Chapter 15

Mycobacteria-Derived Agents for the Treatment of Urological and Renal Cancers

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Additional information is available at the end of the chapter

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Abstract

Mycobacteria are the unique group of bacteria that are currently used in antitumoral immunotherapy. Specifically, intravesical instillation of viable cells of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), after transurethral resection of non-muscle invasive bladder cancer, is the most efficacious treatment for avoiding recurrence and progression of the disease. BCG has been used for the last 35 years for bladder cancer treatment, but other mycobacteria or mycobacteria components are currently under preclinical and clinical studies for the immunotherapeutic treatment of non-invasive bladder cancer and also of other types of tumors located at the urinary system. Those are, for instance, cell wall extracts or heat-killed forms from BCG or other mycobacteria such as *Mycobacterium phlei* or *Mycobacterium indicus pranii* (MIP) or even viable cells from non-pathogenic mycobacteria such us *Mycobacterium brumae*. A review of the literature in which mycobacteria components, non-viable mycobacteria, and viable mycobacteria have been used for these different cancers will be performed. In this chapter, the function of mycobacteria as antitumor agents will be then analyzed, awarding the audience a broad knowledge of one of the beneficial applications of mycobacteria, which are usually introduced as dangerous microorganisms.

Keywords: BCG, bladder cancer, immunotherapy, *Mycobacterium brumae*, *Mycobacterium phlei*

1. Introduction

Since Science journal chose cancer immunotherapy as the breakthrough of the year in 2013, with only a few evidence of its efficacy and consequences, this field is now in fashion [1]. Immunotherapies follow extremely diverse strategies, and the only point they have in common is that all of them activate somehow the cancer patient's immune system to attack tumor

cells. In fact, tumor cells are supposed to be recognized by the immune system as foreign, however, in the cases in which cancer progresses because the tumor and the immune system reach equilibrium that drives to immunotolerance. Immunotherapies include antibodies against tumor epitopes, cytokines, checkpoint inhibitors that break the equilibrium, oncolytic virus, T cell therapy using T cells removed from the patient and modified with chimeric antigen receptors (CARs), and finally, therapeutic vaccines and adjuvants which are the most ancient immunotherapies that exist.

1.1. Prechemotherapeutic era: the first association between mycobacteria and cancer

The first thoughts about a possible intervention of the immune system in the clearance of tumors were made at the beginning of the nineteenth century (**Figure 1**). It was observed that in cancer patients who underwent a gas gangrene, caused by *Clostridium*, their tumors regressed [2]. Later three different physicians independently made the same observations: first Busch in 1868, then Fehleisen in 1882, and, finally, Coley in 1891 observed tumor shrinkage in patients suffering from erysipelas. From their observations, all three had the same idea: exposing their cancer patients to the infectious agent. At that time, Busch ignored that erysipelas was an infectious disease caused by *Streptococcus pyogenes* (Fehleisen would describe this later [3]) which resulted in the death of his first patient due to the infection despite the

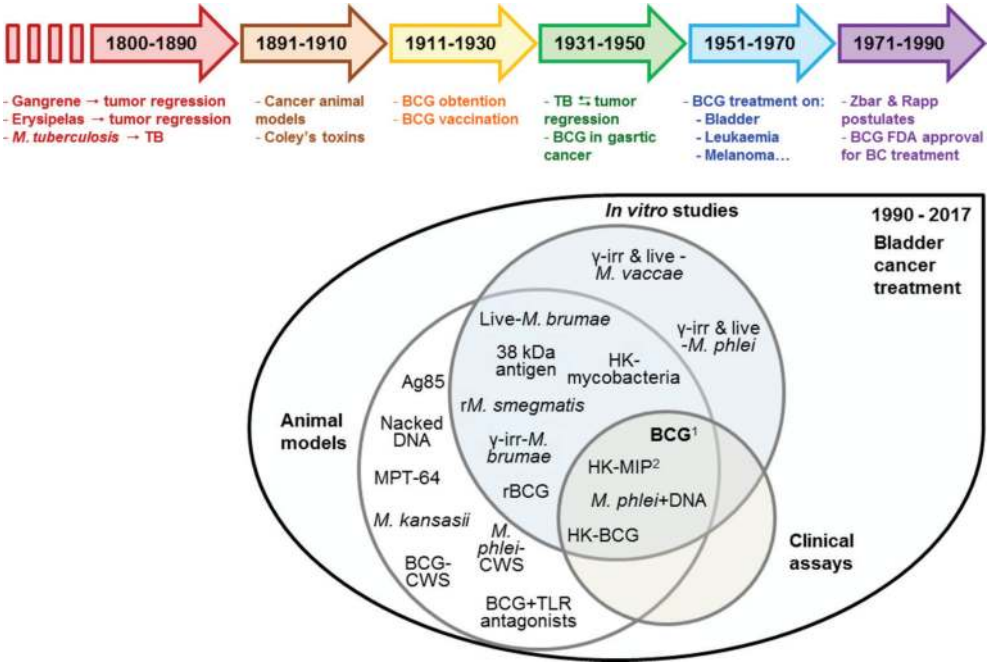

Figure 1. Time course of the development of BCG immunotherapy and studies using mycobacteria-derived agents carried out for BC treatment. [1]Alone or in combination with chemotherapy. [2]In combination with chemotherapy and radiotherapy.

regression of the tumor. Coley was the first one who systematically exposed their patients to infectious agents; he treated more than 1000 patients. The combination of the bacterial products of *S. pyogenes* and *Serratia marcescens* became the well-known Coley's toxins [3]. Although the efficacy of Coley's toxins became later controversial, nowadays it is correctly considered based on an immunotherapeutic effect [4]. Another relevant scientist who extensively contributed to cancer understanding and cure through immunotherapy was Clowes, who systematized the methodology to monitor tumor size in transplantable animal cancer models. He dedicated part of his life in seeking the immunomodulating agent responsible for tumor clearance [5]; nevertheless, as all we know, this "magic" antigen has not still been described.

The phenomenon described in the case of erysipelas disease was also observed for tuberculosis (TB) patients. In 1929, Pearl published a large series of studies describing the inverse relationship between patients suffering from cancer and TB based on the evaluation of hundreds of autopsies [6]. Almost in parallel, 8 years before the publication of Pearl's studies, the first girl was being administered with three doses of an attenuated strain of *Mycobacterium bovis* during the first week of her life. After the discovery of *Mycobacterium tuberculosis* as the causative agent of TB in humans by Koch, scientists raced to find a vaccine against this big killer. Nocard isolated the highly virulent species *M. bovis*, and in 1904, he transferred the strain to Albert Calmette and Camille Guérin who obtained the attenuated strain by subculturing *M. bovis* over bile-potato medium every 3 weeks for 13 years. They demonstrated the avirulence without reversion in a guinea pig model, and the strain received the name of *M. bovis* Bacillus Calmette-Guérin (BCG) [7]. In 1932, the BCG was approved as safe vaccine against TB.

Therefore, studies on the use of BCG for cancer treatment started right after, and in 1935, Holmgren was the first scientist to report success in cancer patients [8]. As a continuation of the experiments he had begun in 1913 in which he evaluated the sensitivity to tuberculin in more than 600 gastric cancer patients, he intravenously injected repeated BCG doses in 28 cancer patients, most of them were gastric cancer patients [8]. In the 1950s, Rosental observed specifically lower incidence of leukemia in people who had received BCG at birth [9]. However, once the patient developed the tumor, there was no chance to previously immunize the patient, so the key point was to know whether the BCG had a curative role [10]. In the following years, many authors proved the efficacy of BCG in several cancer animal models including the bladder [11]. However, the spreading of the modern chemotherapy and radiotherapy for the treatment of cancer weakened the investigations on BCG as tumor treatment.

1.2. Post-chemotherapeutic era: consolidation studies

During the 1970–1980s, hundreds of articles were published in the field of immunotherapy using of BGC or BCG components for the treatment of cancer following different strategies. First, as was done until that moment, BCG was directly used intratumorally, and many studies in different cancers during these decades continued in this line of investigation. Following another strategy, some other scientists assayed the antitumoral effect of BCG as adjuvant in patients undergoing chemotherapy or radiotherapy for the treatment of lung [12], melanoma

[13], cervical [12], head and neck [12], or ovarian cancers [14], for instance. Another completely different strategy was the use of BCG as an adjuvant administering it together with tumor cell lysates [15]. In that period there were some authors who evaluated the antitumor efficacy of some mycobacterial fractions or other mycobacterium species in different cancer models, for instance, of hepatoma or sarcoma [16].

In 1974, Zbar and Rapp established the favorable conditions for obtaining a positive outcome using a guinea pig model [17]. They determined the amount of bacilli that should be administered, that it was a mandatory a close contact between BCG and the tumor, and that BCG worked better against small tumors and immunocompetency of the host which permit to mount an immune response against the BCG [17]. Thanks to this postulates, in 1976, the urologist Morales and collaborators published the results of a successful small clinical trial in which they evaluated the intravesical administration of BCG in bladder cancer (BC) patients. It was not until 1990 that BCG was finally approved by the Food and Drug Administration (FDA) for the treatment of superficial BCG (**Figure 1**).

2. Mycobacteria as antitumor agents in the last 25 years

Although more than a century of research led finally only to the standard use of live BCG for BC treatment, the research trying to use mycobacteria components for cancer treatment has not been abandoned. In fact, in the last 25 years, several attempts for using different mycobacteria as immunotherapeutic agents for bladder cancer treatment have been carried out (**Figure 1**).

Despite the molecule or molecules of BCG responsible for its antitumor effect are still unknown, several genus-specific antigens have been described in mycobacteria cells, and most of them are known stimulators of the immune system. Specifically, the mycobacteria cell wall is rich in a variety of exclusive lipids, glycolipids, lipoproteins, glycans, and proteins that are recognized by immune receptors. Zlotta et al. [18] demonstrated that not only mycobacterial cell wall components are responsible for the antitumor effect but other fractions also triggered the production of Th1 cytokines and stimulated the cytotoxic activity against T24 BC cells by peripheral blood cells. Antigens from different cell fractions are recognized by surface-located receptors present in antigen-presenting cells.

Molecules such as lipoarabinomannan (LAM), phosphatidylinositol mannosides, or heat-shock proteins (HSP) are recognized by Toll-like receptors (TLR) 2 and 4 or mannose receptors; or antigens like trehalose mono- and dimycolate are agonist of C-type lectin receptors. Other mycobacteria antigens interact to specific intracellular immune receptors after being internalized to the cell, such as unmethylated cytosine-guanosine nucleotide (CpG)-rich DNA motifs, muramyl dipeptide (MDP), or cytosolic DNA which bind to TLR-9, NOD2, or cyclic GMP-AMP synthase (cGAS), respectively. After being processed inside the cells, some antigens are presented to T cells via CD1 receptors such as mycolic acids (MA) or trehalose and glucose mycolates. Signaling through these receptors can induce the production of cytokines and/or chemokines favoring a desirable pro-inflammatory profile in tumor microenvironment [19].

Nevertheless, not all mycobacteria possess all the mentioned antigens, and even the structure of each antigen can vary between different species. Although some of these molecules such as LAM, trehalose dimycolate (TDM), or MA are present in all mycobacteria, the structure, for example, the presence or not of mannose residues in LAM structure, the length or the presence of unsaturations or oxygenated groups in the lipidic chains of TDM or MA, etc., determine the interaction to the corresponding immune receptor, and in consequence, the immune response is generated. The complexity of mycobacteria antigenicity is enormous leading even to the fact that different strains of the same species can have different antigenic pattern. The case of the antigenic profile of BCG is a good example. As it is known, from the seed strain originated in France by Albert Calmette and Camille Guérin, different strains were originated after subculturing the original BCG in different countries. Before being preserved by freezing, decades of subculturing originate deletions of some genome regions or even the duplication of some other genome regions. Today, we count about a decennium of different BCG strains [20] that are used broadly for BC treatment as well as for TB vaccination. Each different BCG strain possess or not some immunogenic antigens like phenolglycolipids, phthiocerol dimycocerosates, and MBT64 protein antigen, or even they have two different MA profiles: some strains possess alpha, methoxy- and keto-MA, while some other possesses only alpha- and methoxy-MA. The relevance of these differences has tried to be related to BCG efficacy as antitumor agent or TB vaccine, but until nowadays the critical antigens are still not known.

In the following sections, the use of different mycobacteria or their components in the last 25 years for urinary tract cancers will be reviewed. We will mainly focus on the use of these components as unique antitumor agents, although the inclusion of studies in which mycobacterial antigens are used as adjuvants for tumor antigens or other therapies will also be mentioned.

3. Urinary tract cancers and mycobacteria

3.1. Bladder cancer and live BCG: a fruitful relationship

The immunotherapeutic ability of mycobacteria against cancer has the most successful example in the case of the use of BCG for the treatment of high-risk non-muscle invasive bladder cancer (NMIBC) patients. All the conditions described by Zbar and Rapp are accomplished in intravesical BC treatment: a close cavity where mycobacteria can be loaded and being in close contact to tumor cells triggering also an immune response [17]. BC is one of the most common malignances in urology. The number of new cases and deaths of BC was 20.1 and 4.4 per 100,000 men and women per year, being in 2013 estimated 587,426 people living with BC in the USA [21]. In 2016, 76,960 new cases of BC were estimated, and around 70% of them present as NMIBC. Whether NMIBC patients are not treated after transurethral resection (TUR) of tumor, as much as 80% will experience disease recurrence and/or progression. Therefore, after TUR, the standard treatment for high-risk NMIBC patients consists in the intravesical instillation of live BCG. The bladder cavity allows a closed contact between the possible remaining tumor cells and the bacilli. For a short period of time (approximately 2 h), a high concentration of microorganisms (between 10^7 and 10^9 bacilli depending on the commercially

available preparations of BCG strains), besides being in contact to possible remaining tumor cells, initiates an immune cascade of events. Although the detailed chronogram and magnitude of these events are not totally understood, numerous studies have provided information about the immune cells and signals implicated in the action of BCG. Several excellent reviews have covered this field, explaining what is known until today, see [22, 23]. In summary, BCG firstly interacts with remaining bladder tumor cells inducing apoptosis and/or cell cycle arrest as demonstrated in *in vitro* experiments [24–26]. Moreover, internalization of the mycobacteria triggers the production of cytokines and chemokines [27] that work as initial signals for the host immune system, leading to the infiltration of multiple immune cells into the bladder lumen, as observed in in vivo models of the disease [28, 29] as well as in BCG-treated patients [27]. Although the role of each infiltrated immune subsets—T cells, B cells, natural killer (NK) cells, macrophages, γδ cells, etc.—is still unknown, their presence is critical for an appropriate antitumor response [30]. This BCG-triggered immune response is finally able to fight against the presence of new tumors. BCG significantly reduces the risk of recurrence in treated patients, compared to NMIBC patients to whom only TUR is applied [31]. Moreover, in a recent meta-analysis of the literature, BCG has been confirmed as the only agent associated with decreased progression risk versus TUR alone [32].

Intravesical BCG therapy is then successful. In fact, the same protocol of instillations has been used in high-risk NMIBC patients for the last 30 years. But, although efficacious, BCG is not the perfect treatment. On the one hand, a percentage of patients do not respond to BCG for unknown reasons. On the other hand, a high percentage of patients suffer adverse events during the treatment.

3.2. Increasing efficacy

Several strategies are tried to solve the problem of unresponsive BCG patients. The main strategy consists of improving the immune response triggered by BCG, by combining BCG and immunomodulators or modifying genetically the bacterium for expressing these immunomodulators. For instance, BCG plus an optimized interleukin (IL)-15 mutant significantly increased immune activation and reduced tumor burden and angiogenesis compared to the single agents in the carcinogen-induced rat NMIBC model [33]. The list of modified BCGs is long and comprises the expression of cytokines and chemokines—IL-2, interferon (IFN)-γ, GM-CSF, etc.—or immunodominant mycobacteria antigens like alpha-crystallin antigen (fibronectin-binding protein) complex (Ag85) [9, 22, 34]. These strategies seem to be promising for improving the efficacy of BCG alone as *in vitro* and *in vivo* experiments using animal models of the disease have shown. In that way, intravesical instillation of recombinant BCG strain expressing the fusion protein of IL-15 and Ag85B (BCG-IL-15) in tumor-bearing mice leads to a high neutrophil infiltration, increased presence of chemokines into the bladder, and prolonged survival rates compared to mice treated with BCG alone [35]. Recombinant BCG expressing human interferon-alpha 2b (hIFNα-2b) showed higher antiproliferative capacity on EJ BC cells after infection compared to wild-type BCG. Furthermore BCG-hIFNα-2b-stimulated lymphocytes trigger the production of higher levels of IFN-γ, tumor necrosis factor (TNF)-α, and IL-12, together with higher cytotoxic activity against BC cells, compared to those treated with BCG [36].

The study of the immune response triggered by BCG inside the bladder has revealed that an excess of IL-10 production in tumor microenvironment is detrimental for BCG efficacy. Another successful strategy is then the use of anti-IL-10 antibodies in combination to BCG for an improved effect. To block IL-10 receptor together with intravesical BCG reach high tumor regression rates in the murine orthotopic model of BC, enhancing also a systemic specific antitumor immune response compared to BCG alone [37].

Another strategy is the combination of BCG with TLR agonists. BCG together with TLR4 agonist such as polyporus polysaccharide triggers the expression of activation molecules like CD80 and CD40. In rat BC models, this combination therapy showed a synergic effect reducing invasiveness of cancer together with reduced adverse events originated by BCG alone [38]. Similarly BCG plus a TLR3 agonist (polyinosinic:polycytidylic, acid poly(I:C)) showed to be more efficacious on reducing MBT-2 bladder tumor growth in treated mice than the monotherapies [39].

Finally, it has been also demonstrated that the combination of live BCG together with chemotherapeutic treatments improves disease survival compared to BCG alone. Although some works showed no impact on the progression or survival rates of the patients [16, 17], recent study has demonstrated the beneficial effect of sequential intravesical treatment with BCG and mitomycin C. While in mice experiments an augmentation of beneficial M1 tumor-associated macrophages on tumor-bearing treated mice was observed, in treated patients increasing IL-2, IL-8, IL-10, and TNF-α urine levels during treatment and increased efficacy over BCG treatment alone were observed [40].

3.3. Reducing adverse events

The most critical adverse event regarding intravesical instillations of BCG is the possibility of the patient to be infected. Numerous cases of BCGosis have been described in the literature in the last 5 years [41, 42]. The main reason is a traumatic instillation that can lead to the dissemination of BCG throughout the systemic circulation. As soon as BCG showed to be an excellent option for BC treatment, researchers tried to compare its effect to non-viable mycobacteria, cell wall extracts, or even purified antigens from mycobacteria.

3.3.1. Purified mycobacteria antigens and non-viable mycobacteria

The majority of mycobacteria antigens, which are able to stimulate the immune system, have been widely studied in order to find epitopes for vaccines to prevent TB infection and also to develop immunodiagnostic tests for TB infection or disease. Researchers have taken advantage of the knowledge of these molecules for trying to use them as antitumor agents for BC. In this line of research, MPT-64 antigen, 38 kDa protein, Ag85 antigen, or mycobacterial DNA have been evaluated for their ability to treat BC. For MPT-64 a dose-dependent response in survival rates was observed when instilled intravesically. The higher MPT-64 dose administrated provided higher survival rates in tumor-bearing treated mice than non-treated mice, triggering also a favorable IFN-γ systemic response [43]. When the 38 kDa antigen was studied, a cytotoxic activity against T24 BC cells was observed in 38 kDa antigen-activated peripheral blood mononuclear cells (PBMC), also in a dose-dependent

manner. Again, 38 kDa antigen-intravesically treated tumor-bearing mice survived longer than non-treated tumor-bearing mice [44], triggering a systemic response observed when splenocytes from treated mice respond specifically to the instilled antigen. Different works have evaluated the antitumor capacity of Ag85. Initially, the generation of cytolytic CD8 and an antitumor response was observed when cDNA from *M. kansasii* Ag85 antigen was transferred to MBT-2 BC cells and injected into mice [45]. Dendritic cells (DC) expressing Ag85 have also shown to activate T cells triggering them to be cytotoxic to bladder tumors [46]. Ag85-dendritic cells induce the infiltration of T cells into tumor site triggering also the reduction of BC tumors in in vivo experiments [46]. Finally, mycobacterial DNA, specifically from BCG and from *Mycobacterium phlei*, has proven to have antitumor activity. DNA from BCG activates NK and triggers the production of IFN-α, IFN-β, and IFN-γ in splenocytes [47–49]. DNA from *M. phlei* is the responsible for the induction of cytokine release by monocytes and macrophages and to inhibit BC-cell proliferation by triggering apoptosis [50, 51].

Apart from purified antigens, complex extracts of mycobacteria have also been evaluated for BC treatment. The two principal assayed compositions have been a cell wall extract from BCG (composed by the cell wall skeleton or also called SPM-105) [52] and a mixture of cell wall plus DNA of *M. phlei* (MCNA) mentioned above. The main problem to work using cell wall preparations is the huge hydrophobic character of the mycobacteria cell wall. Thus, in both cases, although early *in vitro* studies showed promising results [51, 53], a reformulation of the cell wall extract was needed in order to optimize the solubility and stability of the preparation and facilitate the contact with target cells.

On the one hand, the BCG-cell wall skeleton (CWS) has been formulated into liposomes in which their surface was modified by an octaarginine (R8) anchor, an efficient cell-penetrating peptide [54–56]. Researchers demonstrated that R8-liposome-BCG-CWS binds to MBT-2 murine BC cells inhibiting its growth in a syngeneic subcutaneous tumor model [57], being also efficacious in an intravesical BC rat model of the disease [58]. Furthermore, the formulated extract is able to inhibit human BC growth *in vitro* [55], activated immune cells to a Th1 profile, and leads to their cytotoxic activity against T24 and RT112 BC tumor cells [58].

On the other hand, *M. phlei* cell wall extract was initially formulated in mineral oil-in-water emulsion to be effective. After showing to induce BC apoptosis *in vitro*, the first clinical trial demonstrated a similar rate of response in NMIBC patients who previously were refractory to BCG treatment and those without previous BCG treatment, indicating a possible role of *M. phlei* as a second-line drug after BCG failure [59]. From the initial composition evaluated, the formulation was improved by considering the inclusion of *M. phlei* DNA that, as previously explained, showed antitumor effect [50, 60]. The new composition, denominated MCNA, triggers both a direct effect by inducing apoptosis on cancer cells and an indirect effect by triggering an immune response [60]. MCNA was therefore evaluated in phase II and phase III clinical trials [61]. Both studies had some drawbacks: the number of patients finally considered was relatively small and, among them, a small percentage (around 2%) of patients showed serious adverse events, and around 60–70% of patients in each study showed mild drug-related adverse events. In the phase III study, there was no signal of cancer in 25% of patients after 1 year and in 19% of patients after 2 years. Thus, MCNA could be a therapeutic option for patients in which the only therapeutic option is cystectomy after being refractory to BCG treatment, although further studies are needed [62].

Finally, whole non-viable mycobacteria have also been evaluated for BC treatment. Contrary to purified antigens or cell extracts, the use of the whole bacteria warrants the presence of the whole antigenic profile, but depending on the inactivation method, some of the possible crucial antigens can be altered or lost. On the one hand, heat-killing form has been the most studied although mycobacteria cells are damaged [63, 64], and on the other hand, γ-irradiation is the treatment which preserves better the integrity of the mycobacteria cell and maintains some metabolic activity [63].

Regarding *in vitro* BC cell growth inhibition, works using T24, J82, RT4, RT112, EJ28, or HT1376 demonstrated that both heat-killed (HK) and γ-irradiated BCG, *M. brumae*, *M. vaccae*, or *M. phlei,* or HK MIP, inhibit BC proliferation similarly to live mycobacteria [63, 65–67], while other works found less growth inhibition of 253J and T24 cells significantly when HK BCG was used [68]. Unlike the observations made by the above mentioned authors, other authors found that HK BCG had no inhibitory effect in MGH BC cells [69]. Moreover, lower cytokine levels were produced by HK and γ-irradiated mycobacteria-infected cells than those infected by live bacteria [63, 69]. Between both treatments, γ-irradiated mycobacteria trigger cytokine levels closer to those induced by live mycobacteria, than those induced by HK mycobacteria. Regarding immune activation: induction of cytotoxicity by activated cells, induction of cytokine production, and expression of activation markers on immune cells, again the immune response induced by live mycobacteria was higher than that triggered by non-viable mycobacteria [63, 66, 70, 71].

Using the orthotopic murine intravesical model of BC, HK BCG-treated mice survived similarly to non-treated tumor-beating mice [72], together with lower production levels of Th1 cytokines [72] that has also been related to inability to trigger T-cell infiltration into bladder cavity [28]. Interestingly, when live BCG or *M. brumae* was instilled in the first week of treatment and subsequent instillations were done using γ-irradiated mycobacteria, significant prolonged survival rates were observed in tumor-bearing mice compared to untreated mice [71].

In BC patients, HK BCG instilled to previously live BCG nonresponders showed reduced toxicity and no increase in the risk of tumor recurrence [73]. Whole HK MIP has been instilled intravesically in five BC patients undergoing radiation therapy [74], maintaining 100% survival rates and recurrence-free rates. It has been also instilled in the treatment of BCG-refractory patients [75].

3.3.2. Live non-BCG mycobacteria

Concluding for the whole work compiled in the literature, it seems that live BCG provides the best option compared to the non-viable mycobacteria or mycobacteria fractions. Therefore, another feasible option as alternative to BCG for BC treatment is to consider the use of live non-pathogenic mycobacteria. However, few studies have considered them for BC treatment. As explained above, the majority of mycobacterium species is saprophytic and potentially share immunomodulatory antigens with BCG.

In a recent work of our research group [76], several non-pathogenic-considered mycobacteria (*M. confluentis*, *M. chitae*, *M. chubuense*, *M. fallax*, *M. gastri*, *M. hiberniae*, *M. mageritense*, *M. obuense*, *M. phlei*, and two strains of *M. vaccae*) were evaluated for their capacity to inhibit BC cell proliferation *in vitro*, compared to the effect of BCG Connaught. Among all the species

studied, *M. brumae* effect highlighted over the rest of results. Among the mycobacteria tested on 7 BC cell lines (T24, J82, RT112, SW780, HG-MG3, 5637, and RT4, belonging to different grades), *M. brumae* stood out for inhibiting BC-cell proliferation at a similar extent to BCG in high-grade cell lines and for showing an improved effect than BCG in low-grade cell lines. *M. brumae* triggered the expression of activation markers on macrophages at higher degree than *M. phlei* or *M. vaccae* did. Moreover, *M. brumae* was able to activate human PBMC *ex vivo* to kill BC cells by both direct contact and using only the soluble factors released by the activated PBMC. *M. brumae* also was able to activate a murine macrophage cell line [66, 76]. Using the murine orthotopic model of BC, a high percentage of live *M. brumae*-treated mice survived compared to BCG-treated and non-treated tumor-bearing mice, being the differences significant only in comparison to non-treated-bearing mice [76, 77].

In vitro experiments have demonstrated that *M. brumae* cells did not persist alive inside both macrophages and BC cells after 72–96 h after infection [76, 77]. Besides *in vivo* experiments in the murine BC model showed that *M. brumae* do not persist neither in the spleen of mice treated intravesically with *M. brumae*, contrary to BCG, which is found in splenocytes after finishing the intravesical treatment [76, 77]. Nevertheless further security studies are needed to confirm the safety of this mycobacterium when it is used in its live form.

Apart from *M. brumae*, only *M. vaccae*, *M. smegmatis*, and *M. kansasii* have been studied in their live forms for BC treatment. According to our results [76], Baran and collaborators show that any of the three different strains of *M. vaccae* they studied was able to inhibit BC proliferation *in vitro* in a better way than BCG [78]. Regarding *M. smegmatis*, a recombinant strain expressing human TNF-α was studied. This strain inhibited EJ18, MGH-U1, RT4, and RT112 human BC-cell growth and triggered higher release of cytokines than the parental strain [79]. Later, inoculated in the heterotopic syngeneic mouse model, TNF-α-expressing *M. smegmatis* induced higher survival rates than the parental strain or BCG [80]. The authors also demonstrated a systemic immune response obtaining higher IFN-α levels in mycobacteria-stimulated spleen cultures from TNF-α-*M. smegmatis*-treated mice. Finally, only one study showed that live *M. kansasii* triggers higher tumor reduction in the orthotopic murine BC model than a range of BC strains [81].

Although antitumor effect of mycobacteria is not considered for muscle-invasive BC, MIP plus radiation therapy in a small number of patients (5) showed disease-free survival more than 2 years [74].

3.4. Other renal and urinary tract cancers

Immunotherapy using mycobacteria components has been also applied in urinary tract cancers other than NMIBC.

For prostatic cancer, studies using BCG, *M. phlei* cell wall extract, MCC (a previous version of MCNA), SLR-172, and HSP65 have been carried out. Intravenous injection of BCG, but not subcutaneous injection, demonstrated to avoid metastasis of prostate adenocarcinoma (PA-III) cells in rats [82]. Intraperitoneal injection of BCG, however, avoids only propagation of PA-III cells in the peritoneal cavity. Thus, BCG and tumor cells have to be in the same compartment to prevent systemic propagation of the tumor [83]. Both live-attenuated BCG and

SRL172 plus autologous whole tumor cell vaccination were effective in the prevention of MAT-LyLu tumors and increase survival rates in the rat model of prostate cancer. SRL172 alone did not provide any effect [84]. In this way, regular vaccination with tumor cells plus SRL172 in hormone-refractory prostate cancer patients demonstrated to be safe and induce cytokine production, specific antibody levels, and evidence of T-cell proliferation in response to the vaccinations [85]. Regular intradermal infection of only SRL172 in patients with advanced hormone-refractory prostate cancer demonstrated its safety and the ability to modulate cytokine changes in these patients. Serum PSA diminished in 5 out of 10 patients, and an increase in IL-2 secreting PBMC was also shown [86]. Finally, MCC also showed an antitumor effect on prostatic cancer. *In vitro*, MCC induced both a dose-dependent proliferation reduction of LNCaP prostate cancer cells and the production of IL-12 and GM-CSF [87]. When administrated in the Dunning R3327-H adenocarcinoma of the prostate in rats, no effect was seen after intraperitoneal administration, but up to 50% of animals showed complete tumor regression after intratumor administration in small-volume tumors [88]. Finally, immunization with a recombinant GnRH vaccine conjugated to *M. bovis* HSP65 prolonged significantly survival, triggering suppression of local tumor growth, strong lymphocyte proliferative responses, and high IFN-γ levels in the orthotopic prostate cancer mouse model [89].

In renal cancer, SRL172 has been administrated in patients suffering from metastasis demonstrating low toxicity and similar survival rates compared to patient treatment with cytokines (IL-2 or IFN-α) [82] and increased rates when synergized both treatments: SRL172 and antibodies [90]. In stage IV renal cell cancer, patients treated with BCG plus irradiated autologous tumor cells, and later infused with autologous activated T cells, showed durable tumor responses [91].

Few studies have evaluated the efficacy of BCG in the treatment of upper tract urothelial cancer, but the conclusions driven from them indicate that there is no role for BCG in these cases [92, 93].

4. Future perspectives: a long history with room for improvement

The potential beneficial effect of mycobacteria as antitumor agents has been clearly demonstrated after almost a century of observations and experimentation. But even in the case of BCG treatment for NMIBC patients, many issues remain under question: the appropriate schedule to reduce recurrence and progression without increasing adverse events [94–96]; the reason why a proportion of patients do not respond to BCG treatment; the detail description of immune mechanism which could permit to predict the response to the treatment; the possibility of using other mycobacteria species that have shown similar or increased effective than BCG but with potential increased safety; etc. In fact, the experience using mycobacteria in BC is permitting novel approaches for improving its efficacy. In this sense, a critical point for mycobacteria efficacy is the delivery of mycobacteria or mycobacteria antigens into the tumor site. The optimization of mycobacteria formulation could be critical for reducing adverse-associated events [97] and/or improving mycobacteria antitumor efficacy [77]. Moreover, the possibility to manipulate genetically mycobacteria for being vehicle for delivery antigens could lead also a chance to get more potent antitumor tools.

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References

[1] Couzin-Frankel J. Cancer immunotherapy. Science. 2013;**342**:1432-1433. DOI: 10.1007/978-1-4614-4732-0

[2] Stephen S. Hall. A Commotion in the Blood: Life, death, and the immune system. Henry Holt. New York. 1997; ISBN: 0-8050-3796-9

[3] Oelschlaeger TA. Bacteria as tumor therapeutics? Bioengineered Bugs. 2010;**1**:146-147. DOI: 10.4161/bbug.1.2.11248

[4] McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. The Iowa Orthopaedic Journal. 2006;**26**:154-158

[5] Krahl ME. George Henry Alexander Clowes 1877-1958. Cancer Research. 1959;**19**

[6] Pearl R. Cancer and tuberculosis. American Journal of Hygiene. 1929;**9**:97-159

[7] Gandhi NM, Morales A, Lamm DL. Bacillus Calmette-Guérin immunotherapy for genitourinary cancer. BJU International. 2013;**112**:288-297

[8] Holmgren I. Employment of BCG especially in intravenous injection. Acta Medica Scandinavica. 1936;**90**:350-361. DOI: 10.1111/j.0954-6820.1936.tb15958.x

[9] Begnini KR, Buss JH, Collares T, Seixas FK. Recombinant *Mycobacterium bovis* BCG for immunotherapy in nonmuscle invasive bladder cancer. Applied Microbiology and Biotechnology. 2015;**99**:3741-3754. DOI: 10.1007/s00253-015-6495-3

[10] Mathé G, Pouillart P, Lapeyraque F. Active immunotherapy of L1210 leukaemia applied after the graft of tumour cells. British Journal of Cancer. 1969;**23**:814-824

[11] Old LJ, Clarke DA, Benacerraf B. Effect of BCG infection on transplanted tumours in the mouse. Nature. 1959;**184**:291-292. DOI: 10.1038/184291a0

[12] Olkowski ZL, McLaren JR, Skeen MJ. Effects of combined immunotherapy with levamisole and Bacillus Calmette-Guérin on immunocompetence of patients with squamous cell carcinoma of the cervix, head and neck, and lung undergoing radiation therapy. Cancer Treatment Reports. 1978;**62**:1651-1661

[13] MacGregor AB, Falk RE, Landi S, Ambus U, Langer B. Oral bacille Calmette Guérin immunostimulation in malignant melanoma. Surgery, Gynecology & Obstetrics. 1975;**141**:747-754

[14] Alberts DS, Mason-Liddil N, O'Toole RV, Abbott TM, Kronmal R, Hilgers RD, Surwit EA, Eyre HJ, Baker LH. Randomized phase III trial of chemoimmunotherapy in patients with previously untreated stages III and IV suboptimal disease ovarian cancer: A SOGS. Gynecologic Oncology. 1989;**32**:8-15

[15] Simmons RL, Rios A. Immunotherapy of cancer: Immunospecific rejection of tumors in recipients of neuraminidase-treated tumor cells plus BCG. Science. 1971;**174**:591-593

[16] Yarkoni E, Rapp HJ. Immunotherapy of experimental cancer by intralesional injection of emulsified nonliving mycobacteria: comparison of Mycobacterium bovis (BCG), Mycobacterium phlei, and Mycobacterium smegmatis. Infection and Immunity. 1980;**28**:887-892

[17] Zbar B, Rapp HJ. Immunotherapy of guinea pig cancer with BCG. Cancer. 1974;**34**:1532-1540. DOI: 10.1002/1097-0142(197410)34:8

[18] Zlotta AR, Van Vooren, Denis O, et al. What are the immunologically active components of BCG in therapy of superficial bladder cancer? International Journal of Cancer. 2000;**87**:844-852

[19] LaRue H, Ayari C, Bergeron A, Fradet Y. Toll-like receptors in urothelial cells—Targets for cancer immunotherapy. Nature Reviews. Urology. 2013;**10**:537-545. DOI: 10.1038/nrurol.2013.153

[20] Chen JM, Islam ST, Ren H, Liu J. Differential productions of lipid virulence factors among BCG vaccine strains and implications on BCG safety. Vaccine. 2007;**25**:8114-8122. DOI: 10.1016/j.vaccine.2007.09.041

[21] SEER Stat Fact Sheets: Bladder Cancer, (n.d.). http://seer.cancer.gov/statfacts/html/urinb.html [Accessed: January 11, 2017]

[22] Yi Luo, Eric J. Askeland, Mark R. Newton, Jonathan R. Henning and Michael A. O'Donnell. Immunotherapy of Urinary Bladder Carcinoma: BCG and Beyond. Prof. Letícia Rangel (Ed.). Cancer Treatment - Conventional and Innovative Approaches. InTech Open; 2013. DOI: 10.5772/55283. https://www.intechopen.com/books/cancer-treatment-conventional-and-innovative-approaches/immunotherapy-of-urinary-bladder-carcinoma-bcg-and-beyond

[23] Fuge O, Vasdev N, Allchorne P, Green JS. Immunotherapy for bladder cancer. Research and Reports in Urology. 2015;**7**:65-79. DOI: 10.2147/RRU.S63447

[24] Schwarzer K, Foerster M, Steiner T, Hermann I-M, Straube E. BCG strain S4-Jena: An early BCG strain is capable to reduce the proliferation of bladder cancer cells by induction of apoptosis. Cancer Cell International. 2010;**10**:21. DOI: 10.1186/1475-2867-10-21

[25] 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. DOI: 10.1186/1471-2490-5-8

[26] See WA, Zhang G, Chen F, Cao Y. p21 expression by human urothelial carcinoma cells modulates the phenotypic response to BCG. Urologic Oncology: Seminars and Original Investigations. 2010;**28**:526-533. DOI: 10.1016/j.urolonc.2008.12.023

[27] 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**:370-378. DOI: 10.1111/j.1365-2249.2006.03288.x

[28] Biot C, Rentsch CA, Gsponer JR, et al. Preexisting BCG-specific T cells improve intravesical immunotherapy for bladder cancer. Science Translational Medicine. 2012;**4**:137ra72. DOI: 10.1126/scitranslmed.3003586

[29] Rentsch CA, Birkhäuser FD, Biot C, et al. BCG strain differences have an impact on clinical outcome in bladder cancer immunotherapy. European Urology. 2014;**66**:677-688. DOI: 10.1016/j.eururo.2014.02.061

[30] 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**:1018-1023

[31] Babjuk M, Burger M, Zigeuner R, et al. EAU guidelines on non-muscle-invasive Urothelial carcinoma of the bladder: Update 2013. European Urology. **64**(2013):639-653. DOI: 10.1016/j.eururo.2013.06.003

[32] Chou R, Selph S, Buckley DI, Fu R, Griffin JC, Grusing S, Gore JL. Intravesical therapy for the treatment of non-muscle-invasive bladder cancer: A systematic review and meta-analysis. The Journal of Urology. 2016. DOI: 10.1016/j.juro.2016.12.090

[33] Gomes-Giacoia E, Miyake M, Goodison S, et al. Intravesical ALT-803 and BCG treatment reduces tumor burden in a carcinogen induced bladder cancer rat model; a role for cytokine production and NK cell expansion. PLoS One. 2014;**9**. DOI: 10.1371/journal.pone.0096705

[34] Begnini KR, Rizzi C, Campos VF, et al. Auxotrophic recombinant Mycobacterium bovis BCG overexpressing Ag85B enhances cytotoxicity on superficial bladder cancer cells in vitro. Applied Microbiology and Biotechnology. 2013;**97**:1543-1552. DOI: 10.1007/s00253-012-4416-2

[35] Takeuchi A, Eto M, Tatsugami K, et al. Antitumor activity of recombinant Bacille Calmette-Guérin secreting interleukin-15-Ag85B fusion protein against bladder cancer. International Immunopharmacology. 2016;**35**:327-331. DOI: 10.1016/j.intimp.2016.03.007

[36] Sun E, Nian X, Liu C, Fan X, Han R. Construction of recombinant human IFNα-2b BCG and its antitumor effects on bladder cancer cells in vitro. Genetics and Molecular Research. 2015;**14**:3436-3449. DOI: 10.4238/2015.April.15.7

[37] Newton MR, Askeland EJ, Andresen ED, Chehval VA, Wang X, Askeland RW, O'Donnell MA, Luo Y. Anti-interleukin-10R1 monoclonal antibody in combination with BCG is protective against bladder cancer metastasis in a murine orthotopic tumour model and demonstrates systemic specific anti-tumour immunity. Clinical and Experimental Immunology. 2014;**177**:261-268. DOI: 10.1111/cei.12315

[38] Zhang G, Qin G, Han B, Li C, Yang H-G, Nie P, Zeng X. Efficacy of Zhuling polyporus polysaccharide with BCG to inhibit bladder carcinoma. Carbohydrate Polymers. 2015;**118**:30-35. DOI: 10.1016/j.carbpol.2014.11.012

[39] Ayari C, Besançon M, Bergeron A, LaRue H, Bussières V, Fradet Y. Poly(I:C) potentiates Bacillus Calmette–Guérin immunotherapy for bladder cancer. Cancer Immunology, Immunotherapy. 2016;**65**:223-234. DOI: 10.1007/s00262-015-1789-y

[40] Gan C, Amery S, Chatterton K, Khan MS, Thomas K, O'Brien T. Sequential Bacillus Calmette-Guérin/electromotive drug administration of Mitomycin C as the standard intravesical regimen in high risk nonmuscle invasive bladder cancer: 2-Year outcomes. The Journal of Urology. 2016;**195**:1697-1703. DOI: 10.1016/j.juro.2016.01.103

[41] Shimura H, Ihara T, Mitsui T, Takeda M. Tuberculous granuloma in the scrotal skin after intravesical Bacillus Calmette-Guerin therapy for bladder cancer: a case report. Urology Case Reports. 2017;**11**:4-6. DOI: 10.1016/j.eucr.2016.11.001

[42] Kaburaki K, Sugino K, Sekiya M, Takai Y, Shibuya K, Homma S. Miliary tuberculosis that developed after intravesical Bacillus Calmette-Guerin therapy. Internal Medicine. 2017;**56**:1563-1567. DOI: 10.2169/internalmedicine.56.8055

[43] Yu DS, Lee CF, Chang SY. Immunotherapy for orthotopic murine bladder cancer using Bacillus Calmette-Guerin recombinant protein Mpt-64. The Journal of Urology. 2007;**177**:738-742. DOI: 10.1016/j.juro.2006.09.074

[44] Sänger C, Busche A, Bentien G, Spallek R, Jonas F, Böhle A, Singh M, Brandau S. Immunodominant PstS1 antigen of mycobacterium tuberculosis is a potent biological response modifier for the treatment of bladder cancer. BMC Cancer. 2004;**4**:86. DOI: 10.1186/1471-2407-4-86

[45] Kuromatsu I, Matsuo K, Takamura S, Kim G, Takebe Y, Kawamura J, Yasutomi Y. Induction of effective antitumor immune responses in a mouse bladder tumor model by using DNA of an alpha antigen from mycobacteria. Cancer Gene Therapy. 2001;**8**:483-490. DOI: 10.1038/sj.cgt.7700330

[46] Zhang P, Wang J, Wang D, Wang H, Shan F, Chen L, Hou Y, Wang E, Lu CL. Dendritic cell vaccine modified by Ag85A gene enhances anti-tumor immunity against bladder cancer. International Immunopharmacology. 2012;**14**:252-260. DOI: 10.1016/j.intimp.2012.07.014

[47] Tokunaga T, Yamamoto H, Shimada S, Abe H, Fukuda T, Fujisawa Y, Furutani Y, Yano O, Kataoka T, Sudo T. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. Journal of the National Cancer Institute. 1984;**72**:955-962

[48] Shimada S, Yano O, Tokunaga T. In vivo augmentation of natural killer cell activity with a deoxyribonucleic acid fraction of BCG. Japanese Journal of Cancer Research. 1986;**77**:808-816

[49] Shimada S, Yano O, Inoue H, et al. Antitumor activity of the DNA fraction from Mycobacterium bovis BCG. II. Effects on various syngeneic mouse tumors. Journal of the National Cancer Institute. 1985;**74**:681-688

[50] Filion MC, Filion B, Reader S, Menard S, Phillips NC. Modulation of interleukin-12 synthesis by DNA lacking the CpG motif and present in a mycobacterial cell wall complex. Cancer Immunology, Immunotherapy. 2000;**49**:325-334

[51] Filion MC, Lépicier P, Morales A, Phillips NC. Mycobacterium phlei cell wall complex directly induces apoptosis in human bladder cancer cells. British Journal of Cancer. 1999;**79**:229-235. DOI: 10.1038/sj.bjc.6690038

[52] Uenishi Y, Kusunose N, Yano I, Sunagawa M. Isolation and identification of arabinose mycolates of Cell Wall Skeleton (CWS) derived from Mycobacterium bovis BCG Tokyo 172 (SMP-105). Journal of Microbiological Methods. 2010;**80**:302-305. DOI: 10.1016/j.mimet.2010.01.003

[53] Chin JL, Kadhim SA, Batislam E, Karlik SJ, Garcia BM, Nickel JC, Morales A. Mycobacterium cell wall: an alternative to intravesical bacillus Calmette Guerin (BCG) therapy in orthotopic murine bladder cancer. The Journal of Urology. 1996;**156**:1189-1193

[54] Homhuan A, Kogure K, Akaza H, Futaki S, Naka T, Fujita Y, Yano I, Harashima H. New packaging method of mycobacterial cell wall using octaarginine-modified liposomes: Enhanced uptake by and immunostimulatory activity of dendritic cells. Journal of Controlled Release. 2007;**120**:60-69. DOI: 10.1016/j.jconrel.2007.03.017

[55] Nakamura T, Fukiage M, Higuchi M, et al. Nanoparticulation of BCG-CWS for application to bladder cancer therapy. Journal of Controlled Release. 2014;**176**:44-53. DOI: 10.1016/j.jconrel.2013.12.027

[56] Nakamura T, Fukiage M, Suzuki Y, Yano I, Miyazaki J, Nishiyama H, Akaza H, Harashima H. Mechanism responsible for the antitumor effect of BCG-CWS using the LEEL method in a mouse bladder cancer model. Journal of Controlled Release. 2014;**196**:161-167. DOI: 10.1016/j.jconrel.2014.10.007

[57] Joraku A, Homhuan A, Kawai K, Yamamoto T, Miyazaki J, Kogure K, Yano I, Harashima H, Akaza H. Immunoprotection against murine bladder carcinoma by octaarginine-modified liposomes incorporating cell wall of Mycobacterium bovis BCG. BJU International. 2009;**103**:686-693. DOI: 10.1111/j.1464-410X.2008.08235.x

[58] Miyazaki J, Kawai K, Kojima T, et al. The liposome-incorporating cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin can directly enhance the susceptibility of cancer cells to lymphokine-activated killer cells through up-regulation of natural-killer group 2, member D ligands. BJU International. 2011;**108**:1520-1526. DOI: 10.1111/j.1464-410X.2010.10056.x

[59] Morales A, Chin JL, Ramsey EW. Mycobacterial cell wall extract for treatment of carcinoma in situ of the bladder. The Journal of Urology. 2001;**166**:1633-1638

[60] Phillips NC, Filion MC. Therapeutic potential of mycobacterial cell wall-DNA complexes. Expert Opinion on Investigational Drugs. 2001;**10**:2157-2165. DOI: 10.1517/13543784.10.12.2157

[61] Morales A, Herr H, Steinberg G, Given R, Cohen Z, Amrhein J, Kamat AM. Efficacy and safety of MCNA in patients with nonmuscle invasive bladder cancer at high risk for recurrence and progression after failed treatment with Bacillus Calmette-Guérin. The Journal of Urology. 2015;**193**:1135-1143. DOI: 10.1016/j.juro.2014.09.109

[62] Morales A, Cohen Z. *Mycobacterium phlei* cell wall-nucleic acid complex in the treatment of nonmuscle invasive bladder cancer unresponsive to bacillus Calmette-Guerin. Expert Opinion on Biological Therapy. 2016;**16**:273-283. DOI: 10.1517/14712598.2016.1134483

[63] Secanella-Fandos S, Noguera-Ortega E, Olivares F, Luquin M, Julián E. Killed but metabolically active Mycobacterium bovis BCG retains the antitumor ability of live BCG. The Journal of Urology. 2014;**191**:1422-1428. DOI: 10.1016/j.juro.2013.12.002

[64] Murata M. Activation of Toll-like receptor 2 by a novel preparation of cell wall skeleton from Mycobacterium bovis BCG Tokyo (SMP-105) sufficiently enhances immune responses against tumors. Cancer Science. 2008;**99**:1435-1440

[65] Kato T, Bilim V, Yuuki K, Naito S, Yamanobe T, Nagaoka A, Yano I, Akaza H, Tomita Y. Bacillus Calmette-Guerin and BCG cell wall skeleton suppressed viability of bladder cancer cells in vitro. Anticancer Research. 2010;**30**:4089-4096

[66] Secanella-Fandos S. Funcionalitat dels micobacteris ambientals de creixement ràpid com a agents antitumorals. Servei de publicacions, Universitat Autònoma de Barcelona. Bellaterra (Barcelona); 2012: ISBN: 978-84-490-3560-9. http://www.tdx.cat/handle/10803/117614

[67] Subramaniam M, In LLA, Kumar A, Ahmed N, Nagoor NH. Cytotoxic and apoptotic effects of heat killed Mycobacterium indicus pranii (MIP) on various human cancer cell lines. Scientific Reports. 2016;**6**:19833. DOI: 10.1038/srep19833

[68] Shah G, Zhang G, Chen F, Cao Y, Kalyanaraman B, See W. Loss of BCG viability adversely affects the direct response of urothelial carcinoma cells to BCG exposure. The Journal of Urology. 2014;**191**:823-829. DOI: 10.1016/j.juro.2013.09.012

[69] Zhang Y, Khoo HE, Esuvaranathan K. Effects of bacillus Calmette-Guèrin and interferon alpha-2B on cytokine production in human bladder cancer cell lines. The Journal of Urology. 1999;**161**:977-983

[70] Yamada H, Kuroda E, Matsumoto S, Matsumoto T, Yamada T, Yamashita U. Prostaglandin E2 down-regulates viable Bacille Calmette-Guérin-induced macrophage cytotoxicity against murine bladder cancer cell MBT-2 in vitro. Clinical and Experimental Immunology. 2002;**128**:52-58

[71] Noguera-Ortega E, Rabanal RM, Secanella-Fandos S, Torrents E, Luquin M, Julián E. γ irradiated mycobacteria enhance survival in bladder tumor bearing mice although less efficaciously than live mycobacteria. The Journal of Urology. 2016;**195**:198-205. DOI: 10.1016/j.juro.2015.07.011

[72] Günther JH, Frambach M, Deinert I, Brandau S, Jocham D, Böhle A. Effects of acetylic salicylic acid and pentoxifylline on the efficacy of intravesical BCG therapy in orthotopic murine bladder cancer (MB49). The Journal of Urology. 1999;**161**:1702-1706

[73] Lamm DL, Iverson T, Wangler V. 1785 clinical experience with heat-inactivated Bacillus Calmette-Guerin (BCG) immunotherapy. The Journal of Urology. 2013;**189**:e733-e734. DOI: 10.1016/j.juro.2013.02.2875

[74] Chaudhuri P, Mukhopadhyay S. Bladder preserving approach for muscle invasive bladder cancer−Role of mycobacterium w. Journal of the Indian Medical Association. 2003;**101**:559-560

[75] Yates DR, Brausi MA, Catto JWF, et al. Treatment options available for bacillus Calmette-Guérin failure in non-muscle-invasive bladder cancer. European Urology. 2012;**62**:1088-1096. DOI: 10.1016/j.eururo.2012.08.055

[76] Noguera-Ortega E, Secanella-Fandos S, Eraña H, Gasión J, Rabanal RM, Luquin M, Torrents E, Julián E. Nonpathogenic Mycobacterium brumae inhibits bladder cancer growth in vitro, ex vivo, and in vivo. European Urology Focus. 2016;**2**:67-76. DOI: 10.1016/j.euf.2015.03.003

[77] Noguera-Ortega E, Blanco-Cabra N, Rabanal RM, Sánchez-Chardi A, Roldán M, Guallar-Garrido S, Torrents E, Luquin M, Julián E. Mycobacteria emulsified in olive oil-in-water trigger a robust immune response in bladder cancer treatment. Scientific Reports. 2016;**6**:27232. DOI: 10.1038/srep27232

[78] Baran J, Baj-Krzyworzeka M, Węglarczyk K, Ruggiero I, Zembala M, Weglarczyk K, Ruggiero I, Zembala M. Modulation of monocyte-tumour cell interactions by Mycobacterium vaccae. Cancer Immunology, Immunotherapy. 2004;**53**:1127-1134. DOI: 10.1007/s00262-004-0552-6

[79] 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**:114-121

[80] 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**:653-660. DOI: 10.1002/ijc.20442

[81] Hudson MA, Ritchey JK, Catalona WJ, Brown EJ, Ratliff TL. Comparison of the fibronectin-binding ability and antitumor efficacy of various mycobacteria. Cancer Research. 1990;**50**:3843-3847

[82] Pollard M, Luckert PH. The anti-metastatic effect of intravenously-inoculated BCG on prostate tumor cells. Anticancer Research. 1993;**13**:705-708

[83] Pollard M, Luckert PH. Immunotherapy by BCG against prostate adenocarcinomas in anatomical compartments. Anticancer Research. 1994;**14**:2691-2694

[84] Hrouda D, Souberbielle BE, Kayaga J, Corbishley CM, Kirby RS, Dalgleish AG. Mycobacterium vaccae (SRL172): A potential immunological adjuvant evaluated in rat prostate cancer. British Journal of Urology. 1998;**82**:870-876

[85] Eaton JD, Perry MJA, Nicholson S, Guckian M, Russell N, Whelan M, Kirby RS. Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. BJU International. 2002;**89**:19-26

[86] Hrouda D, Baban B, Dunsmuir WD, Kirby RS, Dalgleish AG. Immunotherapy of Advanced Prostate Cancer: A Phase I/II Trial using Mycobacterium vaccae (SRL172); Br J Urol. 1998;**82**:568-573

[87] Reader S, Ménard S, Filion B, Filion MC, Phillips NC. Pro-apoptotic and immunomodulatory activity of a mycobacterial cell wall-DNA complex towards LNCaP prostate cancer cells. The Prostate. 2001;**49**:155-165

[88] Morales A, Nickel JC, Downey J, Clark J, van der Linden. Immunotherapy of an experimental adenocarcinoma of the prostate. The Journal of Urology 1995;**153**:1706-1710

[89] Xu J, Zhu Z, Wu J, Liu W, Shen X, Zhang Y, Hu Z, Zhu D, Roque RS, Liu J. Immunization with a recombinant GnRH vaccine conjugated to heat shock protein 65 inhibits tumor growth in orthotopic prostate cancer mouse model. Cancer Letters. 2008;**259**:240-250. DOI: 10.1016/j.canlet.2007.10.011

[90] Patel PMM, Sim S, O'Donnell MA, et al. An evaluation of a preparation of Mycobacterium vaccae (SRL172) as an immunotherapeutic agent in renal cancer. European Journal of Cancer. 2008;**44**:216-223. DOI: 10.1016/j.ejca.2007.11.003

[91] Chang AE, Li Q, Jiang G, Sayre DM, Braun TM, Redman BG. Phase II trial of autologous tumor vaccination, anti-CD3-activated vaccine-primed lymphocytes, and interleukin-2 in stage IV renal cell cancer. Journal of Clinical Oncology. 2003;**21**:884-890. DOI: 10.1200/JCO.2003.08.023

[92] Rastinehad AR, Smith AD. Bacillus Calmette-Guérin for upper tract urothelial cancer: Is there a role? Journal of Endourology. 2009;**23**:563-568

[93] Kita Y, Soda T, Mizuno K, Matsuoka T, Nakanishi S, Asai S, Taoka R, Inoue K, Terai A. Long-term outcome of initial treatment with Bacillus Calmette-Guérin for carcinoma in situ of the upper urinary tract. Hinyokika Kiyo. 2011;**57**:353-357

[94] Martínez-Piñeiro L, Portillo JA, Fernández JM, et al. Maintenance therapy with 3-monthly Bacillus Calmette-Guérin for 3 years is not superior to standard induction therapy in high-risk non-muscle-invasive urothelial bladder carcinoma: Final results of randomised CUETO study 98013. European Urology. 2015;**68**:256-262. DOI: 10.1016/j.eururo.2015.02.040

[95] Kamat AM, Flaig TW, Grossman HB, Konety B, Lamm DL, O'Donnell MA, Uchio E, Efstathiou JA, Taylor JA. Expert consensus document: Consensus statement on best practice management regarding the use of intravesical immunotherapy with BCG for bladder cancer. Nature Reviews. Urology. 2015;**12**:1-11. DOI: 10.1038/nrurol.2015.58

[96] Oddens JR, Brausi M, Sylvester RJ, et al. Final results of an EORTC-GU cancers group randomized study of maintenance BCG in intermediate- and high-risk Ta, T1 papillary carcinoma of the urinary bladder: One-third dose versus full dose and 1 year versus 3 years of maintenance. European Urology. 2013;**63**:462-472. DOI: 10.1016/j.eururo.2012.10.039

[97] Erdoğar N, Iskit AB, Eroğlu H, Sargon MF, Mungan NA, Bilensoy E. Antitumor efficacy of BCG loaded cationic nanoparticles for intravesical immunotherapy of bladder tumor induced rat model. Journal of Nanoscience and Nanotechnology. 2015;**15**:10156-10164