IMMUNOTHERAPY TREATMENTS
ALTERNATIVE BCG IN THE NON-INVASIVE BLADDER CANCER

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ABBREVIATIONS AND ACRONYMS

BCG: Mycobacterium bovis bacillus Calmette-Guérin.
MNU: N-methyl-n-nitrosourea
NMIBC: non muscle invasive bladder cancer
SEB: Staphylococcal enterotoxin B
HP-NAP: Helicobacter pylori protein
CWS: Cell wall skeleton.
TNF-α: Tumor necrosis factor alpha.
IFN: Interferon.
PBS: Phosphate Buffered Saline.
CpG: sites or CG sites are regions of DNA where a cytosine nucleotide.
LAK: limphokine-activated killer cells.
DNA: Deoxyribonucleic acid.
RNA: Ribonucleic acid.
LPS: Lipopolysaccharide.
P-MAPA: Proteic - Magnesium Ammonium Phospholinoleate Anhydride.
TLR: Toll-like receptors.
KLH: Keyhole limpet hemocyanin.
DCs: Dendritic cells.
PBMCs: Peripheral blood mononuclear cells.
MICB: MIHC class I polypeptide-related sequence B.
IL: Interleukine.
ULBP: UL-16-binding protein.
S1PT: Pertussis toxin.
Th: T helper type.
VLA5: very late activation antigen 5.
PstS1: Periplasmic phosphate-binding lipoprotein.
CTLA-4: Cytotoxic T-Lymphocyte Antigen 4.
PD-1: Programmed death receptor 1.
AdCD40L: adenoviral vectors expressing CD40 ligand.
MCWE: efficacy of Mycobacterial Cell Wall Extract.
rBCG: recombinant BCG.
IMMUNOTHERAPY TREATMENTS ALTERNATIVE BCG IN THE NON-INVASIVE BLADDER CANCER

1. ABSTRACT

Immunotherapy with Bacillus Calmette-Guerin (BCG) instillation is recommended for high-risk, non–muscle invasive bladder cancer (NMIBC). However, many patients become refractory to BCG, giving impetus to the development of alternative therapies. Thus, an overarching search of the literature was used to identify relevant studies to analyze alternatives that reduce toxicity BCG and improve their effectiveness. Findings and interpretation the search identified over 37 articles in total, 2 of other mycobacterial, 6 of the other microorganisms, 11 of purified antigens, 5 of CWS, and 13 of rBCG. It shown be more effectiveness that BCG. However, is necessary carry out more experiments in vivo in order to validate these assays.

Keywords: BCG, immunotherapy, bladder cancer.

2. INTRODUCTION

Bladder cancer is one of the most common disorders of the urinary tract, the fourth most common in men and the eleven in women in the world. The 6% of cases occur in developed countries, especially in southern Europe and North America, related to the high proportion of smokers among the population (http://www.cancer.gov). Approximately 70% of all newly diagnosed bladder tumors are non-muscle invasive bladder cancers (NMIBC) and represent pathologic stages Ta, T1, and carcinoma in situ (CIS). Since the 1970s, perioperative instillation of chemotherapy immediately following transurethral resection (TUR) has been advocated to destroy residual microscopic tumor cells, and to prevent re-implantation in an effort to decrease recurrence rates, which approach to 45% following TUR alone (Hall, 2007). Despite the use of adjuvant intravesical therapy for NMIBC, tumor recurrence is still high, mandating lifetime surveillance and high patient and healthcare-related costs (Avritscher, 2006). It is recurrences, which, in time, may lead to invasive disease of the muscle. Recurrence has been attributed to the presence of residual tumour cells after surgery or to the presence of aberrant epithelial cells in the urothelial layer that develop into new tumours.

Bacillus Calmette–Guerin (BCG) instillation following TUR of the bladder tumour, is the gold standard for the treatment of transitional cell carcinoma and results in a reduction of recurrence (Nseyo, 1997). In addition to the direct anti-tumour effect, it is widely recognized that intravesical BCG therapy is more potent in preventing tumour recurrence than intravesical chemotherapy (Martinez-Pineiro, 1990). Although intravesical BCG therapy is effective, it is not free from serious side effects (e.g. high fever, granulomatous prostatitis, pneumonitis, hepatitis, and BCG sepsis in approximately 30-40% of patients (DeHaven, 1992).

To avoid such unfavourable events, it is necessary to develop a more active and less toxic immunotherapeutic agent. In order to reduce the side effects of BCG therapy and to improve their effectiveness, or otherwise replaced, are being made to various studies such as engineering using recombinant BCG and other mycobacteria such as Mycobacterium smegmatis, modified genetically to express cytokines or immunomodulatory molecules to enhance immune response and reduce the recurrence and progression (Haley, 1999). Furthermore, there are investigations of cell wall extracts from different mycobacterial and Lactobacillus species also shown to have antitumor capacity better than BCG in bladder cancer cells in vitro (Seow, 2002). Currently there are also studies with other microorganisms, purified and immunogenic antigens, which could replace BCG.

The purpose of this study was to search and review the literature related to the immunotherapeutic alternatives to intravesical BCG, their safety and efficacy in the treatment of NMIBC patients.

3. MATERIAL AND METHODS

An overarching search of the literature was used to identify relevant studies to improve or replace intravesical BCG therapy, and comparing different alternatives provide immunotherapeutic studies in a systematic review articles. PUBMED and SCOPUS searchers were used, giving results of research articles from 1996-2012 about immunotherapeutic for non-
invasive bladder cancer using the following keywords in PUBMED: "bladder cancer" and "immunotherapy" and "BCG", and SCOPUS "bladder cancer" and "immunotherapy" and "mycobacteria", respectively, giving a result of 570 articles in PUBMED, of which 17 were related to the subject; 230 articles in SCOPUS, in which 6 were related to the subject, and 13 were found in both searchers. Studies of the documents were read and analyzed to meet the objective of this review.

4. RESULTS
4.1 Table 1. Immunotherapeutic alternatives to NMIBC (n= 37):

Search we obtained a total of 36 articles related to the topic, resulting a number of 13 items to rBCG and 2 items in order to other mycobacteria, as upper and lower limit. Included studies from 1987 to 2012, as shown in the table 1.

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<tr>
<th>Publication date</th>
<th>Oldest</th>
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<tr>
<td>Most recent</td>
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<td>Reducing toxicity</td>
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<td>Other mycobacteria</td>
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<td>Other microorganisms</td>
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<td>Purified antigens</td>
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<td>BCG extracts (including CWS)</td>
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<td>Improving BCG efficacy</td>
<td>rBCG 13</td>
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Key: rBCG= bacille Calmette-Guérin; CWS= Cell wall skeleton.

4.2 Table 2. Trials using other mycobacteria than BCG:

Other Mycobacteria:

Two published articles found that using M. smegmatis (table 2). These studies included experiments In vitro and in vivo. In terms of tumour specific responses, both possess significant anti-tumour properties in a model of local tumour immunotherapy. In vitro, one of these shows an increase in monocyte derived DCs with an inflammatory phenotype, which may play an important role in orchestrating the anti-tumor response. While that M. smegmatis/TNFα produced significantly elevated levels of IFNα. It should be noted, that studies have not been compared with BCG. Therefore, it is suggested carry out more experiments to be a safe candidate to replace BCG.

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<thead>
<tr>
<th>Reference</th>
<th>Study/aims</th>
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<tr>
<td>Rich FJ, 2012</td>
<td>Induction of T cell responses and recruitment of an inflammatory dendritic cell subset following tumor immunotherapy with Mycobacterium smegmatis</td>
<td>Treatments were conducted in C57Bl/6 or CD45.1+ B6.SJL/Ptpcr/Pep3b age- and sex-matched mice. The first injection was immediately after tumor challenge and then a further four injections of 2×10^6 CFU were given at the same site 2, 4, 6, and 8 days after the first treatment. Control mice received injections of 100 µl PBS. Mice were euthanized once tumor size exceeded 150 mm^2. Heat-killed M. smegmatis was prepared by incubation at 70 °C for 30 min and was administered in the same way as live M. smegmatis. By day 12, mice treated with M. smegmatis had noticeably smaller tumors than control PBS-treated mice, and a significant reduction in tumor growth was observed until completion of the experiment, at day 35 post-challenge. Tumors in mice treated with M. smegmatis took significantly longer to reach a size of 150 mm^2, at which point the mice were required to be euthanized, than tumors in PBS-treated mice. A analysis of the tumor draining lymph nodes revealed an increase in monocyte derived DCs with an inflammatory phenotype, which may play an important role in orchestrating the anti-tumor response.</td>
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Recombinant M. smegmatis was engineered to secrete human TNFα as previously described (Hayel, 1999). Murine MB49 bladder tumour cells were used. Female mice were used at 6-8 weeks of age. In initial experiments, Mice (n=10) were challenged with 510⁴ MB49 tumour cells s.c. and vaccinated s.c. at an adjacent site with 210⁶ M. smegmatis (wild-type), recombinant M. smegmatis/TNFα, BCG (all re-suspended in 100µl PBS) or saline control. Mice were treated on a total of 5 occasions at the same site with bacteria, every 2 days. Tumour growth was determined by measuring the length and breadth of each tumour nodule at least 3 times per week.

Splenocytes from mice vaccinated with wild-type M. smegmatis produced IFNα in response to mycobacterial antigen early in the immune response. However IFNα production was no longer measurable 20 days after vaccination. In contrast, splenocytes of mice receiving recombinant M. smegmatis/TNFα produced significantly elevated levels of IFNα in response to mycobacterial Ag. Furthermore these mice continued to produce elevated levels of IFNα for up to 27 days after the initial vaccination.

Similar elevated levels of IFNα were seen in lymphocytes from tumour-bearing animals treated with recombinant mycobacteria (>12-fold increase over wild-type). However in addition to responses against mycobacterial antigen, tumour-specific responses were also observed and lymphocytes isolated from draining lymph nodes proliferated in response to both mycobacterial and tumour-derived antigen preparations.

4.3 Table 3. Trials using other microorganisms:

Other microorganisms

In the search six studies have been found, 1 using SEB, 1 using HP-NAP, 2 using Lactobacillus, 1 using reovirus and 1 using Corynebacterium.

HP-NAP: As expected, BCG efficiently counteracted tumour growth. However, the effect of HP-NAP was more pronounced both in terms of tumour volume and tumour necrosis. Accordingly, the percentage of CD4+/CD8+ T cells producing IFN-γ was higher in the local lymph node of HP-NAP-treated animals than in that of BCG-treated ones. Further, none of the HP-NAP-treated animals showed a macroscopic alteration of the urine aspect. However, will be necessary to assess the therapeutic efficacy of HP-NAP.

Reovirus: is a normally benign virus found in the respiratory and enteric tracts (respiratory, enteric and orphan) has been shown to have an oncolytic effect on transformed cells in vitro and in vivo (Strong, 1998). The assays of this study, in vitro and in vivo demonstrated the oncolytic activity and a superior response relative to BCG with none of the side effects.

Corynebacterium: In this study showed that CP and AS appeared equally effective or even slightly more effective than BCG in this model. However, it suggest that clinical evaluation of CP or AS may be worthwhile.

SEB: this study indicates that the associated BCG-SEB treatment was more effective for restoring apoptosis, cellular proliferation, and angiogenic balance and cancer state than isolated BCG or SEB treatment.

Lactobacillus: This first study compared, Live and Lyo LGG. Both preparations produced increased TNF-α, but only Lyo LGG induced significantly more cytokine production. Therefore, intravesical Lyo LGG, like live LGG, induces immune cell recruitment into the bladder. Both Lyo LGG and BCG induced a significant and comparable increase in the number of cured mice. While that intravesical LC9018 reduced the rate of tumor appearance and augmented the local expression of antitumor (INF-ϒ and TNF). Both Lyo LGG and LC9018 are capable of stimulating cytokine production. Only LC9018 was compared with BCG, and BCG had not significant antitumor activity.
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<th>Reference</th>
<th>Study/aims</th>
<th>Included studies</th>
<th>Results</th>
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<tr>
<td>Codolo G, 2011</td>
<td>HP-NAP inhibits the growth of bladder cancer in mice by activating a cytotoxic Th1 response</td>
<td>In a first set of experiments, HP-NAP (50 μg/100 μl/dose) or PBS (100 μl/dose) was administered every 3 days, starting at day 0. Six groups of animals (4 mice for each condition) were killed at different time points, and bladders were isolated. For the evaluation of HP-NAP therapeutic efficacy, mice, instilled with tumour cells at day 0, were treated with HP-NAP or with sterile PBS every 3 days starting at day 3. In the two sets of experiments, the animals (8 mice for each treatment) were killed either at day 7 or at day 13. In the long protocol, a group of mice treated with BCG (ImmuCyst, Sanofi Pasteur S.A. Lione, France) was included. BCG (3 × 10^6 CFU/100 μl/dose) was administered 3 and 9 days after MB49 implant.</td>
<td>Tumour volumes, measured everyday with a caliper, revealed that HP-NAP counteracted the tumour growth. Tumour volume gap between HP-NAP-treated animals and vehicle-treated animals progressively increased, and at the end of the experiment, tumours of the former group were 4 times smaller than those of the vehicle-treated animals (P &lt; 0.009). As expected, BCG efficiently counteracted tumour growth. However, the effect of HP-NAP was more pronounced both in terms of tumour volume and tumour necrosis. Accordingly, the percentage of CD4+/CD8+ T cells producing IFN-γ was higher in the local lymph node of HP-NAP-treated animals than in that of BCG-treated ones. Moreover, it is noteworthy that while the administration of BCG resulted in a strong haematuria, a condition often associated with the therapy (Lamm, 1992), none of the HP-NAP-treated animals showed a macroscopic alteration of the urine aspect.</td>
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<td>Hanel EG, 2004</td>
<td>A novel intravesical therapy for superficial bladder cancer in an orthotopic model: oncolytic reovirus therapy</td>
<td>In vitro: AY-27 cells. Exponentially growing cells were plated on a 96 well plate (10,000 cells per well). Culture medium was removed and virus of incremental titers (up to 3.0 × 10^7 pFU/ml) was added. In vivo: Ten rats per treatment group. Treatment group Reovirus: Low: 5×10^5 pFU/ml, medium: 5×10^6 pFU/ml, high: 5×10^7 pFU/ml; Treatment group BCG: Low: 5×10^5 CFU/ml, medium and standard: 5×10^5 CFU/ml, high: 5×10^7 CFU/ml; and saline control group: 0.9% NaCl.</td>
<td>MTT cytotoxicity assay demonstrated that AY-27 cells were susceptible to reovirus oncolysis. Log rank comparisons between each treatment group and saline controls also showed statistical significance (p 0.02, 0.0004 and 0.0002 for the low, medium and high reovirus group, respectively). However, although dose dependent survival was observed in animals treated with BCG instillations, only the high dose group showed a statistical difference compared with the saline group. Severe complications were observed only in BCG treated groups (necrotic cystitis, nephritis and/or hydronephrosis) and in the saline group (tumor related hydronephrosis) but not in the reovirus treated groups. Survival greater than 90 days was considered long-term survival (LTS). There was 1 LTS in the saline group (the only one without tumor). There were 2 to 5 LTSs in BCG treated groups (20% to 50%) and 5 to 9 in reovirus treated groups (50% to 90%).</td>
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<td>Marsh CL, 1987</td>
<td>Superiority of intravesical immunotherapy with Corynebacterium parvum and Allium sativum in control of murine bladder cancer</td>
<td>In vivo: was studied in mice transplanted intravesically with mouse bladder tumor cells (MBT-2). BCG (2 X 10(6) CFU), CP (250 micrograms), KLH (50 micrograms), or AS (25 mg) was administered directly into the bladder via urethral catheter on day 1, day 6, or days 1 and 6.</td>
<td>The results of the study showed that two treatments given one and six days after tumor transplant yielded the lowest tumor incidence and that CP and AS appeared equally effective or even slightly more effective than BCG in this model.</td>
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<td>Reis LO, 2012</td>
<td>Anti-angiogenic effects of the superantigen staphylococcal enterotoxin B and bacillus Calmette-Guerin immunotherapy for nonmuscle invasive bladder cancer</td>
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<td>A total of 75 female Fisher 344 rats. Of the rats 15 received 0.3 ml saline (control) and 60 received 1.5 mg/kg MNU (N-methyl-n-nitrosourea) intravesically every other week for 6 weeks. The rats were divided into 5 groups. The MNU and control groups received 0.3 ml saline. The BCG group received $10^5$ cfu BCG. The SEB group received $10\mu g/ml$ SEB. The BCG plus staphylococcal enterotoxin B group received the 2 treatments simultaneously. Each group was treated intravesically for 6 weeks. At 15 weeks all bladders were collected for histopathological and immunological evaluation, and Western blot. Papillary carcinoma (pTa) and high grade intraepithelial neoplasia (carcinoma in situ) were more common in the MNU group. Papillary hyperplasia was more common in the BCG and enterotoxin groups. Flat hyperplasia was more common in the BCG plus enterotoxin groups compared to the MNU group. Intensified vascular endothelial growth factor, matrix metalloproteinase-9, Ki-67 and insulin-like growth factor receptor-1 immunoreactivity was verified in the MNU group. Moderate in the BCG and enterotoxin groups, and weak in the BCG plus enterotoxin and control groups.</td>
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<tr>
<th>Seow SW, 2010</th>
<th>Lactobacillus rhamnosus GG induces tumor regression in mice bearing orthotopic bladder tumors</th>
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<td>Bacteria: LGG ($3 \times 10^9$ cfu/mL). For lyophilisation, 10 mL PBS was added to 1 mL LGG. In vitro stimulation of splenocytes with LGG. Splenocytes ($2 \times 10^6$/well) Live LGG ($2 \times 10^5$ cfu) and Lyo LGG were added. After 6, 12, 24, 48, and 72h post-stimulation the following cytokines (IL12p40, TNF$\alpha$, and IL10). Mice were divided into two groups. Control mice were instilled with 100 µL PBS, while the mice treated with LGG were instilled with 100 µL Lyo LGG (n= 10). The amount of Lyo LGG instilled was equivalent to $1 \times 10^8$ cfu. Both groups were instilled once a week for 6 weeks. The experiment was terminated 1 day after the last instillation (day 40). In vitro studies were carried out using splenocytes to determine the ability of live and Lyo LGG to stimulate cytokine production. Both preparations (live LGG and Lyo-LGG) produced increased TNF-$.\alpha$, but only Lyo LGG induced significantly more cytokine production. In vivo: Only one in five control animals had more than 50% neutrophil infiltration compared with three in five LGG-treated animals.</td>
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<tr>
<th>Takahashi T, 2001</th>
<th>Antitumor effects of the intravesical instillation of heat killed cells of the Lactobacillus casei strain shirota on the murine orthotopic bladder tumor MBT-2</th>
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<td>LC9018 or BCG, Tokyo 172 strain, was instilled once daily for 10 days starting on the day after orthotopic implantation of MBT-2. Tumor appearance and mean bladder weight on day 21 after tumor implantation were evaluated. Intravesical LC9018 instillation significantly reduced the rate of tumor appearance in 8 of 38 subjects and mean tumor in controls. BCG had no significant antitumor activity in the orthotopic implantation model. Intravesical instillation of LC9018 augmented the local expression of antitumor cytokine messenger RNA (interferon-y and tumor necrosis factor-$.\alpha$) and induced the infiltration of neutrophils surrounded by macrophages that phagocytosed LC9018 cells at the bladder mucosa.</td>
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4.4 Table 4. Trials using purified antigens:

Purified Antigens:

Ten published articles found that using purified antigens: 5 using CpG, 1 using P-MAPA, 1 Streptavidin-tagged, 1 AdCD40L, 1 PstS1 and 1 Ag85A.

CpG: three studies shown an inhibiting tumor growth and had 40–60% complete tumor regression. While, one study showed that the production of IL-12 was comparable to that achieved following BCG stimulation. Other study to demonstrated that CpGs were superior to BCG.
P-MAPA: the present study showed that P-MAPA increased significantly TLR2, TLR4 and p53 protein levels. In addition, it was demonstrated that this immunomodulator was more effective in the treatment of BC compared to BCG. These results were correlated with the ability of P-MAPA to act as TLR ligand, mainly for TLR2 and TLR4. The increased TLR2 and TLR4 levels were fundamental for anti-tumor immunotherapy of BC.

AdCD40L: This study demonstrated that local administration of AdCD40L vector therapy in patients with bladder cancer is safe and feasible, but is necessary future studies may include an extended duration and intensity of AdCD40L treatment.

PstS1: Intravesical PstS1 immunotherapy induced strong local and systemic immune responses together with substantial anti-tumor activity in a preclinical mouse model. Thus, could recombinant PstS1 antigen as a potent immunotherapeutic drug for cancer therapy. In a model of prime-boost immunotherapy we observed that antigen-specific sensitization might jeopardize the positive effects of topical immunotherapy and therefore has to be considered and evaluated with caution.

Ag85A: This result indicated that Ag85A-DCs have the potential to stimulate T cell activation. It showed significantly higher cytolytic activity when compared with that in other controls. Furthermore, produced a significantly higher level of IFN-γ.

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<th>Reference</th>
<th>Study/aims</th>
<th>Included studies</th>
<th>Results</th>
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<tr>
<td>Atkins H, 2003</td>
<td>Polarization of a T-helper cell immune response by activation of dendritic cells with CpG-containing oligonucleotides: A potential therapeutic regime for bladder cancer immunotherapy</td>
<td>Female C3H/HeSn (TLR4 wild type, LPS responsive) and C3H/HeJ (TLR4 mutant, LPS resistant) mice (8–10 weeks old) and female CD1 mice (8–10 weeks old) were used. Approximately 1–1.5x10⁷ leucocytes were obtained from each C3H mouse. The activating ligands were 50 or 100 ng ml1 LPS (Sigma), 1.25x10⁵ colony forming units (CFU) 1 BCG, 2 or 5 μM phosphorothioate-stabilised CpGrich adjuvant oligonucleotide (ODN) (containing CG motif, ATA ATC GAC GTT CAA GCA AG; TAG Newcastle, UK) or 2 μM phosphorothioate-stabilised non-CpG-rich control-ODN (reversed CG motif, ATA ATG CAG CTT CAA GCA AG; TAG Newcastle).</td>
<td>BCG stimulation of C3H/HeSn and C3H/HeJ cultures showed no difference on the maturation status of DC by examination of CD83 expression, but did result in a dose-dependent increase in the expression of CD86 in DCs from both mouse strains. Following stimulation of C3H/HeSn DCs with adjuvant CpG-oligonucleotides, the production of IL-12 was comparable to that achieved following BCG stimulation and was highly significant compared to control cultures. The amount of secreted IL-12 in response to both BCG or adjuvant CpG did not differ between the two C3H mouse strains.</td>
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<td>Faverow J, 2012</td>
<td>Effects of P-MAPA immunomodulator on toll-like receptors and p53: Potential therapeutic strategies for infectious disease and cancer</td>
<td>In vitro: The activity of P-MAPA in a screening test was assessed in seven different human TLRs (TLR2, 3, 4, 5, 7, 8 and 9). In Vivo: Female C57BL/6 mice, 6 and 8 weeks old. The 60 rats were divided into 4 groups (15 animals per group): The Control (CT) group received 0.30 ml dose of 0.9 % physiological saline intravesically every other week for 8 weeks; The MNU group (Bladder Cancer) received the same treatment as the CT group; The BCG group received 10⁸ CFU (40 mg) dose of BCG intravesically every other week for 8 weeks; The P-MAPA group received 5 mg/kg dose of P-MAPA intravesically every other week for 8 weeks. After 16 weeks of treatment, all animals were submitted to cystography to evaluate the occurrence of tumor.</td>
<td>In vitro: P-MAPA was initially tested using the Rezasurin MIC assay against H37Rv strain and did not show any antimycobacterial activity in vitro at a concentration of 10 μg/mL. Also, negative and sterility control groups did not show any antimycobacterial activity. In vivo: In the urinary bladder, the more frequent histopathological changes in the P-MAPA group were flat hyperplasia (60.0 %) and papillary hyperplasia (20.0 %). The occurrence of urinary calculi and macroscopic haematuria was only observed in the MNU and BCG groups, being absent in the P-MAPA group. The highest TLR4 and TLR2 protein levels were found in the P-MAPA groups compared to the other experimental groups. However, these levels were significantly higher in the BCG group than in the CT and MNU groups. The p53 protein level was significantly higher in the P-MAPA groups than in the BCG and CT groups. Furthermore, this level was significantly higher in</td>
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<td>Reference</td>
<td>Antineoplastic effect of immunostimulatory DNA (CpG) in a murine 57/BLE/MB-49 transitional cell carcinoma model</td>
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<td>Hegel A, 2004</td>
<td>A subcutaneous murine TCC model was established in female C57BL6 mice using the corresponding syngeneic MB49 TCC cell line. Three groups of 5 animals received a cell suspension, standardized for 1x10^6 cells/50 µl, injected s.c. into the right and left flank. Group I received 10 nmol of CpG-ODN only into the right cell depot. Group II received 10 nmol of GpC ODN. Group III served as untreated control and received only PBS. Tumor sizes and weights showed no side differences. The average tumor weight on day 14 was 171 mg, 110 mg and 18 mg, respectively, in groups III, II and I (p&lt;0.05). Histopathology revealed solid vital epithelial tumors in group III and reduced vital tumor mass with central necrosis and moderate mononuclear infiltration in group II. Group I showed almost complete tumor necrosis and a considerable mononuclear inflammatory response.</td>
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<td>Huang X, 2010</td>
<td>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. In vivo: A total of 125 female C57BL/6 mice was divided into five groups: the PBS control group, the soluble IL2 group, the SAGFP group, the SAhIL2 group, and the untreated group (blank control group); each with 25 mice. Then mice of each group received bladder perfusion with 0.1 mL of PBS, hIL2 (0.15 mg/mL), SAGFP (0.15 mg/mL), and SAhIL2 (0.15 mg/mL), respectively. A total of 6 continuous cycles of this treatment were conducted, 3 days per cycle. SAhIL2 could be immobilized efficiently and durably on the bladder mucosal surface for as long as 7 days. Immunohistochemistry suggested that SAhIL2 would significantly decrease 3 days after immobilization. Survival rates were significantly higher in the SAhIL2 group than in the other groups. Immunohistochemistry revealed a significant increase in IL2α receptor-positive lymphocytes at the tumor site in the SAhIL2 group compared with the PBS control group. The results also showed that SAGFP treatment could increase the survival rate of tumor-bearing mice in comparison with the PBS control group.</td>
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<td>Mangsbo SM, 2008</td>
<td>CpG therapy is superior to BCG in an orthotopic bladder cancer model and generates CD4+ T-cell immunity. MB49 tumor-bearing mice were treated with BCG or CpG and survival as well as tumor progression were observed over time. Urine, blood, and tumors were collected and analyzed. Mice were rechallenged and evaluated for tumor-specific immunity. A comparison of CpGs and BCG in both a highly and less aggressive orthotopic tumor model, and in a subcutaneous tumor model, demonstrated that CpGs were superior to BCG.</td>
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| Mangsbo SM, 2010   | Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. In vitro: Herein, explored single or combined antibody blockade of CTLA-4 and PD-1 alone or combined with the toll-like receptor agonists CpG or bacillus Calmette-Guérin for treatment of murine experimental bladder cancer. The tumors were rejected by anti-CTLA-4 (aCTLA-4) while anti-PD-1 (aPD-1) suppressed tumor growth. The combination had no additive effect compared with aCTLA-4 alone. However, elevated levels of circulating CD107a expressing CD8 T cells were found in the aCTLA-4 plus aPD-1 group. In addition, levels of antinuclear antibodies correlated inversely with tumor size. Next, we combined CpG or bacillus Calmette-Guérin with aCTLA-4, aPD-1, or aPD-L1 and found that CpG in combination with aCTLA-4 or aPD-1 increased the survival of mice, with aPD-1 plus CpG being superior to either agent alone. CpG plus aCTLA-4 or aPD-1 increased the numbers of circulating tumor-specific CD107a expressing CD8 T cells as well as activated (CD25FoxP3-) CD4 splenocytes.
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<tr>
<th>Malmström PU, 2010</th>
<th>AdCD40L immunogene therapy for bladder carcinoma—the first phase I/II a trial</th>
<th>Patients in phase I (n = 5) were scheduled for cystectomy because of high-risk tumor, whereas patients in phase IIa (n = 3) had stage Ta tumors. Patients in phase I received three preoperative cycles of AdCD40L at 1 week apart. The three first patients received $1 \times 10^{11}$ vector particles per treatment (low dose), whereas the remaining five patients received $1 \times 10^{12}$ vector particles (high dose). In phase I, patients underwent cystectomy 3 to 5 days after their last treatment. In phase IIa, all tumors were removed at baseline, save for one marker tumor that was removed by transurethral resection 14 days after the last treatment. In three of five phase I patients, there were no detectable malignant cells in the bladder after therapy, although one had scanty malignant cells remaining in the resected ureter. The two patients with refractory tumor and the patient with residual tumor in the ureter received low-dose vector treatment. Although some tumor cells may have been removed during transurethral resection before cystectomy, seven closely matched controls all had remaining high-grade tumor post cystectomy and only two had increased inflammation. The three patients in phase IIa had residual Ta tumors posttherapy but with a 38% reduction of tumor size in one.</th>
</tr>
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<tbody>
<tr>
<td>Ninač C, 2005</td>
<td>CpG oligonucleotide therapy cures subcutaneous and orthotopic tumors and evokes protective immunity in murine bladder cancer</td>
<td>Mice were instilled with MB49 on day 0 and then given orthotopic treatments of PBS or CpG ODN 1668 (100 mg/treatment) on days 1, 8, 15, and 22. Mice died as a result of tumor progression or were euthanized. Subcutaneous model experiments were carried out on 8- to 10-week-old C57BL/6 female mice, where each treatment group consisted of four to five mice. For orthotopic experiments, groups consisted of seven to nine mice 12–14 weeks old. On day 0, 2.5 $3 \times 10^5$ MB49 cells were injected subcutaneously. On days 4, 7, and 10, peritumoral treatments of PBS or CpG ODNs (50 mg/treatment) were given. CpG type B ODNs are more potent than CpG type A against MB49. Mean tumor area is shown until the first mouse in each group reached its end point (144 mm²). Whereas non-CpG control ODN 1982 gave an effect similar to PBS, treatment with CpG type A ODN 1585 resulted in a slight delay of tumor growth. Nonetheless, the CpG-B ODNs 1826 and 1668 generated the most potent response against MB49, inhibiting tumor growth and even leading to complete regression in one of five and five of five cases, respectively. A dose response was seen, with complete tumor regression in two of five (50 mg/treatment), four of five (100 mg/treatment), or five of five mice (200 mg/treatment). Even in the group treated with 200 mg, all mice exhibited signs of well-being during and after the treatment; no side effects were observed. In this subcutaneous model, In vivo studies using CpG ODNs combined with adenoviral vectors immunostimulatory molecules did not demonstrate synergism. When AdCD40L, AdIL-15, or AdMock was combined with CpG, all combination groups had 40–60% complete tumor regression, similar to CpG ODNs alone. As for the rest of the mice in the groups, there was no difference in tumor growth.</td>
</tr>
</tbody>
</table>
Sänger C, 2004

Immunodominant PstS1 antigen of mycobacterium tuberculosis is a potent biological response modifier for the treatment of bladder cancer

In vitro: The human bladder tumor cell line T-24 and The murine bladder tumor cell line MB-49 were used. PBMCS were stimulated for 2 to 7 days with PstS1, BCG, PBS or PHA (Sigma). 2 x 105 cells / well of the stimulated PBMCS. For generation of immature DCs, 2 x 106 monocytes were cultured for seven days.

In vivo: Trial I: 15 animals per group were subcutaneously injected with 250 μg PLG-particles coated with 50 μg PstS1 or 250μg PLG-particles alone. Thereafter 6 x 104 MB-49 cells were instilled into the bladder. Intravesical immunotherapy with 100 μg PstS1 in 100 μl PBS was performed on days 1, 8, 15 and 22 after tumor implantation. Control groups were instilled with PBS (100 μl) alone.

Ag85A-DCs showed stronger capability to stimulate allogeneic T cell proliferation than mock-DCs and DCs. The T cell proliferation, stimulated with Ag85A-DCs began significantly at 48 h as compared with what mock-DCs and DCs did and the highest peak of T cell proliferation was reached at 72 h. This result indicated that Ag85A-DCs have the potential to stimulate T cell activation.

T cells primed with MB49 lysate pulsed Ag85A-DCs showed significantly higher cytolytic reactivity when compared with that in other controls. Furthermore, the T cells primed by MB49 lysate pulsed Ag85A-DCs produced a significantly higher level of IFN-γ than those primed by MB49 lysate pulsed mock-DCs, MB49 lysate pulsed DCs, Ag85A-DCs, MB49 lysate, Ag85A and PBS.

Zhang Y, 2012

Dendritic cell vaccine modified by Ag85A gene enhances anti-tumor immunity against bladder cancer

Were isolated from the spleen of mice inoculated with MB49 cells for 21 days through negative selection using MicroBeads and then, 5x106 T cells were cultured with 5x10⁵ Ag85A-DCs, mock-DCs or DCs in the presence of MB49-lysate for 7 h, 24 h, and 48 h separately. For the negative control, the T cells were cultured with or without MB49 lysate in the absence of DCs. Brefeldin A was added.

To confirm that tumor-specific cytotoxic T lymphocytes (CTLs) can be generated ex vivo, CD3+ T cells (1x10⁶ cells/ml, purity>90%) were isolated from naive C57BL/6 mice through positive selection using MicroBeads by Auto MACS. These cells were cultured in RPMI-1640 containing 10% FCS, then primed ex vivo in the presence of IL-2 (5 ng/ml) at days 0, 7, and 14 with MB49 lysate pulsed Ag85A-DCs, MB49 lysate-pulsed mock-DCs and MB49 lysate pulsed DCs, respectively, at a ratio of 1:10. Unpulsed Ag85A-DCs and DCs, MB49 lysate, Ag85A and PBS were used as controls.

4.5 Table 5. Trials using BCG extracts (including CWS)

BCG extracts (including CWS)

Five published articles found that using BCG extracts (CWS): 3 R8-liposome-BCG-CWS and 2 BCG-CWS.

R8-liposome-BCG-CWS: The first study had a better effect on tumour growth. While that the other assays no differences were observed between. A dependency study of R8-liposome-BCG-CW is planned to elucidate the optimum concentration for BCG-CW incorporation. Therefore, need more research about the subject.

BCG-CWS: Both studies induce cell growth retardation in cells malignant phenotype. One of the studies done in humans and showed excellent tolerance with minimal toxicity was observed.
<table>
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<th>Reference</th>
<th>Study/aims</th>
<th>Included studies</th>
<th>Results</th>
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<tbody>
<tr>
<td>Joraku A, 2009</td>
<td>Immunoprotection against murine bladder carcinoma by octaarginine-modified liposomes incorporating cell wall of Mycobacterium bovis bacillus Calmette-Guerin</td>
<td>In vitro: The R8-liposomes incorporated with CWs derived from <em>Mycobacterium bovis</em> BCG Tokyo 172 (BCG-CW). In vivo: female C3H/HeN mice (7-week-old). MBT-2 cells (7x10^5) were inoculated into the right side back of each mouse with 100μL of PBS alone (group A, six mice), PBS containing 1 mg BCG (group B, six mice), 1 mg BCG-CW (group C, 18 mice), 0.1 mg BCG-CW (group D, six mice), 1 mg R8-liposome-BCG-CW (group E, 18 mice), 0.1 mg R8-liposome-BCG-CW (group F, six mice), or R8-liposomes vehicle alone (group G, six mice). The six mice in group H were not treated. The 0.1 mg R8-liposome-BCG-CW inhibited the growth of all tumours of MBT-2 cells by 4 weeks, while 0.1 mg BCG-CW with no R8-liposomes vector did not. The growth of re-challenged tumour was recorded weekly in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg BCG-CW. The growth of re-challenged tumours of BCG-pretreated MBT-2 cells was suppressed compared with that of MBT-2 cells with no pretreatment. The growth of the re-challenged tumours was recorded weekly in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg R8-liposome-BCG-CW. The growth of the re-challenged tumours of R8-liposome-BCG-CW- and BCG-pretreated MBT-2 cells was suppressed compared with that of MBT-2 cells with no pretreatment.</td>
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<tr>
<td>Kato T, 2010</td>
<td>Bacillus Calmette-Guerin and BCG cell wall skeleton suppressed viability of bladder cancer cells in vitro.</td>
<td>Established bladder cell cancer cell lines T24, HT1376, and RT4. BCG Immunobladder® (Tokyo 172 strain) was used. For blocking experiments, anti-α5β1 mAb, which blocks VLA5 function, was used. Both viable and heat-killed BCG, as well as SMP105 BCG CWS-induced cell growth retardation in UC cells with highly malignant phenotype. Culturing of UC cell lines with BCG resulted in a concentration-dependent decrease of cell viability only in high-grade urothelial cancer expressing VLA5, but not low-grade (RT4) urothelial cancer cells, which lack VLA5 on their surface.</td>
<td>Significant differences were present between groups 1 and 4 in numbers of tumors per rat. The mean number of tumors per rat in group 4 was significantly lower than that in group 1. No significant differences were evident in tumor volume among the four groups, but the total tumor volume in group 4 was less than half that in group 1. The incidence of tumors in rats treated with PBS was higher than that in groups treated with BCG-CW and R8-liposome-CWS. The number of simple hyperplasias in group 4 was significantly lower than that of group 1. The number of PN hyperplasias in group 3 was than that of the others groups, but statistical differences was not found among the groups. In group 1, the number of bladder papillomas and carcinomas were 1.8±2 and 1.1±1.1, respectively. The incidence of urinary bladder carcinoma in group 4 was less than that in group 1, but the difference was not significant.</td>
</tr>
<tr>
<td>Miyazaki J, 2011</td>
<td>The therapeutic effects of R8-liposome-BCG-CWS on BBN-induced rat urinary bladder carcinoma</td>
<td>20 rats were divided into 4 groups according to the treatment administered at 28 weeks: group 1, control (phosphate-buffered saline, only); group 2, BCG-CW only (1.0 mg/rat once weekly for 8 weeks); group 3, R8-liposome-BCG-CWS (0.1 mg/rat once weekly for 8 weeks); and group 4, R8-liposome-BCG-CWS (1.0 mg/rat once weekly for 8 weeks).</td>
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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.

The T24 cells and RT-112 cells were cocultured with R8-liposome-BCG-CWS and BCG for 2, 4, or 6 h, and then the surface expression of NKG2D ligands was analyzed using TaqMan real-time quantitative RT-PCR. Peripheral blood mononuclear cells were obtained with a conventional preparation kit, and then lymphokine-activated killer (LAK) cells were generated from these purified.

Major histocompatibility complex class I-related chain B (MICB) expression was increased ≈1.5-fold on T24 cells and RT-112 cells with BCG. UL-16-binding protein (ULBP) 1 expression was also increased ≈1.5-fold on T24 cells and RT-112 cells with BCG. R8-liposome-BCG-CWS increased the surface expression of MICB 2.2-fold on T24 cells but did not increase it significantly on RT-112 cells. ULBP1 expression was increased ≈2.2-fold on RT-112 cells, although no differences were observed between the expression of ULBP2 and 3 with R8-liposome-BCG-CWS.

In humans: A total of 61 patients with histologically documented carcinoma in situ completed the study. Cell wall extract from M. phlei suspended in oil droplets to form an emulsion were instilled into the bladder at a dose of 4 mg. once weekly for 6 weeks and then monthly for 1 year. Response assessment was performed at 3-month intervals. Complete response to treatment indicated the absence of endoscopic and histological evidence of carcinoma in situ.

Of the 61 evaluable patients BCG therapy for carcinoma in situ had previously failed in 28 (46%), whereas no prior immunotherapy was administered in the remaining 33. Of the 28 patients in whom BCG therapy had failed previously with a minimum of 6 weekly treatments 17 (61%) responded to MCWE. Of the 33 patients who had not received prior BCG therapy 20 (61%) had also responded to MCWE.

The incidence of undesirable local side effects was similar to those reported after BCG intravesical instillations, although they were less severe and of shorter duration.

4.6 Table 6. Trials improving BCG efficacy (recombinant BCG)

Recombinant BCG:
Thirteen published articles found that using recombinant BCG: 2 using S1PT, 1 Ag85B, 7 overexpressing cytokines, and 3 poly-multi-rBCG.

S1PT: BCG and rBCG-S1PT showed a tendency toward Th1 polarization, considering the increased levels of TNF-α in both groups. The findings also showed an adequate therapeutic effect of rBCG-S1PT by significantly reducing the mean bladder tumor weight and showed an increase in the survival curve compared with the BCG and control groups. While that other study to exhibit the adequate therapeutic effect of rBCG-S1PT by significantly reducing the mean bladder tumor weight when compared to BCG and PBS. These results indicate that rBCG could serve as a useful substitute for wild-type BCG, but is necessary carry out more studies that validate this assay.

Ag85B: Ag85B demonstrated a significant in vitro cytotoxic activity in compared to BCG, and this effect may be due to apoptosis and cell-cycle arrest. This is reason that, speculate that BCG ΔleuD/Ag85B treatment represents an improved therapeutic agent for bladder cancer over the current commercially available strains. Nevertheless, need to test in vivo and in humans, in order to demonstrate antitumoral activity.

Overexpressing cytokines: rBCG all, according to that recombine, revealed enhanced levels of cytokines, enhanced by BCG-mediated cytotoxicity. These rBCG strain may be particularly useful for treating diseases that require a desirable Th1 response such as superficial bladder cancer. However, it is important carry out more research to validate their power cytotoxic.

Poly-rBCG and multi-rBCG: the three experiments showed a significantly inhibited tumor growth with increased production of Th1-type cytokines, as well as, induced infiltration of CD4/CD8 T cells. The results of these studies provided a basis for exploring the use of BCG subcomponents, however, to consolidate these findings further, studies in vivo are needed.
<table>
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<th>Reference</th>
<th>Study/aims</th>
<th>Included studies</th>
<th>Results</th>
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<tr>
<td>Andrade PM, 2010</td>
<td>The therapeutic potential of recombinant BCG expressing the antigen S1PT in the intravesical treatment of bladder cancer</td>
<td>In vivo: Six- to 8-week-old female C57BL/6 mice (n = 60). Twenty-four hours following tumor implantation, intravesical BCG therapy was initiated. Mice were randomly assigned to either a control group (receiving PBS) or 2 treatment groups (BCG or rBCG-S1PT). The BCG doses used were 1 × 10^6 CFU/mouse once a week for 1 month, given periurethrally in a volume of 100 µl while the mice were lightly anesthetized.</td>
<td>Both BCG and rBCG-S1PT immunotherapy resulted in bladder weight reduction, and rBCG-S1PT increased survival time compared with the control group. There were increases in TNFα in the BCG treated group, as well as increases in TNFα and IL-10 mRNA in the rBCG-S1PT group. The viability of MB49 cells cocultured with splenocytes from rBCG-S1PT-treated mice was lower than in both the BCG and control groups.</td>
</tr>
<tr>
<td>Arnold J, 2004</td>
<td>Immunotherapy of experimental bladder cancer with recombinant BCG expressing interferon-gamma</td>
<td>MB49 cell lines was used. A rBCG strain secreting murine IFN-gamma (rBCG-IFNgamma) was generated and tested for its immunostimulatory capacity in several in vitro and in vivo test systems.</td>
<td>The instillation of rBCG-IFN-gamma resulted in an enhanced recruitment of CD4+ T-cells into the bladder and further induced the local expression of IL-2 and IL-4 cytokines (mRNA) compared to control rBCG. With a low-dose treatment regimen for murine orthotopic bladder cancer, rBCG-IFNgamma significantly prolonged survival, whereas the therapeutic effect of wild-type control BCG did not reach statistical significance.</td>
</tr>
<tr>
<td>Begnini KR, 2012</td>
<td>Auxotrophic recombinant Mycobacterium bovis overexpressing Ag85B enhances cytotoxicity on superficial bladder cancer</td>
<td>In vitro: Three different BCG strains were used in this study: M. bovis BCG Pasteur 1173P2, M. bovis BCG Pasteur ΔleuD, and M. bovis BCG Pasteur ΔleuD/Ag85B (rBCG), a recombinant BCG ΔleuD overexpressing the Ag85B antigen. Cloning Ag85b gene into mycobacterial expression vector.</td>
<td>The Ag85B gene coding sequence was incorporated immediately downstream of the Ag85B signal sequence. Auxotrophic recombinant BCG strain overexpressing Ag85B (BCG ΔleuD/Ag85B) demonstrated a significant in vitro cytotoxic activity after 48 h of treatment, inhibiting more than 50 % of tumour cells. The inhibition of cell proliferation following BCG ΔleuD/Ag85B treatment was 77.8 %, while for the other strains it was 28 % (BCG ΔleuD) and 38.1 % (BCG Pasteur).</td>
</tr>
<tr>
<td>Chade DC, 2008</td>
<td>Immunomodulatory effects of recombinant BCG expressing pertussis toxin on TNF-alpha and IL-10 in a bladder cancer model</td>
<td>Ninety female C57BL/6 mice aged six to eight weeks and the murine transitional cell carcinoma cell line MB49 were used. For Experiment I (n = 60), 24 hours after tumor implantation, intravesical BCG therapy was initiated. Mice were randomly assigned to either a control group (receiving PBS) or two treatment groups (receiving BCG or rBCG-S1PT). The BCG dose was 1 × 106 CFU/mouse once a week for one month administered periurethrally in a volume of 100 µl while the mice were lightly anesthetized. Experiment II (n = 30) was performed in the same manner as Experiment I, but the animals did not receive bladder tumor implantation.</td>
<td>In Experiment I, mice were challenged with MB49 tumor cells. Treatment with BCG and rBCG-S1PT showed significantly increased level of TNF-α, but only animals that received rBCG-S1PT showed a significantly increase in IL-10 when compared with the control group. Also, in Experiment II, without tumor implantation, the same patterns of expression were observed for TNF-α and IL-10.</td>
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<td>Author</td>
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<td>Research Focus</td>
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<tr>
<td>Ding GQ</td>
<td>2012</td>
<td>Antitumor effects of human interferon-alpha 2b secreted by recombinant bacillus Calmette-Guerin vaccine on bladder cancer cells</td>
<td>The two genes were cloned in <em>Escherichia coli</em>-BCG shuttle-vector pMV261 to obtain a new recombinant plasmid pMV261-Ag85B-IFNα-2b. BCG was transformed with the recombinant plasmid by electroporation and designated rBCG-IFNα-2b. Mononuclear cells were isolated from human peripheral blood (PBMCs) and stimulated with rBCG-IFNα-2b or wild type BCG for 3 d, and then cultured with human bladder cancer cell lines T24 and T5637. A positive acid fast staining indicated that the constructed rBCG-IFNα-2b was anti-acid bacteria rather than random contamination. Western blotting showed the protein expression of IFNα-2b in rBCG-IFNα-2b. PBMC proliferation increased with increasing concentrations of rBCG-IFNα-2b. The rBCG-IFNα-2b concentration of $8 \times 10^4$ CFU/ml exerted the most proliferative effect. The cytotoxicity of PBMCs stimulated by rBCG-IFNα-2b to T24 and T5627 was significantly stronger in comparison to wild type BCG.</td>
</tr>
<tr>
<td>Lee CF</td>
<td>2004</td>
<td>Treatment of bladder carcinomas using recombinant BCG DNA vaccines and electroporative gene immunotherapy</td>
<td>For the transfer of poly-rBCG intratumoral genes, MBT-2 cells (approximately $5 \times 10^6$ cells) were subcutaneously inoculated into the backs of 5-week-old female C3H/HeN mice, and tumors were allowed to develop for about 2 weeks. The mouse serum from four groups (empty vector control, poly-rBCG alone, mIL-12 alone, or poly-rBCG+mIL-12; n=8 each) was collected on days 0, 7, 14, and 21 after vaccine treatment. Of the five BCG antigen-specific genes incorporated in these four vaccine constructs, all have been gene inserts in previous DNA vaccines that have elicited antigen-specific responses. Poly-rBCG was also shown to increase the concentration of macrophages and T cells, in particular CD4$^+$ and CD8$^+$ cells, within treated tumors.</td>
</tr>
<tr>
<td>Lee CF</td>
<td>2004</td>
<td>Immunotherapy for bladder cancer using recombinant bacillus Calmette-Guerin DNA vaccines and interleukin-12 DNA vaccine</td>
<td>Four mycobacteria candidate genes (<em>Ag85A</em>, <em>Ag85B</em>, Mpt64 and PstS3) were cloned, fused with ESAT6 and ligated into eukaryotic expression vectors. Combined poly-rBCG and mIL-12 vaccines were transferred into a murine bladder tumor model. The efficiency of gene expression was detected using Western blotting, flow cytometry and semiquantitative reverse transcriptase-polymerase chain reaction. Systemic cytokine responses, tumor growth and cumulative survival rates were monitored. Mice with tumors injected with poly-rBCG plus mIL-12 produced serum interferon-γ significantly within 21 days but no significant elevations in tumor necrosis factor-α, IL-2, IL-4 or IL-5 were found. On day 28 after electroporation the growth of MBT-2 implants treated with poly-rBCG, mIL-12 or poly-rBCG plus mIL-12 was significantly inhibited. The cumulative survival of mice treated with poly-rBCG plus mIL-12 was significantly higher than that of the other 3 groups.</td>
</tr>
<tr>
<td>Liu W</td>
<td>2009</td>
<td>Recombinant bacillus Calmette-Guerin (BCG) expressing interferon-alpha 2B enhances human mononuclear cell cytotoxicity against bladder cancer cell lines in vitro</td>
<td>Both rBCG-IFNα and control MV261 BCG were developed previously from a BCG Pasteur strain. Human bladder cancer cell lines T24, J82, 5637, CCSUP, and UMUC-3. PBMC were prepared from healthy individuals and stimulated with control MV261 BCG or rBCG-IFNα for 7 days. Both PBMC preparations showed basal killing on all target cells tested. Stimulation with MV261 BCG increased PBMC cytotoxicity by 1.8- to 3-fold for one subject and 2.1- to 4.2-fold for the other subject. Stimulation with rBCG-IFNα further increased PMBC cytotoxicity by up to 2-fold depending on target cells used (except UMUC-3 cells). Have also observed that PBMC stimulated with rBCG-IFNα produce elevated IFNα and IL-2, which correlates with and is required for the induction of enhanced PBMC cytotoxicity by this rBCG.</td>
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### Manipulation and potentation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines

**Murray PJ, 1996**

To construct BCG recombinants capable of secreting cytokines, selected murine cytokine cDNAs were modified by replacing the normal cytokine secretion signal-coding sequence with a mycobacterial secretion signal sequence (Matsuo, 1990). Murine IL-4, IL-6, GM-CSF, and IFN-ϒ cDNAs modified in this fashion were introduced into shuttle vectors in E. coli, and these vectors were subsequently introduced into BCG. Mice were inoculated intravenously with $10^5$ to $10^6$ cfu of bacteria and killed at 6 or 16 weeks postinfection; splenocytes were isolated and placed in culture either with no stimulus or with mycobacterial antigens (PPD).

The antigen-specific response to PPD at 6 weeks postinfection was notable in that the BCG strain with the vector alone produced the highest response compared to the other strains. The cytokine secretion profiles of splenocytes from mice injected with cytokine-secreting BCG revealed enhanced levels of cytokines. Splenocytes from mice injected with cytokine-secreting BCG produced higher levels of IL-2 and IFN-ϒ, as well as IL-10, IL-3, and GM-CSF, relative to cells from mice exposed to the BCG-vector control.

### Murine IL-2 secreting recombinant Bacillus Calmette-Guerin augments macrophage-mediated cytotoxicity against murine bladder MBT-2

**Yamada H, 2000**

pSO246 plasmid vector ligated with mIL-2 gene was introduced into BCG by electroporation. Thioglycollate-elicited murine peritoneal exudate cells (PEC) were stimulated in vitro with parental BCG or rBCG and their cytotoxic activity and the cytokine production was studied. Cytokines were assayed by an enzyme-linked immunosorbent assay (ELISA) and L929 bioassay.

rBCG (α-Ag-IL-2) secreted functional IL-2 and augmented more efficient cytotoxicity to MBT-2 and cytokines such as IL-12, tumor necrosis factor and interferon (IFN)-γ in PEC than parental BCG did. rBCG (α-Ag) had the same activity as BCG. Anti-IL-2 antibody reduced rBCG (α-Ag-IL-2)-mediated cytotoxicity and IFN-γ production. Exogenous IL-2 also enhanced BCG-mediated cytotoxicity, but 100 times more IL-2 was required to express the same activity as rBCG (α-Ag-IL-2). Anti-IL-12 neutralizing antibody and the depletion of T cells and NK cells reduced IFN-γ production by PEC stimulated with rBCG (α-Ag-IL-2), suggesting that T cells, NK cells and IL-12 participate in the enhancement of IFN-γ production.

### Immunotherapy for Orthotopic Murine Bladder Cancer using Bacillus Calmette-Guerin recombinant Protein Mpt-64

**Yu DS, 2007**

Four recombinant BCG genes were cloned from the genomic DNA of *Mycobacterium bovis* BCG. The DNA fragment of ESAT6 was then fused with the templates of Ag85A, Ag85B, Mpt6A, and PstS3, respectively, and subcloned into the eukaryotic vector pCMV-V5-His6. Multi-rBCG (120 µg) in normal saline was injected into the center of the tumor mass. To increase the effectiveness of multi-rBCG, 100 µg of mIL-12 vaccine (pIRES-p40p35).

Treatment with multi-rBCG plus mIL-12 significantly inhibited tumor growth in C3H/HeN mice, with increased production of Th1-type cytokines, including interferon-gamma and IL-12. Treatment with multi-rBCG and/or mIL-12 in C3H/HeN mice induced infiltration of CD4-/CD8- T cells and expansion of natural killer cells within tumors. By contrast, however, athymic nude mice treated in the same way showed no significant immune cells within tumors and died of the fast growing tumors.
### 5. DISCUSSIONS

Either to improve immunogenicity or replace to BCG, are being carry out various researches. What is apparent from the included studies (Tables 3–6) is that the interventions investigate about other microorganisms, CWS, purified antigens, and rBCG could be candidates to replace to BCG. Some of them; showed immunostimulatory properties are superior to BCG (rBCG), furthermore a significant cytotoxic activity as well is also described and minimal toxicity was observed. However, to consolidate these findings further, studies in vivo are needed.

In fact the number of research is extensive and due to the journal limitation on space, many others trials have not been cited in this review. To date, there are a multitude of encouraging in vitro and murine studies; however, no clinical data has yet been reported, which is compelling enough to change the standard of care, yet many practitioners continue to use BCG immunotherapy based on basic science data and theoretical benefit.

### 6. CONCLUSIONS

Evolution of the field of BCG immunotherapy will be towards replace it, with recombinant, purified and other microorganism therapy and such as changes in dose and duration based on patient tolerance and tumor characteristics, but is necessary, as has been suggested carry out more experiments to be sure that are safe candidates to replace BCG. Despite, the wealth articles about alternative immunotherapeutic to BCG, BCG remains is most widely used for the treatment non-invasive bladder cancer. Therefore, currently, the focus will be on replicating and improving the effectiveness of BCG while reducing troubling and harmful side effects.
7. REFERENCES


References


