COMBINATION OF IMMUNE CHECKPOINT INHIBITORS IN METASTATIC UROTHELIAL CARCINOMA: PD-1/PD-L1 WITH CTLA-4

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2022

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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Abstract

Urothelial carcinoma is the most common form of bladder cancer and is the variant with the most immunogenic response. This makes urothelial carcinoma an appropriate candidate for immunotherapy in the form of immune checkpoint inhibitors. Immune checkpoint inhibitors are antibodies directed against immune checkpoint-related molecules expressed on tumor-infiltrating lymphocytes. There have been multiple studies on the role of the immune checkpoint molecule, PD-1 on T-cells and its interaction with the ligand PD-L1. Moreover, CTLA-4 is a different checkpoint also expressed on T-cells in urothelial carcinoma. The blockade of CTLA4 can lead to the reactivation of lymphocytes by preventing apoptosis and anergy. The only FDA-Approved immune checkpoint inhibitors for metastatic urothelial carcinoma target the PD-1/PD-L1 pathway. However, the overall response rate and progression-free survival rates of these drugs are not sufficient in the patient population. In this review, the current immune checkpoint inhibition (ICI) treatment landscape is explored with an emphasis on combination therapy in the form of PD-1/PD-L1 with CTLA-4. Investigation of the current literature on ICI in in-vivo experiments shows a decrease in tumor volumes and size when PD-1/PD-L1 is blocked, and this result is replicated in CTLA-4 blockade. However, there are limited preclinical models testing tumor response in combination blockade of CTLA-4 in the presence of PD-1/PD-L1 blockade. In this review, a proposal on canine organoid bladder samples as a complement to transgenic mice for preclinical drug testing is also explored. We anticipate this review to be a foundation for a deeper experimental investigation into combination therapy in the form of PD-1/PD-L1 and CTLA-4 blockade in metastatic urothelial carcinoma.

Introduction

An estimated 81,180 new cases of bladder cancer will be diagnosed in the United States in 2022 with approximately 17,100 deaths occurring during this same period[1]. According to the American Cancer Society, bladder cancer is the sixth most common cancer in the United States and has a median age at diagnosis of 73 years. Urothelial carcinoma (UC) also known as transitional cell carcinoma is the most common histology type with risk factors being smoking, toxins, age, gender, race, chronic bladder infections, and history of bladder cancer. The other forms of bladder cancer including squamous cell carcinoma and adenocarcinoma are less frequent in incidence. UC affects a significant percentage of the older population, and the incidence rate continues to rise every year. UC can be divided into 3 categories: non-muscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC), and metastatic UC as shown in figure 1. Historically metastatic UC has had high mortality rates[1].

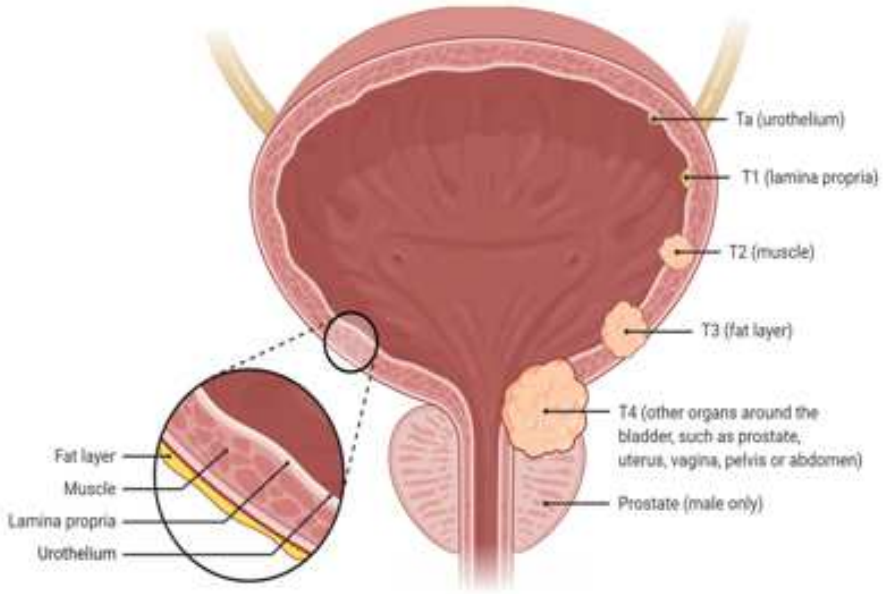

Figure 1*: Graphical illustration of the 4 stages of tumor growth in UC. T4 represents Metastatic UC that has travelled to distal organs. Illustration created with BioRender.com*

The definitive treatment for MIBC per American Urological Association guidelines is surgery in the form of radical cystectomy with or without neoadjuvant chemotherapy[2]. Numerous agents are approved for adjuvant treatment of UC including platinum-based chemotherapy, radiation, and more recently immune checkpoint inhibitors (ICI). Platinum-based chemotherapy has been the standard of care for metastatic UC. However, many patients relapse after chemotherapy or are unable to tolerate platinum chemotherapy due to medical comorbidities[2].

Newer agents, most notably checkpoint inhibitors, have been approved for the treatment of both NMIBC and MIBC. ICI target ligands that lead to inactivation and apoptosis of T-cells, specifically cytotoxic T-cells.[3] Much of the bladder cancer research into ICI has been directed towards preventing programmed-cell-death 1 (PD-1) binding to its ligand, programmed-death ligand 1 (PD-L1) on PD-L1 positive tumor cells. Patients who are treated with ICI for bladder cancer are administered PD-1 or PD-L1 therapy at various stages of the disease. However, ICI in metastatic UC has not achieved a complete overall response rate even among the ones that are FDA approved[1]. Multiple immune checkpoint ligands play a part in immune evasion during bladder tumorigenesis[4]. Understanding the molecular pathway of the ligands these inhibitors act on could be important in increasing the response rate of ICI therapies.

In the bladder tumor microenvironment, some factors promote tumor evasion of the immune system using immune checkpoint-related molecules. The purpose of ICI is to abrogate these interactions and prevent apoptosis of T-cells. Due to the multifactorial aspects of immune

checkpoint molecules, a combinatorial approach using ICI could target different pathways of immune evasion and result in a more robust response.

Given the challenge of tumor resistance to ICI treatment after conventional chemotherapy, there remains a need for novel agents that may bolster the immunomodulating response of currently, available therapies. In this review, we discuss the current landscape of FDA-approved checkpoint inhibitors in the management of advanced UC with the focus on the scientific rationale for combination therapy in the form of PD-1/PD-L1 blockade and CTLA-4.

Tumor Microenvironment in Urothelial Carcinoma

In UC, the tumor microenvironment (TME) consists of stroma, connective tissue, blood vessels, and immune cells. These different cell types provide nutrition and support to the tumor parenchyma. Crosstalk between the tumor cells and their environment can alter the environment and the environment can promote the spread of the tumor[5]. Among the cells in the microenvironment are the stromal cells which make up the connective tissue and extracellular matrix (ECM) of the environment. In UC, cancer-associated fibroblasts (CAF) are the most common stromal cell type present in the TME. Activated CAF expresses vimentin, α-smooth muscle actin, platelet-derived growth factor receptor, and other markers, all of which aid in the remodeling of the ECM[6]. There has been controversy over the exact role of CAF in cancer biology, due to the heterogeneity of these cells they could either promote or inhibit tumors even in the same cancer. Bladder cancer research has found that the frequency of fibroblasts is increased in UC compared to normal uroepithelium[6]. Moreover, a clinical study with 344 bladder cancer patients found that dominant expression of CAF markers is negatively correlated with 5-year survival and positively correlated with muscle-invasiveness of cancer [7], [8]. While

the mechanism behind this is still being explored, one possible explanation would be the role CAF have on immune cells in the TME. Stromal cells in the TME can release soluble factors that alter cell surface proteins and suppress the immune system; for example, they can regulate the expression of PD-L1 on tumor and immune cells[5]. Factors regulated by CAF can lead to the release of anti-inflammatory cytokines like TGFβ, TGFβ can then induce the production of immunosuppressive cells like the T-regulatory cells[9]. The desmoplasia that is promoted by CAF and TGFβ can also contribute to the exclusion of immune cells in the tumor parenchyma and localize them to the stroma[10]. Wang. et al found that bladder cancer samples with high T-cell infiltration and low stroma-related gene expression had longer survival[10]. These modulations by the CAF all have an impact on the immune microenvironment of UC especially the tumor-infiltrating lymphocytes (TIL).

Additionally, another effect CAF has on the TME of UC is the increased expression of the BCL2 gene through the Estrogen receptor alpha (Erα) signaling in vivo[11]. BCL2 is an antiapoptotic gene that prevents the activation of proapoptotic proteins like BAK/BAX. In this way, CAF promotes the growth of UC by constitutively activating antiapoptotic proteins. The constant expression of BCL2 in bladder cancer also has downstream effects on the immune cells in the TME, and this is further discussed in the section on immune cells in the TME.

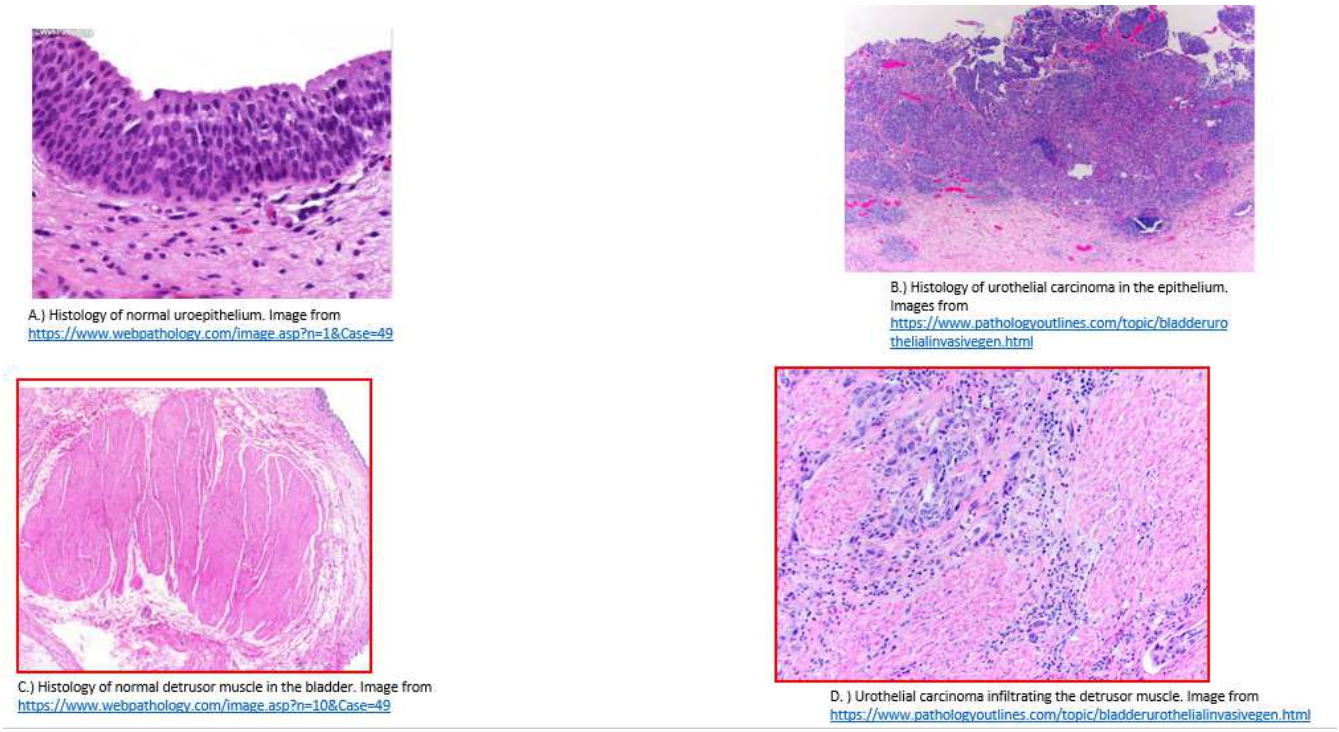

Figure 2: *Histopathology of bladder cancer. This histology image shows the difference between normal uroepithlium and lymphovascular invasion of UC. The neoplastic cells appear pleomorphic, hyperchromatic, and irregular. Invasion of plasma cells and lymphocytes are noted in the stroma as well as the capillaries. Images contributed by Maria Tretiakova on Pathologyoutline.com* https://www.pathologyoutlines.com/topic/bladderurothelialinvasivegen.html.

Tumor Mutational Burden in Bladder Cancer

Infiltration of T-cells into the TME is indicative of cellular immune response to tumor neoantigens as shown in figure 2. UC has been shown to have a robust immune response, based largely on how high the tumor mutational burden (TMB) of the cancer is. Based on work done by the Cancer Genome Atlas, bladder cancer has been shown to frequently have high somatic mutation rates along with lung and skin cancer[12]. Identification of frequent mutations has

allowed several institutions to create molecular subgrouping of UC. Particularly, muscle-invasive bladder cancer (MIBC) has been categorized into 5 subgroups: luminal papillary (LumP), luminal non-specified (LumNS), luminal unstable (LumU), stroma-rich, basal/squamous (Ba/Sq), and neuroendocrine-like (NE-like) subtypes[6]. The most pertinent to this paper is the LumU subtype which has the highest level of genomic instability due to somatic mutations. One of the most common genes mutated in this subtype is the ones that encode apolipoprotein B mRNA editing catalytic polypeptide-like family of proteins (APOBEC)[6]. The APOBEC-specific mutations seen in bladder cancer are commonly known to be in the TERT promoter region, 70-80% of bladder cancers contain this mutation[13]. TERT is the catalytic subunit of the telomerase holoenzyme and it is responsible for preventing telomere shortening[6]. During embryonic development TERT activity is high but in somatic tissues, the expression levels decrease. In somatic tissues, TERT mustn't be overexpressed, or else the limitation of cell division and cell cycle regulation is compromised. Pathological expression of TERT allows for the immortality of tumor cells. Among the patients with UC, those with high expression of TERT have a lower disease survival rate[14]. Other mutations are also common in UC some examples are TP53, RB1, and EGFR[15]. Currently, more research is still being done on the molecular categorization of bladder cancer and its mutations. Bladder cancer specifically UC has a high TMB which in turn means an increase in neoantigen presentation to the cellular immune system. Therefore, the immune environment within UC consists majorly of T-lymphocytes.

Tumor-Infiltrating Immune Cells in Bladder Cancer.

Within the immune microenvironment of UC, there are several immune cells present, the major effector immune cells are the tumor-infiltrating lymphocytes (TILs). Among TIL, cytotoxic T-cells (CD8 cells) are the most important to mounting a response against the

neoantigens presented from the tumor. Studies have shown a positive correlation between the amount of CD8 cells present in tumor tissue and the patient's response to immunotherapy[16]. Due to the effector function of CD8 cells, it is conceivable that most immune evasion tactics from the tumor are directed towards these cell types. CD8 cells are presented with peptide antigens on MHC-I molecules present on antigen-presenting cells (APC). The binding of the T-cell receptor to the antigen is not enough to activate the CD8 cells; other signals like the binding of CD80 on APC to CD28 on T-cells and the presence of cytokines like IFNγ are needed to fully activate the CD8 cells[17]. CD8 cells carryout their functions via the use of catalytic enzymes, this promotes lysis of the cells they target[6]. CD8 cells also release several other cytokines that additionally prime other immune cells towards the tumor microenvironment. These cells are incredibly important in the restraint of tumor growth and are the primary therapeutic targets in this paper. There are cell receptors that are present on CD8 cells that can either be upregulated or downregulated by the tumor cells, this modification of receptors helps the tumor evade the TILs. For example, immune checkpoints are cell-surface proteins that are used by tumor cells in the TME. While CD8 T-cells are important in the direct killing of tumor cells, helper T-cells (CD4 cells) play a supportive role to CD8 cells. CD4 cells can ultimately end up in one of 3 pathways: Th1, Th2, and Th17[18]. The Th1 pathway leads to the release of inflammatory cytokines particularly IFNγ. Studies in bladder cancer cell lines have found that depletion of CD4 cells in the TME increases tumor size and dampens the effect of immune checkpoint inhibition[18]. IFNγ has multiple effects on different cells including activation of CD8 cells and macrophages. Thereby, in this manner, CD4 cells are significant for the differentiation of important immune cells in the TME. Immunotherapy targets multifactorial aspects of the

signaling pathway that leads to CD8 and CD4 cell activation. Moreover, there are other immune cells in the TME that are also important and impact the immune microenvironment in UC.

Other cells in the TME that contribute to the immune microenvironment are macrophages. They also possess the ability to affect the efficacy of cancer therapy including chemotherapy and immunotherapy. Macrophages are phagocytic cells that differentiate from circulation monocytes to become tissue-resident cells. In normal tissues, macrophages act as part of the innate system to fight foreign pathogens through phagocytosis. However, they are also involved in the adaptive immune system, macrophages can present antigens to activated T-cells to modulate cellular immunity. Finally, macrophages are very important for wound healing and coordinating anti-inflammatory cytokines in the body after infection. A key feature of macrophages is their ability to differentiate into one of two phenotypes in the presence of the appropriate cytokines and gene expression[19]. The M1 phenotype is pro-inflammatory and is activated in the presence of cytokines like IFNγ, TNFα, IL-6, IL-1[19]. The endpoint of M1 function is to fight infection, and tumors and prevent wound healing. Conversely, the M2 phenotype is anti-inflammatory and mediates the wound healing process in the body. M2 phenotype is activated in the presence of cytokines like IL-4, IL-3, and IL-10, the M2 macrophages secrete mediators like IL-10 and TGFβ to damper the inflammatory process[19].

During tumorigenesis, the macrophages present in the TME are referred to as tumor-associated macrophages (TAM). These can either be resident in the tissue at the time of neoplasia or they are recruited from circulating monocytes into areas of hypoxia[6]. Tumor cells secrete cytokines that favor the differentiation of TAM into the M2 phenotype thereby dampening inflammation and potentiating tumor growth. In bladder cancer, TAMs found in tumor samples are predominantly of the M2 phenotype and are commonly present in high-grade

diseases [6]. Besides the cytokines secreted by the tumor cells, the overexpression of BCL2 by melanoma cells has been found to aid in the polarization of the M2 phenotype via IL-1β secretion[19]. Since research has found that UC cells also overexpress BCL2, it is conceivable that this is the reason for the M2 phenotype being predominant in the TAMs. Several cytokines are secreted by these M2 macrophages, pertinent to this paper are the IL-10 and TGFβ cytokines. IL-10 inhibits the function of APCs, they suppress the maturation of intratumoral dendritic cells and reduce IL-12 production[20]. All these pathways that are disrupted are needed in the activation of CD8 cells in the TME, therefore M2 macrophages can dampen the cytotoxic T-cell response needed in UC. The effects of TGFβ on T-cell exclusion in UC have been discussed in previous sections of this paper.

The TME of UC is a complex system with multifactorial cells playing different parts in immune evasion, evasion of apoptosis, and cell immortality to aid in the growth and sustenance of the tumor. There are different therapeutic targets within the TME, pertinent to this paper is the use of ICI to target TIL in UC. ICI weaponize CD8 cells against the tumor cells. To understand the mechanism behind ICI, it is important to understand the function of immune checkpoints in normal uroepithelium and conversely how cancer has hijacked this system.

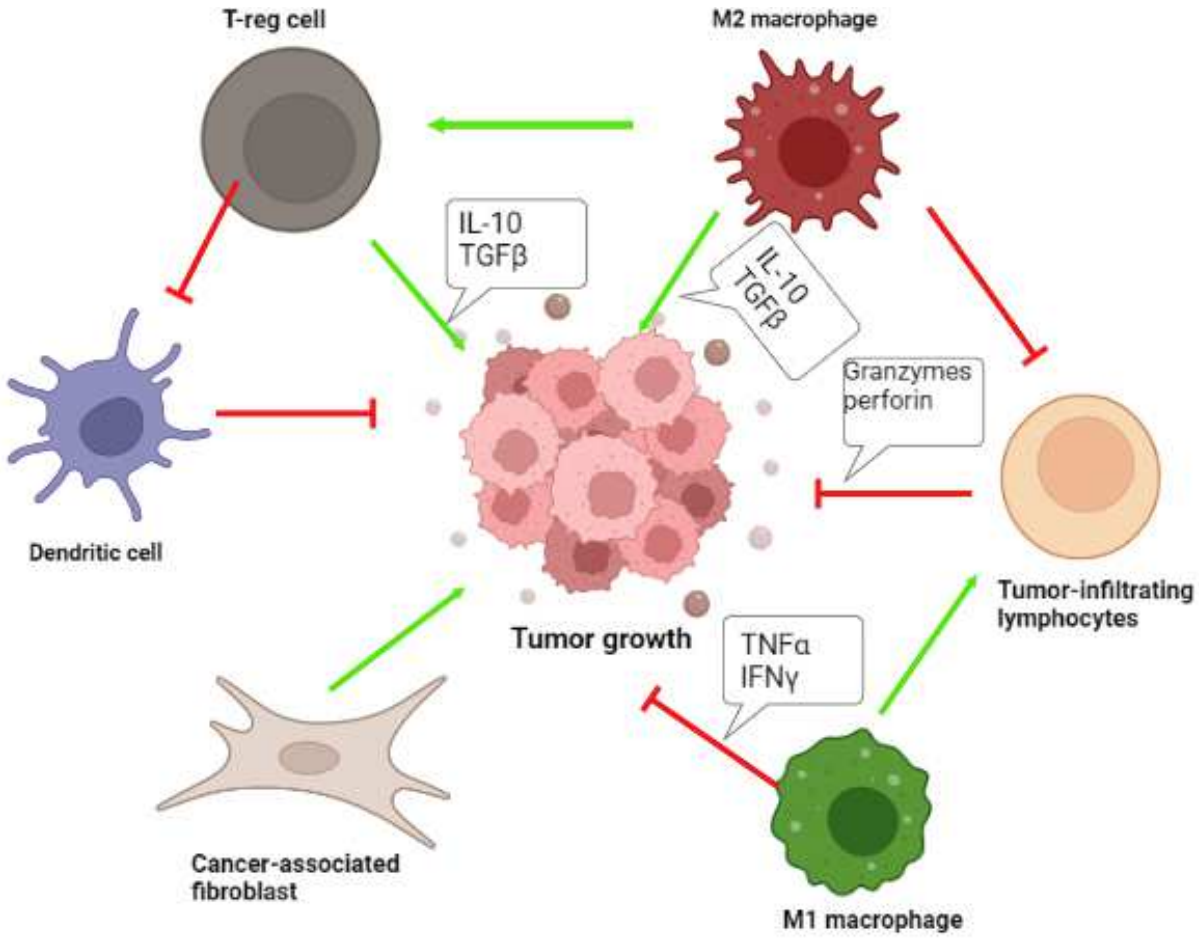

Figure 3: *Illustrates the interactions between different cells in the tumor microenvironment of UC including some cytokines released by these cells. Green arrow represents promotion and red arrow represents inhibition. Illustration created with BioRender.com*

Immune Checkpoints in Urothelial Carcinoma: Mechanism of Action

. PD-L1 is a ligand that can be expressed on tumor cells as well as tumor-associated immune cells. Canonically PD-L1 binds to PD-1, a receptor expressed on effector T-cells. In normal uroepithelium, PD-L1 only binds to PD-1 when the immune system has been stimulated for a prolonged period. Moreover, new research has shown that PD-L1 is also expressed on

antigen-presenting cells (APC) and binds to the ligand CD80 on APC[21]. A different protein that also binds to CD80 is cytotoxic T-lymphocyte antigen-4 (CTLA-4). Tumor cells have adapted these ligands and use them to escape the immune system during tumorigenesis

Program-Death 1(PD-1)

PD-1 is a major cell surface checkpoint receptor present on T-cells in the TME of UC. In tumorigenesis, PD-1 expression is greatly increased on activated T-cells, B-cells, natural killer cells, and myeloid-derived cells to decrease the proliferation and activation of these immune cells[22]. The mechanism of action of PD-1 in the T-cell population including CD4 and CD8 cells is the primary focus of this paper. Studies have shown that PD-1 upregulation is prominent on CD8 cells in UC compared to CD4 cells, although its expression is still overexpressed in both cell lines[23]. PD-1 binds to two ligands PD-L1 and PD-L2 however, PD-L2 is rarely present in high concentrations. Primarily PD-1 asserts its actions by binding to PD-L1, this binding leads to the downregulation and apoptosis of T-cells[22]. The downstream effect of PD-1 signaling is the downregulation of the PI3k/AKT signaling that shuts down cytokine secretion of T-cells as well as the cytolytic effects of CD8 cells[24]. Moreover, tumor cells expressing PD-L1 can escape apoptosis due to the signaling of PD-1/PD-L1 binding[25]. Currently, there have been 2 PD-1 immune checkpoints approved by the FDA for metastatic UC: Pembrolizumab and Nivolumab.

Program-Death Ligand 1 (PD-L1)

PD-L1 is a receptor expressed both on the surface of tumor cells and host immune cells in the TME. It has already been established how PD-L1 binding PD-1 leads to T-cells downregulation, moreover, novel research has found that PD-L1 also binds to CD80 on dendritic

cells. In-vitro experiments done by Mayoux et al. showed that PD-L1 colocalizes with CD80 thereby sequestering it from binding to CD28[21]. In this fashion, PD-L1 can induce anergy of T-cells by inhibiting the appropriate signaling needed for activation. There are several ways that PD-L1 upregulation can be induced in the TME of UC. Some of the ways it can be upregulated are: in response to cytokines in the TME, oncogenic alterations, and hypoxia

During inflammation IFNγ acts as a pro-inflammatory cytokine, it drives T-cell proliferation and activation as well as other immune cells like natural killer cells and M1 macrophages[19]. In the TME of UC, IFNγ has been shown to play a role in the upregulation of PD-L1 during in-vitro cell line studies. One particular study showed that when dendritic cells are co-cultured with IFNγ it led to increased expression of PD-L1[3]. IFNγ binds to interferon-gamma receptor (IFNGR) leading to activation of a JAK/STAT pathway via STAT1[26]. The result of this signaling cascade is the expression of a transcription factor called interferon-responsive factor 1 (IRF1). A study of IRF1-deficient mice in colon mouse models showed inhibition of tumor growth[27].IRF1 mediates the IFNγ-dependent expression of PD-L1 in the TME.

UC is highly mutagenic and has such a high rate of somatic mutations, given this quality, oncogenic alterations would play a major role in PD-L1 upregulation. The p38/MAPK is a signaling cascade that plays a positive role in PD-L1 expression on dendritic cells in bladder cancer[28]. Epigenetic regulations through DNA methylation and histone modification are another way for oncogenic alteration to contribute to PD-L1 expression. A recent study done by Zhang et al. explored the role of an epigenetic protein called WDR5 in immune evasion via PD-L1 expression in bladder cancer[29]. WDR5 is a histone presenter that forms a complex with methyltransferase called MLL1-MLL4 and other protein subunits. This complex plays a vital

role in chromatin remodeling, transcriptional activation of genes, and histone methylation in bladder cancer[30]. Using genomic data from the cancer genome atlas (TCGA), they found that WDR5 is positively correlated with the expression of PD-L1 in different bladder cancer subtypes. The lab then tested their hypothesis and found that competitive inhibition of WDR5 led to a decrease in PD-L1 expression in the TME of bladder cancer cell lines even in the presence of IFNγ[29]. Inhibiting WDR5 led to a decrease in PD-L1 expression at the mRNA level by decreasing RNA polymerase II levels in the PD-L1 promoter region[29]. These experiments show that oncogenic alterations in bladder cancer also impact the upregulation of PD-L1 in the TME.

Patients with UC who have higher PD-L1 expression have been associated with higher stages and lower chances of recovery[21]. Due to the ability of PD-L1 to induce apoptosis and anergy in the TME, it stands to reason that tumor growth is conducive to the environment caused by PD-L1 expression. Currently, Avelumab is still FDA-Approved for maintenance therapy of metastatic UC.

Cytotoxic T-Lymphocyte Associated Protein-4 (CTLA-4)

The first immune checkpoint to be clinically targeted for cancer treatment was CTLA-4. CTLA-4 is expressed on activated CD4 and CD8 T-cells with the former having higher levels of expression[22]. It is also expressed on certain subsets of T-regulatory cells in the TME. CTLA-4 has a similar function to PD-L1 expressed on antigen-presenting cells (APC); CTLA-4 also binds CD80 with approximately ten times more affinity compared to CD28[22]. Without CD80 and CD28 binding, the costimulation of T-cell activation is not achieved[22]. In this way, CTLA-4 can modulate the anergy of T-cells and promote evasion of the immune system in UC. Moreover,

CTLA-4 can also assert its effects by removing CD80 molecules from neighboring APC through transendocytosis[31].

Although some studies have shown that blockade of CTLA-4 can lead to tumor regression in-vitro using cell lines[23], there has not been much research into the in-vivo effects of CTLA-4 inhibition for UC. It is hypothesized that the inhibition of CTLA-4 can prevent anergy during the priming stage of T-cell activation allowing for increased infiltration of lymphocytes into the tumor[32]. Moreover, since CTLA-4 is also expressed on Treg cells, blocking this immune checkpoint could reduce the ratio of Treg to effector T-cells in the TME leading to better control of the tumor by the immune system[32]. There is currently no FDA-Approved ICI for bladder cancer, this highlights the need for better in-vitro models to assess CTLA-4 blockade in UC. In-vitro models that can model the natural TME will be able to provide more accurate

information to conduct in-vivo experiments on CTLA-4

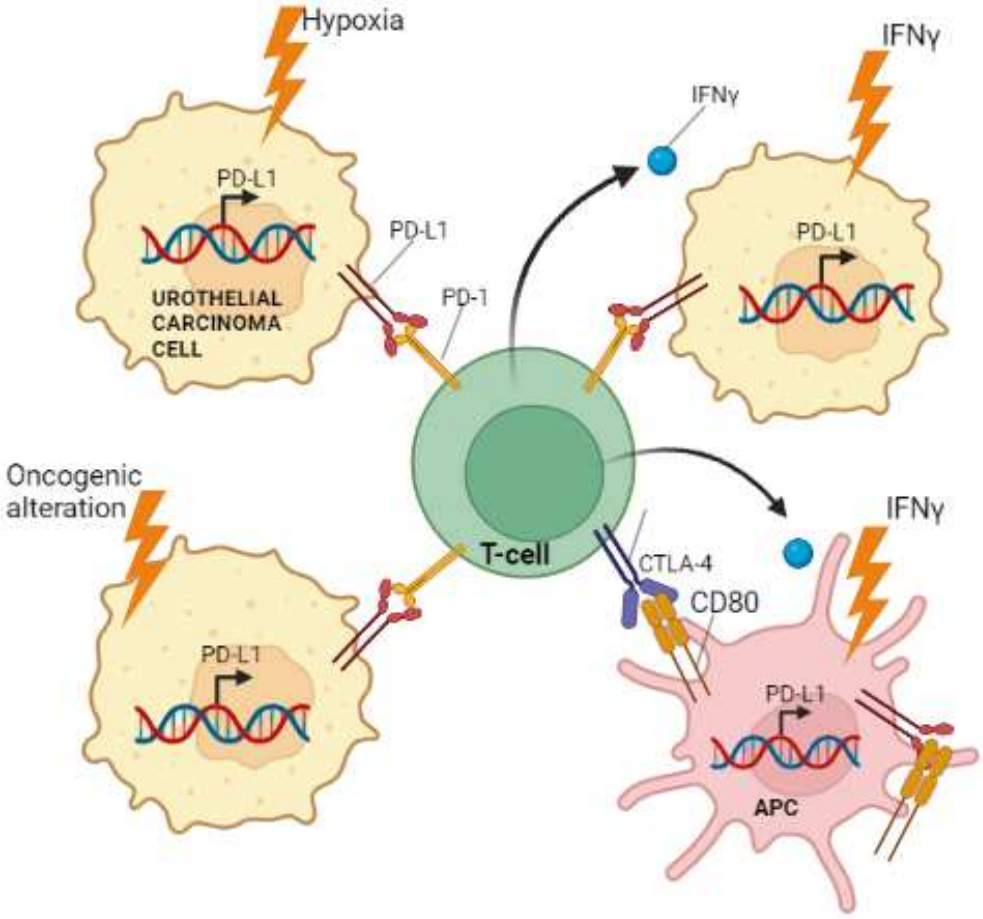

Figure 4: *Graphical illustration of the different immune checkpoint binding in UC. Included are the different pathways by which PD-L1 expression is upregulated both in tumor cells and antigen-presenting cells. Illustration created with BioRender.com*

FDA-Approved Immune Checkpoint Inhibitors in Urothelial Carcinoma

Metastatic UC has been considered responsive to chemotherapy but has a limited responsive duration and will often require second-line treatment upon recurrence[33]. The most common form of chemotherapy used in UC is cisplatin-based chemotherapy however about two-

thirds of patients are ineligible for cisplatin[33]. Historically second-line treatment has had limited benefit for patients with progressive disease after chemotherapy[33]. ICI as a second-line therapy have shown the greatest benefit in terms of overall survival for UC. In this paper, I review the current FDA-approved drugs for metastatic UC: Pembrolizumab, Avelumab, and Nivolumab

Pembrolizumab

Pembrolizumab is a highly selective IgG4 humanized antibody that binds programmed cell death 1[34]. The KEYNOTE-045 was the phase III clinical trial that demonstrated the efficacy of pembrolizumab in the treatment of metastatic UC. It was an open-label, international trial including 542 patients with advanced urothelial cancer that recurred or progressed after chemotherapy [35]. The primary endpoints were the overall survival (OS) and progression-free survival (PFS) which were assessed by calculating the tumor PD-1 ligand positive score, with 10% or more being the cutoff [35]. The cohort was divided into two groups in a 1:1 ratio with one group receiving pembrolizumab at 200mg intravenously every three weeks. For comparison, the other group was given the investigator's choice of paclitaxel, docetaxel, or vinflunine all administered at 3 weeks[35]. To measure tumor size in response to therapy, the participants underwent tumor imaging at week nine and then every six weeks after that for a year and subsequently every 12 weeks. The median duration of study treatment in the pembrolizumab group was 3.5 months while the chemotherapy group was treated for 1.5 months.

The results of the study showed a significantly higher overall survival rate for the pembrolizumab group with a hazard ratio of 0.73[35]. The pembrolizumab group had a median overall survival of 10.3 months compared to the chemotherapy group with 7.4 months. Moreover, the estimated overall response rate in the pembrolizumab group was 43.9% compared to 30.7% in the chemotherapy group. There was no difference in the overall progression-free

survival between the pembrolizumab and the chemotherapy group. However, there was a higher objective response rate in the pembrolizumab groups (21.1%) while the chemotherapy group was at 11.4%. The adverse effects of both drugs were also tested with pembrolizumab having fewer grade 3, 4, or 5 adverse effects. In the pembrolizumab group, the most common treatment-related side effects were pruritus, fatigue, and nausea[35].

Based on these results, pembrolizumab received approval from the FDA for second-line neoadjuvant treatment of metastatic UC in the US. Since then there has been a new clinical trial for pembrolizumab as a first-line treatment for UC. Currently, it has reached a phase II trial called KEYNOTE-052.

Nivolumab

Nivolumab is another humanized IgG4 antibody against PD-1 that was FDA-approved as a second-line treatment for metastatic UC based on the checkmate 275 phase II clinical trial[34]. The checkmate 275 phase II trial was a multicenter, single-arm study of 270 patients eighteen or older with metastatic UC or unresectable local UC[36]. The patient's performance status was rated using the Eastern Cooperative Oncology Group. A status of zero or one received 3mg of nivolumab every 2 weeks until disease progression, clinical deterioration, or unacceptable toxicity[36]. The primary endpoint was the overall objective response (OBR) or PD-L1 positive expression (≥5% and ≥1%). The duration of the trial was 7 months from March 2015 to October 2015 with the last follow-up visit in April 2016.

The overall objective response rate was 20% in the nivolumab group compared to 10% in the control group with no significant difference in OBR for PD-L1 expression between both groups[34]. Moreover, there still was an increase in response rates for patients with higher PD-L1

expression[36]. Treatment-related adverse effects occurred in 64% of the cohort in this clinical trial, with the most common adverse effect of any grade being fatigue which was noted in 17% of the patients[36]. In the cohort, 18% of patients experienced fatigue and diarrhea, 1% suffered from pneumonitis, 1% from pemphigoid, and <1% suffered from lung-related complications and autoimmune reactions like a pruritic rash[36].

Nivolumab was approved for second-line treatment of metastatic or unresectable UC in the US. Since then, Nivolumab was approved as an adjuvant treatment for stage four UC following radical surgical resection. The phase III, multicenter, double-blind, randomized trial gained measured efficacy using disease-free survival as its endpoint[37]. Median disease-free survival in the nivolumab group was 20.8 months while the placebo group was 10.8 months. Moreover, the median survival free from recurrence in the nivolumab group was 22.9 months compared to 13.7 months in the placebo group[37]. The clinical trial proved that nivolumab as an adjuvant treatment after radical surgery for stage 4 UC significantly decreased recurrence.

Avelumab

Avelumab is an IgG1 type antibody against PD-L1, it prevents the binding of PD-L1 to PD-1. In 2020 Avelumab received FDA approval for use as maintenance therapy in locally advanced or metastatic bladder cancer following the JAVELIN 100 trial[34]. It was an international, open-label phase III trial that lasted from May 11, 2016, to June 4, 2019[38]. The inclusion criteria for this trial include locally advanced or metastatic bladder cancer patients who had stable disease after receiving four to six cycles of chemotherapy with gemcitabine and cisplatin or carboplatin[38]. Additionally, patients had to have been treatment-free for at least four

weeks before enrollment into the clinical trial. Patients were randomly selected on a 1:1 ratio into the Avelumab or control group, in the Avelumab group patients received 10 mg per kg of body weight plus best supportive care while the control group received only supportive care[38]. For both groups, PD-L1 expression was assessed using the Ventana PD-L1 assay with a PD-L1 positive score assigned to tumor cells or immune cells staining for at least 25% PD-L1. Tumor samples that had 100% staining for PD-L1 on immune cells when less than 1% of the area had immune cells were also classified as positive[38]. Finally, to measure the primary and secondary endpoints, the tumors were measured using RECIST. The chest, abdomen, and pelvis were imaged every 8 weeks for 12 months and then every 12 weeks until confirmed disease progression[38]. A total of 51.1% of patients had PD-L1 positive tumors with the breakdown being 57.% in the Avelumab group and 56.3% in the control group.

The primary endpoint was overall survival (OS) and the secondary endpoints were progression-free survival (PFS) and safety [38]. The OS at 12 months was 71.3% in the avelumab group compared to 58.4% in the control group with the median OS being 21.4 months and 14.3 months respectively[38]. The PD-L1 positive group also showed longer survival in the avelumab group, at 12 months 79.1% of the avelumab group had OS compared to 60.4% in the control group. Moreover, PD-L1 negative patients in the avelumab had an OS of 18.8 months compared to 13.7 months in the control group[38]. PFS and ORR were also markedly higher in the avelumab group compared to the control group. In the overall population, the avelumab group had a PFS rate of 3.7 months compared to 2.0 months in the control group. The PD-L1 positive patients in the avelumab had a median PFS rate of 5.7 months compared to 2.1 months in the control group[38]. Moreover, PD-L1 negative patients in the avelumab group had a PFS rate of 3.0 months compared to 1.9 months in the control group.

Adverse effects of any grade occurred in 98% of patients in the Avelumab group compared to 77.7% in the control group with the most common adverse effects being fatigue, pruritus, urinary tract infections, and diarrhea[38]. Adverse events of grade 3 or higher occurred in 47.4% of the avelumab group with the control group having 25.2%. The most common adverse effect in the avelumab group was anemia, urinary tract infections, fatigue, and hematuria[38]. Additionally, 29.4% of patients in the avelumab group had an immune-related adverse effect the most common being thyroid disorders.

Based on the OS and PFS rates being significantly higher in the avelumab group it was granted FDA approval for maintenance therapy in locally advanced or metastatic bladder cancer.

Treatment	Trial, year	Phase	Line of therapy	Number	Characteristics
Pembrolizumab vs Chemotherapy	Keynote-045, 2017	3	Second-line	542	mOS: 10.3 vs. 7.4 (P vs chemo, all pts) (p = 0.002) mOS: 8.0 vs. 5.2 (P vs chemo, PD-L1 status CPS ≥ 10%) (p = 0.005)
Nivolumab post platinum-based chemotherapy	Checkmate275, 2017	2	Second-line	270	ORR: 19.6% (52/265)
Avelumab maintenance vs best supportive care	Javelin bladder 100, 2020	3	First-line	700	mOS: 21.4 vs. 14.3 mo (Avelumab vs. BSC, all pts) (p=0.001)

Table 1: Overview of clinical trials for current FDA-Approved ICI for metastatic UC.

The clinical trials testing immune checkpoint inhibition in UC have been successful however there is still a lack of successful clinical trials for PD-1/PD-L1 and CTLA4 combination therapy. A possibillity for this discrepency is a lack of pre-clinical experiments that tests this

form of therapy. In this paper, I propose the use of canine-derived bladder cancer organoids to test the pharmacodynamics of combination ICI

Pre-clinical testing of Combination Therapy

Traditionally, cell lines have been used for in-vitro lab testing of bladder cancer. There are multiple cell lines such as RT4, UMUC3, MB49, and SW780 that have been instrumental in deciphering the use of antibodies against immune checkpoints in bladder cancer. Still, cell lines lack heterogeneity and do not accurately portray the different cell lineages present in the TME in-vivo. Another model used in bladder cancer is called spheroids. Spheroids are aggregates of cells cultured in three-dimensional (3D)[39]. Although, spheroids retain interactions between different cells and the extra-cellular matrix they still lack heterogeneity[39]. In contrast, organoid samples are 3D assays of cells that are differentiated from stem cells. The stem cells can either be induced pluripotent stem cells or embryogenically derived stem cells, they can be forced down different lineages, thereby making organoid models heterogeneous[40]. These organoids can mimic characteristics present in the TME in humans, allowing for opportunities to screen drugs and visualize how they interact with tumors. Organoids can be made from human tumor cells or direct or through animals expressing tumors, however, human tumor cells vary wildly between different patients. Therefore, animal models can provide specific genetic-defined tumor organoids to focus on particular pathways involved in drug response[41]. Ultimately, the more that can be learned from animal-derived organoids, will improve the translation into using patient-derived organoids to understand pharmacodynamics.

In the current literature, mice have been used as the animal model for creating tumors that are then converted into organoids. Unfortunately, they tend not to accurately reflect human bladder cancers. However, canine species have been found to develop bladder cancer naturally and mirror the TME of human bladder cancer thereby making them a good potential animal model for bladder cancer organoids.

Dogs have a 1-2% chance of developing high-stage MIBC, this is about the same rate as humans developing MIBC[42]. The bladder cancers that develop in dogs have been found to mimic the cellular and histopathological characteristic of UC, they also follow the same pattern of metastasis[43]. Due to this similarity, canine models have been proposed as a good potential for testing biomarkers, genetic profiles, and drug sensitivity of UC. The organoid bladder cancer samples are created from urine samples of the affected dogs. Elbadawy et al. have been successful in creating different canine organoids including prostate and bladder cancer[43]. Generally, cells from the urine sample are mixed with Matrigel and then cultured in a medium that stimulates stem cells [43]. When the organoids are created there are different ways to confirm if it was successful, one of those is using biomarkers typically upregulated in cancer. The fibroblast marker vimentin, myofibroblast marker α-smooth muscle actin, and epithelial cell marker E-cadherin[42]. Moreover, canine organoids also express biomarkers specific to the human urothelium. Previous studies have shown superficial and basal cells in the uroepithelium express cytokeratins (CK) and uroplakin (UPK) gene family[44]. CK20, CK7, and UPK3A are commonly expressed in the uroepithelium thereby upregulated in the setting of neoplastic growth.

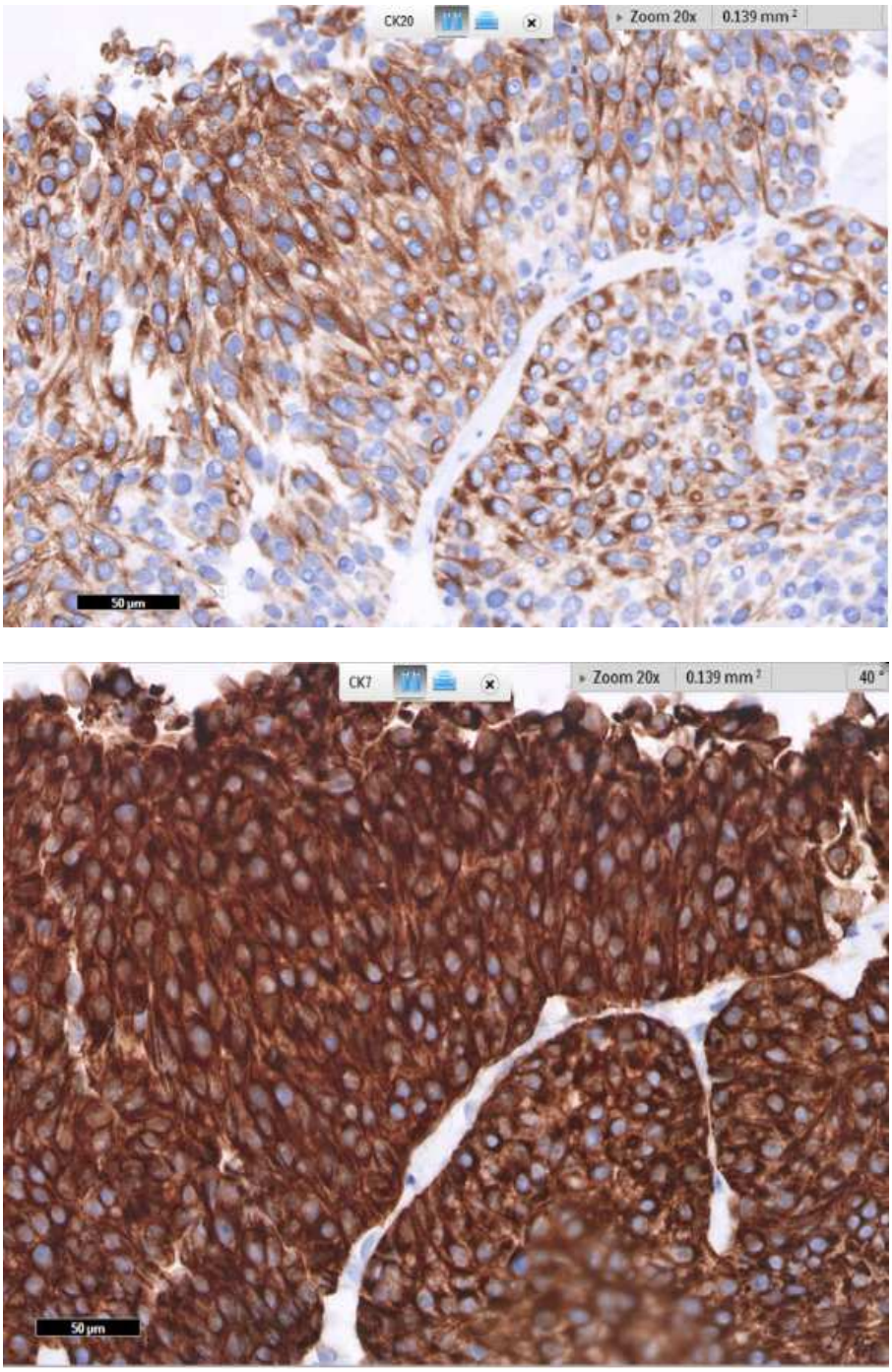

Figure 6*: Histological images from PathologyOutlines.com showing CK20 and CK7 in UC cells, these are cytoplasmic markers which is indicated by the concentration within the cytoplasm and not the nucleus. Images contributed by Andrey Bychkov* https://www.pathologyoutlines.com/topic/bladderurothelialinvasivegen.html

Canine organoids also upregulate these same markers and are used to confirm the successful culturing of organoids.

A valuable aspect of using canine models is it can be transfected into immunodeficient mice and produce a tumor which can be studied in vivo. Abugomaa et al. were able to see 1cm tumor growth in six weeks after injecting immunodeficient mice with organoids cultured from dogs with cancer[42]. Another experiment by Elbadawy et al. showed canine organoid tumors in mice also expressed the same markers as the organoid cells did, they speculated it was due to the organoids secreting cytokines that encouraged mesenchymal expansion within the mice[43]. This opens many possibilities for testing anti-cancer drugs and including combination therapy. Bladder organoids could be cultured with immune cells to recapitulate the immune microenvironment in-vivo and transfected into immune deficient mice. Control mice can then be compared to mice treated with different combination of ICI to measure tumor response.

Discussion

The use of ICI in UC is instrumental in the activation of tumor-infiltrating lymphocytes (TIL) against the tumor. Moreover, TIL interfaces with other immune cells and cytokines in the tumor microenvironment [5]. Due to the crosstalk between these different elements, there will be indirect effects of ICI on the immune microenvironment. A major effect of ICI besides reactivation of T-cells is the increase in IFNγ in the environment. Although IFNγ upregulates PD-L1 expression, it is involved in many of the inflammatory processes of the body[16]. IFNγ is notably involved in the polarization of monocyte into the M1 macrophage lineage, the pro-

inflammatory subtype of macrophages[19]. This means that macrophages can phagocytose neoplastic cells, present neoantigens to T-cells, and provides mediators for inflammation. In this way, ICI will not only affect the lymphocytes but can indirectly prime other immune cells to decrease tumor volume. The upregulation of PD-L1 due to IFNγ is addressed by PD-L1 inhibitors, consequently, it stands to reason that the anti-tumor effects of IFNγ can be achieved while avoiding the pro-tumor effects. The potential for ICI in UC can be transformational if the mechanisms behind resistance are understood and addressed. A way to address this resistance is the combination of PD-1/PD-L1 inhibitors with CTLA-4 inhibitors.

Studies have shown that reactivation of T-cells under PD-1 inhibition potentially stimulates compensatory immune checkpoints one of which is CTLA-4[22]. As discussed previously in this paper, CTLA-4 is capable of inducing anergy of T-cells in the priming stage by binding CD80 on APC. Targeting CTLA-4 at the priming stage would allow for increased de-novo T-cell synthesis as well as proliferation[45]. So, in addition to reactivating existing T-cells using PD-1/PD-L1 inhibitors, CTLA-4 adds the benefit of increasing the number of T-cells in the TME. Therefore, disruption of multiple pathways for immune checkpoints in UC can lead to prolonged activation of T-cells. This can be achieved with combination therapy. However, the use of CTLA-4 for UC has been limited even though it has been used in other highly mutagenic cancers such as melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSLC). In 2010, Ipilimumab a CTLA-4 antibody was approved for the treatment of melanoma following a successful phase III trial[46]. Since then, Ipilimumab combined with PD-1 inhibitors has been approved for melanoma[47] along with NSLC[45] and RCC[48].

Approved Combination Therapies in Other Cancers

The checkmate 9LA clinical trial tested the use of nivolumab and ipilimumab combined with chemotherapy (experimental) vs chemotherapy alone (control) for stage IV or recurrent NSLC. The dosage for nivolumab given was 360mg intravenously every 3 weeks, and ipilimumab was administered at a dose of 1mg/kg. This study used overall survival as its primary endpoint, the experimental group had a longer survival rate at 14.1 months compared to 10.7 months in the control group[45] The safety profile for this trial showed that the most common grade 3-4 adverse effects occurred at a higher percentage in the experimental group, however, there was seven recorded deaths in the experimental group and 6 in the control group[45]. The risk-benefit ratio was favorable to the use of this combination therapy therefore, this combination therapy was approved and has been successfully translated into clinical practice.

In RCC, checkmate 214 was the phase III clinical trial that led to the approval of nivolumab plus ipilimumab for untreated advanced RCC. This trial tested the Nivolumab plus ipilimumab (experimental) vs sunitinib a vascular endothelial growth factor tyrosine kinase inhibitor (control)[48]. The experimental group received 3mg/kg of nivolumab plus 1mg/kg of ipilimumab for three weeks of four doses followed by 3m/kg of nivolumab every 2 weeks[48]. At 18 months, the experimental group had a significantly higher overall survival rate at 75% compared to 60% in the control group, the progression-free survival was also higher in the experimental group at 11.6 months[48]. Grade 3-4 adverse effects occurred in 46% of the experimental group with 22% having to drop out of the study due to treatment-related side effects. The safety profile was favorable for the experimental group leading to FDA approval.

In melanoma, checkmate 067 was a phase III clinical trial that tested nivolumab plus ipilimumab (experimental) vs nivolumab or ipilimumab alone in stage III or IV melanoma[47].

The experimental group received 1mg/kg of nivolumab plus 3mg/kg of ipilimumab every three weeks for four doses followed by 3mg/kg of nivo every 2 weeks. Following the four-year follow up it was determined that nivolumab plus ipilimumab provided a durable, sustained survival benefit with a good safety profile for melanoma[47]. In the experimental group, 59% of patients experienced treatment-related grade 3-4 adverse effects, with the most common grade three side effect being diarrhea and the most common grade four effect being lipase[47]. In 2019 a phase IIIb/IV clinical trial to evaluate a lower dosage of Ipilimumab in advanced melanoma was carried out[49]. Patients who were given 3mg/Kg/d of nivolumab plus 1mg/Kg/d of ipilimumab had a lower incidence of grade 3-4 adverse effects compared to patients given 1mg/kg/d of nivolumab plus 3mg/kg/d of ipilimumab[49]. It was concluded that there was no significant difference in efficacy between the two ipilimumab doses however the lower dose provided fewer adverse effects.

Cancer Type	Treatment	Phase	Characteristics	Adverse Effects
NSLC- Stage IV/recurrent	Nivolumab (360mg IV)+Ipilimumab (1mg/kg)+chemotherapy vs Chemotherapy alone	III	mOS: 14.1 months vs 10.7 months	Grade 3-4: 47% vs 38%

RCC- Advanced stage	Nivolumab (3mg/kg)+Ipilimumab (1mg/kg) vs Sunitinib	III	18-month OS benefit: 75% vs 60% ORR: 42% vs 27%	Grade 3-4: 46% vs 63%
Melanoma- Stage III and IV	Nivolumab (1mg/kg)+Ipilimumab (3mg/kg) vs Nivolumab alone (3mg/kg) vs Ipilimumab alone (3mg/kg)	III	mPFS: 11.5 months vs 6.9 months vs 2.9 months	Grade 3-4: 59% vs 22% vs 28%
Melanoma- Advanced stage	Nivolumab (3mg/kg)+Ipilimumab (1mg/kg) vs Nivolumab (1mg/kg)+Ipilimumab (3mg/kg)	IIIb/IV	ORR: 50.6% vs 48% mPFS: 9.9 months vs 8.9 months	Grade 3-5: 34% vs 48%
Urothelial Carcinoma- Metastatic	Nivolumab (3mg/kg) vs Nivolumab (3mg/kg)+Ipilimumab (1mg/kg) vs Nivolumab	I/II	ORR: 25.6% vs 26.9% vs 38%	Grade 3-4: 26.9% vs 30.8% vs 39.1%

	(1mg/kg)+Ipilimumab (3mg/kg)			

Table 2: Summary of clinical trials of PD-1/PD-L1 and CTLA-4 inhibition combination therapy applied on RCC, melanoma, NSLC, and UC.

A common thread between these three clinical trials is the dosage of ipilimumab and the safety profile of the studies. Although treatment-related side effects were present in the experimental groups, the risk-to-benefit ratio was still favorable. All used 1mg/kg of ipilimumab and observed significant improvement with appropriate safety profiles. However, the same has not been observed in UC even though CTLA-4 is also expressed in this cancer. It is still unclear why it has been difficult to use CTLA-4 in clinical treatment for UC, one ongoing hypothesis is that an increased dosage of CTLA-4 is needed to see benefits leading to severe treatment-related adverse effects. From 2015-to 2017 AstraZeneca ran a clinical trial called DANUBE, this was a phase 3 trial of a PD-L1 inhibitor called durvalumab combined with a CTLA-4 inhibitor called Tremelimumab vs chemotherapy alone in UC[50]. The clinical trial was unsuccessful in reaching its primary endpoint of overall survival. Since then, a phase I/II trial testing nivolumab and ipilimumab have emerged, although it has not reached the phase III trial, the results looked promising. The trial divided patients on a 1:1:1 ratio into three groups: 3mg of nivolumab, 3mg of nivolumab plus 1mg of ipilimumab and 1mg of nivolumab plus 3mg of ipilimumab[51]. The most significant increase in ORR was seen in the group given 3mg of ipilimumab with the safety profile being unfavorable. Therefore, further research needs to be done in-vitro to understand different pathways that could contribute to drug efficacy in UC. The introduction of organoid

models in basic science research has opened a new field of testing for different cancers including bladder cancer.

Proposed Experimental Design

To develop this experiment, the canine bladder organoids must be created first using urine or biopsy. Urine is preferred as this is easier to obtain from the dogs and has a lower risk of harm. Following urine collection from dogs with bladder cancer, stem cells are obtained and used to create multiple spheroid organoids in trans wells[39]. The organoids are then treated with growth factors to allow them to be differentiated. Next, the immune cells such as CD8, CD4, T-regulatory cells, and antigen-presenting cells are created and treated with IFNγ. IFNγ allows for upregulation of PD-L1 in tumor and immune cells via the JAK-STAT pathway[26]. The immune cells and organoids will then be co-cultured together to stimulate the immune microenvironment within UC. These cells can then be tranfected into immunodeficient mice. The experimental groups for the study would be PD-1 antibody, and PD-L1 antibody, with both groups being monitored using flow cytometry to check for upregulation of CTLA-4. To test the efficacy of the drug, cell viability can be measured using CellTiter-Glo luminescent assay. This assay measures ATP from metabolically active cells therefore lower ATP levels mean apoptosis of the neoplastic cells. If the development of resistance and CTLA-4 occurs concurrently, the experimental groups should be treated with CTLA-4 antibodies. Evaluation of apoptosis of neoplastic cells will be done using the luminescent assay. The goal of the experiment would be to evaluate if there is increased destruction of UC cells when CTLA-4 inhibition is added in the setting of resistance. Using this experiment, exploration for reasons why UC requires higher doses of CTLA-4 antibodies and possible pathways involved in the pharmacodynamics could be done. One

possible reason could be the controversy over the depletion of T-reg cells when CTLA-4 is blocked. A study done by Sharma et.al found that Ipilimumab and tremelimumab did not decrease the amount of T-reg in bladder cancer but did increase the infiltration of CD4 and CD8 cells[52]. They suggested that perhaps the Fc regions of the antibodies should be modified to increase efficacy. However, other labs have carried out studies showing that T-reg cells are decreased during CTLA-4 inhibition. It could be due to differences in how different labs measure T-reg cells but clearly, further investigation is needed and can be done using canine bladder organoids.

Although, bladder organoids are an excellent potential they are not perfect and come with their own setbacks, some of which is the cost and time. It costs a lot of money to create bladder organoids using Matrigel and stem cell stimulation. It also takes longer to culture organoids compared to 2D cell lines and requires increased skill set due to the complicated handling of the Matrigel. As more research is done into the use of 3D organoids, it is hopeful that cheaper and faster techniques are developed that makes it more accessible and expands the use globally.

Future Directions

There is still a lot of research to do on different aspects of ICI in UC. Some future directions include examining relevant biomarkers and other immune checkpoints. In the beginning, when PD-1 inhibitors were being developed it was believed that PD-L1 expression levels would be a biomarker for predicting patients that would benefit from PD-1/PD-L1 inhibition therapy[22]. But it has been difficult to establish a correlation, making PD-L1 expression a non-predictive marker for clinical response. Different manufacturers use different

assays to measure PD-L1 expression and each has different thresholds for determining PD-L1 positive tumors. For example, Ventana assay classified ≥5% of PD-L1 expression as positive and this was used by Atezolizumab[22]. However, pembrolizumab used IHC 22C3 PharmDx assay which classifies ≥10% as PD-L1 positive[35]. Similarly, nivolumab and avelumab also used different assays with different thresholds so there is no consensus on what constitutes PD-L1 positive tumors making it difficult to establish a correlation. The only consistent finding from different clinical trials has been that both PD-L1 negative and positive tumors respond to PD-1/PD-L1 for reasons that are still unknown[22]. PD-L1 expression is also limited as a biomarker in combination therapy as other ICI do not specifically target PD-L1. Canine bladder organoids are yet another potential avenue to explore other biomarkers that could be useful in UC. The density of lymphocytes in the TME could be a possible biomarker since PD-1, PD-L1, and CTLA-4 inhibitors have all been shown to increase CD4 and CD8 cell infiltration[52]. This can be studied experimentally in-vitro before being applied in-vivo, if decreasing tumor cells is correlated with increased density of lymphocytes in organoid models it can build the foundation for using this biomarker.

Another prospective biomarker is the use of genetic signatures such as molecular subtypes and tumor mutational burden. The molecular subtype classification of UC done by TCGA has been used in some clinical trials as a possible biomarker[34]. Nivolumab reported response rates based on molecular subtypes in the checkmate275 trial, basal 1 had the highest response rate followed by luminal cluster 2[36]. Since organoid models can be differentiated into tumor cells expressing specific mutations, they can be used to examine the pharmacodynamics of combination therapy in different molecular subtypes. Recent data suggest that TMB might be a more robust biomarker than PD-L1 expression in UC[53]. The higher the titers of neoantigens

present in the patient, it is hypothesized that there is a durable response by the immune system. Additionally, patients in the nivolumab clinical trial with higher TMB had a better response rate as opposed to those with lower TMB[36]. The problem with TMB as a biomarker in UC is that it has not been well researched and just like other biomarkers, there is no "threshold" to indicate high vs low TMB[34].

Lastly, other molecular players involved in tumor promotion in UC need to be investigated for future drug development. CTLA-4 is not the only compensatory checkpoint upregulated during PD-1/PD-L1 resistance, other checkpoints such as TIM-3, LAG3. TIM-3 is expressed on CD4 and CD8 cells, it binds to different receptors such as galectin-9, CEACAM-1, and HMGB-1[22]. The binding of TIM-3 to its receptors leads to defective expression of proinflammatory cytokines, additionally, it signals using a different mechanism of action from PD-1/PD-L1. LAG3 is another checkpoint expressed on multiple cells most notably T-cells and T-reg[54]. LAG3 is highly expressed on T-cells after activation, the role of LAG3 in immune regulation is understudied but important. The main receptor for LAG3 is MHCII however it also binds to other receptors such as galectin-9, FGL-1, and others[54]. There is a massive potential to use canine bladder organoids for future research into the mechanism of action of different checkpoint inhibition.

Conclusion

The use of immune checkpoints in UC has shown to be a promising therapeutic, however, the issue of resistance is an obstacle that still needs to be solved. In this review, the crosstalk between immune cells in the TME and the tumor has shown how PD-L1 is upregulated both on tumor cells and dendritic cells. Simultaneously, comprehensive flow cytometry has shown that CTLA-4 upregulation in the setting of PD-1/PD-L1 resistance is possible. Moreover, this

observation has only been noted in a case study, therefore preclinical studies exploring this trajectory are needed. Given that canine species also develop bladder cancer naturally and genomic sequencing has shown the TME is similar to that of humans, they present as a perfect model for in-vitro studies. Using organoid bladder samples, the hypothesis of upregulation of CTLA-4 in the setting of PD-1/PD-L1 can be tested. This information could lay the foundation for how clinical phase trials are set up to test human candidates. In the end, the goal is a way to increase overall survival and progression-free survival in patients with UC undergoing immune checkpoint inhibition.

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