumor defense, it must co-opt the TME and induce its polarization to a more pro-tumor, immunosuppressive immune cell phenotype, thereby encouraging tumor growth and survival. Specific identification of which cells (immune subtypes or tumor) are exhibiting increased fatty acid metabolism (as well as glycolysis) is therefore an important next step in determining therapeutic strategies for bladder cancer. If the immune cells are exhibiting glycolysis and FAO metabolic signatures, this would signify an activated immune response capable of attacking tumors. If the tumor cells show increased glycolysis and FAO, this may be a sign of the Warburg effect, increased nucleotide, amino acid, and lipid biosynthesis, and ultimately tumor cell proliferation (472). To differentiate metabolic signatures between pro-tumor and anti-tumor immune cells, Liu and colleagues tested preferred utilization of TCA cycle intermediates for M2 pro-tumor macrophages and TLR-stimulated anti-tumor macrophages. M2 macrophages preferred the use of exogenous fatty acids for TCA cycle components (and increased lipid transporter expression), whereas TLR stimulated macrophages showed preference for de novo lipid synthesis, displaying a shift away from complete utilization of carbon from glucose towards glutamine anaplerosis for generating TCA cycle components (471). A similar strategy can be employed to better understand the mixed
metabolic RNA profile seen in the treated MB49 tumors: investigation into ECAR, OCR, and lipid synthesis analysis of intratumoral immune cells and tumor cells will be important for identifying which cell components the metabolic changes are specifically attributed to and can help identify cells that are immunosuppressive, aiding in pinpointing targets for therapeutic intervention.

Combination treatment also showed enrichment of ECM and collagen modification gene expression, potentially hinting at increased cell motility or proliferation. Similar to the uncertain attribution of glycolysis and FAO metabolic genes, which cells contribute to this increased expression remains unclear. As previously stated, upregulation of expression of genes involved in ECM remodeling and mesenchymal transition has been shown to be a marker of tumor resistance to anti-PD-1 therapy in patients with metastatic melanoma (465). Further investigation into anti-PD-1 therapy resistance mechanisms in bladder cancer, and other cancers is an important field to pursue, and has only begun to be understood at the innate and adaptive cellular level (473). It is not currently clear if the increased expression of cell motility and ECM modulation signaling is related to PD-1 resistance in the MB49 model. However, to investigate PD-1 resistance as a possibility in IFN+anti-PD-1 treatment of bladder cancer, carcinogen-induced or transgenic murine models of bladder cancer may provide the best tumor genesis rate to test combination therapy resistance mechanisms, as MB49 tumors are fast-growing and may not provide sufficient time and opportunity to study.

Looking to the future of bladder cancer treatment and considering my findings in this dissertation, I believe that efforts to inhibit IL-6 signaling, because of its role in chronic inflammation and potential relationship with tumor promotion,
are hasty (474). In models which require T cell priming for activation, IL6-/− mice display impaired involvement of innate cells (475). Likewise, inhibition of downstream IL-6-target STAT3, because of its role in regulating cell survival, proliferation, and angiogenesis (476), could negatively preclude the immune response due to STAT3 regulation of granulopoiesis, DC development and function, T cell differentiation and function, Treg development, and anti-inflammatory signaling, among other effects (477). In this work, I show that IL-6 is a necessary component in the IFN-driven antitumor response. Immunotherapy, radiation therapy, and chemotherapy all rely on the immune response, including IFN signaling, to combat tumor growth; consequently, to some degree this will rely on inflammatory cytokines such as IL-6 to potentiate the antitumor immune response. Therefore, focus should be directed towards inciting the immune response in bladder cancer treatment, not inhibiting its effector branches. Ad-IFNα therapy theoretically reaches the goal of activating the immune response. The use of a viral vector delivery system can be difficult in cancer types that are systemic (blood), or those hard to reach without systemic administration (pancreatic, colon, lung, some prostate, etc.), exhibiting potential bystander events and adverse effects; this same reasoning makes viral-mediated therapy in bladder cancer more advantageous because of the ability to locally deliver the drug and avoid systemic toxicities. Because melanoma and other skin cancers are also easily accessible, Ad-IFNα may be utilized in these indications as well.

Despite the advantage of local delivery, treatment with Ad-IFNα still shows less than 50% response rates in NMIBC patients (66, 67). This may be due to mixed ability of the drug to permeate through different patient’s urothelial barrier,
the heterogeneity of the bladder tumor landscape, upregulated immune evasion mechanisms, and inaccurately staged and stratified disease, as previously discussed. However, for BCG unresponsive patients, I think Ad-IFNα is still a promising therapeutic alternative to cystectomy because it can sustain patient quality of life, and the contributing reasons for resistance to IFN therapy can be addressed by more vigilant clinical assessment and combination therapeutic approaches to target immune evasion strategies such as inhibitory checkpoints.

To this end, PD-1 inhibition in combination with IFN therapy may not be the best therapeutic option when considering a more holistic immune therapy approach to bladder cancer. PD-1/PD-L1 pathway inhibition is effective at re-stimulating exhausted T cells already in the tumor, whereas CTLA-4 inhibition has actually been shown to increase T cell activation and chemotaxis to tumors (294). The potential combination of anti-CTLA-4 mAb with viral-mediated IFN may prove to be more successful in inhibiting UC tumor growth though the enhanced recruitment of effector T cells, an additive effect to IFN-I’s diverse antitumor mechanisms. Anti-CTLA-4 therapy has been helpful to disease phenotypes with high mutational loads, due to more probable neoantigen targets for T cell attack (90); thus bladder cancer, having the third highest mutation landscape after melanoma and lung cancers (478), is a prime candidate for CTLA-4 targeted therapy. Focusing more on therapies that are already approved for use in bladder cancer, and have shown efficacy, the combined use of IFN with BCG may be an attractive treatment strategy. Combination BCG + IFNα2b was shown to potentiate the effects of BCG alone with a response rate of about 60% in prior BCG failed patients (479). Because this study was performed with a
recombinant IFN protein, I would speculate that viral-mediated IFN would provide
even greater clinical benefit due to its enhanced durability and permeation into
the bladder wall.

Together, my results provide a preclinical conceptual example for using
type I IFN activation to increase the therapeutic benefit of PD-1 blockade for
bladder cancer patients, as well as a rationale for pursuing further studies in
NMIBC to optimize a treatment protocol (dosing and timing). Recent US Food
and Drug Administration approval of atezolizumab (anti-PD-L1 mAb) and
nivolumab (anti-PD-1 mAb) for treatment of metastatic urothelial carcinoma (63)
has brought into question the role of immune therapy earlier in the treatment of
UC, and thus clinical trials examining the effect of checkpoint inhibitors in earlier
staged cancer is ongoing. To this point, Pietzak and colleagues showed that
chemotherapeutic treatment of “secondary” MIBC, that is, tumors that have
progressed to higher staged MIBC from previously diagnosed and treated
NMIBC, had lower response rates and short survival compared to “primary” de
novo, treatment naïve MIBC (480). Moving forward, I think the bladder cancer
treatment field should focus on implementing a combination innate and adaptive
stimulating immunotherapy strategy at earlier disease stages (not just BCG failed
patients). It can broadly affect more patients rather than personalized targeted
molecular therapies, and lead to better clinical outcomes for many subtypes of
bladder cancer patients.
Chapter 6: Methodology

**Patient Samples**

Patient specimens utilized in this study were collected from previous Phase I, Phase Ib, and Phase II clinical trials with adenoviral interferon-α2b formulated with Syn3 (Ad-IFNα/Syn3) with patient eligibility, treatment, and specimen collection approved by the University of Texas MD Anderson Cancer Center institutional review board (IRB) (66, 67, 408).

Urine samples utilized for preliminary marker identification (Figure 7) were collected for clinical research use from informed and consented patients about to undergo standard TURBT. Specimen collection was approved by the University of Texas MD Anderson Cancer Center IRB.

**Mice**

Wild-type male C57BL/6J mice, IL-6KO mice, RAG-/-, and p53 +/- mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All gene-deficient mice used are on the C57BL/6 background. IFNAR/- (481) were provided by Dr. Paul W. Dempsey (Department Of Microbiology and Molecular Genetics, University of California, Los Angeles) and Dr. Tadatsugu Taniguchi (Department of Immunology, Tokyo University, Japan) to Dr. W. Overwijk and crossed to the C56BL/6 background. IL15Rα/- mice (482) were originally generated by and obtained from Dr. Averil Ma through Dr. Leo Lefrancois and crossed to the C57BL/6 background. All animal experiments were performed according to the institutional guidelines for the care and use of laboratory animals.
Cell Lines and Treatment in vitro

MB49-GFP/luciferase murine bladder cancer cells were generously donated by Dr. Robert Svatek (the University of Texas Health San Antonio). Cells were grown in culture in modified Eagle medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. MB49 bladder cancer cells were seeded in 6-well plates and treated with 0-10,000 IU/mL recombinant murine IFNα (PBL Assay Science, Piscataway, NJ). After 24 hours of stimulation, cell death and apoptosis were analyzed by a combined PI/Annexin V (APC) assay (Invitrogen [Thermo Fisher Scientific], Carlsbad, CA), and analyzed by flow cytometry whereby early apoptosis (Annexin+PI−) and late apoptosis (Annexin+PI+) were quantified (n=2 biological replicates). UPPL1541 and UPPL1595 cell lines are established from a spontaneous primary bladder tumor in an Uroplakin-Cre driven PTEN/P53 knockout genetically engineered mouse model and were generously provided by Dr. William Kim (UNC Chapel Hill).

Tumor Transplantation

Mice were injected subcutaneously into the right flank with 1×10^5 MB49 bladder cancer cells (1×10^6 or 1×10^7 for UPPL1595 and UPPL1541, respectively). For analysis of abscopal effect, following primary tumor injection of MB49 on right flank, a secondary tumor (1×10^5 MB49 cells) was injected in the left flank 4 days later. Tumor development was monitored by palpation and fluorescent imaging with the IVIS Spectrum In Vivo Imaging System and Living Image software (PerkinElmer, Waltham, MA). Mice were randomized into treatment groups on the basis of fluorescent intensity and palpated tumor size at
day 6 after implantation. From day 6 on, tumor size was measured every 3 days by caliper and was recorded as Area [$L \times W$] in millimeters. Mice with tumors exceeding 20 mm in diameter or with large ulcerations were deemed moribund and euthanized. Each point on tumor growth graphs reflects the average area of the total starting number of mice per treatment group; graph lines are stopped when multiple mice in a group are euthanized. Experiments were performed in groups of five or more mice and repeated at least twice.

**Treatment of Mouse Tumors and Depletions**

When transplanted bladder tumors became palpable, poly(I:C) (100 µg; Invivogen, San Diego, CA) was injected peritumorally beginning on Day 6 or 7 and continued every 3 days until mice were deemed moribund, or for tumor analysis after a total of 2, 3, or 4 treatments as denoted in Figure 8, 17. Therapeutic blockade of PD-1 was performed using rat anti-mouse PD-1 mAb (200 µg, i.p., clone RMP1-14; BioXcell, West Lebanon, NH) or control-rat IgG mAb (200 µg, i.p.; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) every 3 days in conjunction with poly(I:C) treatment. Antibody-mediated depletion of T cells or natural killer cells was induced with rat anti-mouse Thy1.2 mAb (300 µg, i.p. clone 30H12; BioXcell), rat anti-mouse CD8 mAb (300µg, i.p. clone 2.43; BioXcell), or rat anti-mouse Nk1.1 mAb (300µg, i.p. clone PH136; BioXcell) delivered two times, 1 week apart. Ly6G and CSFR1 mAbs (400µg, i.p. clones 1A8 and AFS98 respectively; BioXcell) were given three times a week until mouse morbidity. Greater than 85% depletions of target cells were confirmed by flow cytometry of peripheral blood samples taken 1-2 days after Ab treatment. Efficiency of cell depletions is represented in Figure 21. All treatments and
**Figure 21: Efficacy of immune cell subset depletions.** A) mAb depletion of Ly6G⁺ cells; flow plot is of gated lineage (TCRβ⁺CD19⁻NK1.1⁻)⁺CD11b⁺ cells. B) mAb depletion of CD8 T cells; flow plot is of gated TCRβ⁺ cells. C) mAb depletion of NK cells; flow plot is of gated TCRβ⁻ cells. D) mAb depletion of all T cell subsets by Thy1.2 antibody. All samples from peripheral blood. Plots are representative of n=5.
depletions were performed in Wild-type male C57BL/6J mice, male IL-6KO mice (B6.129S2-Il6tm1Kopf/J), and male RAG-/- (B6.129S7-Rag1tm1Mom/J) mice (The Jackson Laboratory; Bar Harbor, ME).

p53+/- mice (8 weeks old) were treated with 0.25% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water for 12 weeks (483). By 16 weeks mice develop carcinoma-in-situ (CIS) which proceeded to invasive cancer by 30 weeks. Mice were treated with LV Ctrl or LV IFN (3X10^7 virus particles) for 4 weeks starting at 16 weeks of age (Lentiviral Vectors [LV-Control and LV-IFN] provided by FKD Therapies, University of Finland). Excipient SYN3 (1mg/ml) was used as vehicle for all treatment. For intravesical treatment, mice were anesthetized and mouse urethra was catheterized with 20G angiocatheter and after emptying the bladder contents, virus was instilled (100 μl) volume and allowed to dwell in the bladder for 40 mins. After instillation of virus, mice were allowed to recover and returned to their cages.

**T cell Stimulation**

For intracellular cytokine and IFNγ staining, MB49 tumor-infiltrating immune cells were isolated by manual homogenization followed by Percoll gradient, and plated into 6-well plates coated with anti-CD3 antibody, incubated in RPMI with 10% fetal bovine serum with protein transport inhibitors GolgiStop and GolgiPlug (2 μg/mL; BD Biosciences, San Jose, CA) for 5 hours (n=4). After incubation, cells were stained for surface markers and then fixed and permeabilized prior to staining for intracellular proteins.

**Murine Cytokine Analysis**
Murine cytokines were measured by ELISA: IFNα (PBL VeriKine Mouse Interferon Alpha ELISA kit, 42120-1; Piscataway, NJ) and IL-6 (R&D Mouse IL-6 Quantikine ELISA Kit, M6000B; Minneapolis, MN). Samples were run in duplicate and ELISA was performed according to manufacturer instructions. Plates were read on Molecular Devices Spectra Max Plus384 plate reader.

**Immunohistochemistry and Immunofluorescence Staining**

Mouse tumors were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned by the Research Histology Core Laboratory at The University of Texas MD Anderson Cancer Center. Patient tumors were isolated and processed according to the phase I trial protocol (66, 408) and obtained from MD Anderson. Immunohistochemistry was performed with either rat anti-mouse CD31 mAb (SZ31, Dianova, Hamburg, Germany) or rabbit anti-human CD3 pAb (A0452, Dako [Agilent], Santa Clara, CA) followed by rabbit-anti rat HRP- or goat-anti rabbit HRP–conjugated secondary antibody respectively (Bio-rad Laboratories, Hercules, CA) and the DAB peroxidase substrate kit (Vector Laboratories, Inc. Burlingame, CA). Sections were then counterstained with hematoxylin. Tissue sections were blindly quantified by manual counting. Representative areas (2-3) from each CD31 stained section (n=12 in total) with most intensive microvessel density was captured under the light microscope at 200x (Leica). MVD per tumor section was calculated from the average count of CD31+ vessels per representative area, averaging total number from n=3 tumors per treatment group. Total image length is 384µm. Immunofluorescence was performed using rat mAb CD4 (GK1.5, Abcam, Cambridge, MA) and rabbit pAb
CD8 antibodies (ab4055, Abcam). Sections were examined with a Nikon microscope and camera and processed in ImageJ.

**Flow Cytometry**

Tumor-infiltrating immune cells were isolated by manual homogenization and digestion of tumors or bladders (BBN-induced), followed by Percoll gradient, and stained with fluorochrome-conjugated mAbs specific for mouse surface markers CD45, CD44, CD8, CD4, Nk1.1, CD11b, CD11c, Ly6C, Ly6G, CD19, TCRβ, F4/80 (BD Biosciences, Ebioscience/Thermo Fisher Scientific) according to standard procedures. Intracellular staining (*T cell Stimulation*) for IFNγ, and Ki-67 and granzyme-B was performed after permeabilization of cell membranes using the Transcription Factor Staining Buffer Set from eBioscience (ThermoFisher).

Human urine samples were collected and stained for immune and epithelial markers for flow cytometry. Briefly, samples were centrifuged at 2000 RPM, washed with DPBS, and centrifuged at 2000 RPM again. Pellets were resuspended in 1mL of DPBS, counted on hemocytometer, and aliquoted to a maximum of $10^6$ cells per tube. Samples were then stained with fluorochrome-conjugated mAbs specific for human markers CD45, cytokeratin, CD8, TCRαβ, PD-1, PD-L1 (Thermo Fisher Scientific) according to standard procedures.

All samples were run on a BD LSRFortessa unit and analyzed by FlowJoX software (Flowjo LLC, Ashland, OR).

**Reverse Transcriptase PCR**
Tumor samples were harvested and immediately snap-frozen in liquid nitrogen. Total RNA was isolated using the mirVana miRNA isolation kit with phenol (Ambion [Thermo Fischer Scientific], Carlsbad, CA) with concentration measured on the NanoDrop ND-1000 Spectrophotometer. One-step quantitative reverse transcriptase PCR was performed with diluted RNA, AgPath-ID One-step reverse transcriptase PCR reagents, and Taqman gene expression assay primers (Thermo Fisher Scientific) for the genes indicated, using relative expression of GAPDH as a reference gene. Samples were analyzed on the StepOnePlus Real-Time PCR System with StepOne Software v2.3 (Applied Biosystems [Thermo Fisher Scientific]).

**RNA-seq of Murine Tumors**

Stranded Total RNA sequencing was performed by the MD Anderson Sequencing and Microarray Facility (SMF) on the Illumina Hi-Seq 4000 platform. RNA samples were isolated from MB49 tumors using the mirVana miRNA isolation kit with phenol (Ambion [Thermo Fisher Scientific]), confirmed purity and concentration by the Agilent 2100 Bioanalyzer (Agilent Technologies [Thermo Fisher Scientific]) and Nanodrop ND-1000 Spectrophotometer, and sent to SMF. Raw reads in FASTQ format were aligned to the mouse reference genome, GRCm38/mm10, using MOSAIK alignment software. Mapped reads were used to generate raw counts for each gene using HTSeq. Counts data were normalized across samples with DESeq (484) and normalized expression values were analyzed by Morpheus matrix visualization and analysis software (Broad Institute), Gene Set Enrichment Analysis (GSEA) software (Broad Institute), and Ingenuity Pathway Analysis (IPA) software (Qiagen). The up-regulated pathways
in GSEA were defined by a normalized enrichment score (NES) >0. Only the top 20 pathways more enriched in each treatment group were listed.

**Urinary Cytokine Analysis**

Frozen patient urines collected from baseline Day 1 (D1) pre-treatment, and Day 4 and Day 12 (D4, D12) post treatment of Phase I and II clinical trials with Instiladrin (Ad-IFNα/Syn3) by the SUOCTC working group were thawed and diluted before analysis with the Bio-Plex Pro™ Cytokine, Chemokine, and Growth Factor Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA). We utilized antibody targets from the Human Cytokine Standard Groups I and II. Samples were run in duplicate and the plate was read with Bio-Plex Manager™ software (Bio-Rad Laboratories, Inc) in the MD Anderson Department of Surgery. Observed concentration was log2 transformed, and graphed with baseline corrected to Day 1 levels of each cytokine. Patients with undetectable levels of cytokines were left out of analyses. **Assessment**: of the 39 total patients, 13 were deemed as “responders” (CR) as defined by no evidence of recurrence of a high grade tumor by cystoscopy, cytology, or if clinically indicated, biopsy at 12 months. The other 26 patients were deemed “non-responders” (NR). Correlation between IFNa2 levels and other cytokines were deemed as moderately positive if r > 0.5 and strongly positive if r >0.7. Positive correlation was considered statistically significant if p value <0.05.

**Analyses of Gene Expression for Patients Treated with Ad-IFNα**

RNA isolation
The tumor areas in formalin fixed, paraffin-embedded (FFPE) human specimens from the Phase I, Ib, and II trials with Instiladrin (Ad-IFNα/Syn3) were reviewed by a genitourinary pathologist. Total RNA from 8 matched (16 total) FFPE tumors was isolated using the High Pure miRNA isolation kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, for the deparaffinization, five to ten (depending on the tumor area) 10 µm sections were incubated with xylene for 5 minutes, followed by two ethanol washes and dried for 10 minutes at 55°C. The dried tissues were incubated with proteinase K for 3 hours at 55°C degree, followed by two washes according to the instructions of the manufacturer. RNA was eluted with water and treated with DNAsé for 30 mins at 37°C. DNase treated RNA was washed twice according to the with manufacturer’s instructions and eluted with water. RNA purity and integrity was measured by NanoDrop ND-1000 and Agilent 2100 Bioanalyzer and only high quality RNA was used for library preparation.

Library preparation and sequencing

Whole transcriptome RNA sequencing was performed using Ion Torrent’s AmpliseqRNA platform (Thermo Fisher Scientific) and an Ion Proton sequencer (Thermo Fisher Scientific). Twenty nanograms of purified RNA was transcribed into cDNA using the SuperScript® VILO™ kit. Then cDNA was amplified using the Ion Ampliseq Transcriptome Human Gene Expression Core panel, followed by ligation of adapters and barcodes to amplicons and purification. Purified libraries were quantified using the Ion Library Quantification kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Libraries were diluted to 100 pM and pooled in sets of 8. Pooled libraries were amplified on Ion Sphere™ particles (ISP) using emulsion PCR and enriched on the IonChef (Thermo Fisher Scientific).
Scientific). Template positive ISPs were loaded into Ion PI chips and run on the Proton instrument in the Genomics Core in the Department of Urology at The University of Texas MD Anderson Cancer Center.

**Bioinformatics Analysis**

RNA-Seq gene expression analysis: Primary analysis of RNA sequencing data was performed using AmpliSeqRNA analysis plugin in the Torrent Suite Software. This plugin aligned the raw sequence reads to a human reference genome that contains 20,802 RefSeq transcripts (hg19 Ampliseq Transcriptome_ERCC_V1.fasta) using the Torrent Mapping Alignment Program (TMAP). Then, the number of reads mapped per gene will be counted to generate raw counts files and normalized reads per gene per million mapped reads (RPM) files. To visualize expression patterns, log ratios of POST/PRE gene expression of matched tumors were used for hierarchical clustering with Cluster and TreeView (485), or log2 normalized expression values were analyzed by Morpheus matrix visualization and analysis software (Broad Institute).

**Assessment:** of the 8 total patients analyzed, none were classified as “responders” (CR) as defined by no evidence of recurrence of a high grade tumor by cystoscopy, cytology, or if clinically indicated, biopsy at 12 months.

**Statistical Analyses**

Statistical analyses of experimental results were evaluated with the GraphPad Prism 7 software. Two-tailed Student t tests, log-rank analyses, or multiple unpaired t tests were performed using averaged treatment group measurements at any one time point, as indicated. One way ANOVA with multiple comparisons was used for patient urine samples for each cytokine.
Results were considered statistically significant when *, P < 0.05; ** P <0.01; *** P<0.001.
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Vita

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