Chapter 15

Immunotherapy of Urinary Bladder Carcinoma: BCG and Beyond

Yi Luo, Eric J. Askeland, Mark R. Newton,
Jonathan R. Henning and Michael A. O'Donnell

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55283

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 disese 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

http://dx.doi.org/10.5772/55283

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 X 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-1β, 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)-1α, 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 succesful 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 deficint 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-1α, IL-1β, IL-8, MIP-1α, MIP-1β, 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

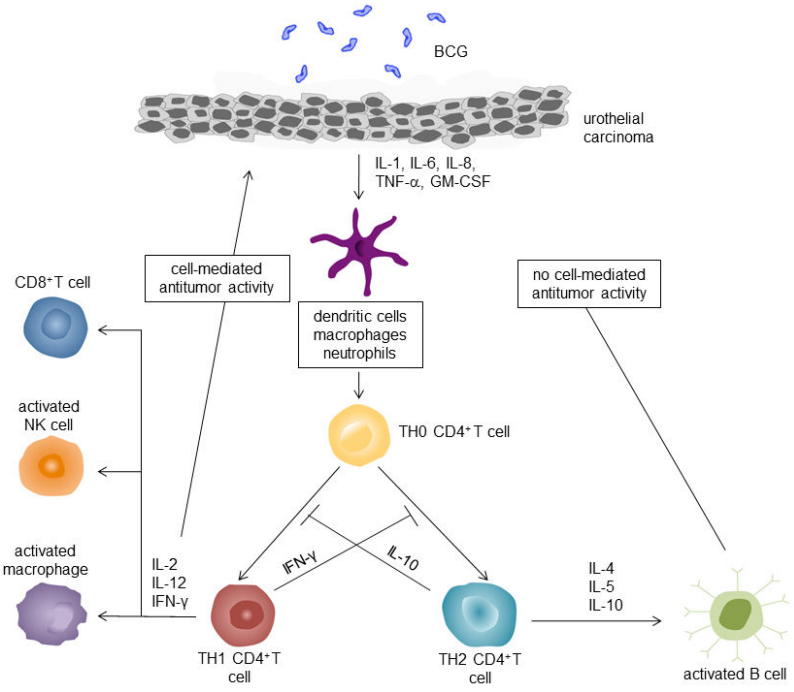

Figure 1. Suggested cascade of immune responses in bladder mucosa induced by intravesical BCG instillation. Attachment of BCG to urothelial cells including carcinoma cells triggers release of cytokines and chemokines from these cells, resulting in recruitment of various types of immune cells into the bladder wall. Activation of phagocytes and the new cytokine environment lead to the differentiation of naïve 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

http://dx.doi.org/10.5772/55283

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 instilation [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-1β, 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 α5β1 integrin receptors, BCG exerts its direct biological effects on UCC, including activation of the signal transduction pathways involving activator protein (AP) 1, NFkB 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-α2a (Roferon-A, recombinant, Roche Laboratories, Nutley, NJ) and IFN-α2b (Intron-A, recombinant, Schering Plough, Kenilworth, NJ), though to date most research involves IFN-α2b. There has been interest in IFN-α2b 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-α2b 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 metalloprotease-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-α2b treatment following TURBT [89].

http://dx.doi.org/10.5772/55283

In vivo monotherapy with IFN-α2b 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-α2b 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-α2b 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-α2b. At 12 months of follow-up, recurrence rates were significantly lower for IFN-α2b 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-α2b 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-α2b 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-α2b 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-α2b by itself generates a negligible Th1 response, as no significant levels of IFN-γ were detected after IFN-α2b 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-α2b 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 faired 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-α2b plus recommended daily vitamins, and BCG plus IFN-α2b plus megadose daily vitamins. At 24 month follow up, median recurrence-free survival was similar across all groups, though the two IFN-α2b groups experienced higher incidence of constitutional symptoms and fever ($p<0.05$).

In general, a BCG/IFN-α2b combination thearpy 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-α2b. 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-α2b. For weeks 2 and 3, the BCG dose is lowered to 1/10 the standard dose plus 50 MU of rIFN-α2b. 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-α2b 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-α2b with other antiproliferative agents has shown *in vitro* promise. Louie and associates reported that a combination of IFN-α2b and maitake mushroom D-fraction (PDF) could reduce T24 bladder cancer cell proliferation by 75%, accompanied by G_1 cell cycle arrest [106]. A recently reported study indicated that adding grape seed proanthocyanin significantly

http://dx.doi.org/10.5772/55283

enhanced antiproliferative effects of IFN-α2b, with >95% growth reduction in T24 bladder cancer cells [107]. Cell cycle analysis also revealed G_1 cell cycle arrest, with Western blots confirming expression of G_1 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-α2b 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 standarized 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 heterogenous 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].

http://dx.doi.org/10.5772/55283

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-α2b 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 administrated, 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-α2b) 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-*M.tb*	[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-α2b	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-*M.tb*	[242]
rBCG-Ag85B-IL15	IL-15	m	CI, Th1 cyt prod, Anti-*M.tb*	[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-*M.tb*	[249]
rBCG-IFN-α–2b	IFN-α2b	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 respnses 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-α2b [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-α2b 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)

http://dx.doi.org/10.5772/55283

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 a solo 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.

Author details

Yi Luo, Eric J. Askeland, Mark R. Newton, Jonathan R. Henning and Michael A. O'Donnell

University of Iowa, Department of Urology Iowa City, Iowa, USA

References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer Journal for Clinicians 2012;62(1):10-29.

[2] Ro JY, Staerkel GA, Ayala AG. Cytologic and histologic features of superficial bladder cancer. The Urologic Clinics of North America 1992;19(3):435-453.

[3] Williams SK, Hoenig DM, Ghavamian R, Soloway M. Intravesical therapy for bladder cancer. Expert Opinion on Pharmacotherapy 2010:11(6):947-958.

[4] Weizer AZ, Tallman C, Montgomery JS. Long-term outcomes of intravesical therapy for non-muscle invasive bladder cancer. World Journal of Urology 2011;29(1):59-71.

[5] Shelley MD, Jones G, Cleves A, Wilt TJ, Mason MD, Kynaston HG. Intravesical gemcitabine therapy for non-muscle invasive bladder cancer (NMIBC): a systematic review. British Journal of Urology International 2012;109(4):496-505.

[6] Dinney CP, Greenberg RE, Steinberg GD. Intravesical valrubicin in patients with bladder carcinoma in situ and contraindication to or failure after bacillus Calmette-Guérin. Urologic Oncology 2012;PMID:22575238.

[7] Yutkin V, Chin J. Apaziquone as an intravesical therapeutic agent for urothelial non-muscle-invasive bladder cancer. Expert Opinion on Investigational Drugs 2012;21(2): 251-260.

[8] Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. The Journal of Urology 1976;116(2):180-3.

[9] Sylvester RJ. Bacillus Calmette-Guérin treatment of non-muscle invasive bladder cancer. International Journal of Urology 2011; 18(2):113-120.

[10] Hall MC, Chang SS, Dalbagni G, Pruthi RS, Seigne JD, Skinner EC, Wolf JS Jr, Schellhammer PF. Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. The Journal of Urology 2007;178(6):2314-2330.

[11] Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, Stanisic TH, Smith JA Jr, Sullivan J, Sarosdy MF, et al. A randomized trial of intravesical doxorubicin and immunotherapy with bacille Calmette-Guérin for transitional-cell carcinoma of the bladder. The New England Journal of Medicine 1991;325(17): 1205-1209.

[12] Morales A, Ottenhof P, Emerson L. Treatment of residual, non-infiltrating bladder cancer with bacillus Calmette-Guerin. The Journal of Urology 1981;125(5):649-651.

[13] Malmström PU, Wijkström H, Lundholm C, Wester K, Busch C, Norlén BJ. 5-year followup of a randomized prospective study comparing mitomycin C and bacillus Calmette-Guerin in patients with superficial bladder carcinoma. Swedish-Norwegian Bladder Cancer Study Group. The Journal of Urology 1999;161(4):1124-1127.

[14] van der Meijden AP, Sylvester RJ, Oosterlinck W, Hoeltl W, Bono AV; EORTC Genito-Urinary Tract Cancer Group. Maintenance Bacillus Calmette-Guerin for Ta T1 bladder tumors is not associated with increased toxicity: results from a European Organisation for Research and Treatment of Cancer Genito-Urinary Group Phase III Trial. European Urology 2003;44(4):429-434.

[15] Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J; European Association of Urology (EAU). EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. European Urology 2008;54(2):303-314.

[16] Lamm DL, Blumenstein BA, Crissman JD, Montie JE, Gottesman JE, Lowe BA, Sarosdy MF, Bohl RD, Grossman HB, Beck TM, Leimert JT, Crawford ED. Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. The Journal of Urology 2000;163(4)1124-1129.

[17] Koga H, Ozono S, Tsushima T, Tomita K, Horiguchi Y, Usami M, Hirao Y, Akaza H, Naito S; BCG Tokyo Strain Study Group. Maintenance intravesical bacillus Calmette-Guérin instillation for Ta, T1 cancer and carcinoma in situ of the bladder: randomized controlled trial by the BCG Tokyo Strain Study Group. International Journal of Urology 2010;17(9):759-766.

[18] Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J, Rouprêt M; European Association of Urology (EAU). EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. European Urology 2011;59(6): 997-1008.

[19] Brausi M, Witjes JA, Lamm D, Persad R, Palou J, Colombel M, Buckley R, Soloway M, Akaza H, Böhle A. A review of current guidelines and best practice recommendations for the management of nonmuscle invasive bladder cancer by the International Bladder Cancer Group. The Journal of Urology 2011;186(6):2158-2167.

[20] Brandau S, Suttmann H. Thirty years of BCG immunotherapy for non-muscle invasive bladder cancer: a success story with room for improvement. Biomedicine and Pharmacotherapy 2007;61(6)299-305.

[21] Alexandroff AB, Nicholson S, Patel PM, Jackson AM. Recent advances in bacillus Calmette-Guerin immunotherapy in bladder cancer. Immunotherapy 2010;2(4)551-560.

[22] Kavoussi LR, Brown EJ, Ritchey JK, Ratliff TL. Fibronectin-mediated Calmette-Guerin bacillus attachment to murine bladder mucosa. Requirement for the expression of an antitumor response. Journal of Clinical Investigation 1990;85(1):62-67.

[23] Becich MJ, Carroll S, Ratliff TL. Internalization of bacille Calmette-Guerin by bladder tumor cells. The Journal of Urology 1991;145(6):1316-1324.

[24] Bevers RF, Kurth KH, Schamhart DH. Role of urothelial cells in BCG immunotherapy for superficial bladder cancer. British Journal of Cancer 2004;91(4):607-612.

[25] Böhle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD. Effects of local bacillus Calmette-Guerin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. The Journal of Urology 1990;144(1)53-58.

[26] Prescott S, James K, Hargreave TB, Chisholm GD, Smyth JF. Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall. The Journal of Urology 1992;147(6)1636-1642.

[27] Saban MR, Simpson C, Davis C, Wallis G, Knowlton N, Frank MB, Centola M, Gallucci RM, Saban R. Discriminators of mouse bladder response to intravesical Bacillus Calmette-Guerin (BCG). BMC Immunology 2007;8:6.

[28] De Boer EC, de Jong WH, van der Meijden AP, Steerenberg PA, Witjes F, Vegt PD, Debruyne FM, Ruitenberg EJ. Leukocytes in the urine after intravesical BCG treatment for superficial bladder cancer. A flow cytofluorometric analysis. Urological Research 1991;19(1):45-50.

[29] De Boer EC, de Jong WH, van der Meijden AP, Steerenberg PA, Witjes JA, Vegt PD, Debruyne FM, Ruitenberg EJ. Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guérin. Cancer Immunology and Immunotherapy 1991;33(6):411-416.

[30] De Boer EC, de Jong WH, Steerenberg PA, van der Meijden AP, Aarden LA, Debruyne FM, Ruitenberg EJ. Leukocytes and cytokines in the urine of superficial bladder cancer patients after intravesical immunotherapy with bacillus Calmette-Guerin. In Vivo 1991;5(6):671-677.

[31] Simons MP, O'Donnell MA, Griffith TS. Role of neutrophils in BCG immunotherapy for bladder cancer. Urologic Oncology 2008;26(4):341-345.

[32] Böhle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG, Flad HD. Elevations of cytokines interleukin-1, interleukin-2 and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guerin immunotherapy. The Journal of Urology 1990;144(1)59-64.

[33] De Reijke TM, De Boer EC, Kurth KH, Schamhart DH. Urinary cytokines during intravesical bacillus Calmette-Guerin therapy for superficial bladder cancer: processing, stability and prognostic value. The Journal of Urology 1996;155(2):477-482.

[34] Taniguchi K, Koga S, Nishikido M, Yamashita S, Sakuragi T, Kanetake H, Saito Y. Systemic immune response after intravesical instillation of bacille Calmett-Guérin (BCG) for superficial bladder cancer. Clinical and Experimental Immunology 1999;115(1):131-135.

[35] Saint F, Patard JJ, Maille P, Soyeux P, Hoznek A, Salomon L, Abbou CC, Chopin DK. Prognostic value of a T helper 1 urinary cytokine response after intravesical bacillus

Calmette-Guerin treatment for superficial bladder cancer. The Journal of Urology 2002;167(1):364-367.

[36] Nadler R, Luo Y, Zhao W, Ritchey JK, Austin JC, Cohen MB, O'Donnell MA, Ratliff TL. Interleukin 10 induced augmentation of delayed-type hypersensitivity (DTH) enhances Mycobacterium bovis bacillus Calmette-Guérin (BCG) mediated antitumour activity. Clinical and Experimental Immunology 2003;131(2):206-216.

[37] Luo Y, Chen X, O'Donnell MA. Mycobacterium bovis bacillus Calmette-Guérin (BCG) induces human CC- and CXC-chemokines in vitro and in vivo. Clinical and Experimental Immunology 2007;147(2):370-378.

[38] Riemensberger J, Böhle A, Brandau S. IFN-gamma and IL-12 but not IL-10 are required for local tumour surveillance in a syngeneic model of orthotopic bladder cancer. Clinical and Experimental Immunology 2002;127(1):20-26.

[39] Bockholt NA, Knudson MJ, Henning JR, Maymi JL, Weady P, Smith GJ 3rd, Eisenbraun MD, Fraser JD, O'Donnell MA, Luo Y. Anti-IL-10R1 monoclonal antibody enhances bacillus Calmette-Guerin induced T-helper type 1 immune responses and antitumor immunity in a mouse orthotopic model of bladder cancer. The Journal of Urology 2012;187(6):2228-2235.

[40] Heldwein KA, Liang MD, Andresen TK, Thomas KE, Marty AM, Cuesta N, Vogel SN, Fenton MJ. TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. Journal of Leukocyte Biology 2003;74(2):277-286.

[41] Pryor K, Goddard J, Goldstein D, Stricker P, Russell P, Golovsky D, Penny R. Bacillus Calmette-Guerin (BCG) enhances monocyte- and lymphocyte-mediated bladder tumour cell killing. British Journal of Cancer 1995;71(4):801-807.

[42] Yamada H, Matsumoto S, Matsumoto T, Yamada T, Yamashita U. Murine IL-2 secreting recombinant Bacillus Calmette-Guerin augments macrophage-mediated cytotoxicity against murine bladder cancer MBT-2. The Journal of Urology 2000;164(2):526-531.

[43] Luo Y, Yamada H, Chen X, Ryan AA, Evanoff DP, Triccas JA, O'Donnell MA. Recombinant Mycobacterium bovis bacillus Calmette-Guérin (BCG) expressing mouse IL-18 augments Th1 immunity and macrophage cytotoxicity. Clinical and Experimental Immunology 2004;137(1):24-34.

[44] Luo Y, Yamada H, Evanoff DP, Chen X. Role of Th1-stimulating cytokines in bacillus Calmette-Guérin (BCG)-induced macrophage cytotoxicity against mouse bladder cancer MBT-2 cells. Clinical and Experimental Immunology 2006;146(1):181-188.

[45] Luo Y, Han R, Evanoff DP, Chen X. Interleukin-10 inhibits Mycobacterium bovis bacillus Calmette-Guérin (BCG)-induced macrophage cytotoxicity against bladder cancer cells. Clinical and Experimental Immunology 2010;160(3):359-368.

[46] Jansson OT, Morcos E, Brundin L, Lundberg JO, Adolfsson J, Söderhäll M, Wiklund NP. The role of nitric oxide in bacillus Calmette-Guérin mediated anti-tumour effects in human bladder cancer. British Journal of Cancer 1998;78(5):588-592.

[47] Godaly G, Young DB. Mycobacterium bovis bacille Calmette Guerin infection of human neutrophils induces CXCL8 secretion by MyD88-dependent TLR2 and TLR4 activation. Cellular Microbiology 2005;7(4):591-601.

[48] Suttmann H, Riemensberger J, Bentien G, Schmaltz D, Stöckle M, Jocham D, Böhle A, Brandau S. Neutrophil granulocytes are required for effective Bacillus Calmette-Guérin immunotherapy of bladder cancer and orchestrate local immune responses. Cancer Research 2006;66(16):8250-8257.

[49] Ludwig AT, Moore JM, Luo Y, Chen X, Saltsgaver NA, O'Donnell MA, Griffith TS. Tumor necrosis factor-related apoptosis-inducing ligand: a novel mechanism for Bacillus Calmette-Guérin-induced antitumor activity. Cancer Research 2004;64(10): 3386-3390.

[50] Kemp TJ, Ludwig AT, Earel JK, Moore JM, Vanoosten RL, Moses B, Leidal K, Nauseef WM, Griffith TS. Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guerin (BCG) results in the release of functional soluble TRAIL/Apo-2L. Blood 2005;106(10):3474-3482.

[51] Suttmann H, Jacobsen M, Reiss K, Jocham D, Böhle A, Brandau S. Mechanisms of bacillus Calmette-Guerin mediated natural killer cell activation. The Journal of Urology 2004;172(4 Pt 1):1490-1495.

[52] Liu W, O'Donnell MA, Chen X, Han R, Luo Y. Recombinant bacillus Calmette-Guérin (BCG) expressing interferon-alpha 2B enhances human mononuclear cell cytotoxicity against bladder cancer cell lines in vitro. Cancer Immunology and Immunotherapy 2009;58(10):1647-1655.

[53] Ratliff TL, Ritchey JK, Yuan JJ, Andriole GL, Catalona WJ. T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. The Journal of Urology 1993;150(3):1018-1023.

[54] Brandau S, Riemensberger J, Jacobsen M, Kemp D, Zhao W, Zhao X, Jocham D, Ratliff TL, Böhle A. NK cells are essential for effective BCG immunotherapy. International Journal of Cancer 2001;92(5):697-702.

[55] Böhle A, Thanhäuser A, Ulmer AJ, Ernst M, Flad HD, Jocham D. Dissecting the immunobiological effects of Bacillus Calmette-Guérin (BCG) in vitro: evidence of a distinct BCG-activated killer (BAK) cell phenomenon. The Journal of Urology 1993;150(6):1932-1937.

[56] Brandau S, Böhle A, Thanhäuser A, Ernst M, Mattern T, Ulmer AJ, Flad HD. In vitro generation of bacillus Calmette-Guérin-activated killer cells. Clinical Infectious Diseases 2000;31(Suppl 3):S94-S100.

[57] Brandau S, Suttmann H, Riemensberger J, Seitzer U, Arnold J, Durek C, Jocham D, Flad HD, Böhle A. Perforin-mediated lysis of tumor cells by Mycobacterium bovis Bacillus Calmette-Guérin-activated killer cells. Clinical Cancer Research 2000;6(9): 3729-3738.

[58] Wang MH, Flad HD, Bohle A, Chen YQ, Ulmer AJ. Cellular cytotoxicity of human natural killer cells and lymphokine-activated killer cells against bladder carcinoma cell lines. Immunology Letters 1991;27(3):191-197.

[59] Jackson AM, Hawkyard SJ, Prescott S, Ritchie AW, James K, Chisholm GD. An investigation of factors influencing the in vitro induction of LAK activity against a variety of human bladder cancer cell lines. The Journal of Urology 1992;147(1):207-211.

[60] Shemtov MM, Cheng DL, Kong L, Shu WP, Sassaroli M, Droller MJ, Liu BC. LAK cell mediated apoptosis of human bladder cancer cells involves a pH-dependent endonuclease system in the cancer cell: possible mechanism of BCG therapy. The Journal of Urology 1995;154(1):269-274.

[61] Kawashima T, Norose Y, Watanabe Y, Enomoto Y, Narazaki H, Watari E, Tanaka S, Takahashi H, Yano I, Brenner MB, Sugita M. Cutting edge: major CD8 T cell response to live bacillus Calmette-Guérin is mediated by CD1 molecules. Journal of Immunology 2003;170(11):5345-5348.

[62] Wang MH, Chen YQ, Gercken J, Ernst M, Bohle A, Flad HD, Ulmer AJ. Specific activation of human peripheral blood gamma/delta + lymphocytes by sonicated antigens of Mycobacterium tuberculosis: role in vitro in killing human bladder carcinoma cell lines. Scandinavian Journal of Immunology 1993;38(3):239-246.

[63] Naoe M, Ogawa Y, Takeshita K, Morita J, Iwamoto S, Miyazaki A, Yoshida H. Bacillus Calmette-Guerin-pulsed dendritic cells stimulate natural killer T cells and gammadeltaT cells. International Journal of Urology 2007;14(6):532-538.

[64] Higuchi T, Shimizu M, Owaki A, Takahashi M, Shinya E, Nishimura T, Takahashi H. A possible mechanism of intravesical BCG therapy for human bladder carcinoma: involvement of innate effector cells for the inhibition of tumor growth. Cancer Immunology and Immunotherapy 2009;58(8):1245-1255.

[65] Emoto M, Emoto Y, Buchwalow IB, Kaufmann SH. Induction of IFN-gamma-producing CD4+ natural killer T cells by Mycobacterium bovis bacillus Calmette Guérin. European Journal of Immunology 1999;29(2):650-659.

[66] von Meyenn F, Schaefer M, Weighardt H, Bauer S, Kirschning CJ, Wagner H, Sparwasser T. Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. Immunobiology 2006;211(6-8):557-565.

[67] Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000;406(6797):782-787.

[68] Bilen CY, Inci K, Erkan I, Ozen H. The predictive value of purified protein derivative results on complications and prognosis in patients with bladder cancer treated with bacillus Calmette-Guerin. The Journal of Urology 2003;169(5):1702-1705.

[69] Reale M, Intorno R, Tenaglia R, Feliciani C, Barbacane RC, Santoni A, Conti P. Production of MCP-1 and RANTES in bladder cancer patients after bacillus Calmette-Guerin immunotherapy. Cancer Immunology and Immunotherapy 2002;51(2):91-98.

[70] Pook SH, Rahmat JN, Esuvaranathan K, Mahendran R. Internalization of Mycobacterium bovis, Bacillus Calmette Guerin, by bladder cancer cells is cytotoxic. Oncology Reports 2007;18(5):1315-1320.

[71] Ikeda N, Toida I, Iwasaki A, Kawai K, Akaza H. Surface antigen expression on bladder tumor cells induced by bacillus Calmette-Guérin (BCG): A role of BCG internalization into tumor cells. International Journal of Urology 2002;9(1):29-35.

[72] Saban MR, Hellmich HL, Simpson C, Davis CA, Lang ML, Ihnat MA, O'Donnell MA, Wu XR, Saban R. Repeated BCG treatment of mouse bladder selectively stimulates small GTPases and HLA antigens and inhibits single-spanning uroplakins. BMC Cancer 2007;7:204.

[73] Chen FH, Crist SA, Zhang GJ, Iwamoto Y, See WA. Interleukin-6 production by human bladder tumor cell lines is up-regulated by bacillus Calmette-Guérin through nuclear factor-kappaB and Ap-1 via an immediate early pathway. The Journal of Urology 2002;168(2):786-797.

[74] Zhang G, Chen F, Cao Y, See WA. Bacillus Calmette-Guérin induces p21 expression in human transitional carcinoma cell lines via an immediate early, p53 independent pathway. Urologic Oncology 2007;25(3):221-227.

[75] Chen F, Zhang G, Iwamoto Y, See WA. BCG directly induces cell cycle arrest in human transitional carcinoma cell lines as a consequence of integrin cross-linking. BMC Urology 2005;5:8.

[76] Ping SY, Wu CL, Yu DS. Sunitinib can enhance BCG mediated cytotoxicity to transitional cell carcinoma through apoptosis pathway. Urologic Oncology 2010;PMID: 20884251.

[77] See WA, Zhang G, Chen F, Cao Y, Langenstroer P, Sandlow J. Bacille-Calmette Guèrin induces caspase-independent cell death in urothelial carcinoma cells together with release of the necrosis-associated chemokine high molecular group box protein 1. British Journal of Urology International 2009;103(12):1714-1720.

[78] See WA, Zhang G, Chen F, Cao Y. p21 Expression by human urothelial carcinoma cells modulates the phenotypic response to BCG. Urologic Oncology 2010;28(5): 526-533.

[79] Jonasch E, Haluska FG. Interferon in oncological practice: Review of Interferon Biology, Clinical Applications, and Toxicities. The Oncologist 2001;6(1):34-55.

[80] Kamat AM, Lamm DL. Immunotherapy for bladder cancer. Current Urology Reports 2001;2(1):62-69.

[81] Luo Y, Chen X, O'Donnell MA. Role of Th1 and Th2 cytokines in BCG-induced IFN-gamma production: cytokine promotion and simulation of BCG effect. Cytokine 2003;21(1):17-26.

[82] Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-alpha in tumor immunity and immunotherapy. Cytokine and Growth Factor Reviews 2002;13(2): 119-134.

[83] Papageorgiou A, Lashinger L, Millikan R, Grossman HB, Benedict W, Dinney CP, McConkey DJ. Role of tumor necrosis factor-related apoptosis-inducing ligand in interferon-induced apoptosis in human bladder cancer cells. Cancer Research 2004;64(24):8973-8979.

[84] Tecchio C, Huber V, Scapini P, Calzetti F, Margotto D, Todeschini G, Pilla L, Martinelli G, Pizzolo G, Rivoltini L, Cassatella MA. IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. Blood 2004;103(10): 3837-3844.

[85] Papageorgiou A, Dinney CP, McConkey DJ. Interferon-alpha induces TRAIL expression and cell death via an IRF-1-dependent mechanism in human bladder cancer cells. Cancer Biology and Therapeutics 2007;6(6):872-879.

[86] Droller MJ, Gomolka D. Enhancement of natural cytotoxicity in lymphocytes from animals with carcinogen-induced bladder cancer. The Journal of Urology 1983;129(3): 625-629.

[87] Parronchi P, De Carli M, Manetti R, Simonelli C, Sampognaro S, Piccinni MP, Macchia D, Maggi E, Del Prete G, Romagnani S. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. Journal of Immunology 1992;149(9):2977-2983.

[88] Slaton JW, Perrotte P, Inoue K, Dinney CP, Fidler IJ. Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. Clinical Cancer Research 1999;5(10):2726-2734.

[89] Giannopoulos A, Adamakis I, Evangelou K, Giannopoulou M, Zacharatos P, Zsantoulis P, Perunovic B, Athanasiou A, Retalis G, Constandinidis C, Gorgoulis VG. Interferon-a2b reduces neo-microvascular density in the 'normal' urothelium adjacent to the tumor after transurethral resection of superficial bladder carcinoma. Onkologie 2003;26(2):147-152.

[90] Glashan RW. A randomized controlled study of intravesical alpha-2b-interferon in carcinoma in situ of the bladder. The Journal of Urology 1990;144(3):658-661.

[91] Hudson MA, Ratliff TL. Failure of intravesical interferon-alfa-2b for the treatment of patients with superficial bladder cancer previously failing intravesical BCG Therapy. Urologic Oncology 1995;1(3):115-118.

[92] Martin B, Hernandez R, Correas M, Gutierrez J, Del Valle J, Roca A, Vega A, Villanueva A, Gutierrez R. Results at 43 months' follow-up of a double-blind, randomized, prospective clinical trial using intravesical interferon alpha-2b in the prophylaxis of stage pT1 transitional cell carcinoma of the bladder. Urology 1997;49(2):187-190.

[93] Gan YH, Zhang Y, Khoo HE, Esuvaranathan K. Antitumour immunity of Bacillus Calmette-Guerin and interferon alpha in murine bladder cancer. European Journal of Cancer 1999;35(7):1123-1129.

[94] Luo Y, Chen X, Downs TM, DeWolf WC, O'Donnell MA. IFN-alpha 2B enhances Th1 cytokine responses in bladder cancer patients receiving Mycobacterium bovis bacillus Calmette-Guérin immunotherapy. Journal of Immunology 1999;162(4):2399-2405.

[95] Stricker P, Pryor K, Nicholson T, Goldstein D, Golovsky D, Ferguson R, Nash P, Ehsman S, Rumma J, Mammen G, Penny R. Bacillus Calmette-Guérin plus intravesical interferon alpha-2b in patients with superficial bladder cancer. Urology 1996;48(6): 957-961.

[96] O'Donnell MA, Krohn J, DeWolf WC. Salvage intravesical therapy with interferon-alpha 2b plus low dose bacillus Calmette-Guerin is effective in patients with superficial bladder cancer in whom bacillus Calmette-Guerin alone previously failed. The Journal of Urology 2001;166(4):1300-1304.

[97] Lam JS, Benson MC, O'Donnell MA, Sawczuk A, Gavazzi A, Wechsler MH, Sawczuk IS. Bacillus Calmete-Guérin plus interferon-alpha2B intravesical therapy maintains an extended treatment plan for superficial bladder cancer with minimal toxicity. Urologic Oncology 2003;21(5):354-360.

[98] Punnen SP, Chin JL, Jewett MA. Management of bacillus Calmette-Guerin (BCG) refractory superficial bladder cancer: results with intravesical BCG and Interferon combination therapy. The Canadian Journal of Urology 2003;10(2):1790-1795.

[99] Smith BJ, O'Donnell MA; National BCG-Interferon Phase 2 Investigator Group. Final results from a national multicenter phase II trial of combination bacillus Calmette-Guérin plus interferon alpha-2B for reducing recurrence of superficial bladder cancer. Urologic Oncology 2006;24(4):344-348.

[100] Bazarbashi S, Soudy H, Abdelsalam M, Al-Jubran A, Akhtar S, Memon M, Aslam M, Kattan S, Shoukri M. Co-administration of intravesical bacillus Calmette-Guérin and interferon α-2B as first line in treating superficial transitional cell carcinoma of the urinary bladder. British Journal of Urology International 2011;108(7):1115-1118.

[101] Gallagher BL, Joudi FN, Maymí JL, O'Donnell MA. mpact of previous bacille Calmette-Guérin failure pattern on subsequent response to bacille Calmette-Guérin plus interferon intravesical therapy. Urology 2008;71(2):297-301.

[102] Nepple KG, Lightfoot AJ, Rosevear HM, O'Donnell MA, Lamm DL; Bladder Cancer Genitourinary Oncology Study Group. Bacillus Calmette-Guérin with or without interferon α-2b and megadose versus recommended daily allowance vitamins during induction and maintenance intravesical treatment of nonmuscle invasive bladder cancer. The Journal of Urology 2010;184(5):1915-1919.

[103] Joudi FN, Smith BJ, O'Donnell MA; National BCG-Interferon Phase 2 Investigator Group. Final results from a national multicenter phase II trial of combination bacillus Calmette-Guérin plus interferon alpha-2B for reducing recurrence of superficial bladder cancer. Urologic Oncology 2006;24(4):344-348.

[104] Luo Y, Chen X, Han R, O'Donnell MA. Recombinant bacille Calmette-Guérin (BCG) expressing human interferon-alpha 2B demonstrates enhanced immunogenicity. Clinical and Experimental Immunology 2001;123(2):264-270.

[105] Liu W, O'Donnell MA, Chen X, Han R, Luo Y. Recombinant bacillus Calmette-Guérin (BCG) expressing interferon-alpha 2B enhances human mononuclear cell cytotoxicity against bladder cancer cell lines in vitro. Cancer Immunology and Immunotherapy 2009;58(10):1647-1655.

[106] Louie B, Rajamahanty S, Won J, Choudhury M, Konno S. Synergistic potentiation of interferon activity with maitake mushroom d-fraction on bladder cancer cells. British Journal of Urology International 2010;105(7):1011-1015.

[107] Fishman AI, Johnson B, Alexander B, Won J, Choudhury M, Konno S. Additively enhanced antiproliferative effect of interferon combined with proanthocyanidin on bladder cancer cells. Journal of Cancer 2012;3:107-112.

[108] Nagabhushan TL, Maneval DC, Benedict WF, Wen SF, Ihnat PM, Engler H, Connor RJ. Enhancement of intravesical delivery with Syn3 potentiates interferon-alpha2b gene therapy for superficial bladder cancer. Cytokine and Growth Factor Reviews 2007;18(5-6):389-394.

[109] Gillis S, Smith KA. Long term culture of tumour-specific cytotoxic T cells. Nature 1977;268(5616):154-156.

[110] Di Sabato G, Chen DM, Erickson JW. Production by murine spleen cells of an activity stimulating the PHA-responsiveness of thymus lymphocytes. Cell Immunology 1975;17(2):495-504.

[111] Chen DM, Di Sabato G. Further studies on the thymocyte stimulating factor. Cellular Immunology 1976;22(2):211-224.

[112] Mizel SB, Farrar JJ. Revised nomenclature for antigen-nonspecific T-cell proliferation and helper factors. Cellular Immunology 1979;48(2):433-436.

[113] Shaw J, Monticone V, Mills G, Paetkau V. Effects of costimulator on immune responses in vitro. Journal of Immunology 1978;120(6):1974-1980.

[114] Yron I, Wood TA Jr, Spiess PJ, Rosenberg SA. In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. Journal of Immunology 1980;125(1):238-245.

[115] Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA. Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor. Cancer Research 1981;41(11 Pt 1):4420-4425.

[116] Rayner AA, Grimm EA, Lotze MT, Chu EW, Rosenberg SA. Lymphokine-activated killer (LAK) cells. Analysis of factors relevant to the immunotherapy of human cancer. Cancer 1985;55(6):1327-1333.

[117] Henney CS, Kuribayashi K, Kern DE, Gillis S. Interleukin-2 augments natural killer cell activity. Nature 1981;291(5813):335-338.

[118] Malkovský M, Loveland B, North M, Asherson GL, Gao L, Ward P, Fiers W. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. Nature 1987;325(6101):262-265.

[119] Waldmann TA, Goldman CK, Robb RJ, Depper JM, Leonard WJ, Sharrow SO, Bongiovanni KF, Korsmeyer SJ, Greene WC. Expression of interleukin 2 receptors on activated human B cells. Journal of Experimental Medicine 1984;160(5):1450-1466.

[120] Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology 1986;136(7):2348-2357.

[121] Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. The New England Journal of Medicine 1987;316(15): 889-897.

[122] Webb DE, Austin HA 3rd, Belldegrun A, Vaughan E, Linehan WM, Rosenberg SA. Metabolic and renal effects of interleukin-2 immunotherapy for metastatic cancer. Clinical Nephrology 1988;30(3):141-145.

[123] Ratliff TL, Haaff EO, Catalona WJ. Interleukin-2 production during intravesical bacille Calmette-Guerin therapy for bladder cancer. Clinical Immunology and Immunopatholgy 1986;40(2):375-379.

[124] Haaff EO, Catalona WJ, Ratliff TL. Detection of interleukin 2 in the urine of patients with superficial bladder tumors after treatment with intravesical BCG. The Journal of Urology 1986;136(4):970-974.

[125] De Jong WH, De Boer EC, Van der Meijden AP, Vegt P, Steerenberg PA, Debruyne FM, Ruitenberg EJ. Presence of interleukin-2 in urine of superficial bladder cancer

patients after intravesical treatment with bacillus Calmette-Guérin. Cancer Immunology and Immunotherapy 1990;31(3):182-186.

[126] Böhle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG, Flad HD. Detection of urinary TNF, IL 1, and IL 2 after local BCG immunotherapy for bladder carcinoma. Cytokine 1990;2(3):175-181.

[127] De Boer EC, De Jong WH, Steerenberg PA, Aarden LA, Tetteroo E, De Groot ER, Van der Meijden AP, Vegt PD, Debruyne FM, Ruitenberg EJ. Induction of urinary interleukin-1 (IL-1), IL-2, IL-6, and tumour necrosis factor during intravesical immunotherapy with bacillus Calmette-Guérin in superficial bladder cancer. Cancer Immunology and Immunotherapy 1992;34(5):306-312.

[128] Balbay D, Ozen H, Ozkardes H, Barut A, Bakkaloglu M, Tasar C, Remzi D. Detection of urinary interleukin-2, interleukin-2 receptor, and tumor necrosis factor levels in patients with superficial bladder tumors after intravesical BCG immunotherapy. Urology 1994;43(2):187-190.

[129] de Reijke TM, De Boer EC, Kurth KH, Schamhart DH. Urinary interleukin-2 monitoring during prolonged bacillus Calmette-Guerin treatment: can it predict the optimal number of instillations? The Journal of Urology 1999;161(1):67-71.

[130] Magno C, Melloni D, Galì A, Mucciardi G, Nicocia G, Morandi B, Melioli G, Ferlazzo G. The anti-tumor activity of bacillus Calmette-Guerin in bladder cancer is associated with an increase in the circulating level of interleukin-2. Immunology Letters 2002;81(3):235-238.

[131] Pizza G, Severini G, Menniti D, De Vinci C, Corrado F. Tumour regression after intralesional injection of interleukin 2 (IL-2) in bladder cancer. Preliminary report. International Journal of Cancer 1984;34(3):359-367.

[132] Lee KE, Weiss GH, O'Donnell RW, Cockett AT. Reduction of bladder cancer growth in mice treated with intravesical Bacillus Calmette Guerin and systemic interleukin 2. The Journal of Urology 1987;137(6):1270-1273.

[133] Ikemoto S, Kamizuru M, Wada S, Nishio S, Kishimoto T, Maekawa M. Combined effect of interleukin 2 and bacillus Calmette-Guérin in the therapy of mice with transitional cell carcinoma. Urologia Internationalis 1991;47(4):250-254.

[134] Riggs DR, Tarry WF, DeHaven JI, Sosnowski J, Lamm DL. Immunotherapy of murine transitional cell carcinoma of the bladder using alpha and gamma interferon in combination with other forms of immunotherapy. The Journal of Urology 1992;147(1):212-214.

[135] Tubaro A, Velotti F, Stoppacciaro A, Santoni A, Vicentini C, Bossola PC, Galassi P, Pettinato A, Morrone S, Napolitano T, et al. Continuous intra-arterial administration of recombinant interleukin-2 in low-stage bladder cancer. A phase IB study. Cancer 1991;68(1):56-61.

[136] Merguerian PA, Donahue L, Cockett AT. Intraluminal interleukin 2 and bacillus Calmette-Guerin for treatment of bladder cancer: a preliminary report. The Journal of Urology 1987;137(2):216-219.

[137] Huland E, Huland H. Local continuous high dose interleukin 2: a new therapeutic model for the treatment of advanced bladder carcinoma. Cancer Research 1989;49(19):5469-5474.

[138] Cockett AT, Davis RS, Cos LR, Wheeless LL Jr. Bacillus Calmette-Guerin and interleukin-2 for treatment of superficial bladder cancer. The Journal of Urology 1991;146(3):766-769.

[139] Gomella LG, McGinnis DE, Lattime EC, Butler K, Baltish M, Thompson I, Marshall ME. Treatment of transitional cell carcinoma of the bladder with intravesical interleukin-2: a pilot study. Cancer Biotherapy 1993;8(3):223-227.

[140] Nouri AM, Hyde R, Oliver RT. Clinical and immunological effect of intravesical interleukin-2 on superficial bladder cancer. Cancer Immunology and Immunotherapy 1994;39(1):68–70.

[141] Ferlazzo G, Magno C, Iemmo R, Rizzo M, Lupo G, Semino C, et al. Treatment of superficial bladder cancer with intravesical perfusion of rIL-2: a follow-up study. Anticancer Research 1996;16(2):979–980.

[142] Den Otter W, Dobrowolski Z, Bugajski A, Papla B, Van Der Meijden AP, Koten JW, Boon TA, Siedlar M, Zembala M. Intravesical interleukin-2 in T1 papillary bladder carcinoma: regression of marker lesion in 8 of 10 patients. The Journal of Urology 1998;159(4):1183-1186.

[143] O'Donnell MA, Aldovini A, Duda RB, Yang H, Szilvasi A, Young RA, DeWolf WC. Recombinant Mycobacterium bovis BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. Infection and Immunity 1994;62(6): 2508-2514.

[144] Murray PJ, Aldovini A, Young RA. Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guérin strains that secrete cytokines. Proceedings of the National Academy of Sciences of the United States of America 1996;93(2):934-939.

[145] Slobbe L, Lockhart E, O'Donnell MA, MacKintosh C, De Lisle G, Buchan G. An in vivo comparison of bacillus Calmette-Guérin (BCG) and cytokine-secreting BCG vaccines. Immunology 1999;96(4):517-523.

[146] Luo Y, Chen X, Szilvasi A, O'Donnell MA. Co-expression of interleukin-2 and green fluorescent protein reporter in mycobacteria: in vivo application for monitoring antimycobacterial immunity. Molecular Immunology 2000;37(9):527-536.

[147] Young SL, O'Donnell MA, Buchan GS. IL-2-secreting recombinant bacillus Calmette Guerin can overcome a Type 2 immune response and corticosteroid-induced immu-

nosuppression to elicit a Type 1 immune response. International Immunology 2002;14(7):793-800.

[148] Li YG, Wang ZP, Tian JQ, Tian BQ, Rodrigues R, Shang PF, Zhang T. Dendritic cell transfected with secondary lymphoid-tissue chemokine and/or interleukin-2 gene-enhanced cytotoxicity of T-lymphocyte in human bladder tumor cell S in vitro. Cancer Investigation 2009;27(9):909-917.

[149] Huang X, Yu HS, Chen Z, Li JL, Hu ZM, Gao JM. A novel immunotherapy for superficial bladder cancer by the immobilization of streptavidin-tagged bioactive IL-2 on the biotinylated mucosal surface of the bladder wall. Chinese Journal of Cancer 2010;29(6):611-616.

[150] Zhang X, Shi X, Li J, Hu Z, Guo F, Huang X, Zhang Z, Sun P, Jing Y, Gao J, Tan W. Novel immunotherapy for metastatic bladder cancer using vaccine of human interleukin-2 surface-modified MB49 cells. Urology 2011;78(3):722.e1-722.e6.

[151] Wong HL, Wilson DE, Jenson JC, Familletti PC, Stremlo DL, Gately MK. Characterization of a factor(s) which synergizes with recombinant interleukin 2 in promoting allogeneic human cytolytic T-lymphocyte responses in vitro. Cellular Immunology 1988;111(1):39-54.

[152] Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. Journal of Experimental Medicine 1989;170(3):827-845.

[153] Stern AS, Podlaski FJ, Hulmes JD, Pan YC, Quinn PM, Wolitzky AG, Familletti PC, Stremlo DL, Truitt T, Chizzonite R, et al. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proceedings of the National Academy of Sciences of the United States of America 1990;87(17):6808-6812.

[154] Gately MK, Desai BB, Wolitzky AG, Quinn PM, Dwyer CM, Podlaski FJ, Familletti PC, Sinigaglia F, Chizonnite R, Gubler U, et al. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). Journal of Immunology 1991;147(3):874-882.

[155] Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proceedings of the National Academy of Sciences of the United States of America 1991;88(10):4143-4147.

[156] Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U. Cloning and expression of murine IL-12. Journal of Immunology 1992;148(11):3433-3440.

[157] Desai BB, Quinn PM, Wolitzky AG, Mongini PK, Chizzonite R, Gately MK. IL-12 receptor. II. Distribution and regulation of receptor expression. Journal of Immunology 1992;148(10):3125-3132.

[158] Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. Journal of Experimental Medicine 1993;177(4):1199-1204.

[159] Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. Journal of Immunology 1995;154(10):5071-5079.

[160] Gately MK, Wolitzky AG, Quinn PM, Chizzonite R. Regulation of human cytolytic lymphocyte responses by interleukin-12. Cell Immunology 1992;143(1):127-142.

[161] Collison K, Saleh S, Parhar R, Meyer B, Kwaasi A, Al-Hussein K, Al-Sedairy S, Al-Mohanna F. Evidence for IL-12-activated Ca2+ and tyrosine signaling pathways in human neutrophils. Journal of Immunology 1998;161(7):3737-3745.

[162] Yeaman GR, Collins JE, Currie JK, Guyre PM, Wira CR, Fanger MW. IFN-gamma is produced by polymorphonuclear neutrophils in human uterine endometrium and by cultured peripheral blood polymorphonuclear neutrophils. Journal of Immunology 1998;160(10):5145-5153.

[163] Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. Journal of the National Cancer Institute 1995;87(8):581-586.

[164] Dias S, Boyd R, Balkwill F. IL-12 regulates VEGF and MMPs in a murine breast cancer model. International Journal of Cancer 1998;78(3):361-365.

[165] Coughlin CM, Salhany KE, Gee MS, LaTemple DC, Kotenko S, Ma X, Gri G, Wysocka M, Kim JE, Liu L, Liao F, Farber JM, Pestka S, Trinchieri G, Lee WM. Tumor cell responses to IFNgamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity 1998;9(1):25-34.

[166] Coughlin CM, Salhany KE, Wysocka M, Aruga E, Kurzawa H, Chang AE, Hunter CA, Fox JC, Trinchieri G, Lee WM. Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. The Journal of Clinical Investigation 1998;101(6):1441-1452.

[167] Hashimoto W, Osaki T, Okamura H, Robbins PD, Kurimoto M, Nagata S, Lotze MT, Tahara H. Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by Fas-Fas ligand- and perforin-induced tumor apoptosis, respectively. Journal of Immunology 1999;163(2):583-589.

[168] Kawamura T, Takeda K, Mendiratta SK, Kawamura H, Van Kaer L, Yagita H, Abo T, Okumura K. Critical role of NK1+ T cells in IL-12-induced immune responses in vivo. Journal of Immunology 1998;160(1):16-19.

[169] Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. Journal of Experimental Medicine 1993;178(4):1223-1230.

[170] Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ, et al. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. Journal of Immunology 1994;153(4):1697-1706.

[171] Teicher BA, Ara G, Buxton D, Leonard J, Schaub RG. Optimal scheduling of interleukin 12 and chemotherapy in the murine MB-49 bladder carcinoma and B16 melanoma. Clinical Cancer Research 1997;3(9):1661-1667.

[172] Teicher BA, Ara G, Buxton D, Leonard J, Schaub RG. Optimal scheduling of interleukin-12 and fractionated radiation therapy in the murine Lewis lung carcinoma. Radiation Oncology Investigations 1998;6(2):71-80.

[173] O'Donnell MA, Luo Y, Hunter SE, Chen X, Hayes LL, Clinton SK. Interleukin-12 immunotherapy of murine transitional cell carcinoma of the bladder: dose dependent tumor eradication and generation of protective immunity. The Journal of Urology 2004;171(3):1330-1335.

[174] Zagozdzon R, Giermasz A, Gołab J, Stokłosa T, Jalili A, Jakóbisiak M. The potentiated antileukemic effects of doxorubicin and interleukin-12 combination are not dependent on nitric oxide production. Cancer Letters 1999;147(1-2):67-75.

[175] Zagozdzon R, Golab J, Mucha K, Foroncewicz B, Jakobisiak M. Potentiation of antitumor effects of IL-12 in combination with paclitaxel in murine melanoma model in vivo. International Journal of Molecular Medicine 1999;4(6):645-648.

[176] Golab J, Zagozdzon R, Kozar K, Kaminski R, Giermasz A, Stoklosa T, Lasek W, Jakobisiak M. Potentiatied anti-tumor effectiveness of combined therapy with interleukin-12 and mitoxantrone of L1210 leukemia in vivo. Oncology Reports 2000;7(1): 177-181.

[177] Teicher BA, Ara G, Menon K, Schaub RG. In vivo studies with interleukin-12 alone and in combination with monocyte colony-stimulating factor and/or fractionated radiation treatment. International Journal of Cancer 1996;65(1):80-84.

[178] Lieu FH, Hawley TS, Fong AZ, Hawley RG. Transmissibility of murine stem cell virus-based retroviral vectors carrying both interleukin-12 cDNAs and a third gene: implications for immune gene therapy. Cancer Gene Therapy 1997;4(3):167-175.

[179] Bramson JL, Hitt M, Addison CL, Muller WJ, Gauldie J, Graham FL. Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and

long-lasting immunity that is associated with highly localized expression of interleukin-12. Human Gene Therapy 1996;7(16):1995-2002.

[180] Addison CL, Bramson JL, Hitt MM, Muller WJ, Gauldie J, Graham FL. Intratumoral coinjection of adenoviral vectors expressing IL-2 and IL-12 results in enhanced frequency of regression of injected and untreated distal tumors. Gene Therapy 1998;5(10):1400-1409.

[181] Meko JB, Yim JH, Tsung K, Norton JA. High cytokine production and effective antitumor activity of a recombinant vaccinia virus encoding murine interleukin 12. Cancer Research 1995;55(21):4765-4770.

[182] Zitvogel L, Tahara H, Cai Q, Storkus WJ, Muller G, Wolf SF, Gately M, Robbins PD, Lotze MT. Construction and characterization of retroviral vectors expressing biologically active human interleukin-12. Human Gene Therapy 1994;5(12):1493-1506.

[183] O'Donnell MA, Luo Y, Chen X, Szilvasi A, Hunter SE, Clinton SK. Role of IL-12 in the induction and potentiation of IFN-gamma in response to bacillus Calmette-Guérin. Journal of Immunology 1999;163(8):4246-4252.

[184] Riemensberger J, Böhle A, Brandau S. IFN-gamma and IL-12 but not IL-10 are required for local tumour surveillance in a syngeneic model of orthotopic bladder cancer. Clinical and Experimental Immunology 2002;127(1):20-26.

[185] O'Donnell MA, Luo Y, Hunter SE, Chen X, Hayes LL, Clinton SK. The essential role of interferon-gamma during interleukin-12 therapy for murine transitional cell carcinoma of the bladder. The Journal of Urology 2004;171(3):1336-1342.

[186] Clinton SK, Canto E, O'Donnell MA. Interleukin-12. Opportunities for the treatment of bladder cancer. The Urologic Clinics of North America 2000;27(1):147–155.

[187] Horinaga M, Harsch KM, Fukuyama R, Heston W, Larchian W. Intravesical interleukin-12 gene therapy in an orthotopic bladder cancer model. Urology 2005;66(2): 461-466.

[188] Zaharoff DA, Hoffman BS, Hooper HB, Benjamin CJ Jr, Khurana KK, Hance KW, Rogers CJ, Pinto PA, Schlom J, Greiner JW. Intravesical immunotherapy of superficial bladder cancer with chitosan/interleukin-12. Cancer Research 2009;69(15): 6192-6199.

[189] Cohen J. IL-12 deaths: explanation and a puzzle. Science 1995;270(5238):908.

[190] Atkins MB, Robertson MJ, Gordon M, Lotze MT, DeCoste M, DuBois JS, Ritz J, Sandler AB, Edington HD, Garzone PD, Mier JW, Canning CM, Battiato L, Tahara H, Sherman ML. Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. Clinical Cancer Research 1997;3(3):409-417.

[191] Robertson MJ, Cameron C, Atkins MB, Gordon MS, Lotze MT, Sherman ML, Ritz J. Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. Clinical Cancer Research 1999;5(1):9-16.

[192] Gollob JA, Mier JW, Veenstra K, McDermott DF, Clancy D, Clancy M, Atkins MB. Phase I trial of twice-weekly intravenous interleukin 12 in patients with metastatic renal cell cancer or malignant melanoma: ability to maintain IFN-gamma induction is associated with clinical response. Clinical Cancer Research 2000;6(5):1678-1692.

[193] Hurteau JA, Blessing JA, DeCesare SL, Creasman WT. Evaluation of recombinant human interleukin-12 in patients with recurrent or refractory ovarian cancer: a gynecologic oncology group study. Gynecologic Oncology 2001;82(1):7-10.

[194] Motzer RJ, Rakhit A, Thompson JA, Nemunaitis J, Murphy BA, Ellerhorst J, Schwartz LH, Berg WJ, Bukowski RM. Randomized multicenter phase II trial of subcutaneous recombinant human interleukin-12 versus interferon-alpha 2a for patients with advanced renal cell carcinoma. Journal of Interferon and Cytokine Research 2001;21(4): 257-263.

[195] Lenzi R, Rosenblum M, Verschraegen C, Kudelka AP, Kavanagh JJ, Hicks ME, Lang EA, Nash MA, Levy LB, Garcia ME, Platsoucas CD, Abbruzzese JL, Freedman RS. Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. Clinical Cancer Research 2002;8(12):3686-3695.

[196] Lenzi R, Edwards R, June C, Seiden MV, Garcia ME, Rosenblum M, Freedman RS. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease < 1 cm) associated with ovarian cancer or primary peritoneal carcinoma. Journal of Translational Medicine 2007;5:66.

[197] Younes A, Pro B, Robertson MJ, Flinn IW, Romaguera JE, Hagemeister F, Dang NH, Fiumara P, Loyer EM, Cabanillas FF, McLaughlin PW, Rodriguez MA, Samaniego F. Phase II clinical trial of interleukin-12 in patients with relapsed and refractory non-Hodgkin's lymphoma and Hodgkin's disease. Clinical Cancer Research 2004;10(16): 5432-5438.

[198] Gollob JA, Veenstra KG, Parker RA, Mier JW, McDermott DF, Clancy D, Tutin L, Koon H, Atkins MB. Phase I trial of concurrent twice-weekly recombinant human interleukin-12 plus low-dose IL-2 in patients with melanoma or renal cell carcinoma. Journal of Clinical Oncology 2003;21(13):2564-2573.

[199] Eisenbeis CF, Lesinski GB, Anghelina M, Parihar R, Valentino D, Liu J, Nadella P, Sundaram P, Young DC, Sznol M, Walker MJ, Carson WE 3rd. Phase I study of the sequential combination of interleukin-12 and interferon alfa-2b in advanced cancer: evidence for modulation of interferon signaling pathways by interleukin-12. Journal of Clinical Oncology 2005;23(34):8835-8844.

[200] Weiss GR, O'Donnell MA, Loughlin K, Zonno K, Laliberte RJ, Sherman ML. Phase 1 study of the intravesical administration of recombinant human interleukin-12 in pa-

tients with recurrent superficial transitional cell carcinoma of the bladder. Journal of Immunotherapy 2003;26(4):343-348.

[201] Giannopoulos A, Constantinides C, Fokaeas E, Stravodimos C, Giannopoulou M, Kyroudi A, Gounaris A. The immunomodulating effect of interferon-gamma intravesical instillations in preventing bladder cancer recurrence. Clinical Cancer Research 2003;9(15):5550-5558.

[202] Stavropoulos NE, Hastazeris K, Filiadis I, Mihailidis I, Ioachim E, Liamis Z, Kalomiris P. Intravesical instillations of interferon gamma in the prophylaxis of high risk superficial bladder cancer--results of a controlled prospective study. Scandinavian Journal of Urology and Nephrology 2002;36(3):218-222.

[203] Serretta V, Corselli G, Piazza B, Franks CR, Palmer PA, Roest GJ, Pavone-Macaluso M. Intravesical therapy of superficial bladder transitional cell carcinoma with tumor necrosis factor-alpha: preliminary report of a phase I-II study. European Urology 1992;22(2):112–114.

[204] Serretta V, Piazza B, Pavone C, Piazza S, Pavone-Macaluso M. Is there a role for recombinant tumor necrosis facto alpha in the intravesical treatment of superficial bladder tumors?—a phase II study. International Journal of Urology 1995;2(2):100–103.

[205] Glazier DB, Bahnson RR, McLeod DG, von Roemeling RW, Messing EM, Ernstoff MS. Intravesical recombinant tumor necrosis factor in the treatment of superficial bladder cancer: an Eastern Cooperative Oncology Group study. The Journal of Urology 1995;154(1):66–68.

[206] Grampsas SA, Kahn K, Crawford ED. Intravesical RTNF therapy of superficial bladder cancer. A phase I study of recombinant tumor necrosis factor administered intravesically to patients with superficial bladder cancer. The Online Journal of Current Clinical Trials 1994; PMID:8136939.

[207] Stravoravdi P, Toliou T, Kirtsis P, Natsis K, Konstandinidis E, Barich A, Gigis P, Dimitriadis K. A new approach in the management of urothelial tumors using GM-CSF on marker lesions: an ultrastructural and immunohistochemical study on the macrophage population in bladder mucosa. Journal of Interferon Cytokine Research 1999;19(3):221-225.

[208] Theano T, Pelagia S, Konstantinos N, Petros K, Alfred B, Konstantinos D, Panagiotis G. Lymphocyte activation by granulocyte macrophage-colony stimulating factor in human bladder cancer. Journal of Experimental Therapeutics and Oncology 2002;2(3):153-157.

[209] Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. Journal of Experimental Medicine 1989;170(6):2081-2095.

[210] Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. The Journal of Immunology 1991;146(10):3444-3451.

[211] Ferguson TA, Dube P, Griffith TS. Regulation of contact hypersensitivity by interleukin 10. Journal of Experimental Medicine 1994;179(5):1597-1604.

[212] Halak BK, Maguire HC Jr, Lattime EC. Tumor-induced interleukin-10 inhibits type 1 immune responses directed at a tumor antigen as well as a non-tumor antigen present at the tumor site. Cancer Research 1999;59(4):911-917.

[213] Lattime EC, Mastrangelo MJ, Bagasra O, Li W, Berd D. Expression of cytokine mRNA in human melanoma tissues. Cancer Immunology and Immunotherapy 1995;41(3):151-156.

[214] Krüger-Krasagakes S, Krasagakis K, Garbe C, Schmitt E, Hüls C, Blankenstein T, Diamantstein T. Expression of interleukin 10 in human melanoma. British Journal of Cancer 1994;70(6):1182-1185.

[215] Huang M, Wang J, Lee P, Sharma S, Mao JT, Meissner H, Uyemura K, Modlin R, Wollman J, Dubinett SM. Human non-small cell lung cancer cells express a type 2 cytokine pattern. Cancer Research 1995;55(17):3847-3853.

[216] Nakagomi H, Pisa P, Pisa EK, Yamamoto Y, Halapi E, Backlin K, Juhlin C, Kiessling R. Lack of interleukin-2 (IL-2) expression and selective expression of IL-10 mRNA in human renal cell carcinoma. International Journal of Cancer 1995;63(3):366-371.

[217] Mosmann TR, Schumacher JH, Fiorentino DF, Leverah J, Moore KW, Bond MW. Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay. Journal of Immunology 1990;145(9):2938-2945.

[218] O'Garra A, Stapleton G, Dhar V, Pearce M, Schumacher J, Rugo H, Barbis D, Stall A, Cupp J, Moore K, et al. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. International Immunology 1990;2(9):821-832.

[219] de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. Journal of Experimental Medicine 1991;174(5):1209-1220.

[220] Qin Z, Noffz G, Mohaupt M, Blankenstein T. Interleukin-10 prevents dendritic cell accumulation and vaccination with granulocyte-macrophage colony-stimulating factor gene-modified tumor cells. Journal of Immunology 1997;159(2):770-776.

[221] Richter G, Krüger-Krasagakes S, Hein G, Hüls C, Schmitt E, Diamantstein T, Blankenstein T. Interleukin 10 transfected into Chinese hamster ovary cells prevents tumor growth and macrophage infiltration. Cancer Research 1993;53(18):4134-4137.

[222] Steinbrink K, Wölfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. Journal of Immunology 1997;159(10):4772-4780.

[223] de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. Journal of Experimental Medicine 1991;174(4):915-924.

[224] Ding L, Linsley PS, Huang LY, Germain RN, Shevach EM. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. Journal of Immunology 1993;151(3):1224-1234.

[225] Willems F, Marchant A, Delville JP, Gérard C, Delvaux A, Velu T, de Boer M, Goldman M. Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. European Journal of Immunology 1994;24(4):1007-1009.

[226] Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389(6652):737-742.

[227] Cenci E, Romani L, Mencacci A, Spaccapelo R, Schiaffella E, Puccetti P, Bistoni F. Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of Candida albicans. European Journal of Immunology 1993;23(5):1034-1038.

[228] Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annual Review of Immunology 2001;19:683-765.

[229] Kong D, Kunimoto DY. Secretion of human interleukin 2 by recombinant Mycobacterium bovis BCG. Infection and Immunity 1995;63(3):799-803.

[230] Ryan AA, Wozniak TM, Shklovskaya E, O'Donnell MA, Fazekas de St Groth B, Britton WJ, Triccas JA. Improved protection against disseminated tuberculosis by Mycobacterium bovis bacillus Calmette-Guerin secreting murine GM-CSF is associated with expansion and activation of APCs. Journal of Immunology 2007;179(12): 8418-8424.

[231] Wangoo A, Brown IN, Marshall BG, Cook HT, Young DB, Shaw RJ. Bacille Calmette-Guérin (BCG)-associated inflammation and fibrosis: modulation by recombinant BCG expressing interferon-gamma (IFN-gamma). Clinical and Experimental Immunology 2000;119(1):92-98.

[232] Young S, O'Donnell M, Lockhart E, Buddle B, Slobbe L, Luo Y, De Lisle G, Buchan G. Manipulation of immune responses to Mycobacterium bovis by vaccination with IL-2- and IL-18-secreting recombinant bacillus Calmette Guerin. Immunology and Cell Biology 2002;80(3):209-215.

[233] Moreira AL, Tsenova L, Murray PJ, Freeman S, Bergtold A, Chiriboga L, Kaplan G. Aerosol infection of mice with recombinant BCG secreting murine IFN-gamma parti-

ally reconstitutes local protective immunity. Microbial Pathogenesis 2000;29(3): 175-185.

[234] Biet F, Kremer L, Wolowczuk I, Delacre M, Locht C. Mycobacterium bovis BCG producing interleukin-18 increases antigen-specific gamma interferon production in mice. Infection and Immunity 2002;70(12):6549-6557.

[235] Biet F, Duez C, Kremer L, Marquillies P, Amniai L, Tonnel AB, Locht C, Pestel J. Recombinant Mycobacterium bovis BCG producing IL-18 reduces IL-5 production and bronchoalveolar eosinophilia induced by an allergic reaction. Allergy 2005;60(8): 1065-1072.

[236] He J, Shen D, O'Donnell MA, Chang H. Induction of MUC1-specific cellular immunity by a recombinant BCG expressing human MUC1 and secreting IL2. International Journal of Oncology 2002;20(6):1305-1311.

[237] Chung MA, Luo Y, O'Donnell M, Rodriguez C, Heber W, Sharma S, Chang HR. Development and preclinical evaluation of a Bacillus Calmette-Guérin-MUC1-based novel breast cancer vaccine. Cancer Research 2003;63(6):1280-1287.

[238] Arnold J, de Boer EC, O'Donnell MA, Böhle A, Brandau S. Immunotherapy of experimental bladder cancer with recombinant BCG expressing interferon-gamma. Journal of Immunotherapy 2004;27(2):116-123.

[239] Fan XL, Yu TH, Gao Q, Yao W. Immunological properties of recombinant Mycobacterium bovis bacillus Calmette-Guérin strain expressing fusion protein IL-2-ESAT-6. Acta Biochimica et Biophysica Sinica (Shanghai) 2006;38(10)683-690.

[240] Chen X, O'Donnell MA, Luo Y. Dose-dependent synergy of Th1-stimulating cytokines on bacille Calmette-Guérin-induced interferon-gamma production by human mononuclear cells. Clinical and Experimental Immunology 2007;149(1):178-185.

[241] Ryan AA, Spratt JM, Britton WJ, Triccas JA. Secretion of functional monocyte chemotactic protein 3 by recombinant Mycobacterium bovis BCG attenuates vaccine virulence and maintains protective efficacy against M. tuberculosis infection. Infection and Immunity 2007;75(1):523-526.

[242] Xu Y, Zhu B, Wang Q, Chen J, Qie Y, Wang J, Wang H, Wang B, Wang H. Recombinant BCG coexpressing Ag85B, ESAT-6 and mouse-IFN-gamma confers effective protection against Mycobacterium tuberculosis in C57BL/6 mice. FEMS Immunology and Medcal Microbiology 2007;51(3):480-487.

[243] Tang C, Yamada H, Shibata K, Maeda N, Yoshida S, Wajjwalku W, Ohara N, Yamada T, Kinoshita T, Yoshikai Y. Efficacy of recombinant bacille Calmette-Guérin vaccine secreting interleukin-15/antigen 85B fusion protein in providing protection against Mycobacterium tuberculosis. Journal of Infectious Diseases 2008;197(9):1263-1274.

[244] Yuan S, Shi C, Han W, Ling R, Li N, Wang T. Effective anti-tumor responses induced by recombinant bacillus Calmette-Guérin vaccines based on different tandem repeats of MUC1 and GM-CSF. European Journal of Cancer Prevention 2009;18(5):416-423.

[245] Yuan S, Shi C, Ling R, Wang T, Wang H, Han W. Immunization with two recombinant Bacillus Calmette-Guérin vaccines that combine the expression of multiple tandem repeats of mucin-1 and colony stimulating-factor suppress breast tumor growth in mice. Journal of Cancer Research and Clinical Oncology 2010;136,(9):1359-1367.

[246] Shen H, Wang C, Yang E, Xu Y, Liu W, Yan J, Wang F, Wang H. Novel recombinant BCG coexpressing Ag85B, ESAT-6 and mouse TNF-alpha induces significantly enhanced cellular immune and antibody responses in C57BL/6 mice. Microbiolgy and Immunology 2010;54(8):435-441.

[247] Deng Y, Bao L, Yang X. Evaluation of immunogenicity and protective efficacy against Mycobacterium tuberculosis infection elicited by recombinant Mycobacterium bovis BCG expressing human Interleukin-12p70 and Early Secretory Antigen Target-6 fusion protein. Microbiology and Immunology 2011;55(11):798-808.

[248] Yang X, Bao L, Deng Y. A novel recombinant Mycobacterium bovis bacillus Calmette-Guerin strain expressing human granulocyte macrophage colony-stimulating factor and Mycobacterium tuberculosis early secretory antigenic target 6 complex augments Th1 immunity. Acta Biochimica et Biophysica Sinica (Shanghai) 2011;43(7): 511-518.

[249] Lin CW, Su IJ, Chang JR, Chen YY, Lu JJ, Dou HY. Recombinant BCG coexpressing Ag85B, CFP10, and interleukin-12 induces multifunctional Th1 and memory T cells in mice. Acta Pathologica, Microbiologica et Immunologica Scandinavica 2012;120(1): 72-82.

[250] Ding GQ, Yu YL, Shen ZJ, Zhou XL, Chen SW, Liao GD, Zhang Y. Antitumor effects of human interferon-alpha 2b secreted by recombinant bacillus Calmette-Guérin vaccine on bladder cancer cells. Journal of Zhejiang University-Science B. 2012;13(5): 335-341.

[251] Duda RB, Yang H, Dooley DD, Abu-Jawdeh G. Recombinant BCG therapy suppresses melanoma tumor growth. Annals of Surgical Oncology 1995;3(6):542-549.

[252] Young SL, Murphy M, Zhu XW, Harnden P, O'Donnell MA, James K, Patel PM, Selby PJ, Jackson AM. Cytokine-modified Mycobacterium smegmatis as a novel anticancer immunotherapy. International Journal of Cancer 2004;112(4):653-660.

[253] Haley JL, Young DG, Alexandroff A, James K, Jackson AM. Enhancing the immunotherapeutic potential of mycobacteria by transfection with tumour necrosis factor-alpha. Immunology 1999;96(1):114-121.