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The immunotherapeutic effects of recombinant Bacillus Calmette-Guérin resistant to antimicrobial peptides on bladder cancer cells

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ABSTRACT

Purpose: Although *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is the most widely used bladder cancer immunotherapy, innate immune responses involving antimicrobial peptides (AMPs) cause BCG failure and unwanted side effects. Here, we generated genetically modified BCG strains with improved immunotherapeutic effects by adding genes that confer evasion of AMPs.

Materials and methods: We constructed recombinant BCG (rBCG) strains expressing Streptococcal inhibitor of complement (*Sic*), which confers resistance to human α -defensin-1 and cathelicidin, and dalanyl carrier protein ligase (*dltA*), which confers resistance to cationic AMPs. *Sic* and *dltA* were separately cloned into the pMV306 plasmid and introduced into BCG via electroporation. Then, the efficacy of the rBCGs was tested in a growth inhibition assay using two bladder cancer cell lines (5637, T24).

Results: We confirmed the presence of cDNA segments corresponding to the *Sic* and *dltA* genes in total mRNA of the rBCG strains containing *Sic* (rBCG-Sic) and *dltA* (rBCG-dltA), and these rBCGs showed higher survival against AMPs. The growth inhibitory effects of rBCGs on bladder cancer cells were significantly enhanced compared to those of the parent BCG, and THP-1 migration also increased. After 8 h of infection, the levels of internalization were higher in rBCG-infected bladder cancer cells than in BCG-infected cells, and cells infected with rBCGs showed increased release of antitumor cytokines, such as IL-6/12, TNF- α , and INF- γ , resulting in inhibition of bacterial killing and immune modulation via antimicrobial peptides.

Conclusions: rBCG-Sic and rBCG-dltA can effectively evade BCG-stimulated AMPs, and may be significantly improved immunotherapeutic tools to treat bladder cancer.

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1. Introduction

Bacillus Calmette-Guérin (BCG) is an attenuated *Mycobacterium bovis* strain that is commonly used as the most effective immunotherapeutics for high-risk non-muscle-invasive bladder cancer

https://doi.org/10.1016/j.bbrc.2018.12.097 0006-291X/© 2018 Elsevier Inc. All rights reserved. patients [1]. The efficacy of BCG has been compared to that of all other forms of intravesical chemotherapy in several trials, which have shown that BCG is more effective than other chemotherapies [2]. Although BCG is currently the best treatment option, some patients do not respond to this treatment, and side effects are common [2]. The prospect of genetically modifying BCG to generate recombinant BCG (rBCG) strains that induce a more specific immune response by overexpressing immunogenic molecules or foreign antigens is a promising approach to improve its efficacy for bladder cancer treatment.

After intravesical administration in the bladder, BCG first establishes direct contact with the bladder urothelium, and the second step is BCG uptake by bladder cancer cells (internalization),

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probably by endocytosis. The innate (non-specific) immune response acts against BCG to sterilize the urinary tract, and some of the effectors involved in this innate response to BCG are antimicrobial peptides (AMPs). Mammalian cells release different kinds of AMPs, such as human beta-defensins (HBD-1 or HBD-4) in the epithelium and leukocytes, histatins in saliva, and cathelicidins (CAP18 or CAMP) in neutrophils and the epithelium [3,4]. Some bacteria protect themselves against AMPs by secreting special proteins that inhibit their function, such as Streptococcal inhibitor of complement (SIC) and D-alanine: d-alanyl carrier protein ligase (DltA) [5–8].

Our hypothesis is that rBCG strains expressing Sic (rBCG-Sic) or DltA (rBCG-dltA) may be more resistant to AMPs, which may improve the internalization and effectiveness of BCG. Therefore, we generated rBCG-Sic and rBCG-dltA strains and investigated the anti-tumor mechanisms of rBCG-Sic and rBCG-dltA in bladder cancer cells.

2. Materials and methods

2.1. Cell cultures and reagents

The 5637 and T24 human bladder cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were grown at 37 °C in a humid, 5% CO₂ incubator, fed every 2–3 days, and passaged when the cells reached confluence. BCG was cultured in 7H9 medium at 37 °C for approximately 15 days, until the optical density at 600 nm (OD₆₀₀) reached 2.0. The BCG cells were then diluted to 1×10^7 cells/ml, which is a multiplicity of infection (MOI) of 100, in phosphate buffered saline and stored at -80 °C until use.

2.2. Construction of plasmids and electroporation of BCG

DNAs encoding Sic (GenBank: AY229858.1) and DltA (GenBank: D86240.2) were synthesized and cloned (Macrogen, Seoul, Korea). Then, Sic and dltA expression vectors were constructed in the pMV306 vector using EcoRI and HpaI restriction enzymes. The primer sequences used in the cloning are shown in Table 1. For electroporation, BCG cells were harvested by centrifugation at $3000 \times g$ for 7 min at room temperature and washed twice with PBS. Then, 2 mm gap cuvettes (BTX, Holliston, MA, USA) were prechilled on ice for about 30 min. 1000 MOI BCG was suspended in 100 µl of 7H9 liquid medium containing OADC (Sigma-Aldrich). Next, the BCG was gently mixed with 2 µg of plasmid DNA in an autoclaved Eppendorf tube, and the mixture was added to the prechilled cuvette and electroporated using an ECM[®] 399 Electroporation system at 2500 V (BTX). After electroporation, the BCG was resuspended in 7H9 medium containing ADC and kanamycin $(10 \,\mu\text{g/ml})$ and cultured at 37 °C for 2 days. After 2 days, the bladder cancer cell lines were treated with the rBCG strains.

2.3. Quantification of BCG cells

The number of BCG cells in infected human cells was measured by a quantitative real-time PCR-based method. To detect BCG in the human cells, BCG-specific primers based on a heat shock protein gene (*hsp65*) were used as previously described [9]. For the BCG DNA amplification, a fragment of *hsp65* was targeted, whereas a fragment of the human β 2-microglobulin (*B2M*) gene was targeted for the human cancer cell DNA amplification. The following human B2M-specific primers (152-bp product) were designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast): forward (HB2M F). 5'-ACTCCACCACCACGAAATGG-3' and reverse (HB2M_R), 5'-AAGACAAAGGGCTCGGCAAT -3'. The real-time PCR was performed in a LightCycler[®] 96 (Roche Diagnostics, Mannheim, Germany). For standard curve generation for the BCG, a BCG DNA stock, which was a gift from professor Bum-Joon Kim at Seoul National University, Korea, was serially diluted (10-fold dilutions) to generate a six-point standard curve containing 10 fg-1 ng of DNA in each reaction [10]. The ratio of the numbers of BCG cells to human cells was determined for each sample using standard curves

2.4. BCG internalization assay

Labelled BCG was prepared according to previously described methods. After treatment, cells were harvested and resuspended in PBS containing 0.2% albumin, 0.02% Na-EDTA, and 0.01% NaN₃. The cells were incubated for 30 min at 4 °C with a polyclonal rabbit anti-*Mycobacterium tuberculosis* antibody (1:100) to distinguish between internalized and surface-bound BCG. At the end of the incubation period, the cells were washed and incubated with a cyanine 5 (Cy-5)-conjugated goat anti-rabbit antibody (1:50) for 30 min at 4 °C. The cells were washed and mounted on cover slides containing mounting medium. Nuclei were counterstained with 4',6-diamidino-2- phenylindole (DAPI) and cover slipped. Fluorescence was observed with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). In each experiment, 100 cells with DAPI-stained nuclei (blue) were counted at 1000 × .

2.5. Cell viability and colony formation assays

Cells were seeded into 96-well plates at 5×10^3 cells per well and treated with various concentrations of rBCG and BCG. After 48 h, cell viability was analyzed using the D-PlusTM CCK kit (Dongins, Seoul, Korea) according to the manufacturer's instructions.

Colony formation assays were performed following treatment with rBCG (rBCG-Sic and rBCG-dltA) at an MOI of 30. On day 1, 1×10^3 cells were reseeded in triplicate into 12-well plates and cultured in complete medium for 14 days to allow colonies to form. Colonies were fixed with 4% paraformaldehyde (Biosesang, Seongnam, Korea) and stained with 0.1% crystal violet (Sigma-Aldrich).

Table 1

Primers used	to engineer	the recombinant	Mycobacterium	bovis Bacillus	Calmette-Guerin strains.
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Primers	Vector	Sequence
Sic	pMV306hsp	Forward: 5'-CGGGCTGCAG <u>GAATTC</u> ATGAAGATCAAGAAGAACATC-3' <i>Eco</i> RI Reverse: 5'-GATCGTACGCTA <u>GTTAAC</u> TCAGGTGGTCGACGGGGTG-3' HpaI
dltA	pMV306hsp	Forward: 5'-CGGGCTGCAG <u>GAATTC</u> ATGACAGATATTATTAAC-3' <i>Eco</i> RI Reverse: 5'-GATCGTACGCTA <u>GTTAAC</u> TCATCCGTTAATTACCTC-3' Hpal

2.6. Cell migration assay and ELISA

Cells were seeded into 24-well plates at 5×10^4 cells per well in complete medium containing 10% FBS and 1% antibiotics. At 24 h after seeding, cells were infected with rBCG, at an MOI of 10 for 8 h. THP-1 cells (3×10^5) in serum free medium were added to the upper chamber of a transwell (Corning, Corning, NY, USA) and incubated for 2 h. The cells on the plate and in the lower chamber were fixed with 4% paraformaldehyde (Biosesang) and stained with 0.1% crystal violet (Sigma-Aldrich).

IL-6 (D6050), IL-12 (D1200), TNF-α (DTA00C), and INF-γ (DIF50) Duo-set ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA); ELISA kits for HBD-2 (cat# 201-12-1937; SunRed Bio, Shanghai, China), HBD-3 (cat# CSB-E14187h; CUSABIO, Houston, TX, USA), and CAMP (cat# 201-12-3404; SunRed Bio).

2.7. Bacterial live and dead assay

BCG and rBCG cells at a density 2×10^7 cells/100 µl were treated with HBD-2 (0.1 ng/ml), HBD-3 (0.5 ng/ml), and CAMP (0.5 ng/ml) for 1 h. Then, the viability of the cells was measured using a bacterial live and dead assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

All data are presented as the mean \pm standard deviation (SD) of at least three separate experiments performed in triplicate. Data were compared by using Student's t-test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. The generation of rBCGs and their resistance to AMPs

We generated rBCG strains expressing Sic (rBCG-Sic) or DltA (rBCG-dltA) by electroporation of integrating expression constructs (Fig. 1A). Total mRNA was extracted from rBCG-Sic and rBCG-dltA and amplified using *Sic* and *dltA* specific primers, and we detected cDNA segments corresponding to these genes (Fig. 1A).

To determine whether the rBCGs were resistant to AMPs, we determined the survival of the rBCGs strains after treatment with AMPs (HBD-2, HBD-3, and CAMP) by using a bacterial live and dead assay, and the rBCGs showed higher survival against AMPs compared to the parent BCG strain (Fig. 1A). The percentages of live BCG after treatment with HBD-2, HBD-3, and CAMP (%, mean \pm SD) were similar (42.5 \pm 1.9%, 40.5 \pm 0.7%, and 43.2 \pm 8.1%, respectively) but significantly lower than the percentages of live rBCG-Sic (59.4 \pm 2.8%, 61.6 \pm 1.8%, and 65.2 \pm 0.2%, respectively) and rBCG-dltA (64.0 \pm 1.0%, 67.6 \pm 1.8%, and 65.6 \pm 0.7%, respectively; Fig. 1B).

3.2. Internalization of the rBCGs in bladder cancer cells

To determine the rate of BCG internalization in bladder cancer cells, we used qRT-PCR to measure the expression of BCG 16S mRNA and a fluorescence confocal microscopy assay to assess BCG internalization. In the BCG internalization assay, 5637 and T24 cells were treated with BCG or rBCG (MOI = 10), and internalization was evaluated after 8 h. In the assay, extracellular BCG was labelled with both fluorescent dyes (FITC, green and Cy-5, red; merge, yellow), and intracellular BCG was labelled with FITC (green). The level of internalization as measured by confocal microscopy was higher for the rBCGs than for BCG, and rBCG-dltA-treated cells showed more BCG internalization than rBCG-sic- and BCG-treated 5637 and T24 cells (Fig. 2A).

Fig. 2B shows the internalization rates of BCG, rBCG-Sic, and rBCG-dltA (at 10 or 30 MOI) in 5637 cells after 8 h of treatment by using quantitative real-time RT-PCR. The internalization rates were determined as the ratio of BCG cells to human cells. The ratio at 10 MOI BCG (mean \pm SD, 0.09 \pm 0.01) was lower than the ratios of 10 MOI rBCG-Sic (1.78 \pm 0.148, p < 0.01) and 10 MOI rBCG-dltA (1.82 \pm 0.185, p < 0.01), and the ratios of 10 MOI rBCG-Sic and 10 MOI rBCG dltA were similar to that of 30 MOI BCG (1.87 \pm 0.185). The ratio for 30 MOI rBCG dltA (3.45 \pm 0.142. p < 0.05) demonstrated a higher internalization rate than that for 30 MOI BCG; however, the ratio for 30 MOI rBCG Sic (2.33 \pm 0.152, p = 0.67) was not increased.

3.3. The effects of rBCG on cytokine production and chemotaxis

We hypothesized that the increased internalization of rBCGs into cancer cells would increase cytokine production. Therefore, we measured the levels of several cytokines (IL-6, IL-12, TNF- α , and INF- γ) in bladder cancer cells after BCG and rBCGs treatment. The rBCG-dltA-treated 5367 and T24 cells showed higher levels of cytokines). In addition, rBCG-Sic-treated cells (p < 0.01 for all cytokines). In addition, rBCG-Sic-treated 5637 cells showed higher secretion of INF- γ (p < 0.05), and rBCG-Sic-treated 5637 and T24 cells showed higher secretion of IL-12 (p < 0.05; Table 2).

To determine the AMP-stimulating effects of rBCG on bladder cancer cells, we measured the levels of AMPs (HBD-2, HBD-3, and CAMP) in 5637 and T24 cells after treatment with rBCG-Sic or rBCG-dltA, and observed that the secretion of HBD-2, HBD-3, and CAMP was significantly higher in rBCG-dltA-treated cells than in BCG-treated cells, whereas rBCG-Sic-treated cells did not show any difference in AMP secretion (Table 2).

The effect of rBCG on chemotaxis was also confirmed in a monocyte (THP-1) migration assay. rBCGs 5637 cells induced a higher migration rate of THP-1 cells compared to BCG 5637 cells (Fig. 3). The number of migrated cells (mean \pm SD) in the BCG group were higher than that in the control group (43.12 ± 2.90 vs. 18.88 ± 2.90 , p < 0.05), and the rBCG-Sic (73.13 ± 4.88 , p < 0.05) and rBCG-dltA (61.63 ± 3.07 , p < 0.05) groups showed higher migration than the BCG group (Fig. 3).

3.4. rBCG-induced bladder cancer cell viability and decreased colony formation

To determine the effect of rBCGs in bladder cancer cells, cell viability was assessed by the CCK-8 assay. The viability of rBCG-infected 5637 and T24 bladder cancer cells was significantly decreased at 10 and 30 MOI in a dose-dependent manner, whereas BCG-infected cells did not show any dose-dependent difference in viability (Fig. 4A).

We also tested the colony formation ability of rBCG- and BCGinfected 5637 cells. Fig. 4B shows that the number and size of colonies formed after 16 days were dramatically lower in rBCG-Sicand rBCG-dltA-infected cells than in BCG-infected cells. The rBCGs and BCG showed dose-dependent inhibitory effects on colony-formation ability (the number of colonies per high power field, mean \pm SD) of 5637 cells. However, rBCG-Sic (67.75 \pm 5.82 [control] vs. 22 \pm 2.62 [rBCG-Sic], p < 0.05) and rBCG-dltA (74 \pm 8.23 [control] vs. 28.75 \pm 2.87 [rBCG-dltA], p < 0.05) at 10 MOI showed greater inhibitory effects when compared with the control and BCG at 30 MOI (67.83 \pm 9.39 [control] vs. 18.25 \pm 2.38 [BCG], p = 0.54).

4. Discussion

In the present study, we generated rBCG strains containing the

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Fig. 1. Construction of recombinant BCG (rBCG) strains and increased resistance of rBCG expressing Sic (rBCG-Sic) or dltA (rBCG-dltA) to AMPs. (A) Experimental schema for the construction of translational fusions of rBCG-sic and rBCC-dltA, and the expression of Sic and dltA in BCC. Reverse-transcribed cDNA of *Sic* and *dltA* present in rBCG. The 16S RNA was included as an internal control. The migration of *Sic* (243 bp), *dltA* (268 bp), and the 16S RNA amplified products are indicated. **(B)** To determine whether the rBCGs were resistant to AMPs, we assessed the survival of rBCG-slic and rBCG-dltA after treatment with three AMPs (HBD-2, HBD-3, and CAMP) by using a bacterial live and dead assay, and the rBCGs when compared to BCG. **p* < 0.05 and ***p* < 0.01.

Sic (rBCG-Sic) or *dltA* (rBCG-dltA) gene by electroporation of an integrating vector, and showed that rBCG-Sic and rBCG-dltA survived in the presence of AMPs, which means that SIC and DltA show biological activity against AMPs and block their antibacterial effects

against BCG. We expect that these rBCG strains will more effectively attach to bladder cancer cells than BCG by avoiding the immune response of AMPs.

The cell wall and membrane of most gram-positive bacteria are

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Fig. 2. Internalization of rBCG in bladder cancer cells. (A) Discrimination between intracellular and extracellular rBCG in 5637 and T24 cells using a dual fluorescence assay and fluorescence confocal microscopy. FITC-labelled rBCG (green) are intracellular and extracellular, Cy-5-labelled rBCG (red) are extracellular, and the nuclei of 5637 and T24 cells are counterstained with DAPI (400 ×). Extracellular rBCG are labelled with both fluorescent dyes (yellow) in the merged image, whereas intracellular rBCG are labelled with FITC (green). **(B)** The 5637 cells infected with 10 or 30 MOI of BCG and rBCG for 8 h were collected, and 5637 cells were analyzed by qRT-PCR. RNA was isolated and reverse-transcribed into cDNA.

p* < 0.05 and *p* < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Table 2	2
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The concentration of cytokines and antimicrobial peptides (AMPs) in bladder cancer cells (5637 and T24) treated with BCG or recombinant BCG (rBCG-Sic and rBCG-dltA).

	5637			T24				
	BCG	rBCG-Sic	rBCG-dltA	BCG	rBCG-Sic	rBCG-dltA		
Cytokine level (pg/mL , mean \pm SD)								
IL-6	120.51 ± 0.14	120.22 ± 1.80	$255.89 \pm 5.05^{**}$	85.44 ± 1.25	82.19 ± 2.26	$214.13 \pm 15.92^{**}$		
IL-12	115.68 ± 2.01	$134.43 \pm 0.20^{*}$	$164.26 \pm 19.09^{**}$	91.13 ± 1.09	$98.96 \pm 1.63^{*}$	$128.87 \pm 2.24^{**}$		
TNF-α	62.50 ± 2.26	68.50 ± 7.79	$87.09 \pm 0.74^{**}$	129.35 ± 17.94	151.59 ± 14.82	$178.12 \pm 0.45^{**}$		
INF-γ	83.86 ± 5.00	$129.76 \pm 0.43^{*}$	$154.67 \pm 1.14^{**}$	93.29 ± 5.57	109.14 ± 2.00	$121.71 \pm 3.81^{**}$		
AMPs level (mean \pm SD)								
HBD-2 (ng/mL)	4.08 ± 0.20	4.67 ± 0.11	$6.33 \pm 0.12^{*}$	3.54 ± 0.01	3.81 ± 0.18	$4.91 \pm 0.01^{*}$		
HBD-3 (ng/mL)	11.25 ± 0.05	11.46 ± 0.32	$14.12 \pm 0.42^{*}$	10.62 ± 0.03	10.63 ± 0.10	$13.51 \pm 0.42^*$		
CAMP (pg/mL)	15.77 ± 0.48	17.08 ± 0.17	$18.41 \pm 0.29^{*}$	14.35 ± 0.17	16.91 ± 0.20	$18.24 \pm 0.06^{*}$		

p* < 0.05 and *p* < 0.01.

IL: interleukin, TNF: tumor necrotizing factor, INF: interferone, AMP: antimictobial peptide, HBD: human beta defensin, CAMP: cathalecidine.



Fig. 3. Chemotaxis of human monocytic THP-1 cells toward bladder cancer cells infected with rBCG-Sic or rBCG-dltA. Bladder cancer cells (5637) were seeded into a 24-well plate. After 24 h, the cells were infected with BCG or rBCG (30 MOI) for 24 h, and then THP-1 cells were added to the upper chamber and incubated for 2 h. Data are the mean \pm SD (n = 3, per group). All expression ratios were normalized to the untreated group.

p < 0.05 and p < 0.01.

composed of teichoic acids (TAs), and the d-alanyl esterification of TA on the cell wall of these gram-positive bacteria increases its positivity [8]. This positive charge can decrease the effectiveness of AMPs, such as CAMPs [6]. The *dlt* operon encodes four proteins, DltA, DltB, DltC, and DltD, that are necessary for the addition of Dalanine esters to TAs in the bacterial cell wall. In particular, a previous study demonstrated that deletion of the *dltA* gene decreased the ability of Streptococcus pneumoniae to evade AMPs [5]. In addition, Sic produced by Streptococcus pyogenes is a hydrophilic secretory protein that sequesters many AMPs and prevents them from reaching their targets on the cell surface [7]. Sic is a 31 kDa extracellular protein produced by a few highly virulent strains of S. pyogenes (predominantly by M1 strains) that was initially identified as an inhibitor of the membrane attack complex of complement [11]. Sic binds to α -defensins, CAMPs, and lysozyme to neutralize them and inhibit their bactericidal activity against S. pyogenes [7]. Sic was also shown to inhibit the action of HBD-1, HBD-2, and HBD-3 [12,13].

In our study, we evaluated the BCG internalization, cytokine production, and monocyte chemotactic effects of rBCG in human bladder cancer cells and showed that the effects of rBCG were greater than those of BCG. Our results suggest that the internalization of rBCG resulted in increased cytokine secretion, which reduced the survival and colony formation of rBCG-infected bladder cancer cells and increased the migration of THP-1 cells. This indicates that rBCG may have improved immunologic activity against bladder cancer cells, and therefore, may have higher therapeutic efficacy, with reduced side effects. BCG immunotherapy plays a crucial role in cytokine secretion through its internalization. This cellular internalization leads to a primary innate immune response involving the secretion of several cytokines, such as IL-1, IL-2, IL-6, IL-8, TNF- α , and IFN- γ [14]. The results showed that cytokine secretion, which is closely related to the immune system, is increased by rBCG internalization.

Our results showed that rBCG treatment induced higher secretion of AMPs by the bladder cancer cells compared to BCG treatment. Our hypothesis to explain this observation is related to the fact that rBCG is less affected by the AMPs than BCG and that internalization is a more frequent event, resulting in increased AMP secretion. As rBCG also interferes with the binding of AMPs to rBCG, higher AMP levels are present in the medium than in the medium containing cells treated with BCG. Moreover, 10 MOI rBCG showed an internalization rate similar to that of 30 MOI BCG, which is the clinical dosage for intravesical instillation. This means that a onethird dose of rBCG showed a similar internalization rate in the urothelium to that of a full dose of BCG, thus increasing therapeutic efficacy and reducing side effects. Moreover, we optimized the concentration of rBCG at 10 MOI, although it is essential to conduct animal studies and clinical trials because the efficacy and toxicity of the materials need to be assessed.

rBCG-dltA showed higher internalization, cytokine production, and AMP induction than rBCG-Sic. The amount of AMP secreted by rBCG-dltA-treated cells was significantly higher than that secreted by BCG- and rBCG-Sic-treated cells. Moreover, 30 MOI rBCG-dltA showed a higher internalization rate than 30 MOI BCG and 30 MOI rBCG Sic. Our hypothesis is the positive charge on the cell wall of rBCG-dltA facilitates tighter binding with the negatively charged bladder cancer cell wall [11]. This higher internalization induces greater cytokine release, and we believe that rBCG-dltA might be a suitable replacement for BCG in bladder cancer immunotherapy.

Moreover, we showed that our rBCGs reduced the viability and colony forming ability of bladder cancer cells at a lower dose than BCG. Several studies have suggested that the anti-tumor activities of BCG are due in part to a direct anti-tumor effect [15-17]. Cellular internalization of BCG, and the resulting alterations in reactive oxygen species and nitric oxide, has been proposed as a mechanism contributing to direct BCG-mediated cytotoxicity. Consistent with

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Fig. 4. Viability and colony formation of rBCG-invaded bladder cancer cells. (A) Bladder cancer cell lines (5637 and T24) were incubated with BCG or rBCG (expressing Sic or dltA) at an MOI of 1, 10, or 30 for 48 h and then evaluated by the CCK-8 assay. Data are the mean \pm SD (n = 6, per group). **(B)** 5637 cells were treated with BCG or rBCG (expressing Sic or dltA) at an MOI of 1, 10, or 30 for 48 h and replated in a 12-well cell culture plate. After 14 days, the number of crystal violet-stained colonies was counted. **p* < 0.05 and ***p* < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

what has been reported by others, the present results showed that rBCG exerts a direct anti-proliferative effect on human bladder cancer cells. Therefore, we believe that the therapeutic efficacy of BCG could be maintained or improved with lower doses of rBCG without the severe side effects or infection associated with the administration of high-dose BCG. Although appropriately powered clinical trials are required to define the true efficacy, optimal dose, levels, and schedules, these studies suggest that rBCG treatment might be a powerful approach to improve the therapeutic efficacy for bladder cancer over the current commercially available strains.

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Author contributions

I.H.C. and Y.M.W. co-designed the experiments; M.J.C. wrote the manuscript and analyzed all the final data; I.H.C. and Y.M.W supervised the research; M.J.C performed all the experiments; and M.J.K. and K.J.K. helped with the in vitro experiments. All authors reviewed and approved the final manuscript for submission.

Additional information

Competing interests: The authors declare no competing interests.

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