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The Dose-Response Relationship of BCG on Urothelial Carcinoma Cell Biology

Gopitkumar Shah*, Guangjian Zhang*, Fanghong Chen*, YanLi Cao*, Balaraman Kalyanaraman[#], and William A See*

*Department of Urology, Medical College of Wisconsin, Milwaukee, WI

[#]Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI

Abstract

INTRODUCTION—The attenuated mycobacterium Bacille Calmette Guerin is widely utilized as intravesical immunotherapy for the treatment of non-muscle invasive urothelial carcinoma. At present, there is limited data on the relationship between BCG dose intensity and tumor response. This study evaluated dose-response relationship of BCG to NMIBC in vitro using urothelial carcinoma cell lines and in vivo using orthotopic mouse model.

MATERIALS AND METHODS—Two human UC cell lines were used to study the effect of BCG dose on the tumor cell response with internalization, activation of signaling pathways, gene transactivation, cell viability, LDH and HMGB1 release as end points. An orthotopic tumor model was used to compare the effect of different dose on antitumor efficacy of Bacillus Calmette-Guérin.

RESULTS—BCG internalization by UC cells increased as a function of both time and BCG dose, with a plateau at higher BCG doses and/or long exposure times. Intracellular signaling demonstrated a similar direct, dose dependent increase. Cytokine expression by UC cells as a function of dose was variable, with some genes increasing progressively while others showing decrease at the highest dose. While non-viable cell number increased in proportion to dose, the number of cells undergoing necrotic cell death decrease at higher doses. Higher dose of BCG (1:200) shows better antitumor effect compared to standard dose (1:50) ($p < 0.01$).

CONCLUSIONS—BCG dose has a direct impact on UC cell biology. Increased dose intensity, particularly for non-responders, may represent a strategy to increase BCG treatment efficacy.

Keywords

Bladder cancer; BCG; dose

Contact Person: Gopitkumar Shah, Ph.D., Assistant Professor, Department of Urology, Medical College of Wisconsin, 9200 W. Wisconsin Avenue, Milwaukee, WI 53226, Phone: (414) 805-0805, Fax: (414) 805-0771.

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1. Introduction

Bacille Calmette Guerin (BCG) is the most effective, currently available treatment for patients with NMIBC.¹⁻³ UC cells internalize BCG via $\alpha 5\beta 1$ integrin, bringing complex change in UC cell biology. Internalized BCG generates oxidative stress which activates multiple intracellular signaling pathways. These signaling pathways upregulate expression of various genes, contributing to changes in tumor biology and phenotype. Along with these direct effects, various cytokines and chemokines released from UC cells contribute to an active immune response by the host. Through a combination of both direct and systemic effects the interaction between UC cell and BCG plays a crucial role in the ultimate response to treatment.⁴

While there is an extensive clinical literature on the consequences of BCG dose reduction, often with the intent of reducing toxicity, the role of BCG dose in mediating direct cellular changes is largely un-quantified.⁵⁻⁶ Given that approximately one third of patients fail to respond to BCG, quantitative insight into the relationship between dose and cellular impact could identify targets for optimizing dose intensity in these patients. The purpose of the current study was to define the relationship between BCG dose and the cellular response to BCG as measured by a panel of intermediate biologic endpoints characterizing the cellular response to BCG.

Our results demonstrate that BCG internalization by UC cells, signaling pathway activation, gene expression, and cytotoxicity of UC cells are significantly correlated with BCG dose. Relative to the BCG dose resulting in peak activity, the highest BCG dose employed in this study (500:1) demonstrating a bell shaped dose response curve. Animals treated with higher dose showed significantly reduced bladder weight compared to untreated and animals treated with standard dose. These findings suggest that there is an “optimal” dose of BCG and that this dose may be higher than currently used in the clinical setting.

2. Methods

2.1 Cell Lines, BCG

The human UC cell line T24 was obtained from American Type culture collection Culture (Rockville, MD). The 253J cell line was a kind gift of Dr. Richard Williams (University of Iowa). 253J and T24 were maintained as described earlier.⁷ MB49 cell line was a kind gift from Dr. Timothy Ratliff (Purdue University College of Veterinary Medicine). MB49-Luc was generated and maintained as described.⁸ Freeze dried BCG was reconstituted in complete media at an estimated concentration of 2.5×10^7 viable organisms/ml (dilution assumed average viability of 4×10^8 organisms per vial based upon manufacturer's specified range of 1 to 8×10^8 per vial) as described earlier.⁷ BCG dose range used for in vitro studies was (Cells: BCG) 1:5 to 1:500.

2.2 Fluoresceination of BCG

BCG (4×10^8 cfu) was incubated with 0.3 mg fluorescein isothiocyanate (FITC; SUPELCO, Bellefonte, PA) for 30 min at 20°C in 1 ml PBS at pH 9.2. After washing with PBS three times and centrifugation for 10 min at 14,000 rpm, FITC-labeled BCG was suspended in

complete medium. The supernatant of the last centrifugation did not contain free fluorescent dye.

2.3 BCG Adherence/Internalization Assay

BCG attachment and internalization in the TCC cell lines were determined by flow cytometry as described previously.⁹

2.4 Luciferase Reporter Assays

UC cell exposure to BCG has been shown to increase the activation of intracellular signaling pathways.¹⁰ 253J and T24 cells were plated at 1×10^5 cells/well in 24-well plate. 24 hours later, the cells were transiently transfected with previously described NF- κ B, and Nrf2 plasmid reporter constructs using lipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.⁹ Duplicate wells were set up for each group. 24 hours after transfection, cells were treated with different dose of BCG. 6 hours later, the cells were washed with PBS and lysed with $1 \times$ reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activities were normalized to protein concentration as measured by BCA protein assay kit (Pierce, Rockford, IL).

2.5 Quantitative rtPCR

Prior work has demonstrated that UC cell exposure to BCG increases the expression of cell cycle regulatory and immune response genes.¹¹ qRT-PCR was done using the LightCycler® 480 Real-Time PCR System (Roche Applied Science, IN). Treatment groups in the comparative analysis included control cells, and cells treated with different dose of BCG. The primer sequence was IL-6, 5'-AGCCGCCCCACACAGA(upstream) and 5'-CCGTCGAGGATGTACCGAAT (downstream); IL-8, 5'-CTGGCCGTGGCTCTCTTG (upstream) and 5'-CCTTGGCAAACTGCACCTT (downstream); CXCL1, 5'-CCACTGCGCCCAAACC (upstream) and 5'-GCAGGATTGAGGCAAGCTTT (downstream); CXCL3, 5'-AATGGGAAGAAAGCTTGTCTCAA (upstream) and 5'-CCTTGTTCAGTATCTTTTCGA (downstream); CCL20, 5'-TCCTGGCTGCTTTGATGTCA (upstream) and 5'-AAAGTTGCTTGCTGCTTCTGAT (downstream); p21, 5'-GCGACTGTGATGCGCTAATG (upstream) and 5'-TGTCTCGGTGACAAAGTCGAAGT (downstream); iNOS, 5'-GGTGAAGCGGTAACAAAGG (upstream) and 5'-TGCTTGGTGGCGAAGATGA (downstream); and β -actin, 5'-ACCGAGCGCGGCTACAG (upstream) and 5'-CTTAATGTCACGCACGATTTC (downstream). Reactions were done using RT-PCR master mix, composed of HotStar Taq™ DNA polymerase, dinucleotide triphosphate, MgCl₂ and ROX™ Dye, and incubated with primers (0.4 μ M), probe (0.2 μ M), QuantiTect® RT Mix and RNA template. The RT-PCR program consisted of 1 cycle at 50°C for 30 minutes for reverse transcription and 1 cycle at 95°C with a 15-minute hold (hot start), followed by 40 cycles of denaturation at 94°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Fluorescence data was collected at the end of each extension phase and β -actin was used to normalize all other genes tested in the same RNA sample.

2.6 LDH Release Assay

LDH is a stable cytosolic enzyme, which is released upon cell lysis and/or injury to the cytoplasmic membrane. The effect of BCG dose on LDH release was measured according to the manufacturer's instruction (CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit Promega, Madison, WI)

2.7 HMGB1 Assay

HMGB1 is a marker of necrotic cell death. HMGB1 level in cell culture supernatant 24 hours following BCG treatment was measured by a commercial available HMGB1 ELISA Kit (Shino-Test Corporation, Sagamihara, Kanagawa, Japan).

2.8 Cell Proliferation

The effect of BCG dose on its anti-proliferative effect was measured using the MTT assay as previously described. Cytotoxic effects of BCG were also confirmed by using Apo-tox-glo kit (Promega) and results were comparable with MTT.

2.9 Tumor Model

All animal experiments were approved by the institutional animal care committee. Orthotopic bladder tumors were generated via intravesical instillation of MB49luc cells as previously described.⁸ Tumor was implanted in C57BL/6 (albino) female mice (14 per group) at ages 7 to 9 weeks on experimental day 0, as described by Ninalga et al.¹² Mice underwent intravesical treatment with BCG (5×10^6 organisms in 50 μ l for standard dose; 2×10^7 organisms in 50 μ l for high dose) or PBS (50 μ l) on days 1, 4 and 8. Biophotonic imaging was performed on days 4, 7 and 11 to assess tumor take and interval growth. Mice were sacrificed on day 12. The bladders were excised and fixed in formalin. At 48 hours the bladders were bisected, blotted dry and weighed. Bladder weight served as the primary end point for the treatment effect. This experiment was repeated twice. Results were consistent between experiments and combined for statistical analysis.

2.10 Statistical Analysis

All experiments were performed in triplicate. The data from individual experiments for assays employing luciferase reporter constructs was subject to arithmetic normalization relative to the highest values among the corresponding replicate experiments. Data was analyzed using two way ANOVA for repeated measures. Results were considered significant at $p < 0.05$. Graphical representation of the data is shown as the mean + S.E.

3 Results

3.1 BCG Adherence/Internalization

Preliminary experiments confirmed that BCG adherence/internalization peaked at 4h. Consequently, all experiments were performed at 4h. Adherence and internalization was evaluated across BCG: cell ratios ranging from 5:1 to 500:1. BCG dose was significantly correlated with adherence/internalization ($p < 0.0001$). While the percentage of "BCG positive" cells increased across the range of doses, there was a dose-adherence plateau

beyond the 100:1 ratio. Approximately 80% of cells were positive at or above this concentration. (Figure 1).

3.2 Activation of Intracellular Signaling pathways

Activation of NF κ B in response to BCG peaked at a 200:1 BCG: cell ratio in both cell lines. NF κ B activation was significantly correlated with BCG dose ($p < 0.05$). There was no difference in the NF κ B - BCG dose response relationship between the two cell lines. CEBP activation, while increased in response to BCG relative to controls, failed to demonstrate a significant correlation with BCG dose ($p = 0.059$). Graphically CEBP activity correlated with dose with peak activity at 200:1 in T24 cells and 500:1 in the 253J line. NRF2 activation significantly increased as a function of BCG dose ($p < 0.0001$). Peak activity was observed at the 200:1 ratio in 253J cells and the 500:1 ratio in the T24 cell line. There was no difference in the NRF2 response to BCG between the 2 cell lines. Graphical representation of the results for NF κ B, CEBP and NRF2 are shown in Figure 2. p21 Activation showed dose depended significant increase ($p < 0.05$) with highest activity observed at 1:200 in 253J and 1:500 dose in T24.

3.3 Gene Expression

The effect of BCG dose on the expression of a panel of BCG responsive genes was evaluated by quantitative rtPCR. The dose response relationship is shown graphically in Figure 3. The dose response relationship between BCG and individual gene expression was assessed using single factor ANOVA. P values for the relationship between dose and gene expression are shown for each gene and cell line in the table embedded in Figure 3. All seven of the genes studied in the 253J cell line demonstrated a significant relationship between BCG dose and gene expression. In the T24 cell line the expression of 4 of 7 genes was significantly correlated with BCG. CD54, IL6 and CCL20 failed to demonstrate a statistically significant dose-response relationship.

3.4 BCG Induced Cytotoxicity

LDH—LDH release by UC cell lines in response to BCG was significantly correlated with both exposure time and BCG dose ($p < 0.0001$ for T24 and 253J, Figure 4).

HMGB1—HMGB1 release by UC cell lines in response to BCG effectively mirrored the LDH response. HMGB1 release was significantly correlated with both exposure time and BCG dose ($p < 0.0001$ for T24 and 253J).

MTT—UC cell viability was measured using MTT in response to different BCG doses at different time points. Cytotoxic response to BCG was significantly correlated with both exposure time and BCG dose ($p < 0.005$) for T24 and 253J (Figure 4).

3.5 In vivo studies using animal model

Based on in vitro results, the dose response relationship demonstrated a modal pattern with peak activity observed at a tumor cell: BCG ratio of 1:200. Consequently, tumor cell: BCG ratio 1:50 was used as standard dose, while 1:200 was used as high dose. A total of 14 animals were included in each study group. Tumor implantation rates were 100 percent.

Animals treated with high BCG dose showed significantly reduced bladder weight compared to untreated group ($p < 0.01$) and animals treated with standard BCG dose ($p < 0.01$). Bladder weight data corresponded with the bio photonic imaging data obtained from implanted MB49-Luciferase cell line on day 7 and day 11 (Figure 5a, 5b). Animals treated with high dose BCG shows significantly reduced fluorescent intensity compared to PBS treated group ($p < 0.001$) and 1:50 dose treatment group ($p < 0.05$).

4. Discussion

Many variables surrounding the clinical use of BCG in the treatment of NMIBC remain empirically based. Dose, dwell time, dosing interval, and duration of induction therapy are among the variables for which there is limited supporting data. With respect to dose, driven by a desire to reduce treatment related toxicity, there has been a relatively extensive literature exploring the question of the minimal effective dose of BCG but virtually no studies exploring dose escalation.^{5–6}

A number of large multicenter, randomized trials have been performed to compare the toxicity and treatment efficacy of “low dose” vs standard dose induction intravesical BCG therapy.^{13–14} With perhaps the exceptions of patients with multifocal tumors reduced dose BCG, 1/3rd the standard dose, appears to have comparable treatment efficacy for recurrence and progression relative to the standard dose. Dose reductions to 1/6th of the standard dose are less effective than 1/3rd dose reductions.¹⁵ A single, small, non-randomized North American trial demonstrated statistical superiority of 120mg BCG relative to a 60 mg induction dose for a combined group of TIS, Ta and T1 patients.¹⁶ With respect to the role of dose in mediating the benefits of maintenance therapy, a recent non-inferiority study by the EORTC was unable to reject the study’s null hypothesis of a 10% decrease in disease free survival at 5 years.¹⁷ Majorities of the studies to date have demonstrated a decrease in toxicity associated with BCG dose reduction.

Defining the minimal effective BCG dose in an effort to reduce treatment related toxicity represents a valid clinical goal. However, an equal if not greater problem in the management of patients with NMIBC is the subset who fails to respond to an induction course of BCG. Clinical experience shows that approximately 1/3 of patients are “non-responders” to an initial six week course of BCG.¹⁸ Where-as dose reduction has been well studied with respect to “treatment comparability” no clinical data exists as to the role of dose escalation as initial therapy or in BCG non-responders.

Despite a paucity of clinical data there is basic research data which hints at a direct BCG dose-treatment response, relationship. Early orthotopic animal model work by Shapiro et al demonstrated a direct, significant relationship between BCG dose and both bladder tumor outgrowth and tumor volume.¹⁹ Subsequent studies found that BCG binding to urothelial carcinoma cells, and direct BCG mediated cytotoxicity, increased as a function of BCG dose.^{20–21} Similar dose-cytotoxicity relationships have been reported for other cell types.²² Finally, a mathematical model based upon clinical and basic research findings found a positive correlation of BCG dose with treatment response.²³

Against a backdrop of potential clinical relevance and suggestive basic research this study was designed to determine the effect of BCG dose on a number of intermediate tumor response endpoints that characterize the direct effect of BCG on UC cell biology. Our experiments confirmed earlier reports of the dose/time relationship of BCG adherence/internalization to UC cells. BCG induced intracellular signaling directly correlated with BCG dose, with the highest dose level reaching plateau or demonstrating a modest decrease. The downstream expression of tumor derived cytokines and other proteins, while varying individually, manifest a dose response relationship analogous to that observed for intracellular signaling. In vivo, the increased dose resulted in lowered tumor weight indicating improved anti-tumor response. Interestingly, rather than plateauing BCG dose response effect on BCG cytotoxicity fell off sharply at doses above those demonstrating peak activity. This is in accordance with earlier study with intradermal injection of MBT2 cell line which showed that higher dose leads to decreased anti-tumor response.²⁴ The relative loss of cytotoxicity at higher BCG:Cell ratios was consistent across the LDH, HMGB1, and MTT endpoints and could not be attributed to an altered response time course or substrate degradation by BCG. At the highest dose, levels of necrosis associated marker HMGB1 decreases, which might indicate a shift in mechanism of cell death.

The clear relationship between BCG dose and direct tumor response would suggest that there is an “optimal” dose for BCG’s treatment effect and to the extent current clinical dosing is below that level, the possibility of clinical BCG dose escalation for therapeutic benefit. Attempting to correlate the BCG doses used in these studies with current BCG clinical doses, as a starting point for dose manipulation, is fraught with difficulty. The number of viable BCG organisms contained in clinical BCG is expressed as a range. Using the ratio of urothelial surface cells in the bladder to BCG number is of limited value given that BCG adherence is relatively selective for areas of UC, and the size/number of umbrella cells per unit of surface area is a poor surrogate for the number of UC cells occupying an identical area. Given these constraints calculations of BCG per unit of bladder surface area may provide a better corollary to the laboratory. An in-vitro ratio of 50:1 BCG:cells represents 2.5×10^6 BCG/cm² (1×10^5 cells, 50:1 ratio, 24 well/cm² culture plates). Using the manufacturer’s average stated viability, 50mg of Tice BCG in 50ml of saline represents approximately 4.0×10^6 BCG/cm² of bladder surface, assuming a decompressed surface area of 100cm². Consequently the in-vitro dose at 50:1 approximates 62% the BCG concentration per surface area used clinically. For both tumor cell lines, across the range of assays, a BCG/cell ratio of up to 250:1 was associated with peak activity suggesting an opportunity to push the current clinical dose up to 3 fold. Given that BCG has never been formally evaluated in a phase I trial, a dose escalation trial in a population of patients refractory to standard doses may be warranted.

5. Conclusions

The effects of BCG on UC cells demonstrate a direct dose-responses relationship. Given the contribution of the direct cellular response to anti-tumor activity dose escalation has the potential to improve response rates. Ultimately the potential for an increased clinical response will need to be evaluated against the risk of increased treatment related toxicity.

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Abbreviations

ANOVA	analysis of variance
BCG	Bacillus Calmette Guérin
CD54	intercellular adhesion molecule 1
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL3	chemokine (C-X-C motif) ligand 3
CCL20	chemokine (C-C motif) ligand 20
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
IL6	interleukin 6
IL8	interleukin 8
iNOS	inducible nitric oxide synthase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NO	nitric oxide
NRF2	nuclear factor (erythroid-derived 2)-like 2
CEBP	CCAAT-enhancer-binding proteins
P21	cyclin dependent kinase inhibitor p21 (cip1; waf1)
PBS	phosphate buffered saline
RNA	ribonucleic acid
rtPCR	reverse transcriptase polymerase chain reaction
q rtPCR	quantitative reverse transcriptase polymerase chain reaction
UC	urothelial carcinoma

References

1. Babjuk M, Oosterlinck W, Sylvester R, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. *Eur Urol*. 2011; 59:997. [PubMed: 21458150]

2. Kassouf W, Kamat AM, Zlotta A, et al. Canadian guidelines for treatment of non-muscle invasive bladder cancer: a focus on intravesical therapy. *Can Urol Assoc J.* 2010; 4:168. [PubMed: 20514279]
3. Hall, MC., Cheng, SS., Dalbagni, G., et al. Guideline for the Management of Nonmuscle Invasive Bladder Cancer: (Stages Ta, T1, and Tis): Update. American Urological Association; 2007.
4. Luo Y, Askeland EJ, Newton M, et al. Immunotherapy of Urinary Bladder Carcinoma: BCG and Beyond. *Advances in Urology.* 2012;181987. [PubMed: 22778725]
5. O'Donnell MA. Optimizing BCG therapy. *Uro Oncol.* 2009; 27:325.
6. Witjes JA. What is the optimal BCG dose in non-muscle-invasive bladder cancer? *Eur Urol.* 2007; 52:1300. [PubMed: 17531374]
7. Zhang G, Chen F, Cao Y, et al. Contributors to HMGB1 Release by Urothelial Carcinoma Cells in Response to BCG. *J Urol.* 2013; 190:1398. [PubMed: 23583857]
8. Zhang G, Chen F, Cao Y, et al. HMGB1 Release by Urothelial Carcinoma Cells is Required for the In Vivo Antitumor Response to Bacillus Calmette-Guérin. *J Urol.* 2012; 189:1541. [PubMed: 23041342]
9. de Boer EC, Bevers RF, Kurth KH, et al. Double Fluorescent Flow Cytometric Assessment of bacterial internalization and binding by epithelial cells. *Cytometry.* 1996; 25:381. [PubMed: 8946146]
10. Shah G, Zhang G, Chen F, et al. Loss of BCG Viability Adversely Affects the Direct Response of Urothelial Carcinoma Cells to BCG Exposure. *J Urol.* 2014; 191:823. [PubMed: 24035882]
11. Chen F, Zhang G, Cao Y, et al. MB49 murine urothelial carcinoma: molecular and phenotypic comparison to human cell lines as a model of the direct tumor response to bacillus Calmette-Guerin. *J Urol.* 2009; 182:2932. [PubMed: 19853870]
12. Ninalga C, Loskog A, Klevenfeldt M. CpG oligonucleotide therapy cures subcutaneous and orthotopic tumors and evokes protective immunity in murine bladder cancer. *J Immunol.* 2005; 28:20.
13. Martinez-Pineiro JA, Flores N, Isorna S, et al. Long-term follow-up of a randomized prospective trial comparing a standard 81 mg dose of intravesical bacille Calmette-Guerin with a reduced dose of 27 mg in superficial bladder cancer. *BJU Int.* 2002; 89:671. [PubMed: 11966623]
14. Martinez-Pineiro JA, Martinez-Pineiro L, Solsona E, et al. Has a 3-fold decreased dose of bacillus Calmette-Guerin the same efficacy against recurrences and progression of T1G3 and Tis bladder tumors than the standard dose? Results of a prospective randomized trial. *J Urol.* 2005; 174:1242. [PubMed: 16145378]
15. Ojea A, Nogueira JL, Solsona E, et al. A multicenter, randomized prospective trial comparing three intravesical adjuvant therapies for intermediate-risk superficial bladder cancer: low-dose bacillus Calmette-Guerin (27 mg) versus very low-dose bacillus Calmette-Guerin (13.5 mg) versus mitomycin C. *Eur Urol.* 2007; 52:1398. [PubMed: 17485161]
16. Morales A, Nickel JC, Wilson JW. Dose-response of bacillus Calmette-Guerin in the treatment of superficial bladder cancer. *J Urol.* 1992; 147:1256. [PubMed: 1569662]
17. Oddens J, Brausi M, Sylvester R, Bono A, et al. Final results of an EORTC-GU cancers group randomized study of maintenance bacillus Calmette-Guerin 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. *Eur Urol.* 2013; 63:462. [PubMed: 23141049]
18. Nepple KG, Lightfoot AJ, Rosevear HM, et al. Bladder Cancer Genitourinary Oncology Study Group Bacillus Calmette-Guerin with or without interferon alpha-2b and megadose versus recommended daily allowance vitamins during induction and maintenance intravesical treatment of nonmuscle invasive bladder cancer. *J Urol.* 2010; 184:1915. [PubMed: 20846688]
19. Shapiro A, Ratliff TL, Oakley DM, et al. Reduction of bladder tumor growth in mice treated with intravesical Bacillus Calmette-Guerin and its correlation with Bacillus Calmette-Guerin viability and natural killer cell activity. *Cancer Res.* 1983; 43:1611. [PubMed: 6339040]
20. Schneider B, Thanhauser A, Jocham D, et al. Specific binding of bacillus Calmette-Guerin to urothelial tumor cells in vitro. *World J Urol.* 1994; 12:337. [PubMed: 7881473]
21. Jackson A, Alexandroff A, Fleming D, et al. Bacillus-calmette-guerin (bcg) organisms directly alter the growth of bladder-tumor cells. *Int J Oncol.* 1994; 5:697. [PubMed: 21559633]

22. Kitamura A, Mastumoto S, Asahina I. Growth inhibition of HeLa cell by internalization of Mycobacterium bovis Bacillus Calmette-Guerin (BCG) Tokyo. *Cancer Cell Int.* 2009; 9:30. [PubMed: 19954548]
23. Rentsch CA, Biot C, Gsponer JR, et al. BCG -mediated bladder cancer immunotherapy: identifying determinants of treatment response using a calibrated mathematical model. *PLoS ONE.* 2013; 8:e56327. [PubMed: 23451041]
24. Lamm DL, Reichert DF, Harris SC, Lucio RM. Immunotherapy of murine transitional cell carcinoma. *J Urol.* 1982; 128:1104. [PubMed: 6757465]

Figure 1. Effect of different BCG dose on adherence and internalization

Cells were treated with different cell:BCG ratios ranging from 1:5 to 1:500 for 4 h. BCG dose significantly correlated with adherence/internalization ($p < 0.0001$). A dose-adherence plateau was observed beyond the 100:1 ratio as approximately 80% of cells were positive at or above this concentration.

Figure 2. Effect of BCG Dose on Intracellular Signaling

T24 and 253J cell lines were exposed to different dose of BCG for 6h. NF κ B activation was highest at 1:200 cell:BCG ratio and was significantly correlated with BCG dose ($p < 0.05$). Even though CEBP activation showed increasing pattern with BCG relative to controls, it did not with dose with highest activity at 1:200 in T24 cells and 1:500 in the 253J line. NRF2 activation showed significantly increased as a function of BCG dose ($p < 0.0001$). Highest activity was observed at the 1:200 ratio in 253J cells and the 1:500 ratio in the T24 cell line. p21 activation also showed dose dependent increase ($p < 0.05$) with peak activity observed at 1:200 and 1:500 dose for 253J and T24 cell lines, respectively.

Figure 3. Effect of BCG Dose on UC cell Gene Expression in Response to BCG

T24 and 253J cell lines were exposed to different dose of BCG for 6h and the expression of various genes were measured using q-RT-PCR. The dose response relationship between BCG and individual gene expression was assessed using single factor ANOVA. In 253J cell lines, all seven genes showed a significant relationship between BCG dose and gene expression, while in T24 cell line, the expression of 4 of 7 genes was significantly correlated with BCG. CD54, IL6 and CCL20 did not show a statistically significant dose-response relationship.

Figure 4. Effect of dose on cytotoxicity

LDH release: Both cell lines were exposed to various dose of BCG for 24h. LDH release by UC cell lines in response to BCG was significantly correlated with BCG dose ($p < 0.0001$ for T24 and 253J).

MTT: Both cell lines were treated with different BCG dose for up to 6 days. UC cell viability as measured MTT by in response to BCG was significantly correlated with both exposure time and BCG dose ($p < 0.005$ for T24 and 253J for both exposure time and dose).

HMGB1: Both cell lines were treated with various BCG dose for 24h following which supernatant was used to measure HMGB1 levels. HMGB1 release by UC cell lines in response to BCG effectively mirrored the LDH response. HMGB1 release was significantly correlated with BCG dose ($p < 0.001$ for T24 and 253J).

Figure 5. Effect of standard and high dose BCG on bladder weight

14 Animals were treated with PBS (control group), standard BCG dose (1:50, cell:BCG ratio) or high dose (1:200, cell:BCG ratio) on day 1, 4 and 7 post tumor cell implantation. Animals treated with high dose BCG showed significant reduction in bladder weight compared to animals treated with standard dose ($p < 0.01$) and control PBS treated group ($p < 0.001$) (Figure 5a). Biophotonic imaging of tumor cells on day 11 showed significant difference in fluorescent intensity between groups. Animals treated with high dose BCG shows significantly reduced intensity compared to PBS treated group ($p < 0.001$) and standard dose treatment group ($p < 0.05$) (Figure 5b).