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Immunotherapy of Urinary Bladder Carcinoma: BCG and Beyond

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1. Introduction

Urothelial carcinoma of the bladder is the second most common urologic neoplasm after prostate carcinoma in the United States, with an estimated 70,510 new cases and 14,880 deaths in 2012 [1]. Global prevalence of bladder cancer is estimated at >1 million and is steadily increasing. This disease places enormous economic burden on the U.S. health care system due to its requirements of surgical resection, repeated intravesical therapies, and lifelong medical follow-up. Urothelial carcinoma accounts for 90% of bladder tumors. At the time of diagnosis, 20-25% of cases are muscle invasive (stage T2 or higher) and are typically treated with surgical resection (radical cystectomy) [2]. The remainders are confined to layers above the muscularis propria – so-called nonmuscle invasive bladder cancer (NMIBC). These cancers (also termed “superficial bladder cancer”) include tumors confined to the urothelium (Ta), tumors invading the lamina propria (T1), and carcinoma *in situ* (Tis, a flat erythematous lesion), occurring in 70%, 20% and 10% of NMIBC cases, respectively [2]. Transurethral resection of bladder tumor (TURBT) is the standard primary treatment for Ta and T1 lesions; however, recurrence rates for TURBT alone can be as high as 70% with up to 30% progressing to muscle invasive disease requiring cystectomy [3]. The high rates of recurrence and significant risk of progression in higher grade tumors mandate additional therapy with intravesical agents. While limiting the systemic exposure, intravesical therapy allows the destruction of residual microscopic tumor and circulating tumor cells after TURBT by exposure to therapeutic agents, thereby preventing reimplantation. To date, intravesical therapy has been used as an adjuvant treatment after TURBT to prevent recurrence and progression of the disease and is also the treatment of choice for Tis that is not feasible for TURBT.

Chemotherapeutic agents such as mitomycin C, doxorubicin and epirubicin have long been used as intravesical therapies for NMIBC [3,4]. Recently, intravesical use of gemcitabine, valrubicin, and apaziquone have also been evaluated [5-7]. With respect to immunotherapy, BCG, a live attenuated strain of *Mycobacterium bovis* widely used as a vaccine against tuberculosis, was first introduced as an intravesical therapy for bladder cancer in 1976 by Morales and associates [8]. Since then, BCG has been extensively evaluated and demonstrated to be superior to any other single chemotherapeutic agent for reducing recurrence and preventing progression of the disease [3,9]. To date, BCG has become the mainstay of therapy for NMIBC and remains the most effective treatment [3,9]. However, despite its favorable effects, a significant proportion of patients do not respond to BCG or tolerate treatment. In addition, recurrence and side effects are common. Therefore, research has been pursued and efforts made to improve BCG therapy. During the past decades, cytokine-based therapies have been developed. To date, multiple cytokines with Th1 stimulating properties, such as IFN- α , IL-2 and IL-12, have been evaluated, alone or in combination with BCG for the treatment of bladder cancer. In addition, pre-clinical research continues, aiming to identify new BCG therapeutic modalities. This chapter reviews the progress of bladder cancer immunotherapy, focusing on the clinical use of BCG and cytokines. In addition, we describe our own experience with BCG and cytokine therapies as well as research on BCG combination therapy and genetic engineering of BCG to secrete Th1 cytokines. Finally, we describe the future directions for research with regard to BCG immunotherapy.

2. BCG immunotherapy of bladder cancer

2.1. Clinical use of BCG in bladder cancer treatment

Intravesical administration of BCG is currently the most common therapy employed for NMIBC. Since its advent in 1976, BCG has been extensively used to reduce recurrence and progression of NMIBC in an attempt to preserve the bladder. Although various BCG strains (e.g. Pasteur, Tice, Connaught, Frappier, RIVM and Tokyo) have been used, there is no evidence of a difference in efficacy or toxicity profile among these strains [9]. Many prospective randomized studies and meta-analyses have demonstrated the effectiveness of intravesical BCG therapy. Typical complete response rates are 55-65% for papillary tumors and 70-75% for Tis, which inversely indicates that 30-45% of patients will fail BCG treatment [3,9-12]. Adjuvant intravesical therapy was noted by the 2007 American Urological Association (AUA) panel to reduce recurrences by 24% and treatment with BCG was recommended by the panel [10]. Unfortunately, of complete responders, up to 50% will develop recurrent tumors within the first 5 years [13]. Furthermore, up to 90% of patients experience side effects ranging from cystitis and irritative voiding symptoms to much more uncommon life-threatening BCG sepsis. Up to 20% of patients are BCG intolerant due to these side effects [14].

The optimum dosing, schedule and duration for BCG treatment of NMIBC are unknown. Both induction and maintenance courses are largely empirical. According to the AUA's 2007 clinical practice guidelines [10], BCG therapy should be initiated 2-3 weeks following TURBT to allow

healing of the urothelium and reduce the risk of side effects. The induction course consists of six weekly intravesical instillations. The recommended dose varies in weight from strain to strain, but each provides approximately $1-5 \times 10^8$ colony-forming units (CFU) of viable mycobacteria. Lyophilized powder BCG is reconstituted in 50 ml of saline and administered via urethral catheter into an empty bladder with a dwell time of 2 hours. Maintenance is given as three weekly intravesical instillations at 3 and 6 months and then every 6 months for up to 3 years. Maintenance BCG is more effective in decreasing recurrence as compared to induction therapy alone. Multiple meta-analyses support BCG maintenance and it is now firmly established in clinical practice. The European Association of Urology (EAU) and the AUA recommend at least one year of maintenance for high risk patients [10,15]. The optimum schedule and duration of therapy have yet to be determined; however, most who use maintenance follow some permutation of the Southwest Oncology Group (SWOG) program, a 3-week “mini” series given at intervals of 3, 6, 12, 18, 24, 30 and 36 months for a total of 27 instillations over 3 years [3,9,16]. Other schedules, such as single maintenance instillations of BCG at 3, 6, 9 and 12 months after induction therapy, have also produced promising results [17]. Recently, the EAU updated its guidelines on NMIBC and recommended a minimum of one year of intravesical BCG therapy for intermediate or high risk disease [18]. The International Bladder Cancer Group (IBCG) also reviewed the current guidelines and recommended the use of intravesical BCG with maintenance for intermediate or high risk disease [19]. Intravesical BCG is contraindicated under the following situations: TURBT within the past 2 weeks, traumatic catheterization, macroscopic hematuria, urethral stenosis, active tuberculosis, prior BCG sepsis, immunosuppression, and urinary tract infection.

At our own institution, a BCG induction course is typically initiated at 2-3 weeks post-TURBT with six weekly installations and a 1-2 hour dwell time. For patients with Tis, severe dysplasia, Grade 3/high grade or poorly differentiated pathology, and/or stage T1 disease, formal restaging under anesthesia is performed 6 weeks later and includes bilateral upper tract cytology, retrograde pyelograms, 4-5 random bladder biopsies, and prostatic urethral biopsies. If this pathology and restaging is negative, maintenance cycles may be initiated in 6 weeks. We classify three maintenance cycles A, B and C. Maintenance A consists of 3 weekly instillations followed by cystoscopy 6 weeks later. Cytology and fluorescence *in situ* hybridization (FISH) in urine specimens may be obtained at this time. If cystoscopy/cytology is negative, maintenance B may be initiated 6 months after the conclusion of cycle A, again for three weekly treatments. Maintenance C is initiated 6 months after the conclusion of cycle B. Following cycle C, cystoscopy/cytology is repeated every 3 months for 2 years from the original diagnosis at which time it is extended to every 6 months for 2 years, and then annually.

2.2. Mechanism of BCG action

Understanding of the mechanisms of BCG action is critical to improving the efficacy of BCG therapy. Although the exact mechanisms of BCG action currently remain elusive, many details have been discovered during the past decades. It has become clear that a functional host immune system is a necessary prerequisite for successful BCG therapy. It has also been known that the effects of intravesical BCG depend on the induction of a complex inflammatory cascade

event in the bladder mucosa reflecting activation of multiple types of immune cells and bladder tissue cells [20,21] (Figure 1). The initial step after BCG instillation is binding of BCG to fibronectin expressed on the urothelial lining through fibronectin attachment protein (FAP) [22]. Attached BCG is then internalized and processed by both normal and malignant cells, resulting in secretion of an array of proinflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony stimulating factor (GM-CSF) [23,24]. Following urothelial cell activation, an influx of various leukocyte types into the bladder wall occurs including neutrophils, monocytes/macrophages, lymphocytes, natural killer (NK) cells, and dendritic cells (DC) [25-27]. These infiltrating leukocytes are activated and produce a variety of additional proinflammatory cytokines and chemokines and also form BCG-induced granuloma structures in the bladder wall [25,27]. Subsequently, a large number of leukocyte types such as neutrophils, T cells and macrophages are expelled into the bladder lumen and appear in patients' voided urine [28-31]. In addition, transient massive cytokines and chemokines can be detected in voided urine including IL-1 β , IL-2, IL-6, IL-10, IL-12, IL-18, IFN- γ , TNF- α , GM-CSF, macrophage colony-stimulating factor (M-CSF), macrophage-derived chemokine (MDC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , interferon-inducible protein (IP)-10, monokine induced by γ -interferon (MIG), and eosinophil chemoattractant activity (Eotaxin) [30,32-37]. The urine of animals treated with intravesical BCG also showed increased levels of numerous cytokines and chemokines [27]. It has been noted that the development of a predominant Th1 cytokine profile (e.g. IFN- γ , IL-2 and IL-12) is associated with the therapeutic effects of BCG, whereas the presence of a high level of Th2 cytokines (e.g. IL-10) is associated with BCG failure [33,35, 36]. Thus, a shift of the cytokines produced towards a Th1 milieu is necessary for successful BCG immunotherapy of bladder cancer. To support this, it has been observed that both IFN- γ and IL-12 but not IL-10 are required for local tumor surveillance in an animal model of bladder cancer [38]. Mice deficient in IL-10 genetically (IL-10^{-/-}) or functionally via antibody neutralization or receptor blockage can also develop enhanced anti-bladder cancer immunity in response to intravesical BCG [36,39].

Multiple immune cell types participate in the inflammatory response induced by BCG in the bladder. It is well accepted that macrophages, an indispensable cellular component of the innate immune system, serve as the first line of defense in mycobacterial infection. Activation, maturation and cytokine production of macrophages are primarily induced by Toll-like receptor (TLR) 2 ligation [40]. Following BCG instillation, an increased number of macrophages can be observed in bladder cancer infiltrates and the peritumoral bladder wall. Voided urine after BCG instillation also contains an increased number of macrophages and the cytokines and chemokines predominantly produced by macrophages such as TNF- α , IL-6, IL-10, IL-12 and IL-18 [28,30,32,35-37]. In addition to presenting BCG antigens, macrophages are capable of functioning as tumoricidal cells toward bladder cancer cells upon activation by BCG [41-45]. The killing of bladder cancer cells by macrophages relies on direct cell-to-cell contact and release of various soluble effector factors such as cytotoxic cytokines TNF- α and IFN- γ and apoptotic mediators such as nitric oxide (NO) [43-45,46]. Th1 cytokines (e.g. IFN- γ) enhance the induction of macrophage cytotoxicity whereas Th2 cytokines (e.g. IL-10) inhibit the induction of macrophage cytotoxicity [44,45].

Neutrophils also compose the early responding cells to BCG instillation of the bladder and can be observed in the bladder wall and urine shortly after BCG instillation [27,28,30]. Neutrophils are central mediators of the innate immunity in BCG infection and are activated by signalling through TLR2 and TLR4 in conjunction with the adaptor protein myeloid differentiation factor 88 (MyD88) [47]. In addition to secretion of proinflammatory cytokines and chemokines (e.g. IL-1 α , IL-1 β , IL-8, MIP-1 α , MIP-1 β , MCP-1, transforming growth factor (TGF)- β , and growth-related oncogene (GRO)- α) that lead to the recruitment of other immune cells [48], recent studies revealed that neutrophils are the primary source of TNF-related apoptosis-inducing ligand (TRAIL) found in the urine after BCG instillation [49,50]. TRAIL is a member of the TNF family that induces apoptosis in malignant cells but not in normal cells. Studies have indicated that the neutrophil TRAIL response is specific to BCG stimulation rather than nonspecific immune activation. Studies have also revealed a positive correlation between urinary TRAIL level and a favorable response to BCG treatment [49]. These observations suggest an important

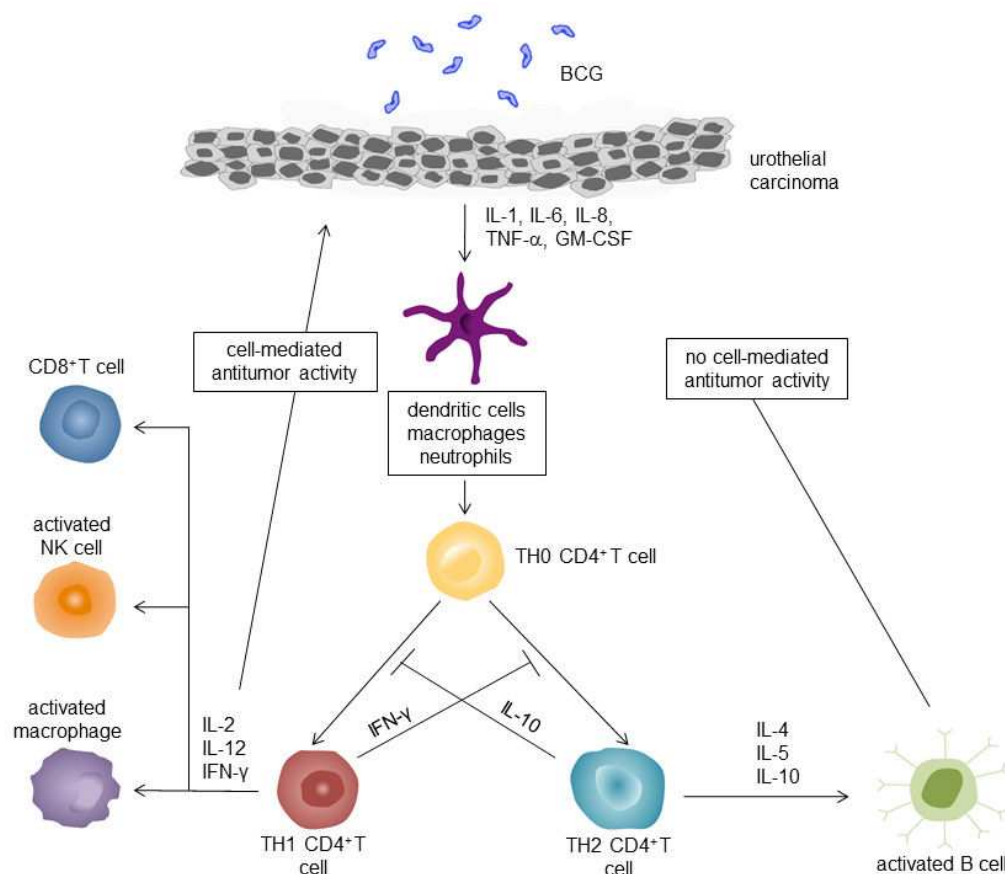


Figure 1. Suggested cascade of immune responses in bladder mucosa induced by intravesical BCG instillation. Attachment of BCG to urothelial cells including carcinoma cells triggers release of cytokines and chemokines from these cells, resulting in recruitment of various types of immune cells into the bladder wall. Activation of phagocytes and the new cytokine environment lead to the differentiation of naive CD4⁺ T cells into TH1 and/or TH2 cells that direct immune responses toward cellular or humoral immunity, respectively. The therapeutic effect of BCG depends on a proper induction of TH1 immune responses. IL-10 inhibits TH1 immune responses whereas IFN- γ inhibits TH2 immune responses. Blocking IL-10 or inducing IFN- γ can lead to a TH1 dominated immunity that is essential for BCG-mediated bladder cancer destruction.

role of neutrophils in BCG-induced anti-bladder cancer immunity. Indeed, it has been observed that depletion of neutrophils resulted in a reduced BCG-induced anti-bladder cancer response in a mouse model of bladder cancer [48].

Following the activation of macrophages and neutrophils in the bladder wall, driven by chemoattractants, recruitment of other immune cell types including CD4⁺ T cells, CD8⁺ T cells, NK cells, and DC takes place [25,26]. As for neutrophils and macrophages, these cell types can be found in the voided urine of patients after BCG instillation [28-30]. These effector cells produce various cytokines and chemokines to further promote BCG-induced anti-bladder cancer immune responses in the local milieu. In addition, DC, together with macrophages, trigger an anti-BCG specific immune response via antigen presentation to T cells that also amplifies the BCG-induced antitumor immunity. Like neutrophils and macrophages, both T cells and NK cells are cytotoxic toward bladder cancer cells upon activation. They kill target cells via the major histocompatibility complex (MHC) restricted (e.g. for cytotoxic T lymphocytes (CTL)) and/or MHC non-restricted pathways (e.g. for NK cells) [41,51,52]. Perforin-mediated lysis and apoptosis-associated killing (e.g. via Fas ligand and TRAIL) have been implicated as the major molecular effector mechanisms underlying the eradication of bladder cancer cells. These effector cell types are crucial for BCG immunotherapy of bladder cancer, as depletion of these cell types failed to develop effective anti-bladder cancer responses *in vivo* and kill bladder cancer cells *in vitro* [53,54].

It has been shown that stimulation of human peripheral blood mononuclear cells (PBMC) by viable BCG *in vitro* leads to the generation of a specialized cell population called BCG-activated killer (BAK) cells [55,56]. BAK cells are a CD3⁺CD8⁺CD56⁺ cell population whose cytotoxicity is MHC non-restricted [56,57]. BAK cells kill bladder cancer cells through the perforin-mediated lysis pathway and effectively lyse NK cell-resistant bladder cancer cells [55-57]. Macrophages and CD4⁺ T cells have been found to be indispensable for the induction of BAK cell killing activity but have no such activity by themselves [56]. Th1 cytokines IFN- γ and IL-2 have been found to be required for the induction of BAK cell cytotoxicity, as neutralizing antibodies specific to these cytokines could inhibit BCG-induced cytotoxicity [56]. BAK cells, together with lymphokine-activated killer (LAK) cells, a diverse population with NK or T cell phenotypes that are generated by IL-2 [58-60], have been suggested to be the major effector cells during intravesical BCG therapy of bladder cancer. Other potential cytotoxic effector cells include CD1 restricted CD8⁺ T cells [61], $\gamma\delta$ T cells [62-64], and natural killer T (NKT) cells [63-65].

Activation of the innate immune system is a prerequisite for BCG-induced inflammatory responses and the subsequent eradication of bladder cancer by intravesical BCG. In BCG instillation, TLRs participate in neutrophil, macrophage and DC recognition, maturation and activation. Both TLR2 and TLR4 appear to serve important but distinct roles in the induction of host immune responses to BCG or BCG cell-wall skeleton [40]. TLR9 also contributes to DC recognition of BCG [66]. Like other microbes, BCG has surface components called pathogen-associated molecular patterns (PAMPs) that are recognized by cells of the innate immune system through TLRs during infection [67]. It is this interaction between TLRs and PAMPs that activates the cells of the innate immune system, leading to BCG-induced inflammatory responses and subsequent eradication of bladder cancer. It is known that the antitumor effect

of intravesical BCG depends on its proper induction of a localized Th1 immune response. However, a systemic immune response appears also to be involved in intravesical BCG therapy. It has been documented that purified protein derivative (PPD) skin test often converts from negative to positive after BCG instillation and the effective treatment is associated with the development of delayed-type hypersensitivity (DTH) reaction to PPD [68]. Animal studies have also demonstrated the importance of DTH in the antitumor activity of intravesical BCG therapy [36]. Moreover, studies have shown increased levels of cytokines and chemokines in the serum (e.g. IL-2, IFN- γ , MCP-1 and RANTES), along with production of these cytokines and chemokines in the urine and/or bladder, during the course of BCG instillation [34,69]. Furthermore, studies have also shown an increase in PBMC cytotoxicity against urothelial carcinoma cells (UCC) after BCG instillation [34].

In addition to the ability of BCG to elicit host immune responses, evidence supports a direct effect of BCG on the biology of UCC. *In vitro* studies have shown that BCG is anti-proliferative and even cytotoxic to UCC [41,70], and induces UCC expression of cytokines and chemokines (e.g. IL-1 β , IL-6, IL-8, TNF- α and GM-CSF) [24], antigen-presenting molecules (e.g. MHC class II, CD1 and B7-1) [71], and intercellular adhesion molecules (e.g. ICAM-1) [71]. Analysis of tumor biopsy specimens from bladder cancer patients who underwent intravesical BCG therapy further supported the ability of BCG to induce UCC expression of these molecules *in vivo* [26]. Moreover, the bladder urothelium of animals treated with intravesical BCG shows upregulation of HLA antigens (e.g. MHC class I and II) and changes of many other molecules [72]. Recent studies have revealed that by cross-linking $\alpha 5\beta 1$ integrin receptors, BCG exerts its direct biological effects on UCC, including activation of the signal transduction pathways involving activator protein (AP) 1, NF κ B and CCAAT-enhancer-binding protein (C/EBP) [73], upregulation of gene expressions such as IL-6 and cyclin dependant kinase inhibitor p21 [73,74], and cell cycle arrest at the G1/S transition [75]. Although some studies showed the ability of BCG to induce apoptosis in UCC [76], other studies showed no such an ability or even induction of apoptotic resistance in UCC [77]. Further studies revealed that BCG induced UCC death in a caspase-independent manner [77] and that p21 played an important role in modulating the direct effects of BCG on UCC [78].

3. Recombinant cytokines for bladder cancer treatment

Prompted by the burden of patients either with BCG refractory disease or intolerance to BCG treatment, the search goes on for therapeutic improvements. Given that the effect of BCG depends on a proper induction of Th1 immune responses, decades of research have focused on enhancing the BCG induction of Th1 immune responses. Th1 stimulating cytokines, such as IFN- α , IL-2, IL-12, IFN- γ , TNF- α and GM-CSF, have been used alone or in combination with BCG and demonstrated to be favorable in the treatment of bladder cancer. Particularly, combination therapies potentially allow the use of a lower and safer dose of BCG while preserving or even enhancing BCG efficacy.

3.1. Recombinant IFN- α

IFNs are glycoproteins initially isolated in the 1950s and valued for their anti-viral properties. Three types have been isolated, IFN- α (which is actually a family of interferons), IFN- β , and IFN- γ . IFN- α and IFN- β are grouped as “Type I” interferons whereas IFN- γ is a “Type II” interferon. The Type I interferon receptor has 2 components, IFNAR-1 and IFNAR-2, which subsequently bind and phosphorylate Jak molecules initiating a cascade resulting in gene transcription [79]. The IFN- α family is well known to stimulate NK cells, induce MHC class I response, and increase antibody recognition [80]. They have antineoplastic properties by direct antiproliferative effects and complex immunomodulatory effects [79], both of which could be advantageous for bladder cancer treatment. Clinically available preparations include IFN- α 2a (Roferon-A, recombinant, Roche Laboratories, Nutley, NJ) and IFN- α 2b (Intron-A, recombinant, Schering Plough, Kenilworth, NJ), though to date most research involves IFN- α 2b. There has been interest in IFN- α 2b both alone and combination with BCG, where a synergistic response has been described. Conceptually, combining BCG and IFN makes sense. BCG efficacy depends on the induction of a robust Th1 cytokine profile and IFN- α 2b has been shown to potentiate the Th1 immune response [81]. However, despite theoretical promise, data after translation to clinical practice has been mixed.

For many years, IFN- α was thought to exert antitumor activity primarily through direct antiproliferative properties [82]. At least part of this effect has been shown to be mediated by directly inducing tumor cell death. IFN- α has been documented to independently induce tumor necrosis factor related apoptosis inducing ligand (TRAIL) expression in UM-UC-12 bladder cancer cells [83], which subsequently triggers apoptosis in cells expressing the appropriate cell death receptor. Cell death occurs ultimately by Fas-associated protein with death domain (FADD) dependent activation of the death inducing signaling complex (DISC) followed by activation of caspase-8. Furthermore, Tecchio and associates have demonstrated that IFN- α can stimulate TRAIL mRNA as well as the release of a bioactive soluble TRAIL protein from neutrophils and monocytes, which induces apoptotic activity on TRAIL sensitive leukemic cell lines [84]. It also appears that IFN- α apoptotic effects may not be limited to TRAIL; rather it may trigger caspase-8 via both cell death receptor dependent and independent pathways [85]. Much like IFN- α , BCG has also been shown to induce TRAIL [49,50], which has correlated with patient response to BCG therapy and has been a source of overlapping research interest. Other direct IFN- α effects include enhancing cytotoxicity of CD4⁺ T cells, increasing antigen detection by up-regulating MHC class I expression [82,86,87]. Direct suppression of proliferation by induction of tumor suppressor genes or inhibition of tumor oncogenes has also been described [82]. Also contributing to antiproliferative properties, IFN- α has been documented to decrease angiogenesis and basic fibroblast growth factor. Additionally, it down-regulates matrix metalloproteinase-9 (MMP-9) mRNA as well as the MMP-9 translational protein in murine bladder tumors [88]. Interestingly, it has also been demonstrated that an optimal biologic dose with higher frequency, rather than maximal tolerated dose, produced the most significant decreases in angiogenesis. Significantly decreased angiogenesis has also been documented in human urothelium during and after IFN- α 2b treatment following TURBT [89].

In vivo monotherapy with IFN- α 2b for bladder cancer has been explored by multiple groups. In 1990, Glashan published data from a randomized controlled trial evaluating high dose (100 million unit) and low dose (10 million unit) IFN- α 2b regimens in patients with Tis [90]. Patients were treated weekly for 12 weeks and monthly thereafter for 1 year. The high and low dose groups had complete response rates of 43% and 5%, respectively. Of the high dose patients achieving a complete response, 90% remained disease-free at a notably short 6 months of follow-up. The primary side effects of treatment were flu-like symptoms (8% low dose, 17% high dose) but without the irritative symptoms seen so often in BCG therapy. When IFN- α 2b was investigated alone to treat BCG failures, eight of twelve patients had recurrence at initial three-month evaluation and only one of twelve was disease-free at 24 months [91]. Another trial conducted by Portillo and associates randomized 90 pT1 bladder cancer patients to either intravesical treatment or placebo groups as primary prophylaxis after complete resection [92]. They utilized a similar dosing schedule but used 60 million units IFN- α 2b. At 12 months of follow-up, recurrence rates were significantly lower for IFN- α 2b group than placebo, 28.2% vs 35.8%, respectively. However, after 43 months rates were similar - 53.8% and 51.2% respectively, indicating that treatment benefit of IFN- α 2b alone may not be durable.

Given the described antiproliferative and immunomodulatory effects of IFN- α , combination therapy with BCG has held tantalizing promise. Gan and associates found significantly greater antitumor activity with combination therapy than BCG alone: 14/15 mice receiving BCG/IFN- α 2b versus 8/15 mice receiving only BCG became tumor-free after 5 weekly intralesional treatments [93]. In an *in vitro* study comparing BCG plus IFN- α 2b to BCG alone, our group demonstrated a 66-fold increase in IFN- γ production in peripheral blood mononuclear cell (PBMC) cultures [81]. Since IFN- γ is a major Th1 restricted cytokine found in patients responding to BCG therapy, it has been used routinely as a surrogate marker for Th1 immune response in studies examining effect of IFN- α [81]. It appears that IFN- α 2b by itself generates a negligible Th1 response, as no significant levels of IFN- γ were detected after IFN- α 2b was incubated alone with the PBMCs. We have also demonstrated that the augmented IFN- γ production persisted even with reduced doses of BCG. These findings give credence to the idea that adding Th1 stimulating cytokines may allow for a decrease in BCG doses, thereby decreasing side effects thought to be directly related to BCG. Further augmenting Th1 differentiation, IFN- α was found to increase levels of several Th1 cytokines, including IL-12 and TNF- α as well as decreasing known Th1 inhibitory cytokines IL-10 and IL-6 by 80-90% and 20-30%, respectively [94].

Clinical investigations with the combination of IFN- α 2b and BCG began initially in BCG refractory patients but were subsequently expanded to BCG naïve patients. Stricker and associates found the combination to be safe, with a similar side effect profile to BCG alone [95]. In 2001, O'Donnell and associates reported on combination therapy administered to 40 patients who had failed at least 1 course of BCG alone [96]. At 24 months, 53% of patients were disease-free. Patients with two or more prior BCG failures fared similarly to patients with only one. Lam and associates in 2003 reported on the treatment of 32 patients, of which 20 (63%) were BCG failures. At 22 months' median follow-up, 12 of the 20 BCG failure patients (60%) remained disease-free [97]. In a smaller trial, Punnen and associates documented a 50%

disease-free rate after combination therapy at 12 months' follow-up in 12 patients with BCG refractory disease [98]. A subsequent large community based phase II clinical trial examined 1106 patients from 125 sites with NMIBC, which were split into BCG naïve and BCG refractory groups [99]. At median 24 months' follow-up, tumor-free rates were 59% and 45%, respectively. In this larger trial, patients who had two or more courses of prior BCG therapy had a worse outcome when compared to patients who had 1 or less, likely indicating more resistant disease. A recent study limited to BCG naïve patients demonstrated similar disease-free rate of 62% but with much longer median follow-up of 55.8 months [100]. Furthermore, after evaluating failure patterns and response rates to BCG plus IFN- α , Gallagher and associates found that patients who recurred more than 12 months after initial BCG treatments had similar tumor-free rates at 24 months when compared to BCG naïve patients [101]. However, patients who recurred within a year of receiving their initial BCG treatments did significantly worse, with disease-free rates of 34-43% at 24 months, indicating that additional immunotherapy may not be appropriate. Overall, while promising, these data are unable to define any treatment benefit of combination therapy over BCG alone in previously BCG untreated patients.

To date, the only randomized trial comparing BCG alone to BCG plus IFN was a multi-center study of 670 BCG naïve patients with Tis, Ta, or T1 urothelial carcinoma [102]. This was a four-arm trial evaluating efficacy of megadose vitamins as well as BCG and IFN. Patients were randomized to 1 of 4 groups: BCG plus recommended daily vitamins, BCG plus megadose daily vitamins, BCG plus IFN- α 2b plus recommended daily vitamins, and BCG plus IFN- α 2b plus megadose daily vitamins. At 24 month follow up, median recurrence-free survival was similar across all groups, though the two IFN- α 2b groups experienced higher incidence of constitutional symptoms and fever ($p < 0.05$).

In general, a BCG/IFN- α 2b combination therapy is appropriate for patients with previous BCG failures, those with Tis, and the elderly [103]. Optimal dose and schedule have yet to be defined in controlled trials and debate continues on the subject. At our institution, we use 1/3 the standard dose of BCG plus 50 MU of IFN- α 2b. The dose may be lowered for those patients experiencing lower urinary tract symptoms or low grade fever. For maintenance cycle A, we adjust the BCG dose for week 1 consisting of 1/3 the standard dose of BCG plus 50 MU of IFN- α 2b. For weeks 2 and 3, the BCG dose is lowered to 1/10 the standard dose plus 50 MU of rIFN- α 2b. Maintenance cycles B and C utilize similar dosing.

There are multiple areas where additional research is warranted. A recent evolution in combination therapy has been the development of an IFN- α 2b expressing strain of recombinant BCG (rBCG-IFN- α) from the Pasteur strain of BCG. An initial *in vitro* study documented enhanced IFN- γ expression in PBMCs after incubation with rBCG-IFN- α as compared to standard BCG [104]. A subsequent study reported that rBCG-IFN- α increased cytotoxicity up to 2-fold over standard BCG in PBMC cultures. Both CD56⁺CD8⁺ NK cells and CD8⁺ T cells were identified as primary contributors to the increased cytotoxicity [105]. Combining IFN- α 2b with other antiproliferative agents has shown *in vitro* promise. Louie and associates reported that a combination of IFN- α 2b and maitake mushroom D-fraction (PDF) could reduce T24 bladder cancer cell proliferation by 75%, accompanied by G₁ cell cycle arrest [106]. A recently reported study indicated that adding grape seed proanthocyanin significantly

enhanced antiproliferative effects of IFN- α 2b, with >95% growth reduction in T24 bladder cancer cells [107]. Cell cycle analysis also revealed G₁ cell cycle arrest, with Western blots confirming expression of G₁ cell cycle regulators. Lastly, several groups have investigated gene therapy with a recombinant adenovirus delivery system (rAd-IFN/Syn3), which could potentially result in sustained therapeutic IFN- α 2b levels for long periods of time. Nagabhushnan and associates were able to demonstrate delivery and expression of IFN in the bladder as well as significant tumor regression in mice. Phase I trials with rAd-IFN/Syn3 were ongoing at the time of their publication in 2007 [108].

3.2. Recombinant IL-2

The discovery and characterization of IL-2 was one of the most important breakthroughs in the field of immunology. Prior to its discovery, lymphocytes were thought to be terminally differentiated and incapable of proliferation [109,110]. In 1975 it was discovered that the supernatant of murine splenic cell cultures could stimulate thymocytes, suggesting a native effector protein was responsible for this mitogenic activity [110,111]. When initially examined independently by different investigators, this “effector protein” was given multiple working names including thymocyte stimulating factor (TSF), thymocyte mitogenic factor (TMF), T cell growth factor (TCGF), co-stimulator, killer cell helper factor (KHF), and secondary cytotoxic T cell-inducing factor (SCIF) [112]. In 1979 it was recognized that these factors likely represented the same entity and the nomenclature was standardized with the term “interleukin” (between leukocytes). Thus, the “effector protein” was named IL-2, differentiating it from the only other interleukin known at that time, IL-1 [112]. Regardless of the nomenclature, this protein was recognized to promote proliferation of primary T cells *in vitro* which revolutionized the experimental armamentarium in the field of immunology [109,111,113].

Since the discovery of IL-2 mediated control of T cell growth in culture, there has been much progress in elucidating its mechanisms. It was discovered relatively early that IL-2 enhances the production of cytotoxic lymphocytes which are capable of lysing tumor cells while leaving normal cells unharmed [113-116]. These IL-2 activated lymphocytes became known as “lymphokine-activated killer” (LAK) cells and were thought to play a large role in antitumor immune function [113-116]. Additionally, it was noted that IL-2 functions to augment the cytotoxic activity of NK cells and monocytes [117,118]. It has even been discovered that IL-2 is important for the activation of B cells [119]. As the CD4⁺ Th1 and Th2 cell cytokine profiles were defined, it became clear that IL-2 is predominantly a Th1 secreted cytokine [120].

The cytotoxic antitumor capabilities induced in lymphocytes by IL-2 make it a potential cancer immunotherapeutic agent. To date, multiple studies have demonstrated regression of metastatic disease following systemic IL-2 treatment in some cancers [121]. Rosenberg and associates reported on 157 patients with a heterogeneous mix of metastatic cancers refractory to other treatments including renal cell, colon cancer, breast cancer and lymphoma. Patients were treated with either IL-2 and LAK cells or IL-2 alone. Between the two groups, 9 complete and 20 partial responses were obtained. Significant morbidity has been reported with systemic IL-2 much of which is secondary to increased capillary permeability [121,122] and includes weight gain, hypotension, oliguria, elevated creatinine and bilirubin. These tend to resolve with

cessation of IL-2 therapy [121]; however, Rosenberg reported 4 treatment related deaths among their 157 patients. Despite the reports of morbidity, IL-2 seemed to offer hope to patients with few treatment options.

With regard to bladder cancer, interest was stimulated after multiple investigators identified elevated IL-2 levels (as well as other cytokines) in urine of patients following BCG, suggesting an immunomodulatory effect of BCG [30,32,33,123-129]. Additionally, an elevation in IL-2 receptor expression has been documented on T cells in voided urine after BCG therapy [30,128]. Increased levels of urinary IL-2 have also been found to correlate with BCG response, which supports the concept that a Th1 cytokine profile confers a favorable response to BCG [35]. Furthermore, elevated IL-2 has been reported in the serum of patients following BCG instillation, which suggests both a local and systemic immune response to therapy [34,130]. These findings led to the conclusion that IL-2 may have a therapeutic use in bladder cancer.

One of the first clinical trials reported evidence of bladder tumor regression following intralesional injections of IL-2, with no adverse events recorded [131]. Multiple murine studies have demonstrated that systemic administration of IL-2, with or without BCG, can significantly decrease tumor size, suppress tumor growth and improve mean survival [132-134]. A small clinical study investigating systemic IL-2 administration effects on low stage bladder cancer found a complete and partial response rate in 5 of 12 patients, though 2 patients discontinued therapy due to toxicity [135]. The poor side effect profile of systemic IL-2 administration subsequently prompted a shift to utilize IL-2 as an intravesical therapy. Reports of intravesical use revealed a much improved side effect profile as well as some efficacy alone or when combined with BCG [136-141]. Den Otter and associates administered intravesical IL-2 alone after incomplete transurethral resection of grade 1-2, T1 papillary urothelial carcinoma, and documented "marker lesion" regression in 8 of 10 patients [142]. Additional experiments have focused on developing recombinant-IL-2 secreting strains of BCG [42,143-147]. Animal models using this approach have shown that compared to native BCG, IL-2 secreting BCG strains have increased IFN- γ production, induced a more favorable IFN- γ to IL-4 ratio, improved antigen-specific proliferation, enhanced antitumor cytotoxicity, and mounted a Th1 cytokine profile even in immunosuppressed or IL-4 transgenic mice (two conditions which favor a Th2 response) [42,143-147]. More recent animal and *in vitro* studies have investigated IL-2 transfecting dendritic cells (DCs), immobilized streptavidin-tagged bioactive IL-2 on the biotinylated surface of murine bladder mucosa, and development of a murine IL-2 surface modified bladder cancer vaccine [148-151]. Since IL-2 plays a crucial role in the Th1 response, it will continue to be a source of interest for immunotherapy of bladder cancer.

3.3. Recombinant IL-12

IL-12 has been the focus of significant cancer research among cytokines as well. In 1987, it was discovered through *in vitro* experiments that there existed a factor which synergized with IL-2 in promoting a CTL response [151]. This factor was given the name cytotoxic lymphocyte maturation factor (CLMF) [151]. Shortly thereafter a factor was discovered that induced IFN- γ production, enhanced T cell responses to mitogens, and augmented NK cell cytotoxicity [152]. This factor was provisionally called natural killer cell stimulatory factor (NKSF) [152].

It didn't take long to discover that these factors represented the same entity, thus the nomenclature converged and this protein was termed IL-12 [153-157].

Although initially discovered in a B cell lymphoma, it was subsequently found that IL-12 is primarily involved with the regulation of T cells, causing proliferation of both activated CD4⁺ and CD8⁺ T cell subsets while causing minimal proliferation of resting PBMCs [152,154]. This concept is supported by studies demonstrating that the IL-12 receptor is upregulated in activated T and NK cells, but not in activated B cells [157]. IL-12 potentiates a Th1 specific immune response, and it was later discovered that DCs produce IL-12 and thus direct the development of Th1 cells from naïve CD4⁺ T cells [158,159]. Additionally, IL-12 can, by itself, stimulate the activation of nonspecific LAK cells and facilitate the generation of an allogeneic CTL response [160]. IL-12 has even been found to play a role in the activation of neutrophils [161,162]. Multiple studies have shown that IL-12 strongly inhibits neovascularization, thought to be mediated through its induction of IFN- γ [163,166]. Furthermore, the mechanism by which IL-12 enhances the cytolytic effect of NK cells has been found to be via the perforin pathway [167,168].

Multiple animal studies have shown tumor responsiveness to immunomodulation with IL-12. Using systemic or peri-tumoral injections, IL-12 showed antitumor properties in murine sarcoma, melanoma, renal cell carcinoma, lung cancer, colon cancer, breast cancer, and bladder cancer models [164,169-173]. Increases in serum IFN- γ were observed in mice treated with IL-12 [170]. Antitumor efficacy was lost in CD8⁺ depleted mice, but not CD4⁺ depleted mice or NK deficient mice, suggesting that the primary mediators of the antitumor IL-12 effect are CD8⁺ T cells [169,170]. Some of these studies saw effectiveness even with metastatic disease, including bladder cancer [169,170,173]. Multiple murine studies have also revealed added effectiveness with IL-12 administered in combination with chemotherapeutic agents [171,174-176]. Additionally, IL-12 therapy has shown synergistic activity when combined with radiation therapy in mice [172,177]. Various delivery systems for IL-12 therapy have been tested in mice using viral and retroviral vectors to elicit an IL-12 response [178-182]. These constructs have shown some effectiveness as antitumor therapeutics [178-181]. IL-12 as intravesical therapy for bladder cancer has shown great success in mouse models. BCG was found to be a potent stimulus for IL-12 expression, and neutralization of IL-12 significantly dampened the induction of IFN- γ by BCG [183]. BCG therapy for murine bladder cancer was essentially found to be ineffective in IL-12 knock-out mice, suggesting a crucial role for IL-12 in the BCG response [184]. When IL-12 is used as a therapy with BCG it causes a synergistic induction of IFN- γ [183]. Intravesical IL-12 treatment alone was found to be effective for the treatment of orthotopically placed bladder tumors in mice, and urinary IFN- γ was subsequently found to be significantly elevated [173,185,186]. These observations further support to importance of IFN- γ induction for effective immunotherapy of bladder cancer. More recently, multiple attempts have been made to improve the delivery of intravesical IL-12 to the bladder mucosa to improve efficacy. One method utilized cationic liposome-mediated IL-12 gene therapy which showed improved survival and tumor-specific immunologic memory in mice [187]. Another method utilized chitosan, a mucoadhesive biopolymer, to increase IL-12 delivery to urothelial surfaces [188]. This method showed improved efficacy over IL-12 alone in a mouse model [188].

The great success of IL-12 in treating various murine cancers subsequently led to experiments testing its use on human cancers, though with mixed success. Initial trials focused on systemic IL-12 treatment for metastatic cancer, though progress was initially halted when several patients suffered severe toxic effects from the treatment and two patients died from the therapy [189]. A phase I trial of systemically administered IL-12 in 40 patients with advanced malignancy found a dose-dependent increase in circulating IFN- γ with administration [190]. Experiments on the peripheral blood of these patients showed augmented NK cell cytolytic activity and enhanced T cell proliferation [191]. Unfortunately, of these 40 patients there was only one partial response and one transient complete response [190]. Further studies looking at chronic administration of twice weekly IL-12 in patients with metastatic cancer found that it is well tolerated and induces co-stimulatory cytokines (including IFN- γ) [192]. However, in a cohort of 28 patients there was only one patient with a partial response and two with prolonged disease stabilization, with one of these patients eventually exhibiting tumor regression [192]. Similar low response rates have been seen with systemic IL-12 in other studies of advanced malignancies [193-197]. Various combinations of immunotherapy have been tested with systemic IL-12 in humans. A phase I study examined systemic IL-12 with low dose IL-2 and showed it was well tolerated, and the addition of IL-2 significantly augmented IFN- γ production as well as the NK response [198]. Of 28 patients there was one partial response and two pathologic responses [198]. Another phase I study using systemic IL-12 with IFN- α 2b showed acceptable toxicity, but with no response in 41 patients [199]. As discussed previously, intravesical IL-12 showed great promise for the topical treatment of bladder cancer in a mouse model, however this success has not translated clinically. A phase I study of intravesical IL-12 therapy in patients with superficial bladder cancer showed minimal toxicity, but disappointing efficacy [200]. A total of 15 patients were enrolled in this study, of which 12 had no visible pretreatment lesions [200]. Of these 12 patients, 7 remained disease-free and 5 had recurrence within 4 weeks. The remaining 3 patients with pretreatment lesions had persistent disease at follow-up [200]. Perhaps the most disparaging results were that there was negligible IFN- γ induced in the urine and serum of these patients post-treatment, suggesting minimal immunologic effect from intravesical IL-12 therapy [200]. Despite the disappointing results from human studies, IL-12 remains an important target for the treatment of bladder cancer.

3.4. Other recombinant cytokines

In addition to the above-mentioned cytokines, several phase I and II trials have shown that other Th1 stimulating cytokines such as IFN- γ , TNF- α and GM-CSF, when intravesically administered, are well tolerated and effective in the treatment of bladder cancer. Giannopoulos and associates conducted a study of 123 patients with stage Ta/T1, grade 2 tumors who were followed for a median of 26.5 months. They demonstrated that intravesical IFN- γ therapy prevented tumor recurrence after TURBT and was more effective than intravesical mitomycin C therapy [201]. The effect of IFN- γ was associated with significant increases of leukocytes in the bladder wall including CD4⁺ T cells, CD8⁺ T cells, NK cells and B cells, suggesting the involvement of a primary cellular immune response in the mechanism of IFN- γ action. A separate study consisting of 54 patients with stage Ta/T1 tumors also supported the safety and anti-bladder cancer activity of intravesical IFN- γ therapy in preventing tumor recurrence after

TURBT during a mean follow-up time of 12.1 months [202]. Serretta and associates demonstrated in two studies that intravesical TNF- α therapy was well tolerated and resulted in approximately a 24.5% complete response rate in 42 patients with superficial bladder cancer [203,204]. Two separate studies also supported the excellent tolerability and some antitumor effects of intravesical TNF therapy in patients with superficial bladder cancer [205,206]. Studies demonstrated that intravesical administration of GM-CSF for patients with stage Ta/T1 tumors after TURBT induced immunomodulatory effects on macrophage activities [207]. In correlation with regression of marker lesions, migration of macrophages to the surface layer was observed. Macrophages showed an extensive lysosomal system and pseudopodia. In addition, intravesical GM-CSF therapy was also observed to enhance lymphocyte recruitment into the bladder wall and activation in the bladder mucosa [208]. These clinical trials suggest that intravesical use of recombinant cytokines are favorable for the treatment of bladder cancer and further investigations are warranted.

4. Advances in BCG immunotherapy research

4.1. BCG therapy in conjunction with IL-10 blockage

Unlike Th1 stimulating cytokines discussed above, IL-10 is distinct in that its primary effect is to promote a Th2 response and thus dampen the immunotherapeutic effects of BCG for the treatment of bladder cancer [36,45]. As a result, it may have therapeutic value not by its native function, but by abrogation of its native function. IL-10 was first characterized in 1989. It was initially termed cytokine synthesis inhibitory factor (CSIF), a rather fitting name, because it was found to inhibit the production of several cytokines produced by Th1 clones [209]. The most important of these cytokines was IFN- γ , which was recognized as an important player in the Th1 response. As discussed previously, it is a key contributor in the immunotherapeutic effectiveness of BCG [209,210]. Further studies showed that IL-10 prevented DTH response to BCG and the neutralization or abrogation of IL-10 prolonged a response, thus further supporting its role in the Th1/2 response [36,211]. Several human tumors, including melanoma, non-small cell lung carcinoma, renal cell carcinoma and bladder cancer, have been found to have elevated expression of IL-10 [212-216]. It is speculated that production of IL-10 by tumor cells may represent an “escape mechanism” whereby tumor cells avoid Th1 immune mediated tumoricidal effects [212].

There has been significant progress in determining the regulation and mechanism of IL-10 function since its discovery, particularly with regard to its role in tumor immunology. It is secreted by multiple cell types including Th2 cells, B cells and monocytes/macrophages [209,217-219]. Like many other cytokines, IL-10 is known to auto-regulate itself by down-regulating its own mRNA synthesis [219]. It has been shown to block the accumulation of macrophages and DCs at tumor sites, which are important players in the cellular immune response [220,221]. Additionally, it compromises DCs ability to stimulate T cells causing induction of antigen-specific anergy of T cells [222]. Furthermore, it down-regulates the expression of MHC class II and co-stimulatory molecules, thus preventing a cellular immune

response to tumor cells [223-225]. During activation of CD4⁺ T cells, the presence of IL-10 can cause them to differentiate into T regulatory cells 1 (Tr1), leading to peripheral tolerance [226]. IL-10 further reduces cellular tumoricidal activity by preventing release of reactive nitrogen/oxygen intermediates by macrophages and NK cells, a key step in their efficacy during cellular immune defense [45,227].

Successful treatment of bladder cancer with BCG, as discussed previously, requires a Th1 cytokine profile. IL-10 antagonizes the production of a Th1 milieu, thus its neutralization has been targeted as a potential means to augment the BCG response. Several murine studies have demonstrated that after IL-10 knock-out mice are inoculated with bladder cancer, they have improved BCG response with amplified local immune response, increased bladder mononuclear infiltrate, enhanced DTH responses, greater antitumor activity, and prolonged survival [36,212]. Although murine IL-10 knock-out studies are conceptually important, studies focused on IL-10 neutralization hold more promise as clinically useful therapeutics. Murine bladder cancer studies utilizing anti-IL-10 neutralizing monoclonal antibody (mAb) have shown similar results, with BCG treatment inducing an enhanced DTH response and increased bladder mononuclear infiltrate [36,211]. More recent efforts have been placed at targeting the IL-10 receptor. The IL-10 receptor is composed of two known subunits (IL-10R1 and IL-10R2) and the IL-10R1 subunit plays the predominant role in signal transduction [228]. In *in vitro* studies we have recently shown that splenocytes incubated with BCG and anti-IL-10R1 mAb produced significantly higher IFN- γ than those incubated with BCG plus anti-IL-10 neutralizing mAb [39], suggesting that interference with IL-10 signal transduction may be more effective than neutralizing IL-10 protein. In *in vivo* studies mice treated with BCG and anti-IL-10R1 mAb showed increased urinary IFN- γ production compared to BCG controls [39]. In a similar murine experiment, there was improved overall and tumor-free state in mice treated with BCG plus anti-IL-10R1 mAb compared to BCG treatment controls, though this difference did not reach statistical significance [39]. Most recently, in an experiment designed to follow murine survival after inoculation with a luciferase-expressing MB49 bladder cancer cells, we discovered that control mice and BCG only treated mice developed histologically confirmed lung metastasis, whereas mice treated with BCG and anti-IL-10R1 mAb developed no metastasis [unpublished data]. This difference was statistically significant and raises questions as to anti-IL-10R1 mAb's role as more than just an augmentation to BCG for local bladder cancer control. Confirmatory experiments and mechanistic studies are necessary, but anti-IL-10R1 mAb shows great potential in not only local bladder cancer control, but also possibly systemic immunomodulation for the prevention of metastatic bladder cancer.

4.2. Development of recombinant BCG strains

BCG in combination with Th1 stimulating cytokines (e.g. IFN- α 2b) has demonstrated to improve BCG efficacy in the treatment of bladder cancer. However, these strategies require multiple applications and a large quantity of recombinant cytokines. Genetic manipulation of BCG to secrete Th1 stimulating cytokines provides an opportunity to overcome the drawbacks. To date, numerous recombinant BCG (rBCG) strains capable of secreting cytokines or chemokines, mainly Th1 stimulating cytokines such as IL-2, IL-12, IL-18, IFN- γ and IFN- α , have been developed

[229-250] (Table 1). Most of these rBCG strains have demonstrated to be superior to BCG in the induction of Th1 immune responses and antitumor immunity in pre-clinical settings.

Strain	Cytokine	Species	Immunological Effect	Reference
IL-2 BCG (RBD)	IL-2	m	Th1 cyt prod, Antitumor, Cytotoxicity	[143]
IL-2 BCG (MAO)	IL-2	r	Th1 cyt prod	[143]
BCG-CI	IL-2	h	Anti-BCG	[229]
BCG-CII	IL-2	h	Anti-BCG	[229]
BCG-IL-2	IL-2	m	CI, Th1 & Th2 cyt prod	[144]
BCG-GM-CSF	GM-CSF	m	CI, Th1 & Th2 cyt prod, DC act, Anti- <i>M.tb</i>	[144, 230]
BCG-IFN- γ	IFN- γ	m	CI, Th1 & Th2 cyt prod, Anti-BCG	[144, 231]
rBCG/IL-2	IL-2	m	CI, Th1 cyt prod, Anti-BCG	[145, 147, 232]
rBCG-IL-2/GFP	IL-2	m	CI, Th1 cyt prod, Anti-BCG	[146]
rBCG(α -Ag-IL-2)	IL-2	m	Th1 cyt prod, Cytotoxicity	[42]
BCG-IFN- γ	IFN- γ	m	Th1 cyt prod, Anti-BCG	[233]
rBCG-IFN- α	IFN- α 2b	h	Th1 cyt prod, Cytotoxicity	[104]
rBCG/IL-18	IL-18	m	no clear effect	[232]
BCG IL-18	IL-18	m	Th1 & Th2 cyt prod	[234, 235]
BCG-hIL2MUC1	IL-2	h	CI, Th1 cyt prod, Antitumor	[236, 237]
rBCG-IFN- γ	IFN- γ	m	CI, Th1 cyt prod, Antitumor	[238]
rBCG-IL-18	IL-18	m	Th1 cyt prod, Anti-BCG, Cytotoxicity	[43]
rBCG-huIL-2-ESAT6	IL-2	h	CI, Th1 cyt prod, Cytotoxicity, HI	[239]
rBCG-IL-2	IL-2	h	Th1 cyt prod	[240]
BCGMCP-3	MCP-3	m	CI, Anti-BCG	[241]
rBCG-AEI	IFN- γ	m	CI, HI, Anti- <i>M.tb</i>	[242]
rBCG-Ag85B-IL15	IL-15	m	CI, Th1 cyt prod, Anti- <i>M.tb</i>	[243]
rBCG-MVNTR4-CSF	GM-CSF	h	CI, Th1 cyt prod, Antitumor	[244, 245]
rBCG-MVNTR8-CSF	GM-CSF	h	CI, Th1 cyt prod, Antitumor	[244, 245]
rBCG-Ag85B-Esat6-TNF- α	TNF- α	m	CI, HI	[246]
rBCG-IE	IL-12	h	CI, Th1 cyt prod	[247]
rBCG:GE	GM-CSF	h	CI, Th1 cyt prod, HI	[248]
rBCG::Ag85B-CFP10-IL-12	IL-12	h	CI, HI, Anti- <i>M.tb</i>	[249]
rBCG-IFN- α -2b	IFN- α 2b	h	CI, Cytotoxicity	[250]

Anti-BCG: anti-BCG infection; Anti-*M.tb*: anti-Mycobacterium tuberculosis infection; CI: cellular immunity; DC act: dendritic cell activation; h: human; HI: humoral immunity; m: mouse; r: rat; Th1 cyt prod: T helper type 1 cytokine production; Th2 cyt prod: T helper type 2 cytokine production.

Table 1. Cytokine- and chemokine-expressing rBCG strains

BCG is a potent immunoadjuvant and induces a primary Th1 immune response that is required for effective treatment of most cancer types. Genetic manipulation of BCG to secrete Th1

stimulating cytokines with simultaneous coexpression of tumor-associated antigens may therefore potentiate the induction of specific antitumor immune responses. Early studies demonstrated that IL-2 secreting rBCG was at least equally effective to wild-type BCG when used as an intratumoral injection or a vaccine therapy in conjunction with irradiated tumor cells in a murine melanoma model [251]. However, it was not until recently that the potential of rBCG for treating cancer has gained further appreciation. We and others have developed rBCG strains that deliver the breast cancer-associated antigen mucin-1 (MUC1) in a form of multiple tandem repeats with coexpression of human IL-2 or human GM-CSF [236,237,244,245]. Severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood lymphocytes (PBL) followed by immunization with the rBCG strains developed MUC1-specific cellular immune responses and enhanced protection against MUC1-positive human breast cancer xenografts, compared to control mice reconstituted with human PBL and immunized with non-cytokine secreting BCG. Studies have also demonstrated that the antitumor effects of the rBCG strains were correlated with the number of MUC1 tandem repeats delivered by BCG [244,245]. These results suggest that these MUC1 rBCG strains coexpressing Th1 stimulating cytokines are promising candidates as breast cancer vaccines and thus warrant further investigation.

It has been known that BCG stimulation of human PBMC leads to the generation of effector cells cytotoxic to bladder cancer cells *in vitro* [55,56]. We recently demonstrated that stimulation of human PBMC with rBCG-IFN- α , a rBCG strain secreting human IFN- α 2b [104], *in vitro* for 7 days induced enhanced PBMC cytotoxicity toward human bladder cancer cell lines T24, J82, 5637, TCCSUP and UMUC-3 by up to 2-fold compared to control BCG carrying an empty vector [105]. This induction of enhanced PBMC cytotoxicity was correlated with increased production of IFN- γ and IL-2 by rBCG stimulated PBMC. Studies further revealed that this enhancement in PBMC cytotoxicity was dependent on BCG secreted IFN- α as well as endogenously expressed IFN- γ and IL-2, as blockage of IFN- α , IFN- γ or IL-2 by neutralizing antibodies during BCG stimulation reduced or abolished the induction of this enhanced PBMC cytotoxicity. Studies using NK and CD8⁺ T cells isolated from human PBMC revealed that both cell types were responsible for the enhanced PBMC cytotoxicity induced by rBCG-IFN- α with the former cell type being more predominant [105]. A similar rBCG strain secreting human IFN- α 2b has also been recently demonstrated to stimulate PBMC proliferation and cytotoxicity toward bladder cancer cell lines T24 and 5637 [250].

An early study demonstrated that human peripheral monocytes/macrophages were capable of functioning as tumoricidal cells toward bladder cancer UCRU-BL-17 cells upon activation by BCG *in vitro* [41]. It was observed that the cytotoxic activity of human monocytes/macrophages was significantly enhanced after BCG stimulation, while the naïve cells exhibited only minimum cytotoxicity. Later, more studies including ours further demonstrated that murine macrophages could also function as tumoricidal cells toward bladder cancer cells upon activation by BCG *in vitro* [42-45]. Stimulation of thioglycollate-elicited peritoneal macrophages by BCG for 24 hour resulted in macrophage-mediated killing of bladder cancer MBT-2 (C3H background) and MB49 (C57BL/6 background) cells in a dose-dependent manner [44,45]. Studies also revealed that endogenous Th1 cytokines (e.g. IL-12, IL-18, IFN- γ and TNF- α)

played an important role in BCG-induced macrophage cytotoxicity, as blockage of these cytokines during BCG stimulation led to substantially reduced macrophage cytotoxicity toward bladder cancer cells [44]. In contrast, supplementation of BCG with Th1 cytokines (e.g. IL-2, IL-12 or IL-18) increased macrophage cytotoxicity by approximately 2-fold. Consistent with these observations, rBCG strains secreting murine IL-2 or IL-18 showed enhanced macrophage-mediated killing on bladder cancer MBT-2 cells, which was correlated with increased expression of IFN- γ , TNF- α and IL-6 by rBCG stimulated macrophages [44]. The effect of murine IL-2 secreting rBCG strain on the induction of macrophage cytotoxicity toward bladder cancer MBT-2 cells was also demonstrated by a separate study [42].

Although the *in vitro* studies have suggested the potential usefulness of Th1 cytokine-secreting rBCG strains for the treatment of bladder cancer, unfortunately, the effect of rBCG on treating bladder cancer *in vivo* has not well been studied. Up to date, only an rBCG strain secreting IFN- γ (rBCG-IFN- γ) has been studied in a murine MB49 syngeneic orthotopic tumor model [238]. This study showed that, with a low-dose treatment regimen, intravesical administration of rBCG-IFN- γ significantly prolonged animal survival compared to medium-treated controls, whereas BCG carrying an empty vector only slightly increased survival. In a similar experiment using the MB49 syngeneic orthotopic tumor model in IFN- γ knockout mice, intravesical treatment with rBCG-IFN- γ failed to prolong survival of mice, indicating that rBCG-derived IFN- γ had no measurable antitumor effect in the absence of endogenous IFN- γ . Studies also provided the mechanisms underlying the effect of rBCG-IFN- γ on treating bladder cancer. As demonstrated, this rBCG-IFN- γ strain could specifically upregulate the expression of MHC class I molecules on MB49 cells *in vitro* compared to control BCG, as the MHC class I upregulation could be blocked by an inhibitory antibody to IFN- γ . This rBCG strain also enhanced recruitment of CD4⁺ T cells into the bladder and further induced the local expression of IL-2 and IL-4 mRNA compared to control BCG. In addition, we have also evaluated the effects of rBCG strains secreting murine IL-2 or IP-10 (a Th1 chemokine) on treating bladder cancer in the MB49 syngeneic orthotopic tumor model and observed survival benefits of these rBCG strains [unpublished data]. All these observations suggest that rBCG strains secreting Th1 cytokines or chemokines possess improved antitumor properties and may offer new opportunities for the treatment of bladder cancer.

Supporting Th1 cytokine-secreting rBCG, *Mycobacterium smegmatis* (*M. smegmatis*), a closely related non-pathogenic mycobacterial organism, has been engineered to secrete murine TNF- α (*M. smegmatis*/TNF- α) and tested in a transplantable MB49 tumor model [252]. Studies demonstrated that lymphocytes from tumor-bearing mice vaccinated with *M. smegmatis*/TNF- α produced elevated and prolonged IFN- γ but no IL-10 in response to mycobacterial antigen or tumor lysate stimulation *in vitro*. Histopathology revealed significantly increased infiltrating CD3⁺ lymphocytes in the tumor nodules of mice receiving the recombinant vaccine compared to those of mice receiving wild-type bacteria. These observations indicated that *M. smegmatis*/TNF- α induced cell-mediated immunity. Importantly, mice implanted subcutaneously with MB49 tumor and treated at an adjacent site with the recombinant vaccine exhibited significantly reduced tumor growth with a 70% durable tumor-free survival compared to those treated with wild-type bacteria or BCG (a 10-20% long-term survival). Interestingly, treatment

with *M. smegmatis*/TNF- α also resulted in similar tumor growth inhibition in T cell-deficient athymic nude mice and reduced but not abolished tumor growth inhibition in NK cell-deficient Beige mice. These observations indicated that NK cells contribute to the antitumor effect of *M. smegmatis*/TNF- α but are not solely responsible for the eradication of tumor. Like immunocompetent mice, Beige mice also developed tumor specific memory after treatment with *M. smegmatis*/TNF- α . A study also demonstrated enhanced immunotherapeutic potential of a human TNF- α secreting recombinant *M. smegmatis* for treating bladder cancer [253]. The ability to deliver immunomodulatory cytokines with no pathogenic effects makes *M. smegmatis* attractive as an alternative intravesical mycobacterial agent for bladder cancer treatment.

5. Conclusion and future perspectives

Intravesical administration of BCG for NMIBC represents one of the most successful immunotherapies for solid malignancy. However, BCG therapy is associated with a considerable side-effect profile and is ineffective in a significant proportion of patients. Therefore, multiple Th1 stimulating cytokines (e.g. IFN- α , IL-2 and IL-12) have been investigated either as adjuncts with BCG or as sole replacement therapy in both clinical and pre-clinical studies. Combination of BCG with IL-10 blocking mAb and genetic engineering of BCG to secrete Th1 cytokines have also been conducted in pre-clinical studies. These treatment strategies potentially allow the use of a lower and safer dose of BCG while preserving or even enhancing BCG efficacy. Despite a multitude of encouraging *in vitro* and murine studies, no clinical data has yet been reported which is compelling enough to change the current standard of care, yet many practitioners continue to use adjunctive immunotherapy based on basic science data and theoretical benefit. Further studies are needed and should focus on the optimization of combination therapies including dosing, schedule and duration. The mechanisms through which supplemental agents enhance BCG-induced Th1 immune responses and antitumor immunity need to be explored in both effector and memory phases. In addition to classical effector cells, influence of combination therapy on Th17 and regulatory T (Treg) cells should be evaluated, as the importance of these cell types in bladder cancer has emerged. Today, research continues and efforts have been made to increase our understanding of tumor biology, human immunology, and the treatment of urothelial carcinoma. The pace of research must be maintained if we are to improve this gold standard therapy for bladder cancer. BCG combination therapy merits further appraisal as an improved modality for the treatment of bladder cancer.

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