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Immunological Basis in the Pathogenesis and Treatment of Bladder Cancer

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Abstract

The pathogenesis and transition of normal urothelium into bladder carcinoma are multifactorial processes. Chronic inflammation causes initiation and progression of the underlying pathophysiology of invasive and metastatic cancer. A dichotomy is observed in the role of immune cells in bladder cancer. While the immune response defends the host by suppressing neoplastic growth, several immune cells, including neutrophils, macrophages, and T-lymphocytes, promote tumor development and progression. The levels of human neutrophil peptide-1, -2, and -3, produced by neutrophils, increase in bladder cancer and might promote tumor angiogenesis and growth. The effect of macrophages is primarily mediated by pro-inflammatory cytokines, IL-6 and TNF- α . Additionally, the underlying immunological mechanisms of two treatments, BCG and cytokine gene-modified tumor vaccines, and future directions are critically discussed.

Keywords

BCG; Bladder cancer; dendritic cells; macrophages; neutrophils; T cells; Urothelial cell carcinoma; Tumor vaccines

Introduction

Bladder cancer, the second most common urological malignancy, is responsible for 5% of all cancer related deaths and accounts for \$4 billion worth of healthcare costs in the US alone [1]. It is estimated that in 2014 there will be 74,690 new diagnoses of bladder cancer and approximately 15,580 deaths [201]. Men are four times as likely to develop the disease compared to women with the incidence double in white men relative to their black

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counterpart. The most common form of this neoplasm is urothelial cell carcinoma (UCC), which accounts for 90% of bladder cancers [2]. The initial signs seen in cancer of the urinary bladder include hematuria along with urinary frequency, discomfort, and pain during urination [201]. Many risk factors have been identified (Figure 1). The primary factor is smoking, which is known to increase the incidence of bladder cancer four-fold relative to nonsmokers. Nearly half of all bladder cancers can be attributed to smoking due to its induction of chronic inflammation. Individuals at additional risk include those who work in the dye, rubber, leather, and aluminum industries. Diagnosis often requires cytology of the urine or cystoscopy of bladder tissue. Treatment is difficult due to the high recurrence rate of nearly 80%. Thus, long-term follow-up is necessary [3]. Surgical removal of either cancerous tissue or the entire bladder is performed in 90% of cases. Often surgery is combined with additional therapies, such as immunotherapy, chemotherapy and radiation, to improve outcomes. The 5-year mean survival for all bladder cancer stages is 78%. The 5-year survival rate of bladder cancer patients increases to 96%, if noninvasive, *in situ* carcinomas are detected in its early stage [201]. Clinicians and researchers continue to search for the proteins, transcription factors, and genes that would be helpful in differentiating patients with non-muscle-invasive bladder cancer from those with muscle-invasive cancer. The following discussion highlights major genes and factors in this regard.

Pathogenesis of Bladder Cancer

The mechanisms underlying the formation of urothelial carcinoma are many and complex, the details of which are still not fully understood. Several mechanisms, however, have been elucidated. Two pathways are used to describe the development of bladder cancer, the invasive pathway and the noninvasive papillary pathway [4]. It has been suggested that the slow-cycling urothelium of the bladder gives rise to the papillary pathway. The urothelium is the epithelial layer composed of basal cells, intermediate cells, and umbrella cells that line the bladder tissue, which is normally replaced every 6 to 12 months [3,5]. This tissue contains urothelial stem cells with self-renewing capabilities, making it much more susceptible to malignant transformation [3]. Cancer develops when hyperplastic urothelium begins to grow towards the lumen of the bladder. This is often the result of genetic alterations in several proto-oncogenes, including fibroblast growth factor receptor-3 (FGFR3) and Harvey rat sarcoma viral oncogene (HRAS) [3,6,7]. Mutations in FGFR3 are found in 75% of papillary tumors while HRAS mutations are present in 30% of cases [3]. Both oncogenes are known to play a role in the activation of the Ras/MEK/ERK pathway. Non-muscle invasive cancers are troublesome due to their high recurrence rate. However, they are often associated with a positive prognosis. Conversely, invasive urothelial cancers originate from one of two mechanisms, severe dysplasia or carcinoma *in situ*. Often they are the result of inactivation of TP53, RB1, or PTEN, pathways with a tumor inhibiting effect. Mutations in PI3K, TSC1, PTCH, CDKN2A, and DBC1 are prevalent in both invasive and noninvasive cancer types. Thirty percent of bladder cancers are muscle-invasive, which have a poor prognosis with death occurring 2–3 years after diagnosis [3,8]. Additionally, 60% of cases die within 5 years of diagnosis even with treatment [9]. More than 90% of muscle-invasive cases are incurable once local or distal metastasis has occurred [2,5].

Many cell types promote the development, invasion, proliferation, and metastasis of bladder cancer. Cancers alter their microenvironment by recruiting and activating stromal cells, adipocytes, fibroblasts, inflammatory cells, as well as mesenchymal and endothelial progenitors [3,10]. They alter the environment so it resembles periods of wound-healing induced by released factors, including VEGF, EGF, CSF, and TGF- β [11]. Mesenchymal stem cells in the bladder have been implicated in tumor growth and metastasis due to their role in tissue repair [3]. By differentiating into multiple bladder cell types, these precursors are able to replace damaged fibroblasts and endothelial cells. They contribute to tumor development by differentiating into endothelial cells, which enhance angiogenesis into and around cancerous tissues. UCC cells secrete G-CSF and express G-CSF receptors, thus promoting autocrine growth [12]. In few cases of muscle-invasive bladder cancers, stages 2 and 3, a high number of tumor-associated fibroblasts are found. It has been suggested that tumor-associated fibroblasts may contribute to tumor invasion and metastasis [3].

Epithelial plasticity, the ability of cells to switch between phenotypic states, plays an important role in the transformation of healthy urothelial tissue into malignant neoplasm [3]. This plasticity is a transient but reversible process that allows host tissue to revert to stem cells and adapt to changes in its microenvironment. This results in the differentiation of bladder urothelium to become invasive, immune to apoptotic triggers, and resistant to therapy. Three families of epithelial-mesenchymal transition (EMT) transcription factors, ZEB, SNAIL and TWIST, have been shown to initiate endothelial plasticity by inducing changes in cell migration, invasive capabilities, and survival phenotypes [13]. The question that remains to be answered is at what stage in the metastatic cascade does EMT begin? This would require monitoring cells in each stage of their transition. This is indeed a challenging task because this would require multiple tissue biopsies at different intervals. Additionally, EMT can be initiated via a number of unique cellular pathways. None-the-less, future studies should explore these pathways in order to find patterns of specificity and regulatory mechanisms.

Inflammation in Pathogenesis of Bladder Cancer

Inflammation is often seen in bladder cancer along with the presence of many immune cells at cancer sites [14]. However, the normal function is disrupted due to a complex interplay of chemokines, proteases and non-chemotactic cytokines, resulting in the survival and proliferation of cancer cells [15]. Furthermore, resultant alterations in tissue architecture are hypothesized to provide a route for both local spread and distant metastasis. Inflammation results in the release of several pro-tumor cytokines and growth factors that support tumor angiogenesis, proliferation, and survival. The production of oxygen species in inflamed tissues has also been shown to promote the formation of mutations in cancer cells.

Inflammation of the urethra and bladder is seen in infection, like schistosomiasis, but there are several non-infectious causes, including benign prostatic hyperplasia (BPH), extended use of indwelling catheters and bladder, kidney and ureter stones [14,16–19]. Both schistosomiasis and indwelling catheters are believed to promote the development of bladder cancer due to the induction of epithelial hyperplasia, which results from chronic irritation (Figure 1). Hyperplasia leads to the emersion of cancer cells in the bladder. Altogether, it is known that chronic inflammation has a serious effect in the development and metastasis of

cancer [3]. However, the mechanisms that make bladder cancer sensitive to acute inflammation remain unknown [20].

At sites of inflammation neutrophils and monocyte/macrophages produce several reactive oxygen species, including superoxide anion, hydrogen peroxide, and nitric oxide [14]. These reactive species create breaks in both double and single-stranded DNA, as well as modify RNAs, proteins, and lipids (Figure 2). The enzyme that produces nitric oxide, inducible nitric oxide synthase (iNOS), along with its RNA products are found in UCC bladder tumors, but they are not found in healthy bladder tissue. Additionally, microvessel density, an effect seen in angiogenesis, was significantly increased in bladder UCC tumors that contained iNOS. Secondary amines interact with the products of nitric oxide to form N-nitrosamines, a carcinogenic compound. N-nitrosamines, therefore, may be directly involved in the development of bladder cancer (Figure 2). Nitrosamines are elevated in the urine of paraplegic patients [21]. These patients are known to have increased risk of chronic urinary tract infections (UTI), and it has been suggested that the resulting amines increase the risk of bladder carcinoma. Chronic inflammation is observed in patients with long term use of indwelling catheters [22]. This promotes the release of iNOS from inflammatory macrophages to synthesize nitric oxide, one of the precursors required for N-nitrosamine formation [14]. The hydrogen peroxide produced during inflammation may support urothelial cell tumorigenesis, and the growth and development of tumor cells are mediated by the cytokines released as a result of chronic inflammation.

Due to an established positive correlation between inflammation and bladder cancer, it is expected that an inverse association between the use of anti-inflammatory drugs and the incidence of bladder cancer would also exist. Animal studies found that the use of the non-steroidal anti-inflammatory drug (NSAID), aspirin, can decrease the risk of developing some carcinogen-induced bladder tumors [14,23]. Furthermore, individuals who were long-term aspirin users have significantly decreased risk of developing bladder cancer relative to people who did not take aspirin or were irregular users [14,24]. A case control study in a large population size (n = 1514) compared the risk of developing bladder cancer in individuals who did and did not regularly take analgesics. A relative risk of 0.81 (95% CI 0.68–0.96) was found in those who regularly took analgesics, again demonstrating the cancer-suppressive effect of some analgesics [23].

Urinary Tract Infections and Bladder Cancer

Urinary tract infections (UTI) account for a major health and financial burden and are a known initiating factor of bladder cancer (Figure 1) [14,25]. It is estimated that 1% of boys and 3–5% of girls will experience a UTI in childhood with 30–50% recurrence rate [26]. UTI accounts for an annual healthcare cost of \$8 billion and 150 million diagnosis [25,27]. Currently, there are no effective treatments to prevent UTI complications, including bladder scarring, chronic kidney disease, and hypertension. *Escherichia coli*, originating from the rectal flora, is most often responsible for UTI and accounts for 70–90% of diagnoses cases [27]. Host defense of the bladder is performed primarily by the innate immune system. In the bladder this is composed of epithelial surface barriers, tight junctions, cytokines, phagocytes, antimicrobial molecules, receptors designed to recognize pathogenic

microorganisms, and the intense flushing action of urine through the urinary tract [28,29]. Once pathogens breach through the urothelium, toll-like receptors (TLRs) register their presence and activate an immune response [25]. Myeloid cells play a major role in the elimination of pathogens from the urinary system in murine models. However, uropathogens are also known to downregulate the action of the innate immune system, primarily by inhibiting the action of PMNs. Upon inoculation with uropathogenic *E. coli* (UPEC), neutrophils were quickly found in the bladder and kidneys [30]. Additionally, the most abundant immune cell found in the kidney are dendritic cells (DCs) [31]. Following inoculation with UPEC, DCs were shown to leave their resident network in the kidney and infiltrate the bladder. UPEC inoculation also leads to the activation of T-lymphocytes in the bladder and the release of IgG specific to the pathogen, resulting in long term resistance against reinfection [32]. Furthermore, IL-10 knockout mice were found to have 10-fold higher bacterial loads in the bladder and 30-fold elevated loads in the urine relative to healthy controls [33]. These elevated bacterial counts are significant for their potential ability to promote UTI and thus bladder cancer. Finally, cystitis is a known promoting factor of bladder cancer. Mutations in the p53 gene are suggested to play a role in bladder cancer formation resulting from cyclophosphamide-induced cystitis [34]. However, the role of hemorrhagic cystitis in secondary bladder cancer remains unclear. Some studies have shown an increased incidence of developing bladder cancer in individuals with cystitis, while others have demonstrated conflicting results. Therefore, the association between the two should be explored further.

Immune System in Bladder Cancer

Immune cells play opposing roles in the pathogenesis of bladder cancer. The adaptive immune system seems to impart an anti-tumor effect while the innate immune system produces a pro-tumor effect. In the following sections, we have critically reviewed the role of key immune cells in the regulation of pathophysiology in bladder cancer.

Myeloid Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are important immune progenitors for the generation of granulocytes, macrophages, and dendritic cells (Figure 2). MDSCs play a role in tumorigenesis and metastasis by supporting angiogenesis and lymphangiogenesis as well as promoting tumor cell survival [35]. MDSCs have been shown to stimulate angiogenesis through the release of factors, including VEGF, TNF, IL-1 β , and basic fibroblast growth factor (bFGF) [202]. Eruslanov and colleagues [36] found that CD11b myeloid cells play a role in the immune suppression observed in bladder cancer. They characterized two myeloid subsets primarily responsible for producing inflammatory cytokines and chemokines in bladder cancer; these are granulocytic CD15^{high}CD33^{low} neutrophil-type cells and monocytic CD15^{low}CD33^{high} myeloid cells (Figure 2). These cells are highly inflammatory in nature and release cytokines and chemokines, like CCL2, CCL3, G-CSF, IL-8, and IL-6, which contribute to the pathogenesis of inflammation and immunosuppression in bladder cancer. Many individuals with bladder cancer have significantly increased granulocytic CD15^{high}CD33^{low} cells, which produce a strong local immunosuppressive effect. Since the CD15^{high}CD33^{low} cells secrete CCL2, CCL3, G-CSF, IL-8, and IL-6, this could be one of

the underlying mechanisms of immunosuppressive effects of CD15⁺ cells. This could be further supported by the finding that the presence of CD15⁺ cell populations significantly reduced the proliferation of T lymphocytes [36]. Therefore, part of the immunosuppressive action of these CD15⁺ cells is their inhibition of T-cell proliferation. Based on these observations, Eruslanov and colleagues [36] proposed that MDSCs might develop an immunosuppressive pro-tumor phenotype due to changes in the microenvironment of cancerous bladder tissue, at least in part, due to local inflammation. The tumor microenvironment has been described in many ways and is often said to be composed of macrophages, endothelial cells, and tumor cells together with blood vessels, infiltrating leukocytes, and stromal cells [37,38]. Increased populations of CD15^{high}CD33^{low}neutrophil-type cells have also been found in cancers of the pancreas, colon, breast, and kidney [39,40]. Therefore, CD15⁺ myeloid cells mediate several responses in the development of bladder cancer including the suppression of T cell proliferation, production of immunosuppressive proteins, and the secretion of pro-inflammatory cytokines.

Neutrophils

A dichotomy is observed in the effects of neutrophils on bladder cancer. On one hand, neutrophils target cancerous cells serving several important anticancer functions. But, they also stimulate excessive inflammation, release growth factors to malignant cells, secrete matrix-degrading enzymes, and they are the major VEGF containing cells [41]. The long term effects of all these factors promote cancer [42].

Gunes *et al.* [43] found that patients with bladder cancer have increased levels of 3 neutrophil proteins, human neutrophil protein-1, -2, and -3 (HNP 1–3). All three HNPs are subtypes of α -defensins, proteins that aid in the recruitment of leukocytes and might contribute to metastasis. The direct mechanism of action of HNPs in bladder cancer remains to be elucidated. The indirect effects of HNPs 1–3 include stimulation of tumor cell proliferation and potentially tumor angiogenesis. This may be accomplished as a result of HNPs promoting cytokine release, stimulating monocytes, and inhibiting the fibrinolytic system. The HNPs found in cancer cells are primarily derived from tumor invading neutrophils and eosinophils [43,44]. Gunes and colleagues [43] found that the levels of all three HNPs were overexpressed in the plasma of the tested bladder cancer patient population, potentially due to neutrophil damage (n=30). Additionally, Vlahou et al. [45] found elevated levels of HNPs 1–3 in the urine of bladder cancer patients. At lower levels HNPs are cytotoxic to monocytes, elevated HNP levels results in cellular damage, and significantly elevated levels are cytotoxic to all cell lines. Therefore, neutrophils may promote tumor invasion and development in the bladder due to their potential role in the production and secretion of human neutrophil proteins. None-the-less, the causative or consequential role of HNPs and the underlying mechanisms in the pathogenesis of urothelial carcinomas warrant further attention.

The neutrophil-to-lymphocyte ratio is used as a measure of systemic inflammation [46]. Studies have shown that NLR may be predictive of poor prognosis and may promote tumor progression due to a decreased immune response from lymphocytes. Additionally, Gondo and colleagues [47] found that the combination of neutrophil-to-lymphocyte ratio, tumor

size, hydronephrosis, and hemoglobin levels were predictive of disease specific survival in bladder cancer patients who had undergone radical cystectomy [47].

Macrophages

Tumor-associated macrophages (TAM) have been shown to promote and may even be required for angiogenesis to occur in bladder carcinomas [3,48]. Macrophages can be differentiated into M1 and M2 subsets based on the type of cytokines present. Under inflammatory conditions, macrophages change their phenotype and become more M1-like or classically activated macrophages ($CD86^+$ $iNOS^+$ $TLR2^+$ $MHCII^{high}$) that produce reactive oxygen species and release cytokines, including $TNF-\alpha$ and IL-12, and activate T_H1 cells to release $IFN-\gamma$ and IL-2 to activate cytotoxic T-cells [49,50] and are deleterious to body homeostasis. M2-phenotype (alternatively-activated macrophages; $Arg1^+$ $CD163^+$ $CD206^+$) is anti-inflammatory IL-4 or IL-13-polarized macrophage that produces IL-10 to suppress inflammation. In most cases M1 macrophages slow cell proliferation and lead to damage of tissues, whereas M2 macrophages stimulate the proliferation of cells and lead to tissue repair. Interestingly, the M1-phenotype macrophages elicit an antitumor effect. However, stimulation of M1 macrophages with $TGF-\beta$ initiates their conversion to pro-tumorigenic macrophages by promoting tumor invasion and metastasis (Figure 3). On the other hand, M2 macrophages promote T_H2 cells while suppressing T_H1 cells via the release of IL-4 and IL-10 [49,51]. However, the M2 macrophages in tumors have been shown to produce an immunosuppressive, pro-angiogenic, and cell invasive function [51]. The increased number and function of M2 macrophages in bladder carcinoma correlate with increased tumor size, stage, and grade [51]. TAMs ($CD163^+$) release cytokines that stimulate tumor growth. These cytokines include vascular endothelial growth factor (VEGF), $TNF-\alpha$, IL-8, epidermal growth factor, and basic fibroblast growth factor (b-FGF), all of which may promote vascularization in tumors. Extensive angiogenesis in the bladder results in the formation of highly vascularized tumors. This is the product of factors like VEGF, b-FGF, and platelet-derived endothelial cell growth factor [48].

The number of tissue-infiltrated macrophages is significantly increased in invasive bladder cancer relative to superficial cancers. Hanada and colleagues [48] found a weak correlation between the density of TAMs and the degree of angiogenesis in bladder tumors, based on microvessel density. Additionally, a correlation was found between elevated TAM counts and poor patient outcomes [48]. A study by Ajili et al [52] found higher cancer recurrence in patients with elevated TAM counts, as well as a decreased response to BCG treatment in patients with increased TAM infiltration. One theory suggests that macrophages promote tumor angiogenesis due to the hypoxic environment surrounding tumor tissues. Macrophages in this environment release angiogenic factors in an attempt to reverse the hypoxia, but in doing so they support tumor growth and invasion [48]. In other tumor types, Granata *et al.* [53] found that in this hypoxic state adenosine levels become elevated, which promotes the release of angiogenic and lymphogenic factors from macrophages. It has also been suggested that the hypoxic tumor microenvironment may promote the buildup of M2 macrophages in bladder tumors [51]. The phenotype of these M2 macrophages also seems to be changed by the hypoxic conditions, resulting in the release of $TNF-\alpha$, IL-1 β , COX2, and macrophage migration inhibitory factor (MIF) (Figure 3) [51,54]. Conversely, during states

of normoxia, M2 macrophages appear to be immunosuppressive, resulting in the inhibition of IL-12, IL-18, TNF- α , and IL-1 β [51,55]. These factors appear to improve the outcome of BCG treatment in hypoxic states. Another theory suggests that bladder tumors secrete monocyte chemotactic factors, which direct macrophages to enter the cancerous tissue and release angiogenic factors. Hanada and colleagues [48] suggest that TAM counts should be used as a prognostic indicator in patients with bladder cancer, because they may require more aggressive therapies than traditional transurethral resection. Patients with tumor vascularization are often advised to undergo chemotherapy combined with MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin) due to improved outcomes. Therefore, TAM count and degree of angiogenesis, based on microvessel density, may be important prognostic factors in bladder cancer. Finally, CD47 expression increases in tumor-initiating cells [5]. This protein is a ligand for signal-regulatory protein- α (SIRP α), and is known to inhibit macrophage-mediated phagocytosis [5,56]. This is potentially significant in the pathogenesis of bladder carcinoma because CD47 is an indicator for bladder cancer stem cells, and is expressed on both the urothelium and on bladder cancer cells.

While many researchers have explored the role of M1 and M2 macrophages in tumors, several questions still remain. Firstly, the mechanisms involved in the maintenance of macrophage levels within cancer tissue have yet to be identified. Secondly, the molecular mechanisms involved in the polarized activation of macrophages are not fully understood. Therefore, future studies investigating these mechanisms will allow for targeted therapies focusing on M1, M2, and TAM function.

Interleukin 6

IL-6 is the primary pro-inflammatory cytokine in humans and is produced primarily by T-lymphocytes and macrophages. Levels of IL-6 are elevated in both the urine and serum of patients with urothelial bladder carcinoma of the bladder relative to controls [57]. IL-6 is also increased in the urine of UCC patients, relative to individuals with less malignant non-muscle-invasive bladder cancer. This suggests that IL-6 may promote more malignant cancer phenotypes. Poor prognosis and increased metastasis have been linked to elevated serum levels of IL-6 in bladder, ovarian, and prostate cancers. *In vitro* and *in vivo* studies have demonstrated that inhibition of IL-6 release suppressed bladder cancer development [57]. One explanation for the pro-tumorigenic effect of IL-6 may be the fact that the cytokine is the main activator of the JAK/STAT3 signaling pathway. STAT3 contributes to tumor proliferation and EMT, a key step in tumor invasion [57,58]. EMT is regulated in part by the transcription factor TWIST [59]. It is the effect of cigarette smoking on TWIST that is believed to be one part of the pro-tumorigenic effect of smoking in the bladder. Furthermore, IL-6 promotes angiogenesis and vascular modeling via VEGF and STAT3, which affects the genes mediating angiogenesis. IL-6 is associated with a poor prognosis in bladder cancer. This is demonstrated by the correlation of IL-6 with decreased response following treatment, shorter length of survival, and increased disease failure rates [57].

IL-6 is also elevated in some patients with muscle invasive bladder cancer. Ho *et al.* [60] found that STAT3-activation in murine models lead to invasive cancer development from carcinoma *in situ*, without first entering the noninvasive papillary tumor stage. Furthermore,

STAT3 may play a role in tumor cell survival through its anti-apoptotic promotion of Bcl-XL expression [60]. A study by Wu and colleagues [61] demonstrated that STAT3 plays an important role in UCC cell growth via Jak2. They found that by inhibiting Jak2, cell growth was suppressed.

TNF- α

TNF- α is primarily produced by activated macrophages, and two polymorphisms of the TNF- α genes have been found to increase the risk of developing bladder cancer [18]. Potentially, this may be due to TNF- α -induced regulation of thymidine phosphorylase, an enzyme that has been shown to promote bladder cancer development. TNF- α also promotes angiogenesis and development of several tumor types. The TNF- α genes are located on chromosome 6 in a region of the major histocompatibility complex (MHC) adjacent to the lymphotoxin locus. Sixteen polymorphisms of the gene have been identified, two of which are TNF +488A and TNF -859T. The TNF +488A polymorphism is found in 28.1% of bladder cancer cases and 14.9% of healthy individuals, while TNF -859T is present in 26.6% of bladder cancers and only 14.4% of healthy patients. The role of these various polymorphisms in cancer are not well understood due to conflicting results from various studies. However, TNF +588A has been implicated in rheumatoid arthritis along with common variable immunodeficiency. TNF polymorphisms have been linked to several cancers including non-Hodgkin's lymphoma, myeloma, and prostate carcinoma [18].

TNF- α levels increase in the urine of patients undergoing BCG treatment. Indeed, an improved understanding of the +488 and -859 polymorphisms may allow researchers to elucidate the role of TNF polymorphisms in the manifestation of several diseases. For example, TNF +488 is located within an intron and is thus noncoding. Still, polymorphisms in this location impact disease states despite the fact that TNF- α production is not changed by this polymorphism. It has, therefore, been suggested that these polymorphisms are the result of linkage disequilibrium in the various alleles surrounding TNF and the adjacent lymphotoxin locus. The association of disease with pro-inflammatory polymorphisms is known to be the result of free radicals, DNA damage, elevated tumor angiogenesis, and immunosuppression.

Several other cytokines have been studied in regard to bladder cancer including IL-8. Both IL-8 and TNF- α correlate with tumor development and poor prognosis. TNF- α stimulates the secretion of matrix metalloproteinase-9 (MMP-9), an enzyme which has been shown to promote tumor invasion and spread [57,62]. In fact, a 17-fold increase in the activity of MMP-9 has been found in patients with invasive bladder tumors relative to healthy individuals. Also, inhibition of IL-8 has been shown to limit MMP-2 and MMP-9 expression, both of which stimulate tumor invasion. Therefore, IL-8 indirectly promotes bladder carcinogenesis and correlates with poor prognosis.

Dendritic Cells

There are three types of antigen presenting cells (APCs), dendritic cells (DCs), macrophages, and B cells. Of the APCs, dendritic cells are the most effective inducers of anticancer immunity [63]. Immature DCs have been found in the urine of individuals with

bladder cancer [64]. Dendritic cells are also present and minimally activated in bladder tumor tissue. Beatty *et al.* [64] suggested that DCs are found in the urine because they migrate there from bladder tumors *ex vivo*. In fact, immature and minimally activated DCs are found in the urine and bladder of cancer patients. One theory suggests this may be the result of tumor cells inhibiting the release of DC stimulation and maturation factors. This results in the promotion of tumor development because immature DCs are less capable of initiating T lymphocyte activation resulting in T cell anergy. While DCs have been used to treat tumors through various immunotherapies, their precise function in cancer remains poorly understood.

Recent studies have characterized two cytokines which play an important role in the prevention of bladder cancer, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF- α [65]. The action of these cytokines is the stimulation of dendritic cells. Rossi *et al.* [2] found that dendritic cell counts are significantly decreased in the blood of patients with superficial bladder cancer relative to healthy individuals. It was suggested that this decrease in DCs may impede the antitumor response, due to a lack of normal cross-talk between DCs, NK cells and T lymphocytes [66,67]. In contrast to the study by Beatty *et al.* [64], Rossi and colleagues [2] did not find DCs in the urine of either healthy or cancer donors who were not undergoing BCG, but DC levels were elevated in the urine of patients undergoing BCG treatment [2]. This increase in DCs may help explain the immunological mechanism for BCG [64]. In fact, DC levels in the urine could be used to monitor the efficacy of BCG and other bladder immunotherapies. Very recently, the findings of Ayari *et al.* [68] suggest that the presence of mature tumor-infiltrated DCs and potentially tumor-associated macrophages may be helpful in stratifying the patients at risk at the time of initial diagnosis of non-muscle-invasive bladder cancer and thus could assist in developing treatment strategies in the follow-up of the bladder cancer patient.

One study explored the role of DCs in the action of probiotics [69]. Probiotics have been shown to decrease the incidence of urinary tract infections and may delay bladder cancer recurrence. Probiotics act on the body and immune system in several ways. Their effects include increasing mucous production, preventing bacteria from binding to epithelial cells, inhibiting bacterial invasion, and altering monocyte, macrophage, DCs, and lymphocyte activity. The role DCs play in bladder cancer during probiotic use is unclear. However, probiotics have been shown to have an effect on DC activity, which is vital to the activation of the adaptive immune system [69]. The tumor development and the size in murine models was reduced by the administration of one probiotic strain, *lactobacillus*, and the anti-tumorigenic effect of *lactobacillus* could be due to the activity of DCs [69]. With increased understanding of the interaction between DCs and probiotics, probiotics may one day be used as a therapy for non-muscle-invasive bladder tumors.

T-lymphocytes

T-lymphocytes are commonly found in the tumor microenvironment and may contribute to tumorigenesis in the bladder [35]. Indeed, T cell subsets may exert both tumor-promoting and tumor-suppressive roles in bladder carcinoma. Levels of the T_H17 subset are elevated in bladder tumors relative to the peripheral blood [35]. Conversely, T-regulatory lymphocyte

(Treg) levels were higher in the peripheral blood compared to bladder tumors [70]. T-regulatory cells assist in self-tolerance and immune homeostasis. They help to modulate the immune system by monitoring and maintaining self-tolerance of the body. However, Tregs have also been shown to downregulate essential antitumor responses [71]. IL-2 from T-lymphocytes can downregulate the suppressive ability of Tregs, as well as promote the conversion of Tregs to T_H17 cells. It has been suggested that the balance between T_H17 cells and Tregs may contribute to tumorigenesis. The presence of elevated levels of Tregs in tumor tissue is also associated with poor prognosis. The granulocytic and monocytic cells seen in bladder cancer express elevated levels of PD-1, believed to be an immunosuppressive protein, compared to healthy individuals. PD-1 is secreted by Tregs, again indicating their potential role in bladder cancer. Still, the mechanisms underlying the effects of Tregs and T_H17 in bladder cancer remain to be elucidated. As discussed above, BCG ultimately leads to the activation of T cells and NK cells, which display cytotoxic activity against bladder cancer. Furukawa *et al.* [72] showed that IL-21 has an antitumor effect in MBT-2 cells. They found that the effect of IL-21 in bladder cancer is mediated by cytotoxic T lymphocytes [72]. Satyam *et al.* [73] found that T_H1 cytokines were suppressed in bladder cancer, while T_H2 cytokines appeared to be preferentially expressed.

T-lymphocytes are the primary producers of IL-2 which, in an autocrine manner, proliferates T-lymphocytes [74]. IL-2 can be found in the urine of patients undergoing BCG treatment, but not in the plasma. Levels of IL-2 have been shown to correlate significantly with the high recurrence rate of bladder cancer within 6 months after BCG treatment. Therefore, Reijke *et al.* [74] suggest that IL-2 and IL-6 should be the preferred cytokines used to monitor BCG treatments and the resulting immune response.

Bladder carcinomas have developed a mechanism to avoid immune-induced apoptosis [75]. Under normal conditions the Fas/Fas-ligand system mediates programmed cell death in infected and cancerous tissues. Fas ligand is found primarily on T-lymphocytes and natural killer (NK) cells [76,77]. Fas activation via binding of Fas ligand results in apoptosis of the cell bearing the receptor. Bladder carcinomas have developed a mechanism to avoid this immune response by removing Fas, effectively evading apoptosis [75]. Additionally, it has been shown that poorly differentiated bladder cancers have developed resistance to Fas-ligand-induced apoptotic events downstream of Fas. One potential mechanism to explain this phenomenon involves X-chromosome-linked-inhibitor of apoptosis protein (XIAP), which is a member of the IAP gene family. XIAP binds to caspase-3 and caspase-7 proteases effectively blocking the induction of apoptosis by Fas. Several cancers, including liver, colon, and pancreatic carcinomas, make use of similar mechanisms. It has been suggested that the production and secretion of soluble Fas (sFas), produced by all bladder cancer cell lines may be able to block the action of T-lymphocytes and even induce apoptosis in immune cells [75,78].

Immunological Basis of the Treatment Strategies in Bladder Cancer

Several treatment options are available for individuals with bladder cancer. Currently, guidelines have been put in place describing the preferred treatment method for each stage of cancer development. According to the American Cancer Society, the primary therapies

for bladder carcinoma include surgery, intravesicular therapy, chemotherapy, and radiation therapy [203]. Newer treatments include photodynamic therapy, gene therapy, and targeted drug therapies [79,203].

The most common treatment remains surgery, specifically transurethral resection of the bladder tumor (TURBT) [80]. TURBT involves the insertion of a resectoscope fitted with a wire loop into the urethra. Tumor tissue is then removed via the loop. A second form of surgery includes partial and radical cystectomy. These are often required for invasive bladder cancers. Intravesicular therapies involve the administration of either immunotherapies or chemotherapies directly into the bladder. Immunotherapies introduced include BCG and interferon, while the most common chemotherapies include mitomycin and thiotepa [81–83]. The primary therapy for advanced bladder cancers that have spread throughout the body include various chemotherapies. Some of the drugs used are cisplatin, mitomycin, gemcitabine, MVAC, and carboplatin [48,84,85]. The most commonly used form of radiation therapy is external beam therapy [86]. Often a combination of radiation, chemotherapy, and surgery is employed for improved results. While surgery activates stress-sensing pathways, necrosis of cancer cells is promoted by both chemotherapy and radiation therapy. This highly inflammatory process results in the release of ATP, heat shock protein (Hsp70), calcium binding proteins, danger associated molecular patterns (DAMPs), and IL-1 α [20]. It remains unclear whether the inflammation induced by these therapies supports or inhibits future tumor development. One theory suggests that necrotic inflammation may stimulate the remaining malignant cells promoting cancer recurrence. Alternatively, this inflammation may deter cancer regrowth improving outcomes. This is another area where future research could advance and improve upon current therapies.

Two immunotherapies will be discussed in detail to demonstrate their reliance on immune cells in their mechanism of action. These therapies are the immensely successful BCG treatment and the more recent cytokine gene-modified tumor vaccine.

BCG Treatment

As stated, TURBT remains the treatment of choice for superficial urothelial cell carcinoma (UCC) [2]. TURBT offers patients an 80% survival rate with a 50–70% risk of relapse. Due to the high rate of recurrence, a novel immunotherapy was introduced in 1976, which was derived from an attenuated tuberculosis vaccine [1,2].

Mycobacterium Bovis Bacillus Calmette-Guerin (BCG), a bacteria administered into the bladder, has long been the treatment of choice for superficial bladder cancer (Figure 4) [1,63]. BCG is often administered via 6-weekly intravesicular instillations to treat and prevent the recurrence of bladder cancer with a response rate of 70% [42,51,87]. Increasing the treatment length to 12 weeks has an improved response rate of 82% [88]. BCG usually begins 2–4 weeks after TURBT treatment and involves lyophilized BCG powder, reconstituted in saline, being administered into the urinary bladder through a urethral catheter [89]. The bacteria attaches to the urothelium of the bladder through a fibronectin-dependent process and enters both healthy and malignant cells [89]. This process initiates a significant immune response in the bladder, and induces the release of such cytokines as

IL-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, TNF- α , IFN- γ , and GM-CSF resulting in urothelial infiltration by inflammatory immune cells [88–90]. Pyuria sets in 4–6 hours post BCG instillation with an average of 10^7 WBCs/ml in the urine [1]. Side effects of BCG are often minimal with the most common presentation including urinary urgency and frequency in 95% of patients [89]. Since its introduction, BCG has become one of the most successful immunotherapies for solid cancers [42].

Jackson *et al.* [88] monitored the cytokines secreted following BCG instillation in an attempt to understand the various immune cells present. IL-4 was not found in the urine of any BCG treated patient. This would suggest that T_H2 cells do not play a role in BCG. However, IL-10 levels were elevated. While T_H2 cells primarily produce IL-10, other cells also secrete IL-10, allowing for the absence of T_H2 cells in the BCG response. Additional evidence to support this theory is the lack of a decrease in IL-2 and IFN- γ levels despite the elevation in IL-10. Traditionally, the cytokines IL-2 and IFN- γ are inhibited by IL-10 [88].

Polymorphonuclear neutrophil leukocytes (PMNs) play the primary immune role in BCG treatment and account for 75% of the cells recruited to the bladder following BCG instillation [42,91]. TNF-related apoptosis-inducing ligand (TRAIL) is an apoptotic initiating molecule, which plays an important role in the beneficial effect of BCG treatment. TRAIL, a member of the TNF family, is located on chromosome 3 and contains domains homologous to FasL. While its position on the chromosome (3q26) is not near any known TNF genes, it is very similar to both TNF and lymphotoxins- α and - β [1,92]. TRAIL is known to induce apoptosis only in cancerous or transformed cells while leaving healthy tissues untouched. After stimulation primarily by IFN- α and IFN- γ many cell types express TRAIL including DCs, macrophages, T cells, B cells, and NK cells. Expression of TRAIL allows these immune cells to gain significant antitumor potential. Neutrophils are known to contain high quantities of TRAIL and TNF- α [91]. Following BCG instillation, TRAIL is released from activated neutrophils resulting in TRAIL-induced death of cancerous cells [1]. It was noted that patients who responded positively to BCG had nearly 3 fold greater levels of TRAIL than individuals who did not respond to treatment. Following BCG stimulation, neutrophils also produce IL-8, GRO- α , MIP-1 α , and MIF [93]. The chemokines released by BCG-activated neutrophils have been shown to recruit macrophages, which then recruit T cells to the bladder. Simons *et al.* [93] suggest that these T cells are directly responsible for antitumor effect of BCG, while neutrophils indirectly contribute through the recruitment of other immune cells [93]. The overall mechanism described by Simons suggests that BCG infects the bladder epithelium and leads to the release of IL-8 to attract neutrophils. BCG-activated neutrophils in the bladder produce sTRAIL and chemokines. These chemokines recruit monocytes that release IFN and other chemotactic factors, which induce T cells to express TRAIL. The combination of sTRAIL from neutrophils and TRAIL from T cells results in apoptosis of bladder tumor cells. Interestingly, while macrophages play an integral part in BCG's antitumor effect, elevated TAM levels have been associated with an increased risk of failure for BCG treatment [94].

Along with PMN activation, BCG provokes the secretion of many pro-inflammatory cytokines primarily from T_H1 cells [42]. Alternatively, elevated levels of IL-4 and IL-10, T_H2 cytokines, have been associated with failure of BCG [95]. IL-8, the primary cytokine

released, is seen in the urine after the first BCG instillation. The concentration of IL-8 correlates with the successful outcome of BCG treatment, and has been associated with the development of the immune response in the bladder and a decrease in cancer recurrence [42]. T_H1 lymphocytes migrate to the bladder following induction by BCG-activated neutrophils and primed monocytes [42]. Multiple BCG instillations result in the accumulation of NK cells, CD8⁺ T lymphocytes, and macrophages in the suburothelial stroma [42,96]. Furthermore, Lima and colleagues [51] hypothesized that M2 macrophages may enter an angiogenic phenotype under hypoxic BCG conditions, resulting in improved outcomes. However, normoxic conditions appear to have the opposite effect, potentially promoting immunosuppression during BCG treatment and increasing treatment failure. Takeuchi, *et al.* [97] found that T_H17 cells may play an important role in BCG by recruiting the initial neutrophils to the bladder via IL-17 secretion.

Due to the success of BCG, it has been suggested that tumor-specific immunotherapies may be successful in the treatment of bladder cancer [63]. Melanoma antigen (MAGE) genes are expressed in several cancers but are not found in healthy tissue, excluding the testes. Due to their prevalence in cancerous tissues alone, they may potentially be used as targets for tumor-specific immunotherapy. One study demonstrated this when dendritic cells, pulsed with MAGE peptide, induced cytotoxic T lymphocytes to attack bladder tissue that expressed MAGE proteins [63]. Thus, DCs pulsed with MAGE peptide may be beneficial in the treatment of advanced bladder cancers that are no longer responsive to surgery, radiation, or chemotherapy. However, the safety and practicality of this novel therapy require further attention.

Not all cancers are receptive to BCG treatment, such as RT4v6 bladder cancer lines [91]. One study, however, demonstrated that BCG combined with Smac mimetic compound resulted in the eradication of RT4v6 cells. Smac is a protein that degrades IAP, inhibitor of apoptosis, resulting in the release of caspases and ultimately the death of effector cells [98]. BCG combined with Smac lead to increased secretion of several death ligands including TNF- α , TRAIL, and FasL by BCG-activated neutrophils. Jinesh *et al.* [91] demonstrated that of these death ligands, TNF- α plays the largest role in eliminating cancerous bladder tissue.

Twenty-five percent of patients do not respond to BCG, and it remains unclear why treatment is unsuccessful in these individuals. Future studies should explore this phenomenon. Research to understand why treatment is unsuccessful in certain people would allow clinicians to identify and avoid the use of BCG in patients for which treatment would be unsuccessful. Additionally, many questions remain regarding the underlying mechanisms of BCG therapy. For example, we still do not understand the kinetics of chemokine secretion, immune cell recruitment, or TRAIL buildup in the bladder. Further exploration of these questions could allow for advances in BCG treatment with improved outcomes and reduced complications.

Cytokine Gene-Modified Tumor Vaccines

For decades research has sought to understand the role cytokine vaccines may one day play in the treatment of various carcinomas [99]. This treatment involves inserting cloned cytokine genes into genetically modified tumor cells, which then constitutively produce the cytokine [100]. Cytokine-secreting tumor cells are used in the treatment of cancer because the administration of cytokines directly into the circulation would require toxic doses [99]. The MBT-2 murine cell line is used as a model for bladder cancer due to its histological and etiological resemblance to human urothelial carcinoma [99,101,102]. Saito *et al.* [99] demonstrated that gene-modified MBT-2 cells, altered to constitutively produce irradiated IL-2, are capable of curing preexisting tumors as well as induce memory and resistance against the future development of tumors. Saito tested several other cytokine vaccines as well including IL-1 α , IL-1 β , and IFN- γ . It was found that these other cytokines are only weakly effective therapies. Granulocyte-macrophage colony stimulating factor (GM-CSF) was extremely effective therapeutically, however, its effects remained less pronounced than those of IL-2. Dranoff *et al.* [103] found that GM-CSF secreting tumor cells were the most effective in treating B16.F10 melanoma tumor cells. The discrepancy in the results of Saito *et al.* [99] and Dranoff *et al.* [103] could be due to cytokine-specific responses in tumor cell types. This must be taken into account when choosing an appropriate treatment. In vitro studies have also demonstrated that dendritic cells can be treated with tumor specific antigens and used in immunotherapy [63].

In these studies the survival of the animal was used to determine the effectiveness of various cytokine vaccines. It was originally suggested that the induction of cytotoxic T lymphocytes (CTLs) could be used to gauge the success of cytokine vaccine, because CTLs are an essential part of the antitumor immune response. The level of induction, however, did not correlate with the therapeutic benefit of cytokine therapy. This suggests that CTL induction might not be a good measure of gene-modified tumor vaccine success. While these cytokine secreting tumors have been extensively studied, continued investigation will only improve outcomes. Primarily, treatment efficacy is dependent on understanding which combination of cytokines and treatments will be the most therapeutic for patients with bladder cancer as well as other diseases.

Expert commentary & five-year view

Many factors contribute to the pathogenesis of bladder cancer. Firstly, inflammation is known to play a significant role in cancer development, invasion, and metastasis and this holds true in carcinomas of the bladder. Reactive oxygen species form in sites of inflammation, and these species induce mutations and modify proteins and lipids. The mutation in various genes, including HRAS and FGFR3, is seen in a high percentage of bladder cancers, suggesting their potential role in the differentiation of normal urothelium into cancerous tissue. Additionally, increased activity of EMT transcriptional factors, ZEB and SNAIL, likely induces transformation of healthy cells and thus epithelial plasticity in the pathogenesis of bladder cancer.

A dichotomy has been observed in the role of various immune cells in bladder cancer. One of the functions of the immune system is to identify and destroy cancerous cells by apoptosis or phagocytosis. However, in an attempt to eliminate tumor cells, the immune system often promotes the formation of various factors that induce tumor invasion by the induction of angiogenesis and the release of tumor growth factors. Myeloid derived suppressor cells, including neutrophils, monocyte/macrophages, and dendritic cells, are known to be elevated in bladder cancer. Furthermore, neutrophils and macrophages release factors that promote tumor angiogenesis and cancer cell proliferation. The balance between the subsets of T lymphocytes is critical in maintaining normal homeostasis and the environment in tissues. In bladder carcinoma, this balance is in flux due to the effect of cytokines that drives the differentiation of Tregs to T_H17 cells. BCG and cytokine gene-modified tumor vaccines are effective therapeutic modalities in the treatment of bladder cancer, and exemplify the use of the immune system in their efficacy.

Despite extensive research in bladder cancer, the underlying cellular and molecular mechanisms remain unclear. Furthermore, the role of individual cell type, cytokine, and chemokine in the pathogenesis of bladder cancer remains to be elucidated. It is critical to characterize the factors that are primarily involved in the initiation of the disease process, invasion, proliferation and metastasis of the tumor. At the same time, the identification of regulatory mediators that suppress differentiation of urothelial cells and maintain normal structure is key to the development of better and more effective therapeutic approaches. Over the last four decades a great deal of study has gone into BCG treatment, however, the mechanisms underlying the therapy are still not fully understood. The immune response elicited by BCG is extraordinarily complex making it difficult to form a comprehensive understanding. Therefore, future research should explore the mechanism underlying the antitumor effect of BCG, including the kinetics of chemokine release and cell recruitment.

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Key Issues

- Further investigations are warranted on the proteins, transcription factors, and genes that would help identify patients who will develop incurable muscle-invasive bladder cancer.
- EMT is an important part of bladder carcinogenesis, but the stage in the metastatic cascade during which EMT begins remains to be elucidated.
- Several cellular pathways initiate EMT, however, the patterns of specificity and mechanisms of regulation for these pathways remain unknown.
- The effectiveness of therapies targeting M1, M2, and TAM function requires an increased understanding of the mechanisms underlying polarized macrophage activation as well as macrophage level maintenance in cancer tissue.
- Chemotherapy and radiation are effective treatments for bladder cancer, but it is unknown whether the necrosis and inflammation resulting from these procedures promotes cancer recurrence or remission.
- Twenty-five percent of patients are unresponsive to BCG. Further study should seek to characterize various factors that might be used in the identification of patients for which treatment would be unsuccessful and should thus be avoided.
- BCG has been extensively studied, however, the kinetics of chemokine secretion, immune cell recruitment, and TRAIL buildup are unknown

Figure 1. The various initiating and promoting factors of urothelial cell carcinoma

This figure depicts several of the diverse infectious and noninfectious agents that have been associated with the development of bladder cancer. *TWIST expression is associated with bladder cancer, but its causative role has yet to be determined. **p53 mutations may be the link between cyclophosphamide-induced cystitis and bladder cancer, but this remains to be confirmed.

Figure 2. Hypothetical pathway including immune cells and their mediators in the pathogenesis of bladder cancer

This figure shows how the microenvironment can transform the phenotype of MDSCs into pro-tumor cells. Pro-tumor neutrophils have been shown to release factors such as HNPs 1–3, CCL2, CCL3, CCL4, G⁺CSF, IL-8 and IL-6, all of which promote bladder carcinogenesis. Pro-tumor monocyte/macrophages release several factors that result in bladder tumor angiogenesis as well as tumor cell protection against apoptosis. Several reactive oxygen species are released resulting in DNA, RNA, protein, and lipid damage. MDSC: Myeloid Derived Suppressor Cells; HNP: Human Neutrophil Protein

Figure 3. M1 and M2 macrophages support or inhibit bladder cancer development depending on the tumor microenvironment

M1 macrophages appear to promote carcinogenesis in the bladder. This occurs through activation of the Stat3 pathway as well as MMP-9 expression, resulting in EMT and basement membrane disruption respectively. M2 macrophages have varying effects on bladder tumors depending on their microenvironment. In hypoxic conditions M2 cells promote the increased production of VEGF, TNF- α , COX-2, IL-1 β and MIF, which improve BCG outcomes. Alternatively, under normoxic conditions M2 cells inhibit IL-12, IL-18, IL-1 β and TNF- α secretion, resulting in immunosuppression.

Figure 4. Mechanism underlying anti-tumor effect of BCG in the bladder

Mycobacterium Bovis Bacillus Calmette-Guerin is introduced into the lumen of the bladder. The bacteria is taken up by the bladder epithelium, resulting in the release of IL-8. IL-8 acts as a chemoattractant for polymorphonuclear leukocytes to the bladder. The PMNs release TRAIL, which promotes apoptosis of tumor cells. Additionally, PMNs release factors that recruit monocytes to the bladder. These monocytes promote both membrane TRAIL expression and a T_H1 mediated response, which results in bladder tumor cell apoptosis and a reduction in cancer recurrence.